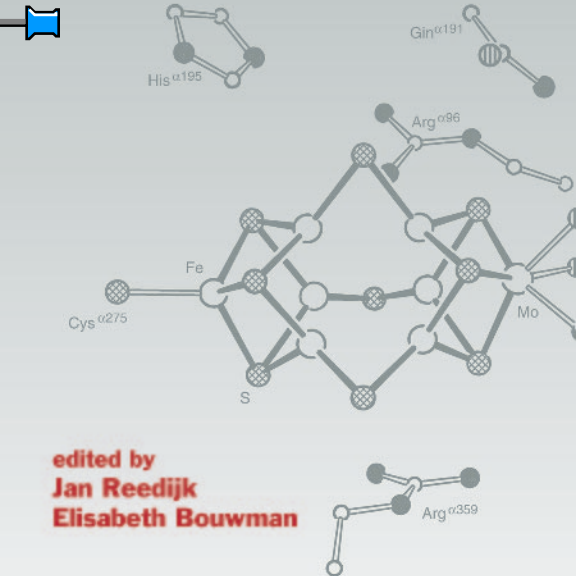


Bioinorganic Catalysis

Second Edition,
Revised and Expanded



edited by
Jan Reedijk
Elisabeth Bouwman

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Preface

Catalysis has been an extremely important area within chemistry and has been well described over the course of the last few decades. Biocatalysis is a more recent branch of catalysis in which the catalyst and the process originate from the biological sciences and deals with enzymes. In the previous decade, a monograph dealing with bioinorganic catalysis would have seemed an unusual collection. The research programs in inorganic chemistry, biochemistry and catalysis have only recently met one another, and the results are exciting. This development resulted in the first edition of this monograph. Even a cursory examination of the most recent chemical literature will indicate a significant increase in the field of bioinorganic chemistry, especially the catalytic aspects of this field.

In many biocatalytic systems, the metal plays an important role at the active site (more than 50% of all known enzymes need a metal ion to be active), and usually the reaction intermediates reside on the metal ion in the enzyme. Bioinorganic catalysis is defined as a branch of catalysis dealing with processes performed with the aid of metalloenzymes, modified enzymes, and synthetic metal-containing molecules resembling the active site of metalloproteins.

The aim of this book is to provide the interested reader with the most recent research results in bioinorganic catalysis and to suggest interesting new topics for interdisciplinary research dealing with metal ions, catalysis, and biochemical systems. A brief introduction in homogeneous and heterogeneous catalysis is provided for readers who are new in the field or who originate from the biological sciences. The book describes the current status of bioinorganic catalysis in the perspective of biochemistry/enzymology, inorganic chemistry, biophysics, organic chemistry, and homogeneous catalysis. Attention has been given to the structures of the catalytic sites at atomic resolution, the specificity of the reac-



tions, the kinetics of the processes, and the possible practical uses. Strategies to design and synthesize analogs of the enzymes are discussed in some detail. The final chapter discusses recent results and elements not treated in the body of the monograph.

The approach of this monograph is directed toward the molecular level, with special attention given to the direct environment of the metal ion at the active site. During the last decade, developments in crystallography, spectroscopy, and structure determination have led to many new insights in structure and activity of biocatalysts. Moreover, enzymologists are continuously isolating new catalytic systems from biological material. However, the interest in this type of work is not only fundamental. The possibility of using enzymes, and the chemical systems based on them, in catalytic processes is increasing every day, as will be seen from several contributions in the book.

It is felt that an updated comprehensive description of both the metalloenzymes and the active-site analogs appears to be highly relevant for many researchers in this area and the respective subareas, and, therefore, the present monograph will benefit many current and potential new workers in this field.

*Jan Reedijk
Elisabeth Bouwman*



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Contributors

Pierrette Battioni, Ph.D. University of Paris V, Paris, France

Elisabeth Bouwman, Ph.D. Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

Alison Butler, Ph.D. Professor, Department of Chemistry, University of California, Santa Barbara, California

Richard Cammack, Ph.D. Professor of Biochemistry, Division of Life Sciences, King's College London, London, England

Johannis A. Duine, Ph.D. Professor, Department of Microbiology and Enzymology, Kluyver Institute for Biotechnology, Delft University of Technology, Delft, The Netherlands

David J. Evans, Ph.D., F.R.S.C. Nitrogen Fixation Laboratory, John Innes Centre, Norwich, England

Sergiu M. Gorun, Ph.D. Associate Professor, Department of Chemistry, Brown University, Providence, Rhode Island

Wilfred R. Hagen, Ph.D. Professor, Laboratory for Biochemistry, Wageningen Agricultural University, Wageningen, and Department of Molecular Spectroscopy, University of Nijmegen, Nijmegen, The Netherlands



Richard A. Henderson, Ph.D. Nitrogen Fixation Laboratory, John Innes Centre, Norwich, England

Kenneth D. Karlin, Ph.D. Professor, Department of Chemistry, The Johns Hopkins University, Baltimore, Maryland

Eiichi Kimura, Ph.D. Professor, Department of Medicinal Chemistry, School of Medicine, Hiroshima University, Hiroshima, Japan

Tohru Koike, Ph.D. Associate Professor, Department of Medicinal Chemistry, School of Medicine, Hiroshima University, Hiroshima, Japan

Daniel Mansuy, Ph.D. Director of Research C.N.R.S., Laboratory of Chemistry and Biochemistry, University of Paris V, Paris, France

Luigi G. Marzilli, Ph.D. Dobbs Professor of Chemistry, Department of Chemistry, Emory University, Atlanta, Georgia

Kenichi Oyaizu, Ph.D. Lecturer, Advanced Research Institute for Science and Engineering, Department of Polymer Chemistry, Waseda University, Tokyo, Japan

Robert S. Pilato, Ph.D. Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland

Lawrence Que, Jr., Ph.D. Professor, Department of Chemistry and Center for Metals in Biocatalysis, University of Minnesota, Minneapolis, Minnesota

Jan Reedijk, Ph.D., F.R.S.C. Professor, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

Roger A. Sheldon, Ph.D., F.R.S.C. Professor of Organic Chemistry, Kluyver Institute for Biotechnology, Delft University of Technology, Delft, The Netherlands

Barry E. Smith, Ph.D. Professor, Nitrogen Fixation Laboratory, John Innes Centre, Norwich, England

Edward I. Stiefel, Ph.D. Scientific Advisor, Corporate Research, Exxon Research and Engineering Company, Annandale, New Jersey



Eishun Tsuchida, Ph.D., F.R.S.C. Advanced Research Institute for Science and Engineering, Department of Polymer Chemistry, Waseda University, Tokyo, Japan

Pieter van Vliet, Ph.D. Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, Leiden, The Netherlands

Jan Wikaira, Ph.D. Department of Chemistry, Brown University, Providence, Rhode Island

Kimihisa Yamamoto, Ph.D.* Advanced Research Institute for Science and Engineering, Department of Polymer Chemistry, Waseda University, Tokyo, Japan

Andreas D. Zuberbühler, Ph.D. Professor, Institute of Inorganic Chemistry, University of Basel, Basel, Switzerland

* *Current affiliation:* Keio University, Yokohama, Japan.





Abbreviations^{a,b}

AANH ₂	2-aminoadipic acid 1-amide
Ac	acetyl
acac	acetylacetonato
ACS	acetyl CoA synthase
Ade	adenine
Ado	adenosine
ADP	adenosine 5'-diphosphate
Ala	alanine (A) ^b
Arg	arginine (R) ^b
Asp	aspartic acid (D) ^b
ATP	adenosine 5'-triphosphate
BDE	bond dissociation enthalpies
bf	benzoylformate
BIPhMe	2,2'-Bis(1-methylimidazolyl)phenylmethoxymethane
BLM	bleomycine
BNP	bis(4-nitrophenyl)phosphate
bpea	bis(pyridylethyl)amine
bpqa	bis(2-pyridylmethyl)(2-quinolylmethyl)amine

^a Ligand abbreviations with the format H_nabc may lose one (or more) hydrons as H⁺ upon metal binding. Combined abbreviations such as Me₃tacn have not been included, and their meanings have to be derived from the subdivisions.

^b For a complete list of one-letter abbreviations of the amino acids, see C. Liébecq, Ed., IUPAC-IUBMB, Recommendations 1992 *Biochemical Nomenclature and Related Documents*, 2nd ed. (1992).



bpy	2,2'-bipyridine
bqpa	bis(2-quinolylmethyl)(2-pyridylmethyl)amine
BrPO	bromoperoxidase
CAPS	3-(cyclohexylamino)propanesulfonic acid
Cba	cobamide
Cbi	cobinamide
Cbl	cobalamin
CcO	cytochrome-c oxidase (EC 1.9.3.1)
CD	circular dichroism
CEPT	coupled electron proton transfer
CHES	2-(cyclohexylamino)ethanesulfonic acid
ClPO	chloroperoxidase
CoA	coenzyme A
CoB	coenzyme B, 7-thioheptanoyl-threonine-O-phosphate
CODH	carbon monoxide dehydrogenase
CoM	coenzyme M, 2-thioethane sulfonate
Cp	cyclopentadienyl
cyclam	1,4,8,11-tetraazacyclotetradecane
cyclen	1,4,7,10-tetraazacyclododecane
Cys	cysteine (C) ^b
DβH	dopamine β-hydroxylase; dopamine β-monooxygenase (EC 1.14.17.1)
Da	dalton; unit of molar mass
DD	propane-1,2-diol dehydratase (EC 4.2.1.28)
Δ9D	stearoyl acyl carrier protein Δ9-desaturase
ΔG°	standard Gibbs free energy change at pH = 7 (also called ΔGθ)
ΔH‡	activation enthalpy of transition state (earlier called ΔH*)
dmf	dimethylformamide
dmsO	dimethylsulfoxide; Me ₂ SO
DNA	deoxyribonucleic acid
DNP	diphenyl 4-nitrophenylphosphate
DOPA	3,4-dihydroxyphenylalanine (also dopa)
dppe	1,2-bis(diphenylphosphino)ethane
EAL	ethanolamine ammonia-lyase (EC 4.3.1.7)
EC	enzyme classification
ENDOR	electron-nuclear double resonance
E°'	redox potential in mV vs NHE
EPR	electron paramagnetic resonance
ES	enzyme-substrate complex
ESE	electron spin-echo spectroscopy
ESEEM	electron spin-echo envelope modulation
Et	ethyl



Abbreviations

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EXAFS	extended X-ray absorption fine structure
FAD	flavin-adenine dinucleotide
FeMoco	cofactor containing Fe and Mo in nitrogenases
FMN	flavin mononucleotide
FTIR	fourier transform infrared spectroscopy
FT-Raman	fourier transform-Raman
GDH	glucose 1-dehydrogenase (EC 1.1.1.47)
Glu	glutamic acid (E) ^b
Gly	glycine (G) ^b
Hbpg	<i>N,N</i> -bis(2pyridylmethyl)glycine
Hc	hemocyanin
HCM	5-methyltetrahydrofolate-L-homocysteine-S-methyltransferase (EC 2.1.1.13)
H ₂ dbc	3,5-di- <i>tert</i> -butylcatechol
Hdmbim	5,6-dimethylbenzimidazole
Hdmg	dimethylglyoximato(1-)
Hdmg ₂ pn	propylene-bridged bis(dimethylglyoximato)(1-)
H ₂ dpa	pyridine-2,6-dicarboxylic acid (also H ₂ dipic)
H ₄ edta	ethylenediaminetetraacetic acid
Hhptb	<i>N,N,N'</i> -tetrakis(2-benzimidazolylmethyl)-1,3-diamino-2-propanol
Hhptp	<i>N,N,N',N'</i> -tetrakis(2-pyridylmethyl)-1,3-diamino-2-propanol
Him	imidazole
His	histidine (H) ^b
H ₄ MPT	tetrahydromethanopterin
H ₂ pc	phthalocyaninic acid
Hpma	2-(2',5'-diazapentyl)-5-bromopyrimidine-6-carboxylic acid N-2-(4'-imidazolyl)ethyl amide
Hpz	pyrazole
Hr	hemerythrin
HRP	horseradish peroxidase (EC 1.11.1.7)
HSAB	Hard and Soft Acids and Bases
H ₂ salen	<i>N,N'</i> -ethylenebis(salicylideneamine); bis(salicylidene)ethylene-diamine
H ₂ salpn	<i>N,N'</i> -propylenebis(salicylideneamine); bis(salicylidene)propyl-enediamine
H ₂ tccat	3,4,5,6-tetrachlorocatechol
IPNS	isopenicillin N synthase (EC 6.3.2 group)
<i>i</i> -Pr	isopropyl
LMCT	ligand-to-metal charge transfer
Lys	lysine (K) ^b
MADH	methylamine dehydrogenase (EC 1.4.99.3)



mcd	2-chloro-5,5-dimethyl-1,3-dimedone
MCD	magnetic circular dichroism
MCR	methyl-CoM reductase
MDH	methanol dehydrogenase
Me	methyl
Met	methionine (M) ^b
MetH	methionine synthase
MFR	methanofuran
MMO	methane monooxygenase (EC 1.14.13.25)
MMOH	methane monooxygenase hydroxylase component
Moco	a cofactor containing molybdenum found in Mo enzymes other than nitrogenase
MRF	methyl reducing factor
MV	methylviologen
mw	M_r , relative molecular mass (molecular weight in dalton)
N.I.H.	1,2-H aromatic ring migration shift induced by certain metalloenzymes
N4py	[bis(2-pyridyl)methyl]bis(2-pyridylmethyl)amine
NA	4-nitrophenyl acetate
NADH	reduced nicotinamide-adenine dinucleotide
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
NHE	normal hydrogen electrode (sometimes called Standard Hydrogen Electrode, SHE)
NMR	nuclear magnetic resonance
NOE	nuclear Overhauser effect
OAc	acetate
OBz	benzoate
OEC	oxygen-evolving complex
OEP	octaethylporphyrin
PAH	phenylalanine 4-hydroxylase; phenylalanine 4-monooxygenase (EC 1.14.16.1, also abbreviated as PheH)
PAM	peptidylglycine (α -amidating) monooxygenase (EC 1.14.17.3)
PCD	protocatechuate 3,4-dioxygenase (EC 1.13.11.3)
4,5-PCD	protocatechuate 4,5-dioxygenase (EC 1.13.11.8)
PGG ₂	prostaglandin G ₂
PGH ₂	prostaglandin H ₂
PGHS	prostaglandin H synthetase (EC 1.14.99.1)
Phe	phenylalanine (F) ^b
phen	<i>o</i> -phenanthroline
PPS	poly(<i>p</i> -phenylenesulfide)
PQQ	pyrroloquinoline quinone (2,7,9-tricarboxy-1 <i>H</i> -pyrrolo[2,3- <i>f</i>]quinoline-4,5-dione)



Abbreviations

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PQQH ₂	dihydro-PQQ
Pro	proline (P) ^b
PSII	photosystem II
py	pyridine
QH-ADH	quinohemoprotein alcohol dehydrogenase
RDPR	ribonucleoside-diphosphate reductase (EC 1.17.4.1)
RNR	ribonucleotide reductase (EC 1.17.4.1)
RR	resonance raman
RTPR	ribonucleoside-triphosphate reductase (EC 1.17.4.2)
SCE	saturated calomel electrode
SCF	self-consistent field
Ser	serine (S) ^b
SOD	superoxide dismutase
tacn	1,4,7-triazacyclononane
teen	<i>N,N,N',N'</i> -tetraethylethylenediamine
terpy	2,2',6',2''-terpyridine
thf	tetrahydrofuran
tmpa	tris(2-pyridylmethyl)amine
TNP	tris(4-nitrophenyl)phosphate
Tp ^{3,5-X2}	hydrotris(3,5-dialkylpyrazolyl)borate monoanion
tpen	<i>N,N,N',N'</i> -tetrakis(2-pyridylmethyl)-1,2-diaminoethane
TPP	<i>meso</i> -tetraphenylporphyrin
TPQ	topaquinone (6-hydroxy-phenylalanine-3,4-dione)
triphos	tris(diphenylphosphinomethyl)methane
Trp	tryptophan (W) ^b
TTQ	tryptophyl tryptophan quinone (4-(2'-tryptophyl)-tryptophan-6,7-dione)
Tyr	tyrosine (Y) ^b
XANES	X-ray absorption near edge structure
XAS	X-ray absorption spectroscopy

ABBREVIATIONS FOR BACTERIAL GENUS NAMES

A., *Azotobacter*; *Al.*, *Alcaligenes*; *As.*, *Ascomyllum*; *Asp.*, *Aspergillus*; *B.*, *Brevibacterium*; *C.*, *Chromatium*; *Ce.*, *Cerarium*; *Cep.*, *Cephalosporium*; *Ch.*, *Chlorella*; *Cl.*, *Clostridium*; *Co.*, *Corallina*; *D.*, *Desulfovibrio*; *Dm.*, *Desulfomicrobium*; *E.*, *Escherichia*; *F.*, *Fucus*; *K.*, *Klebsiella*; *L.*, *Laminaria*; *La.*, *Lactobacillus*; *M.*, *Methanobacterium*; *Ma.*, *Macrocystis*; *Mc.*, *Methanococcus*; *Ms.*, *Methanosarcina*; *Mx.*, *Methanoxthrix*; *N.*, *Neurospora*; *P.*, *Pyrococcus*; *Pa.*, *Panulirus*; *R.*, *Rhodospirillum*; *Rb.*, *Rhodobacter*; *Sp.*, *sphaeroides*; *T.*, *Thiocapsa*; *Th.*, *Thermoleophilum*; *Thr.*, *Thermus*; *W.*, *Wolinella*; *X.*, *Xanthoria*





1

Introduction to Bioinorganic Chemistry

Elisabeth Bouwman and Jan Reedijk
Leiden University, Leiden, The Netherlands

I. HISTORY AND SCOPE

Metal ions are known to play an essential role in living systems, both in growth and in metabolism. In fact the role of iron as an essential metal has been known since the eighteenth century, whereas the importance of other elements, such as cobalt, copper, manganese, and zinc, has only been known for more than half a century. It has also been known for a long time that an excess of these elements can be very dangerous, and, as a matter of fact, a narrow concentration window exists for most of the so-called trace elements. Even though many elements of the periodic table are nowadays known and accepted to be essential or beneficial for life on earth, the molecular role of these elements is only beginning to be understood [1–4].

Many other elements of the periodic table, although nonessential for biological systems, do have an influence on the quality of life, either as toxic pollutants or as drugs to treat certain diseases. Examples of these are well known from the general literature, but again little is understood about the details of their mode of action on the molecular level. The major challenges of modern bioinorganic chemistry are to understand the molecular basis of all the possible interactions between biological tissues and metal ions and to apply this knowledge in medicine, biology, environmental sciences, catalysis, and technology.

This monograph focuses on the catalytic aspects of the essential metals, both in their natural environments and in synthetic, biomimetic catalytic systems. The first three chapters comprise a general introduction to the later chapters. A



very brief review of homogeneous catalysis from a bioinorganic perspective is given in Chapter 2, whereas a detailed comparison between enzymatic and heterogeneous catalysis is presented in Chapter 3. From Chapter 4 onward the monograph covers both enzymatic catalysis and catalysis and substrate binding by so-called biomimetic compounds.

In this introductory chapter, some general aspects of bioinorganic chemistry will be dealt with. In Chapter 2 a section of the periodic table of elements is presented, indicating the transition metals that are catalytically active *in vivo*. Table 1 lists several elements that are essential to life, together with some statistical information and a few comments about their biological role. The compilation is limited and restricted to some of the most important transition elements, the nonmetal Se, and the alkaline earth metals Ca and Mg.

In a relatively short period, bioinorganic chemistry has grown to a large field of science, and it is already divided into several subfields. Scientists from a variety of disciplines contribute to this research. In all cases the role of metals, metal ions, and metal compounds in relation to living systems is a subject of common interest. Classification of the various subfields is not easy because of overlapping research interests. Nevertheless, the research activities can be grouped as follows:

- Study of the metal coordination environment in metalloproteins, nucleic acids, carbohydrates, membranes
- Study of the mechanism of reactions occurring at a metal center in an enzyme
- Study of synthetic analog for the active sites in metalloproteins (design, synthesis, structure, spectroscopy, and applications like metal sequestering from waste water and deposits, catalytic reactions)
- Design and study of metal-containing drugs to cure or prevent diseases (synthesis, mechanism of action)
- Removal of metal ions and metal compounds from living systems (detoxification)
- Study of the process of biomineralization

In all these subfields both the metal and the surrounding neighbors (called ligands) are of importance for the structure, the stability, and the processes that are regulated and catalyzed by the metal species.

The research field that studies metal ions and their interactions with ligands is usually called coordination chemistry. Such metal–ligand interactions in biological systems play a key role in almost every event that takes place during biological processes, both natural and human-induced. Metals are kept in the right location through coordination bonds with the ligands. Ligands are primarily oriented, polarized, activated, etc., through specific interaction with certain metal ions. Excesses of (toxic) metals can be removed by chelation with natural or



Table 1 Some Important Elements in Biology: Occurrence, Concentration of Element, and Biological Role

Element name	Concentrations in		Daily required (mg)	Some comments about role in biology
	Human (mg)	Sea water (ppm)		
Iron	4500	5×10^{-2}	10	Many enzymes; respiratory proteins
Copper	100	3×10^{-1}	2	Many enzymes; dioxygen transport
Zinc	2000	4×10^{-1}	12	Hydrolytic enzymes; nucleic acid synthesis
Manganese	20	2×10^{-2}	3	Enzyme activation, photosynthesis
Molybdenum	5	10	0.2	Many redox enzymes; nitrogenase (plants)
Cobalt	1	1×10^{-3}	0.3	Vitamin B-12
Selenium	0.5	2×10^{-1}	0.1	Glutathione peroxidase; antioxidant
Magnesium	4×10^4	10^6	350	Photosynthesis; nucleic acid processes
Calcium	10^6	4×10^5	800	Bones, teeth; muscle activation
Nickel	<0.1	5×10^{-1}	<0.1	Role in hydrogenases; urease
Vanadium	<0.1	2	<0.1	Role in bromoperoxidases
Tungsten	<0.1	1×10^{-4}	<0.1	Role in dehydrogenases

specifically designed chelating ligands. Certain ligands may interact with metals in living tissue, thereby blocking coordination sites and hampering normal reactions. Toxic metals may substitute nontoxic metal ions in metalloproteins or may bind to ligands in vivo (protein side groups, nucleic acids), thereby changing the reactivities or securing certain conformations. Metal compounds may be added as drugs with the specific purpose of introducing the metal or the ligand, or both, to certain positions in the living system (metal-containing drugs; metalloradio-pharmaceuticals). For a better understanding of these processes, some classification of metals, ligands, and their possible interactions is required.

II. COORDINATION CHEMISTRY IN BIOLOGY

II.A. General Coordination Aspects

The coordination behavior of metals can be categorized into covalently binding metal ions and ionic binding metal ions. The underlying principles have been known for years as the so-called hard and soft acids and bases (HSAB) theory [5,6]. For a general understanding it is sufficient to know that ions with small ionic radii and/or high oxidation states like Ca, Mg, Na, K, and Mn belong to the ionic (hard, class A) group, whereas metal ions with larger ionic radii, which are more readily polarizable, like Pt, Hg, Cd, and Pb, belong to the more covalent group (soft, class B). Transition-metal ions like Zn, Cu, and Co are considered as intermediates between A and B. Ligands are usually arranged according to their donor sites and polarizability: ionic (hard) ones are those with an oxygen-donor group (like carboxylate, alcohol); covalent ones (soft) are those with sulfur or phosphorus donor atoms (thioethers, thiolates, phosphanes). Nitrogen-donor ligands such as imidazole are considered as intermediates. The basic rule is that ionic metals preferentially bind with ionic ligands and that covalent metal ions preferentially bind with covalent ligands.

II.B. Oxidation States and Geometry

The nature of the metal ion that is used by an enzyme may have several origins. Of course, the first reason for the selection of a certain metal is the availability of the various metals at the time and place of evolution of the particular enzyme or protein. When this restriction has been met, the choice of metal is determined by the role that it has to play in the activity of the enzyme; if it is just needed to act as a Lewis acid to bind and activate the substrate, then a metal with limited redox activity such as zinc may be used. If, on the other hand, apart from substrate binding, redox activity is needed to perform the catalytic function of the enzyme, then other metals, which have different oxidation states readily available, are needed.



The specific function of the enzyme results in certain requirements for the oxidation states, the ligands, and the coordination geometries of the metal ion in the active site. In oxidation catalysis, usually the harder, more ionic ligands are used in order to stabilize the higher oxidation states of the metal ion, thereby tuning the redox potential of the enzyme into the appropriate region. The specific geometry in which the coordinating ligands are arranged around the metal ion is of meaningful importance for the catalytic activity of the metal ion. In low-molecular-weight coordination compounds usually there are few or no constraints dictating the geometry, and therefore the arrangement of the ligands around the metal ions in these complexes usually relies on the preference of the metal ion. This preference, however, is dependent on the kind of donor groups and the oxidation state of the metal ion: e.g., copper(II) is generally found in a square planar, or a tetragonal 5- or 6-coordinate geometry, whereas copper(I) is often found in a tetrahedral geometry. This flexibility in inorganic coordination compounds is in contrast with the irregular geometries that are found in the active sites of the enzymes; such sites are enforced by the rigidity of the protein backbones. These enforced geometries also have an influence on the activity of the enzyme. For example, the irregular geometry around the copper ion in blue copper enzymes facilitates the redox switches of the copper ion, which otherwise would require a significant change in geometry.

For the bioinorganic chemists who are trying to mimic the activity of metalloenzymes, these irregular geometries, which are probably crucial for the specific activity of the enzyme, are the most difficult to model in low-molecular-weight coordination complexes.

II.C. Ligands That Bind to Metals in Biological Systems

Ligands that are frequently found in biological systems are as follows:

1. Protein side groups, such as thiolate, imidazole, carboxylates; some examples are given in Figure 1; in many cases dehydronation (i.e., loss of a hydron, H^+) takes place upon binding to a metal.
2. Prosthetic groups, such as tetrapyrroles (Figure 1) and cofactors such as flavin-adenine dinucleotide (FAD) and pyrroloquinoline quinone (PQQ) (see Chapter 16).
3. Small ions, such as OH^- , $O_2^{\cdot-}$, S^{2-} , $OOH^{\cdot-}$, CO_3^{2-} , and molecules (O_2 , H_2O).
4. Bases of nucleic acids, such as guanine.
5. Phosphates and diol groups in nucleic acids.

Table 2 gives an overview of the ligands with examples of their biological presence (ligand abbreviations and other abbreviations used throughout this monograph are given in the Abbreviations section).



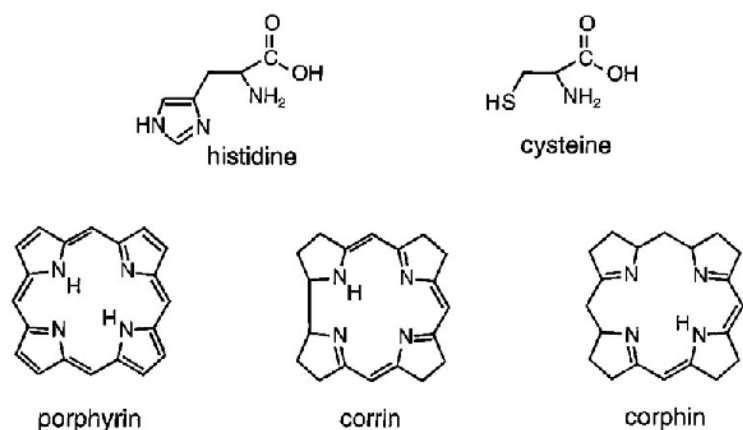


Figure 1 Schematic structure of some amino acids with coordination side chains, and prosthetic groups found in metalloproteins.

In addition to the natural ligands, externally added molecules may act as a ligand to the metal ions. These (usually) nonbiological ligands in general can be classified into two main groups, namely, ligands in drugs and toxic ligands.

Ligands in drugs usually have the purpose of chelating *in vivo* a metal that is present in an excess (i.e., toxic) amount. Selectivity of this ligand toward the toxic metal over the other essential metals is of great importance. Successful ligands have been known for a long time (e.g., D-penicillamine, desferrioxamine, $\text{Na}_2\text{H}_2\text{edta}$, British Anti-Lewisite). In a few cases essential elements need to be removed, such as molybdenum by diethylthiuramdisulfide (dtds). In other situations the ligands present in a drug play an indirect role, such as keeping a metal soluble or stable or influencing a metal that in turn is used only to transport a certain biologically active ligand.

Toxic ligands (CO , NO , CN^- , F^- , H_2S) can bind to metals in the active site of enzymes, thus inhibiting substrate binding, or coordinate to more readily available metal ions, thereby blocking the proper biological function of the metal. However, recent research suggests that such molecules are essential to several hydrogenases (see Chapters 8 and 9).

II.D. Ligands in Synthetic Analogues and Catalysis

Ligands used in synthetic analogues and catalysts usually resemble the naturally occurring ones. Examples of donor groups present in such ligands are thiols, imidazoles, and carboxylates. They are commonly used with the aim of better understanding the natural system and/or making use of certain biological princi-



Table 2 Bioligands and Their Presence in Natural Systems

Group	Metal	Found in
$=\text{O}$	Fe, V	P-450 enzymes
$-\text{OH}$	Fe, Zn	Carbonic anhydrase
H_2O	Fe, Zn, Ca	Many proteins; additional ligand
O_2	Fe, Cu	Hemoglobin, hemocyanin, hemerythrin
$\text{O}_2^{\cdot-}$	Cu, Fe	Superoxide dismutase
$-\text{OOH}^{\cdot-}$	Fe	Hemerythrin
$-\text{NHCO}_2^-$	Ni	Urease
$-\text{F}^-$	Fe	Inhibitor of catalase
$-\text{Cl}^-$	Mn	Mn cluster in photosynthesis
$-\text{S}^{2-}$	Fe, Mo	Ferredoxin; nitrogenase
$-\text{SR}^-$ (cysteine)	Fe, Cu	Ferredoxin, plastocyanin, P-450, azurin
Me-S-R (methionine)	Cu, Fe	Plastocyanin, cytochromes, azurins
CO	Fe	Toxic for myoglobin; cytochrome oxidase
CN^-	Co, Fe	Vitamin B-12; hydrogenase
$(\text{CH}_2-\text{R})^-$	Co	Vitamin B-12
Imidazole	Cu, Zn, Fe, Mn, V	Plastocyanin, insulin, etc.
Benzimidazole	Co	Vitamin B-12
Tyrosine	Fe	Oxidases
Glutamine (and Asp)	Fe	Hemerythrin
OPO_2R	Ca, Mg	Nucleic acids; adenosine triphosphate (ATP)
NO_3^- , SO_3^{2-}	Mo	Reductases
$(\text{N}<)^-$ (peptide)	Cu	Albumin
Tetrapyrroles	Fe, Co, Ni, Mg	Prosthetic group; hemoglobin
Pterin	Mo, W	Cofactor in oxidases

ples (i.e., in metal removal or transport). These donor groups are usually bound together in a chelating form; i.e., two or more ligand donor groups are linked in such a way that all of them can simultaneously bind to the metal, resulting in a very stable species.

Substrates and reagents in catalytic reactions can vary from simple species like O_2 , H_2O , NH_3 , H_2 , N_2 , CO_2 , and NO_2^- to larger molecules such as ethanol, alkenes, and phenols. Many of these substrate molecules will be dealt with in detail in later chapters.

III. REACTIVITY OF METALS AND LIGANDS IN VIVO

III.A. General Aspects

Reactions involving metal ions and ligands that occur in vivo are part of a complex system of equilibria, transport, and storage. Kinetics and thermodynamics



play key roles in these reactions. In other words, the simple presence of a metal and a ligand does not necessarily yield the desired product. For example, the reaction can be far too slow, such as introduction of a metal into a tetrapyrrole system, or the number of competing reactions with other metal ions or ligands can lead to different products. Reactions dealing with Fe, Cu, and Co and the kinetics of these reactions are beginning to be somewhat understood. Reactions of other metal ions are far less understood. An example of a biomedical reaction that is beginning to be understood is the mechanism of action of platinum antitumor compounds. Starting with drug administration and transport through the body, the drug enters the cell, is hydrolyzed, and binds mainly to deoxyribonucleic acid (DNA). However, the several side reactions that occur are still far from being understood.

III.B. Structural Role of the Metal Ions

The role of a metal ion in a biological system can be limited to a structural function, such as holding certain biomolecules (or parts of them) together in a more or less fixed structure. Well-known examples are calcium in thermolysin (a protein with Zn at the active site) and in solid structures such as bone and teeth, and zinc in superoxide dismutase and alcohol dehydrogenase (noncatalytic site). Related to this structural role is the magnetite (Fe_3O_4) structure found in bacteria, honeybees, and probably also pigeons and higher organisms. It appears that the single crystals of Fe_3O_4 are used for orientation, probably in combination with other objects, using the earth magnetic field.

III.C. Transport and Storage of Metal Ions

In living systems that use metal ions in several places, transport of metal ions is an important process, for which efficient systems are in operation. Well-known examples are transferrin for iron transport in humans, albumin for copper transport, and ferritin for iron storage. In addition to these natural transporting proteins, nature makes use of other systems to remove excess of toxic metal ions. The metallothionines, for example, take care of transport and excretion of metal ions such as cadmium, and excesses of zinc and copper. A complete crystal structure of a metallothionine has been published. The protein structure contains seven metal ions coordinated by 20 cysteine residues in two regions of the protein.

III.D. Catalytic Role of Metal Ions

The catalytic role of the enzyme is influenced by the metal ion in the active site of the proteins and the binding mode of the ligands to this metal ion, and the study of this influence is the main theme of the present monograph. The catalytic



role can be in electron transfer only, in a complicated redox reaction, or in a relatively simple acid–base reaction. The presence of the metal at the particular site usually results in activation of the substrates by the metal and the surrounding ligands. Examples of enzymes catalyzing each of these types of reactions are cytochrome *c* oxidase, laccase, carbonic anhydrase, and alcohol dehydrogenase. The recent results from the studies of these enzymes are fascinating, and details are to be found in later chapters.

Enzymes have been classified for many years in six major groups [7], depending on the reactions that are catalyzed, namely:

1. Oxidoreductases (catalyzing oxidation–reduction reactions); in this category one usually distinguishes between dehydrogenases (all used dioxygen or hydrogen peroxide is converted into water), (mono)oxy-
genase (one of the oxygens is introduced into the substrate), and dioxy-
genases (both oxygens are introduced into the substrate)
2. Transferases (transferring a group from a donor molecule to an ac-
ceptor molecule)
3. Hydrolases (hydrolytic cleaving of C—O or C—N bonds)
4. Lyases (cleaving C—N, C—O, or C—C bonds by elimination)
5. Isomerases (causing geometric or structural change within a molecule)
6. Ligases (joining two molecules; often coupled with pyrophosphate hy-
drolysis)

Usually the enzymes are given an Enzyme Classification (EC) number, consisting of three or four figures separated by dots, to indicate subgroup and subsubgroup (e.g., bovine superoxide dismutase [SOD], EC 1.15.1.1). The enzymes discussed in the present monograph are given with their EC numbers in the Abbreviations section.

This classification is perfectly valid for the metalloenzymes, even though most of them fall in group 1, such as the several dioxygenases, (mono)oxy-
genases, and oxidases (dehydrogenase). Many examples will be discussed in the later chapters of the book. The enzymes known and classified [7] so far very often involve a metal, either directly at the active center or indirectly at another place, for instance, in the electron transfer process [8].

III.E. Other Functions of Metal Ions in Vivo

This monograph primarily focuses on redox reactions, but a chapter on the hydrolytic role of zinc enzymes has been added in this second edition (Chapter 4). The reader is referred to other overviews for further details concerning other enzymatic functions. An interesting example is the role in gene expression as a result of zinc-induced protein folding. This topic has been receiving increasing attention during the last few years, when high-resolution nuclear magnetic reso-



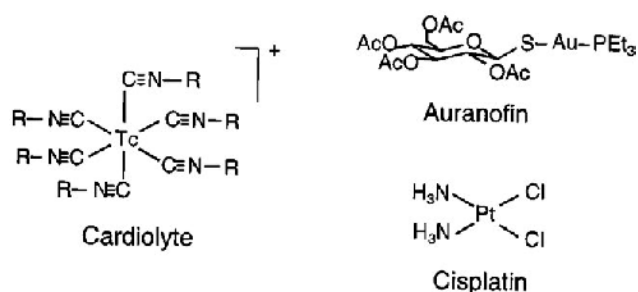


Figure 2 Structures of some important metal-containing drugs (R = organic fragment).

nance (NMR) structures and even a few x-ray structures of the so-called zinc fingers and their adducts with pieces of DNA became available.

Apart from playing a role in structure, transport, or catalysis, metal ions from outside may have deleterious effects as toxic metals (see earlier discussion) or play an important role as metal-containing drugs. Examples of the last category are (1) gold-thiolate compounds in the treatment of arthritis; (2) platinum-amine compounds as antitumor drugs; (3) technetium coordination compounds in radio-pharmaceutical imaging. Examples of a few such compounds are schematically presented in Figure 2.

IV. APPLICATIONS OF BIOINORGANIC CHEMISTRY

The exciting results concerning the role of metal ions in enzymatic systems, which have been unraveled during the last decades, have raised the question, How can we make use of what nature is teaching us, through bioinorganic systems? What can we learn about electron transfer, transport of dioxygen, combustion and consumption of dioxygen, dinitrogen fixation, conversion of solar energy, communication between cells with electric signals, and ways to apply this knowledge? Before application is possible, understanding is necessary. For better understanding, mimicking of biological systems is important; after that reproduction of the building blocks and reaction steps should come within reach. An important question will be, How simple or how complicated will our biomimetic models have to be in order to accomplish in vitro what nature does in vivo? These studies are most likely to be stimulated from several directions; most probably, catalysis and energy research will be strong stimulators (small molecule activation and photoconversion). In this respect, recently an exciting report of a mimic for the reductive acetyl coenzyme A pathway has been published [9]. A



simple aqueous slurry of nickel and iron sulfides was shown to be able to convert a mixture of carbon monoxide and methanethiol to methyl thioacetate, which then hydrolyzes to acetic acid.

Many other areas will stimulate bioinorganic research; one thinks of medical research, where better chelating agents for selective removal of excesses of toxic metal ions will remain of great importance; biomineralization processes (surface coordination); improved diagnostic reagents (monoclonal antibodies with an attached metal); environmental cleaning processes (metallothioninelike chelating ligands); and mutation research (role of certain metal ions in mutation and repair mechanisms).

For a more general set of references, with many excellent review papers on bioinorganic chemistry, the reader is referred to the series of books edited by Sigel [10], Lippard [11], and Sykes [12]. A recent overview of "bioinorganic enzymology" describes the current knowledge of the biochemistry and structures and mechanisms of (metallo)enzymes [13].

It goes without saying that answers to the important questions raised earlier will develop during the next decade. It is hoped that the present monograph will provide a significant contribution to better understanding and will help lead to novel applications of bioinorganic chemistry.

V. NOMENCLATURE AND TERMINOLOGY

A book devoted to the interdisciplinary field of bioinorganic catalysis is directed to readers in the fields of biochemistry, (bio)inorganic chemistry, and catalysis. The terminology used throughout is that of bioinorganic chemistry. Readers who are not familiar with this jargon are referred to the "glossary of terms used in bioinorganic chemistry" [14]. Some specific remarks concerning the terminology used in this book are given here.

We have chosen to use most of the terminology of reference [14], like cytochrome P-450 and vitamin B-12. However, in Chapter 13 we have used B_{12} throughout to reach consistency with most authors in this field.

The word *hydron* is used as a general name for the H^+ ion, where it is not desired to distinguish among the isotopes. If one wishes to discriminate among the isotopes, the words *proton*, *deuteron*, and *triton* are used for $^1H^+$, $^2H^+$, and $^3H^+$, respectively. The editors have attempted to use this terminology throughout this volume, as in the first edition. In Chapter 6 with special terminology for coupled electron proton transfer, proton terminology has been retained where required.

The abbreviations that are used throughout are collected in a table at the beginning of this book. It should be noted that many of these are not generally accepted, although those recommended by IUPAC and IUB and relevant to the



book are included. Following IUPAC guidelines, lowercase abbreviations have been used for most ligands.

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2

Introduction to Homogeneous Catalysis

Roger A. Sheldon

Delft University of Technology, Delft, The Netherlands

I. INTRODUCTION

Bioinorganic chemistry, insofar as it involves catalytic transformations, is largely the chemistry of metalloenzymes (i.e., metalloproteins). Although the complex three-dimensional structure of the protein ligands obviously plays a vital role in determining the activity and stability of metalloenzymes, at the heart of the matter their reactions involve coordination catalysis by metal ions. Thus, at the molecular level we are dealing with homogeneous catalysis in aqueous media.

However, as Dewar has pointed out [1], since a proper substrate of an enzyme fits tightly into its active site, this precludes the presence of water molecules in the active site, except when the water takes part in the reaction. Consequently, any subsequent reaction takes place in the absence of solvent and may be compared with bimolecular reactions in the gas phase. The specificity and high rates of enzymatic processes are readily understood on the basis of this analogy.

In order to gain insights into the molecular mechanisms of metalloenzyme catalysis it is necessary to have an understanding of the fundamental processes involved in catalysis by simple metal salts and their coordination complexes.

II. METAL IONS IN BIOLOGICAL CATALYSIS

The metal ions of major significance for biological catalysis [2] are shown in Figure 1. The majority are first-row transition elements. It is, of course, no mere



Sc	Ti	V	Cr	Mn	Fe	Co	Ni	Cu	Zn
Y	Hf	Nb	Mo	Tc	Ru	Rh	Pd	Ag	Cd
La	Zr	Ta	W	Re	Os	Ir	Pt	Au	Hg

Figure 1 Metal ions at active sites in biological catalysis.

coincidence that it is precisely these metals that are readily available in nature and bind strongly to biological ligands, such as amino acid residues of proteins.

This contrasts with the man-made chemistry of the laboratory and the industrial plant, which often employs the more reactive, but less readily accessible, second- and third-row transition elements. For example, nature chose nickel for the active site of many hydrogenases. Catalytic hydrogenations in the laboratory, on the other hand, are usually performed with palladium or platinum on charcoal.

Similarly, many industrially important homogeneous catalytic processes involve the activation of the small molecules O_2 , CO , and H_2 . Nature, in contrast, generally employs those small molecules, CO_2 , N_2 , and O_2 , that are abundantly available in the oxidizing atmosphere of the earth. However, one should be careful about sweeping generalizations. According to a recent report [3] cell extracts of *Desulfovibrio desulfuricans* are able to catalyze the formation of a mixture of hydrocarbons and oxygen-containing compounds from CO and H_2 in aqueous solution at ambient temperature. Interestingly, the major hydrocarbon products were C_{14} – C_{18} alkanes, which form the basis of high-quality diesel fuel. Moreover, it is becoming increasingly apparent that the formation of metal–carbon bonds in biological processes is more widespread than heretofore imagined [4]. For example, a key step in the synthesis of acetyl coenzyme A from carbon monoxide, mediated by anaerobic bacteria, was shown to involve the formation of a methylnickel intermediate in a bimetallic mechanism [5].

III. BIOLOGICAL LIGANDS

The dichotomy encountered in the choice of metals for biological catalysis versus that in man-made homogeneous catalysis also applies to the choice of ligands. The man-made homogeneous catalysts involved in industrial processes are often triaryl(alkyl)phosphane complexes of noble metals (e.g., $(Ph_3P)_3RhCl$). Such complexes are not thermodynamically stable under the oxidizing conditions where most biological catalysts operate. Hence, the latter are usually bound by



ligands containing nitrogen, oxygen, and sulfur as the coordinating groups. For example, certain amino acid residues in proteins, notably histidine, cysteine, and aspartic acid, are particularly important ligands [6]. In addition many metalloenzymes contain macrocyclic tetrapyrrole ligands as a prosthetic group (some details of biological ligands were presented in Chapter 1, Figure 1).

The nature of these ligands plays a crucial role in determining the specificity and activity of metalloenzymes. The ligand may control the coordination number, spin state, and redox potential of the metal ion and the precise coordination geometry of the complex. Moreover the superstructure of a protein ligand can impose an unusual (high-energy) geometry at a metal site, thus enhancing its reactivity. In bioinorganic chemistry this is referred to as the entatic state [1,6], and it is generally believed to play a major role in determining the activity of, for example, redox metalloenzymes, by modifying the redox potential of the central metal ion.

IV. COORDINATION CATALYSIS VERSUS ORGANOMETALLIC CATALYSIS

Certain properties of transition metal complexes are common to both biological and man-made homogeneous catalysis. For example, an important property of transition metal complexes is their ability to coordinate groups in a specific array (template) that is conducive to high stereo- and/or regioselectivity. This is illustrated in the manganese-catalyzed decarboxylation of dimethyloxaloacetic acid (Figure 2). In this case, the formation of a chelate complex with manganese (II) is essential in promoting the regioselective decarboxylation reaction.

Coordination can also lead to activation of the substrate molecule via cleavage (e.g., H_2 , O_2) or via redistribution of electron density (e.g., CO, olefin, RCN).

Catalysis by transition metal complexes can be further divided into two

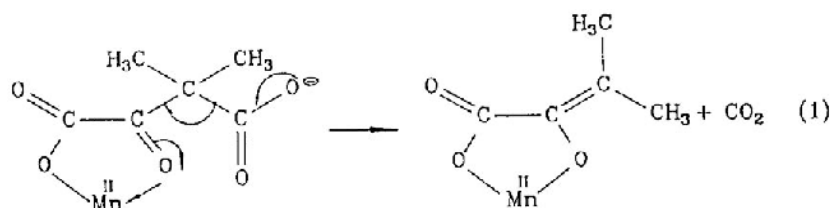


Figure 2 Mechanistic representation of the manganese-catalyzed decarboxylation of dimethyloxaloacetic acid.



types: coordination catalysis and organometallic catalysis. Their major characteristics are outlined below.

Coordination Catalysis	Organometallic Catalysis
Hard metal center	Soft metal center
Strong σ donors as ligands (e.g., RNH_2 , RCO_2^-)	Weak σ and π donors and acceptors (soft ligands) (e.g., R_3P , CO , alkyl, hydride)
Water-soluble (e.g., $\text{Co}(\text{OAc})_2$)	Soluble in organic solvents (e.g., $\text{HRh}(\text{CO})(\text{R}_3\text{P})_2$)

The majority of biocatalytic processes fall into the category of coordination catalysis, although there are a few that involve organometallic catalysis e.g., reactions mediated by nickel-dependent (see earlier discussion and Chapter 9) and vitamin-B-12-dependent enzymes (see Chapter 13).

V. THE FUNCTION OF THE METAL

In addition to providing a template for reaction, the metal can activate the substrate via the transfer of electrons. In this context three different types of fundamental interaction can be identified: electrophilic, nucleophilic, and homolytic.

V.A. Electrophilic Catalysis



In electrophilic catalysis, the metal ion acts as a Lewis acid. An example from organic chemistry is the formation of an acylium ion from aluminum chloride and an acid chloride in Friedel–Crafts acylation reactions (Figure 2). In this case substrate activation results in cleavage of the C—Cl bond. In most cases, however, substrate activation by Lewis acids involves electron redistribution without bond breaking (Figure 3).

Thus, many metal ions catalyze the hydrolysis of esters [7,8], amides [9], and nitriles [10] via electrophilic activation of the $\text{C}=\text{O}$ or $\text{C}\equiv\text{N}$ group. This type of catalysis is characteristic of coordination complexes and is very common in metalloenzyme-mediated processes. Zinc(II), for example, is a key structural component of more than 300 enzymes, in which its primary function is to act as a Lewis acid (see Chapter 4). The mechanism of action of zinc proteases, e.g., thermolysin, involves electrophilic activation of an amide carbonyl group by coordination to zinc(II) in the active site (Figure 4).

In metal ion-catalyzed reactions, peptides have the advantage over simple



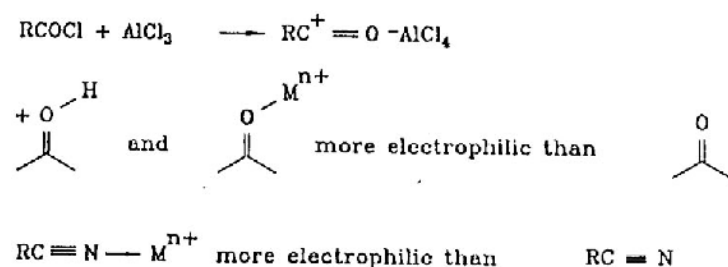


Figure 3 Substrate activation in electrophilic catalysis.

amides that they have more sites that can bind to the metal. Examples of both peptide formation [7] and peptide hydrolysis [8] catalyzed by simple metal complexes are known.

V.B. Nucleophilic Catalysis



In nucleophilic catalysis the metal ion is generally in a low oxidation state, and this type is common in organometallic catalysis, for example, in many processes involving an oxidative addition step (Eq. 3).

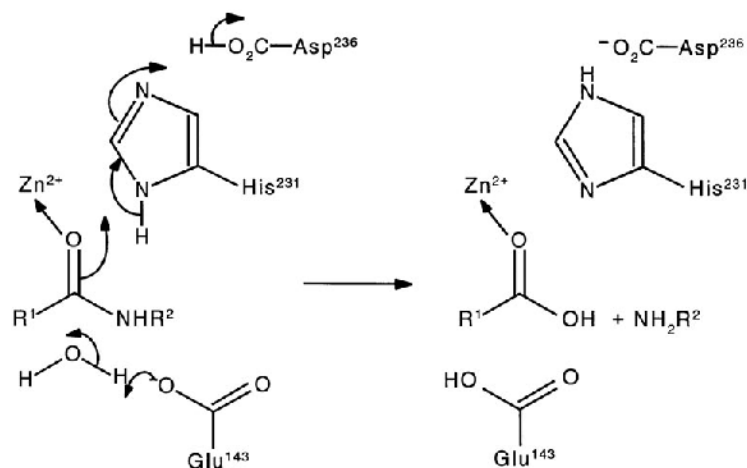


Figure 4 Electrophilic catalysis by Zn^{II} in thermolysin.

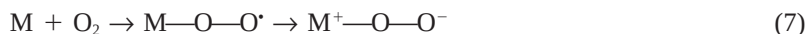
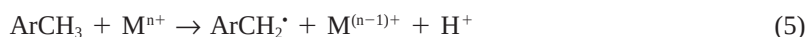


Although rather rare in metalloenzyme chemistry, nucleophilic catalysis can be involved in reactions mediated by metalloporphyrin and metallocorrin complexes (e.g., vitamin B-12-dependent enzymes).

V.C. Homolytic Catalysis



Homolytic catalysis is observed with both organometallic and coordination complexes. It is involved in a wide variety of metal-mediated transformations, often in competition with electrophilic or nucleophilic catalysis [11]. For example, many metal-catalyzed oxidations involve substrate activation by homolytic catalysis (Eq. 5) [12]. Similarly, oxidative additions (Eq. 6) and dioxygen activation (Eq. 7) can proceed via two-step homolytic mechanisms.

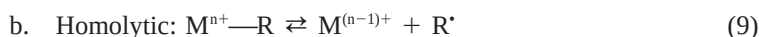


Furthermore, it should be noted that the same metal can often play different roles in different enzymes. For example, nickel(II) displays electrophilic catalysis in urease and redox catalysis in hydrogenase.

VI. BASIC STEPS IN TRANSITION METAL CATALYSIS

A *conditio sine qua non* for catalytic activity (with the exception of outer sphere electron transfer reactions) is that the species present in solution should be *coordinatively unsaturated*; it must possess a vacant coordination site for attachment of the substrate to the metal center. Hence, the first steps in many catalytic processes are ligand dissociation and ligand (substrate) coordination (Equations 8–14).

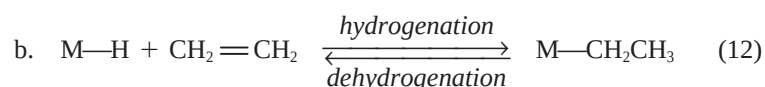
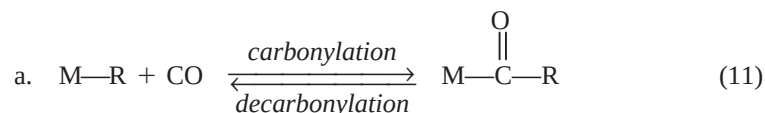
1. Ligand dissociation and coordination



2. Oxidative addition and reductive elimination



3. Insertion and deletion



4. Electron transfer

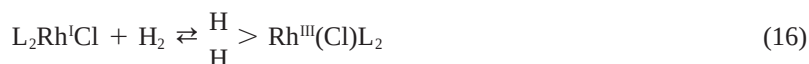


Ligand dissociation can involve either heterolytic or homolytic processes. Most biological processes involve the former, although examples of the latter are known. For example, adenosylcobalamin (vitamin B-12 coenzyme), in association with an appropriate apoenzyme, catalyzes a variety of rearrangements via a 1,2-hydride shift (see Chapter 13). The first step in these reactions is thought to be homolytic dissociation of a Co^{III} —alkyl bond as shown:



where L_5 = vitamin B-12 nucleus; Ad = adenosyl. Ligand dissociation is followed by substrate coordination and activation. This can involve simple electron redistribution or cleavage via oxidative addition or one-electron transfer processes (Eqs. 8–14). The former generally pertain to organometallic-type catalysis (e.g., hydrogenation, carbonylation, polymerization) and the latter to catalytic oxidations. Activation of molecular hydrogen can involve different mechanisms, of which oxidative addition is the most common (Eqs. 16–18). Activation of hydrogen by nickel in hydrogenases probably involves heterolytic cleavage. In organometallic catalysis substrate activation is usually followed by migratory insertion (e.g., in a metal–alkyl or metal–hydride bond) (Eqs. 16–18).

Oxidative addition



Homolytic cleavage



Heterolytic cleavage



VII. COORDINATIVE UNSATURATION AND THE 16/18 ELECTRON RULE

A convenient tool for understanding organometallic catalysis mechanisms is the 16 and 18 electron rule, whereby valence electrons are counted in order to ascertain whether or not complexes are coordinatively unsaturated. An 18 electron complex possesses an inert gas configuration and must first undergo dissociation to achieve the coordinative unsaturation necessary for reactivity. The number of valence electrons for various transition metals is readily seen from their position in the periodic table (e.g., Mn has 7, Fe has 8). The number counted for a particular metal is independent of its oxidation state.

Covalent (e.g., alkyl, hydride) and ionic (e.g., chloride) ligands count for one electron, and coordinative ligands (e.g., CO, Ph_3P , R_3N) for two electrons per coordination site. A mechanistic pathway in organometallic catalysis generally

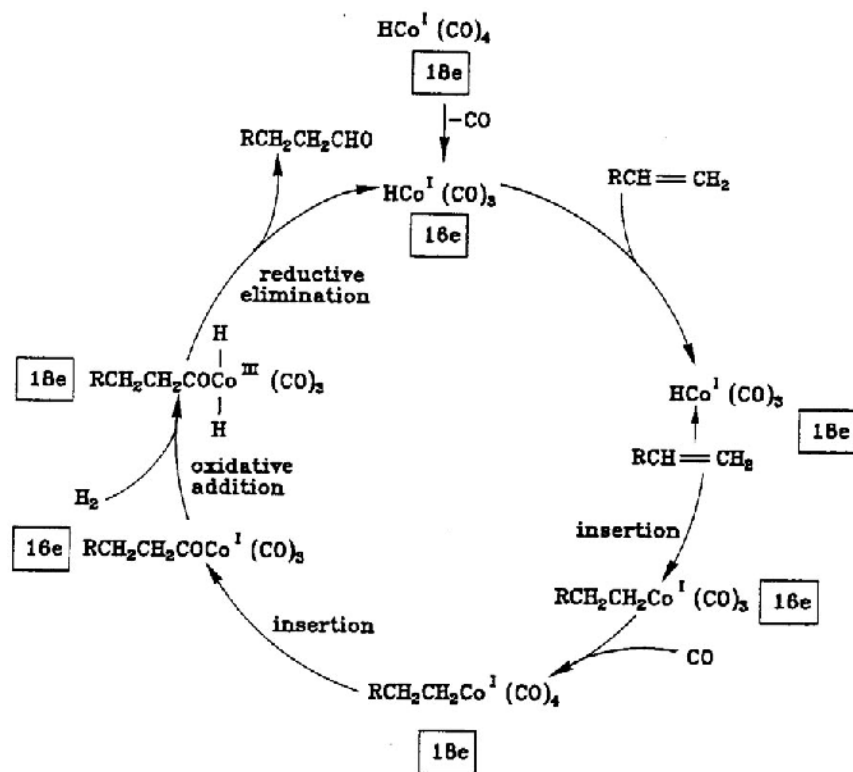


Figure 5 Mechanism of olefin hydroformylation.



proceeds alternatively via 16 and 18 electron complexes as illustrated for HCo(CO)_4 -catalyzed hydroformylation (Figure 5).

Although the 16/18 electron rule is a very useful tool for constructing mechanistic pathways, it should be noted that it completely ignores homolytic mechanisms. It is also noteworthy that it has seldom been applied to coordination and metalloenzyme catalysis. In this context it is interesting to note that the homolytic cleavage of adenosylcobalamin shown in Equation (15) involves the creation of coordinative unsaturation by conversion of an 18 electron to a 17 electron complex. (N.B.: The vitamin B-12 nucleus is a corrin ring containing an attached axial benzimidazole group and is an 8 electron ligand.) In vitamin B-12-mediated rearrangements further reaction presumably alternates between 17 and 18 electron complexes (as described in detail in Chapter 13). Similarly, chloroperoxidase (ClPO)-catalyzed oxygen transfer processes involve shuttling between a 15 electron iron(III) porphyrin complex (porphyrins are 6 electron ligands) and a 17e oxoiron(V) porphyrin (Figure 6) [13].

VIII. CONCLUDING REMARKS

In conclusion, insights into the catalytic mechanisms of metalloenzyme-mediated transformations have evolved considerably in recent years. An appreciation of

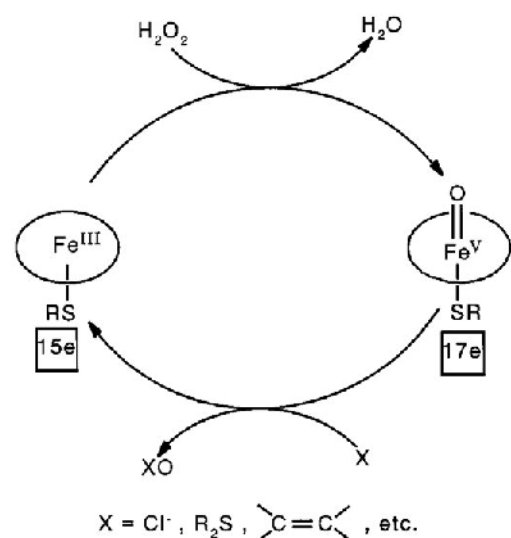


Figure 6 Catalytic cycle of oxygen transfer catalyzed by ClPO (Source: Adapted from Ref. 13).



the fundamental processes involved in homogeneous catalysis by transition metal complexes provides a solid basis for further elaboration of these insights. For more detailed introductions to homogeneous catalysis and its industrial applications, the reader is referred to Ref. [14–18].

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3

Relationships Between Enzymatic and Heterogeneous Catalysis

Edward I. Stiefel

Exxon Research and Engineering Company, Annandale, New Jersey

I. INTRODUCTION

Enzymes, solids, and homogeneous solution systems have all been known to display catalytic activity for over 100 years. However, the fields of homogeneous, heterogeneous, and enzymatic catalysis have developed almost independently. Until recently, seldom have the practitioners of one of these areas had contact with, much less worked in, the other disciplines. Nevertheless, we feel that there is much to learn from the juxtaposition of information and ideas from these seemingly disparate areas of study. The previous chapter discusses the relationship between homogeneous and enzymatic catalysis. The specificity and high reactivity characteristic of enzymes and homogeneous catalysts make the comparison seductively simple. However, it is the contention of the short discussion in this chapter that the similarities between enzymatic and heterogeneous catalysis are at least as great as, if not greater than, those between enzymatic and homogeneous catalysts. Moreover, the thinking developed in one of these fields has potential to impact the other in an efficacious way and to generate creative approaches [1–5].

In the present section, analogies and similarities will be noted between enzymes and heterogeneous catalysts in the concept of the active site and metal–protein/metal–support analogies; the possession of size and shape selectivity; the similarity or identity in kinetics between the two processes; the use of electrochemical organization on a molecular or supramolecular level; the possibility of



active-site energy trapping; and the methodology, both classical and modern, used to discover, understand, and improve upon the catalysts.

II. ACTIVE SITES AND METAL-SUPPORT/ PROTEIN INTERACTION

The study of both heterogeneous catalysts and enzymes is dominated by the concept of the active site. Specifically, in enzymes the active site is known to represent only a small portion of the large protein molecule that is the enzyme [6]. The active site may lie at or near the surface, but it may also be buried in an active site groove or crevice that limits access of all but the desired substrate. Clearly, the total surface area of the protein is significantly larger than that of the active site.

In heterogeneous catalysis, the term *active site* is also used extensively [7,8]. The density of active sites per unit surface area of the catalyst is an important parameter in catalyst analysis and development [9]. However, whereas the surface area is relatively easily determined experimentally [10], the number of active sites in heterogeneous catalysts is not easily estimated. Therefore, although both fields use turnover numbers (reactant converted per unit time per active site) to describe activity, only the enzymologists can be sure that the quantitation of this parameter is adequate.

In both heterogeneous and enzyme catalysts, the active site is diluted by material that is not part of the active site per se. Solid heterogeneous catalysts convert molecules whose bulk (as opposed to adsorbed or surface) presence is in the gas or solution phase. Although single-phase metallic and oxide catalysts are in use in important processes, many modern catalysts are “supported” materials, meaning that the key catalytic entity—the active site—is diluted by a material that prevents the catalytic units from agglomerating or deactivating [11]. In the classical view, this protection or preservation of the active site is the main function of the support. However, it is now clear that the support’s role is anything but passive in many reactions. For example, the acidity or basicity of the support material can have a crucial effect on the overall reaction to be catalyzed [12]. Moreover, the phenomenon of *strong metal-support interaction* (SMSI) [13] has been shown to have a profound effect upon the reactivity and chemical nature of certain reactive sites.

In metalloenzymes, inorganic chemists sometimes view the protein as analogous to the heterogeneous support, in sequestering of the metal atoms to prevent aggregation and/or inactivation. However, as is the case for heterogeneous supports, the protein often plays a very specific role as an acid, base, ligand, or nucleophile. Protein residues in the vicinity of the active site crucially influence



the structural, spectroscopic, and catalytic properties of the metal center [14,15]. Thus, in both supported metal-containing heterogeneous catalysts and metalloenzymes the matrix that binds the metal ions is not a passive but an active component of the active site.

III. SIZE AND SHAPE SELECTIVITY

Enzymes are known for their exquisite shape selectivity and regio- and stereoselectivity with respect to their substrates [16,17]. For example, different versions of the cytochrome P-450 enzyme family catalyze the hydroxylation of substrates as diverse as β -naphthylamine and vitamin D [18,19]. The specificity of the P-450 enzymes derives from the shape of the substrate-binding cavity, which is contiguous with the active site. (Unfortunately, in heterogeneous catalysis, “substrate” is often the term used to describe the stratum or support upon which the catalyst is mounted. The analog of the enzymatic substrate in heterogeneous catalysis is called the *feed* or *reactant*.)

In heterogeneous catalysts, illustrative examples of shape selectivity are found in zeolites, both synthetic and natural, which by their channeled and porous nature allow access and egress only of molecules of certain sizes and shapes [20]. For example, zeolites catalyze the conversion of methanol to a variety of chemicals [21]. Such zeolites have pores that prevent larger molecules from escaping, and these molecules further react to form smaller molecules in the gasoline range, which are able to escape the zeolite structure [21]. Thus, the size and shape selectivity of both zeolites and enzymes derives from a structure in the catalyst that is complementary to that of the reactant, substrate, feed, or product. A potentially major difference lies in the degree of rigidity, with the enzyme being far more flexible and more significantly dynamic [22] during the course of the reaction than is the zeolite.

IV. KINETICS OF REACTION

The kinetics of enzyme reactions was first established by Michaelis and Menten, following the earlier work of Henri [23]. The famous Michaelis–Menten equation for the kinetics of an enzyme reaction with a single substrate is often written [23]

$$\text{Rate} = \frac{V_{\max}[\text{S}]}{[\text{S}] + K_m} \quad (1)$$



where $[S]$ is the concentration of the substrate, $V_{\max}$ is the maximal rate achievable when $[S] \gg K_m$, and K_m , the Michaelis constant, is the concentration at which the rate is half-maximal. At low $[S]$ the kinetics are simple first order in $[S]$, but at high $[S]$ there is saturation, the reaction is zero order in $[S]$, and $V_{\max}$ is achieved. The simplest interpretation of the equation involves saturation of the active sites with the substrate, which makes K_m an effective dissociation constant. The smaller the K_m , the greater the affinity of the substrate for the enzyme (i.e., the enzyme will achieve half-maximal—and maximal—velocities at low substrate levels).

In heterogeneous catalysis the simplest equation that is used to describe the kinetics of a reaction of a single reactant is due to Langmuir and Hinshelwood [24]. It bears the functional form

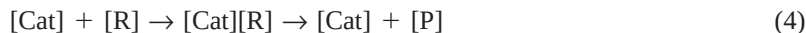
$$\text{Rate} = \frac{k[P_A]}{[P_A] + 1/K} \quad (2)$$

This form is recognized as identical to that of the Michaelis–Menten equation. Clearly, k is equivalent to the enzymological $V_{\max}$; P_A —the reactant pressure in a gas phase reaction—is equivalent to $[S]$; and $1/K$ is equivalent to K_m .

The identity in kinetic expression between enzymatic and heterogeneous reactions is not coincidental. Rather, it is due to the underlying similarity of the two processes. Both involve the adsorption of a reactant to achieve saturation of a limited number of available active sites. The adsorption isotherm of a heterogeneous reaction is analogous to the substrate binding curve of an enzymatic reaction; both represent saturation behavior of the active site. Both rate equations represent a reaction order between limiting zero- and first-order regimes. At saturation the reaction is zero order in the reactant, and at low concentrations (pressures) the reaction is first order. The adsorption/saturation followed by the conversion reaction is illustrated in the following enzymatic equations:



In the heterogeneous equations they are



The 1:1 correspondence of the elementary chemical steps in these reactions provides the underlying basis for the functional identity of the historically distinct kinetic equations for heterogeneous and enzymatic reactions.

V. ELECTROCHEMICAL ORGANIZATION

Many of the reactions in both heterogeneous and enzymatic catalysts are redox in nature. Some catalysts (such as dioxygenases and monooxygenases) work by



bringing together the oxidant and reductant in appropriate states of juxtaposition and activation to facilitate the preferred reaction [25]. Other catalysts do not facilitate such direct reactions of redox partners. Rather, these latter catalysts react with (adsorb) only a single reactant at a particular active site. A redox reaction occurs at this site, and the second redox partner accepts or donates electrons at a site that is both spatially and chemically distinct from the first. The catalyst, enzyme or solid, is configured to allow electron or atom transfer through the catalyst to complete the circuit (reaction). For example, in the plant enzyme nitrate reductase, nitrate is reduced at the Mo site of the enzyme [26,27]. However, NADH, the physiological reductant for nitrate, is oxidized at the flavin site of the enzyme. The enzyme also contains heme, which facilitates the intraenzymatic flow of electrons from flavin to Mo to complete the reaction. The enzyme xanthine oxidase [28] provides another example. The substrate xanthine interacts with the Mo site, but here the oxidant, O_2 , reacts with the flavin site in the enzyme. Iron sulfide clusters provide the pathway (the wire) by which electrons move from the anode (Mo site) to the cathode (flavin site).

The related phenomenon of spillover is well known in heterogeneous catalysts [29–31]. For example, MoO_3 alone does not activate H_2 at low temperature [32,33]. However, addition of a small amount of Pt to the MoO_3 allows H_2 activation at the Pt, with subsequent reduction of the MoO_3 as the hydrogen (most likely now hydrogen atoms) spills over from the Pt onto the molybdenum oxide, where it is available to oxidants in catalytic reactions. Fuel cells, which carry out catalytic combustion-like reactions, use anode and cathode compartments and electronic or ionic conductivity to complete the reaction [34]. The separation of oxidant and reductant allows capture of the energy of reaction to produce electricity (in fuel cells) or ionic imbalances (in some biological cases). The electrochemical principles behind the operation of these systems are clearly similar.

VI. ACTIVE-SITE ENERGY TRAPPING

In heterogeneous catalysts, the notion has been put forward that the energy of reaction to form a surface intermediate can be trapped in that intermediate. Such an intermediate is referred to as “unaccommodated,” meaning that it has not achieved thermal equilibration with the surrounding medium and therefore can be considered “hot” or “energized.” The reactivity pathways of the unaccommodated species (for example, alkyl radicals or H atoms) can be significantly different from those of their thermally equilibrated analogues [35–40].

A similar restriction in intramolecular energy flow has been postulated for metalloenzyme systems [41]. In particular, in the formation of the enzyme substrate complex, the energy of binding can be trapped at the metal active site. The



metal, in view of its heavy mass, is associated with lower-frequency vibrations than the surrounding protein matrix. The active-site metal or metals thus serve as a barrier to energy flow between the enzyme–substrate complex and the remainder of the enzyme. The localization of energy at the active site increases the effective vibrational temperature of the enzyme–substrate complex, producing a “hot” active site [41].

Recently, it has been suggested [42] that the iron–molybdenum–cofactor (FeMoco) of nitrogenase, which contains an MoFe_7S_9 cluster [43], can be vibrationally isolated from the protein and therefore able to trap the energy of adenosine triphosphate (ATP) hydrolysis or H_2 evolution [42]. The cluster, which is only covalently bound to the enzyme by two protein ligands [43], may thereby be able to achieve vibrational, structural, or electronic states that differ from the crystallographically elucidated ground state [43] in their ability to bind and reduce the recalcitrant N_2 substrate molecule. This speculative idea could help explain some of the unusual aspects of the nitrogenase enzyme [42]. Moreover, should the N_2 be shown to bind to the central Fe_6 unit of the FeMoco cluster, then the reactivity of the hot nitrogenase site may resemble that of the iron in Haber process ammonia synthesis catalysts, where an equilibrated hot active site is present at the high temperature of normal operation [44]. This analogy could provide an interesting example whereby nature and industry (i.e., enzymatic and heterogeneous catalysis) each achieve a difficult chemical conversion by producing a hot multi-iron site to bind and activate the substrate.

VII. DISCOVERY AND OPTIMIZATION OF CATALYTIC ACTIVITY

Classically, catalysts of all kinds were discovered more or less empirically [45]. Heterogeneous catalysts were generally invented or discovered by mixing the elements or their salts or oxides, heating the mixture, and running on feed. If the catalyst was active, then variations in composition, temperature regime of formation, and addition of inert materials were tried empirically to improve the catalyst.

In the isolation of enzymes, activities were usually discovered by the provision of the substrate to trial organisms, organs, or organelles, which are candidates for possession of the desired activities. The enzyme so identified could be utilized in the intact organism (dead or alive) but could also be exploited in cell free extracts or in pure form. Further improvements in activity were incremental and were obtained through strain selection, strain improvement, alteration of growth conditions, adjustment of pH, or change of temperature (within a limited



range) [46]. Nevertheless, until recently one took what the organism gave, much as the early workers in heterogeneous catalysis took the catalyst that came out of the preparation or reaction.

Nowadays, spectroscopic, magnetic, and scattering techniques (including X-ray crystallography) are capable of telling us a great deal about the chemical nature of active sites [47]. These techniques allow us to monitor chemical changes that occur in heterogeneous [48] or enzymatic systems [49]. In the past, active sites were chemically changed without much control. The only guide or target was the activity of the final product. Fortunately, one can now often correlate reactivity with directly monitored spectroscopic, magnetic, and/or scattering properties. Moreover, the active sites can be altered with a greater degree of control.

An example of active site control in heterogeneous catalysis lies in the templating that is used to produce desired zeolite structures [50]. Another approach involves the production of specific sites from defined molecular precursors, where the structural details can be more finely controlled [51]. These approaches to new heterogeneous catalysis involve the use of diffraction, spectroscopy, and microscopy to allow exquisitely detailed probing of the active site and accessibility of the reactant in its surroundings.

Control in biocatalytic systems comes from such new techniques as directed evolution [52], the formation of catalytic antibodies using transition state templates [53], and the powerful approach of site-directed mutagenesis [54]. In both heterogeneous and enzymatic catalysis, rather than “take what you get,” we now try to “design what we want” and devise clever chemical and biological approaches to get it.

The rationale for the improvement of both enzymatic and heterogeneous systems is similar. First, one learns more about the reaction chemistry and the electronic and structural nature of the active site. Then, one uses this information to develop working hypotheses for the action of the site. Finally, one changes the site systematically in conjunction with spectroscopic, magnetic, or scattering and/or catalytic monitoring to improve the site. These approaches are of value both for attaining a detailed knowledge of an existing catalytic system and for designing, discovering, and inventing new systems.

Recently, the complementary approach of combinatorial synthesis has been increasingly applied to the generation of biologically active molecules, including potent enzyme inhibitors [55]. Interestingly, the first reports of the application of combinatorial chemistry to heterogeneous catalysts have also recently appeared [56,57].

The similarities discussed here between heterogeneous and enzymatic systems show that we have much to learn by looking at the parallel approaches and results that are being obtained in these closely related fields.



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4

Lewis Acid Properties of Zinc and Its Development to Phosphotriester Detoxifying Agents

Eiichi Kimura and Tohru Koike

Hiroshima University, Hiroshima, Japan

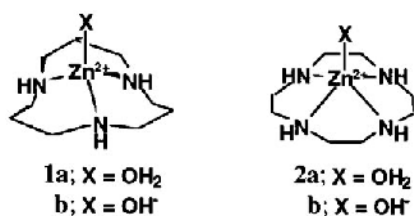
I. INTRODUCTION

The zinc(II) ion is a biologically essential element. Knowledge of its importance is increasing, as more and more enzymes are discovered as zinc(II)-containing bioinorganic catalysts. Carbonic anhydrase, carboxypeptidase, aminopeptidase, superoxide dismutase, yeast aldolase, β -lactamase, alcohol dehydrogenase, alkaline phosphatase, phospholipase C, nucleic acid polymerase, and P1 nuclease may be classified as classic zinc enzymes [1]. More recently added are phosphotriesterase (or “organophosphorus hydrolase,” EC group 3.1.8), which hydrolyzes pesticides and nerve gases [2]; the *hedgehog* family of secreted signaling proteins [3]; collagenase (a member of the matrix metalloprotease family) [4]; and so on. There are now more than 300 known zinc enzymes. The x-ray crystallographic structures of a considerable number of zinc enzymes have now been established.

Transition metal ions (e.g., Cu^{2+} , Co^{2+} , Co^{3+} , Ni^{2+} , and Zn^{2+}) have long been known to hydrolyze esters and amides catalytically in aqueous solution [5,6]. Among phosphate esters the ease of the hydrolysis of neutral triesters is in the order $(\text{RO})_3\text{PO} > \text{monoanionic diesters } (\text{RO})_2\text{PO}_2^- > \text{dianionic monoester } \text{ROPO}_3^{2-}$. Accordingly, they are good candidates for active decontaminants of neutral organophosphoesters, nerve gases, and phosphotriester insecticides. Al-



though classical chemical reactions, such as base-catalyzed hydrolysis or oxidation, may be exploited, the reagents employed may usually be too corrosive to permit their use under certain conditions. For milder decontaminants capable of more general application, metal complexes may be considered as highly efficient general base catalysts for the hydrolysis of toxic phosphates. At a pH near 7 the rates may be increased by several orders of magnitude. One theory was that complexation of water to transition metal ions that are strong Lewis acids markedly induces its dehydronation so that hydroxide-bound metal complexes exist at neutral pH to act as effective nucleophiles toward esters and amides. More typically, metals with chelating ligands have been used to prevent precipitation of metal oxides or hydroxides at neutral pH. As a result, extensive work has been reported on optimization of the ligands and metal ions. The most general problem encountered in the past was that chelating ligands, which leave the catalytic sites vacant, tend to dissociate from the metals at neutral pH in aqueous solution. Among the catalytic metal complexes studied so far, the zinc(II) complexes with macrocyclic 12-membered triamine ([12]aneN₃, 1,5,9-triazacyclododecane) **1** and tetraamine (cyclen, 1,4,7,10-tetraazacyclododecane) **2** ligands provided the best systems for mechanistic and practical probes (Scheme 1) [7–15]. These complexes originally were used to elucidate the role of zinc(II) in the active center of carbonic anhydrase, where the zinc(II) ion is coordinated with three histidine imidazoles [7]. Thanks to the “macrocyclic effect,” the zinc(II) complexes **1** and **2** are very stable at neutral pH and yet provide a vacant site for catalysis. By using these macrocyclic models, we now can explain the hydration of CO₂ ($\text{CO}_2 + \text{H}_2\text{O} \rightarrow \text{HCO}_3^- + \text{H}^+$) catalyzed by Zn²⁺—OH[−] species **1b** and **2b**, which are good nucleophiles toward electrophilic CO₂ [10,11]. It has been well demonstrated that the zinc(II) ions in the macrocyclic polyamine complexes (**1** and **2**) are relatively strong acids, which dehydronate the Zn²⁺-bound water and generate the latent nucleophiles at neutral pH: e.g., the pK_a values are 7.3 for **1a** → **1b** + H⁺ and 7.9 for **2a** → **2b** + H⁺ at 25°C [7]. Thus, CO₂ becomes subject to the Zn²⁺—OH[−] nucleophilic attack at physiological pH, which otherwise occurs only at high alkaline pH. The mild conditions in such a nucleophilic reaction



Scheme 1 Zinc(II) complexes with [12]aneN₃ and cyclen.

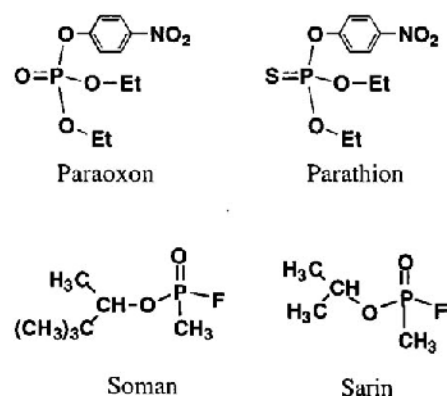


were then successfully applied to catalytic hydrolysis of esters such as acetate [7], phosphates [9], and β -lactam [13]. Independent studies of organophosphate hydrolysis with **2** [16] and *bis*(1-methylimidazol-2-ylmethyl)aminoethane metal (Zn^{2+} and Cu^{2+}) complexes [17] were earlier reported with a specific aim of developing decontaminants. The exploration of good metal catalysts for lipophilic phosphates may also be useful for hydrolysis of phospholipids, which is relatively unexplored.

II. ORGANOPHOSPHORUS HYDROLASE

When the lack of persistence of organophosphate pesticides in the soil was discovered, it was eventually attributed to their susceptibility to microbial transformation. From the soil bacterial *Pseudomonas diminuta* and *Flavobacterium* sp., an organophosphorus hydrolase was isolated [2,18,19] that catalyzes hydrolysis of organophosphate triesters such as pesticides (parathion and paraoxon) or nerve gases (soman and sarin) (Scheme 2). The enzyme requires the presence of a zinc(II) ion bound to the protein. The organophosphate-degrading enzymes then became of considerable interest in light of their ability to detoxify such lethal compounds. The structure of the active center and elucidation of the hydrolysis mechanism will offer a good guide in designing metal complex catalysts.

A site-directed mutagenesis study disclosed two zinc(II) ions at the active center: Zn(1) surrounded by three histidine (His) residues, His55, His57, and His201, and Zn(2) by His254, His257, and an undefined ligand (Figure 1) [20]. Single mutations at His55, His57, His201, and His230 strongly reduced the k_{cat}



Scheme 2 Pesticides and nerve gases.



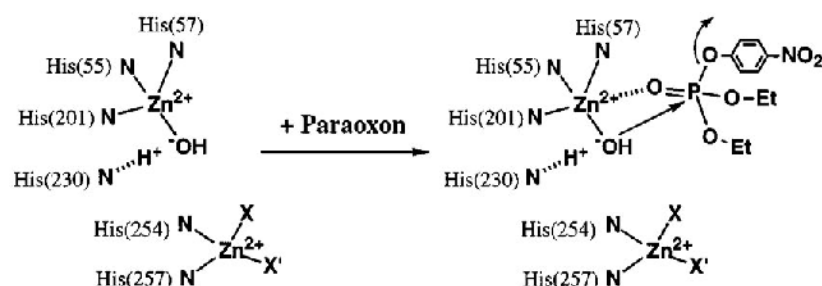


Figure 1 Proposed mechanism for paraoxon hydrolysis by organophosphorus hydrolase (X and X' are unspecified ligands).

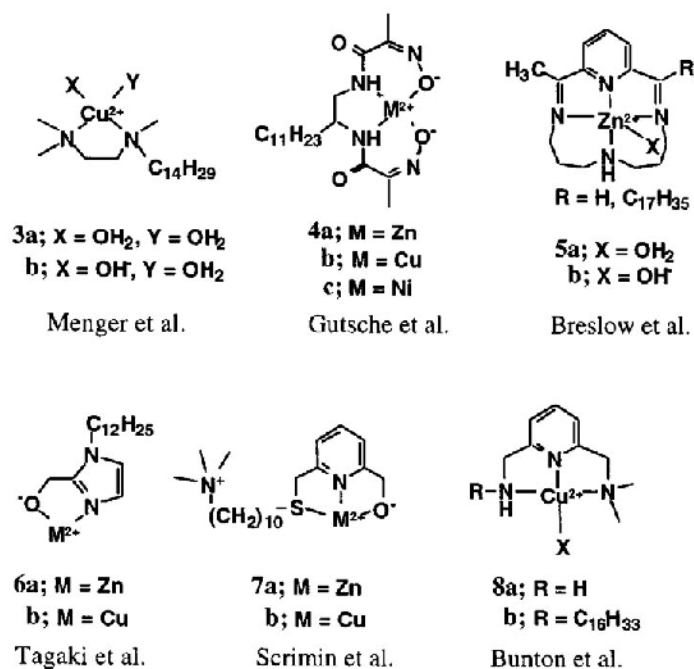
value from 3170 sec^{-1} in the wild-type enzyme to less than 10 sec^{-1} , whereas substitution for the other histidines reduced the activity 2- to 15-fold. These results suggest that His55, His57, His201, and His230 were directly involved in catalysis or that they provided essential functions in active site conformation. In contrast, the less significant effect caused by His254 and His257 substitutions suggests a conformational role for the active site. The substitution of asparagine for His230 decreased the k_{cat} approximately 1000-fold and increased the catalytic pK_a value to 8.0 from 5.6 for wild type. These results suggest that the primary role of His230 in catalysis is to act as a general base, activating a water bond to Zn^{2+} . Since the wild-type enzyme had substantial activity with only one equivalent of zinc(II) ion per molecule, the second independent metal center would play a minor role, possibly indirectly participating in a dinuclear cocatalytic center. It is not yet known how the very lipophilic organophosphates bind to the active center.

The enzyme catalyzes the hydrolysis with overall inversion of stereochemistry at the phosphorus center, which is consistent with a chemical mechanism that utilizes an activated water molecule to attack the phosphorus center directly.

III. PREVIOUS LIPOPHILIC METAL COMPLEXES FOR LIPOPHILIC PHOSPHATE HYDROLYTIC CATALYSIS

For effective catalytic hydrolysis of lipophilic phosphate esters in aqueous solution, metallomicelles such as **3** [21], **4** [22], **5** [23], **6** [24], **7** [25], and **8** [26] have been designed (Scheme 3). These complexes are essentially metal ions chelated with ligands that are attached with lipophilic long alkyl chains. Some of these were used together with surfactants to make water-miscible solutions.





Scheme 3 Lipophilic metal complexes.

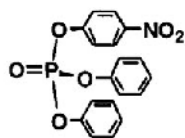
The common problems with those metallomicelles may be summarized as follows: (1) Most of these complexes were prepared in situ and often were not isolated. Hence, the intended structures of the metallomicelles in solution or in the solid state were not verified. (2) The metal complexes in solution were not identified or characterized in rigorous thermodynamic senses by potentiometric pH titration, etc. The complexation constants and possible species distribution at various pH's⁻ were totally unknown. (3) Possible catalytically active species L-Mⁿ⁺-OH⁻ were not identified by means of the thermodynamic pK_a values. Those described were all obtained merely in kinetics. (4) The product (phosphate anion) inhibition was not determined. Accordingly, it often was not clear whether it was catalytic or not. (5) Often, the substrates studied were limited. (6) The kinetics was complex, probably as a result of the existence of various species in solution. Thus, in most of the cases only pseudo-first-order rates (e.g., with excess metal complexes) were given. No solid kinetic studies combined with thermodynamic studies have been presented. It is thus impossible to compare the catalytic efficiency of these metallomicelles with that of the natural system. Besides, different



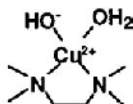
substrates were often employed. Because of these difficult problems, we see that the hydrolysis by metallomicelles awaits a full quantitative mechanistic study for the design of more efficient and practical hydrolytic catalysts.

Menger et al. synthesized a $C_{14}H_{29}$ -attached copper(II) complex **3** that possessed a remarkable catalytic activity in the hydrolysis of diphenyl 4-nitrophenyl phosphate (DNP) and the nerve gas "Soman" (see Scheme 2) [21]. When **3** was used in great excess (ca. 1.5 mM, which is more than the critical micelle concentration of 0.18 mM), the hydrolysis of DNP (0.04 mM) was more than 200 times faster than with an equivalent concentration of the nonmicellar homologue, the Cu^{2+} -tetramethylethylenediamine complex **9**, at 25°C and pH 6 (Scheme 4). The DNP half-life is calculated to be 17 sec with excess 1.5 mM **3** at 25°C and pH 6. The possible reasons for the rate acceleration with **3** were the enhanced electrophilicity of the micellized copper(II) ion or the acidity of the Cu^{2+} -bound water and an intramolecular type of reaction due to the micellar formation. On the basis of the pH(6–8.3)-insensitive rates, Cu^{2+} -OH⁻ species **3b** (generated with $pK_a < 6$) was postulated to be an active catalytic species. In this study, the stability constants for **3** and **9** and the thermodynamic pK_a value of the Cu^{2+} -bound water for **3a** $\rightarrow$ **3b** + H⁺ were not measured, probably because of complexity and/or instability of the metal compounds. Therefore, the question remains as to whether or not **3b** is the only active species in the reaction solution. Despite the lack of a detailed reaction mechanism, **3** seems to be the best detoxifying reagent documented in the literature.

Gutsche et al. observed that a lipophilic dioxime (the ligand in **4**) accelerates by a factor of 60 to 140 the hydrolysis of acetyl phosphate in the presence of an equimolar amount of metal ($M = Cu, Zn, Ni$) under comicellar conditions with cetyl trimethylammonium bromide (CTAB) at pH 11.5 [22]. The hydrolysis reaction was followed by pseudo-first-order kinetics at [ligand] = $[M] = 2.5$ mM and [CTAB] = 250 mM. Although a possible complex **4** was proposed to exist in the hydroxamate form, there were no experimental data for the stability and structure of metal complexes at pH 11.5 (e.g., it is not known whether the



Diphenyl 4-Nitrophenyl Phosphate
DNP



9

Scheme 4 Phosphotriester with 4-nitrophenyl group (DNP) and Cu^{2+} -tetramethylethylenediamine complex.



oximes are dehydronated). The acetyl phosphate hydrolysis mechanism is proposed to be either by acetyl transfer to the oxime oxygen, followed by rapid hydrolysis of the oxime acetate, or by a general base mechanism, as shown in Figure 2a and b, respectively. However, no evidence was given for either mechanism. The role of the metal ions in the hydrolysis reaction is not clear, whether or how they activate the oxime for nucleophilic attack.

Gellman et al. [23] tested a hydrophilic macrocyclic tetraamine zinc(II) complex **5** ($R = \text{CH}_3$) [27] and a lipophilic homologue **5** ($R = \text{C}_{17}\text{H}_{35}$) to find effective catalysts for the hydrolysis of DNP and to unravel its mechanism, using the same substrate as in Menger's study. The lipophilic complex was much more effective than the hydrophilic homologue in the comicellar reaction with the neutral surfactant Brij 35 ($\text{C}_{12}\text{H}_{25}(\text{OCH}_2\text{CH}_2)_{23}\text{OH}$). All the reactions with the lipophilic **5** ($R = \text{C}_{17}\text{H}_{35}$) were carried out at low ionic strength and 20 mM surfactant Brij 35 concentration, well above the critical micellar concentration of about 0.1 mM. The rate-pH profile studies showed that the catalytic zinc(II) species probably is a $\text{ZnL}-\text{OH}^-$ complex (**5b**) with $\text{p}K_a$ values of 8.7 ($R = \text{CH}_3$) and 9.1 ($R = \text{C}_{17}\text{H}_{35}$) for $\mathbf{5a} \rightarrow \mathbf{5b} + \text{H}^+$. The DNP hydrolysis kinetics in comicellar solution of 20 mM Brij 35 and 0.25 mM $\text{ZnL} \cdot \text{Br}^- (\text{ClO}_4)$ ($R = \text{C}_{17}\text{H}_{35}$) was determined at 25°C; the solution behavior seemed a little peculiar and different from that of $(\text{ZnL})_2 \cdot \text{OH}^- \cdot 3(\text{ClO}_4)$ separately synthesized. From the pH rate profile the second-order rate constant was estimated to be $18 \text{ M}^{-1} \text{ sec}^{-1}$ for the active species **5b** ($R = \text{C}_{17}\text{H}_{35}$), which is much larger than that of $0.28 \text{ M}^{-1} \text{ sec}^{-1}$ for the hydrophilic homologue **5b** ($R = \text{CH}_3$) in aqueous solution. The enhanced catalytic activity may result from the aggregation effect in the comicelles. Since the second-order rate constant for the $\text{ZnL}-\text{OH}^-$ compound ($R = \text{CH}_3$) is 10 times greater than that for a OH^- ion, a bifunctional (or "push-pull") mechanism was

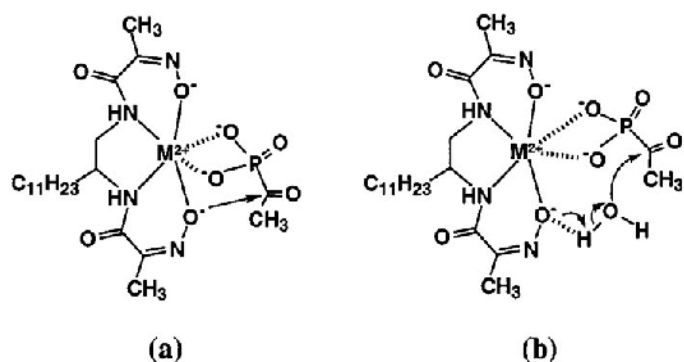


Figure 2 Proposed mechanism for acetyl phosphate hydrolysis by Gutsche's metallomicelle **4**: (a) acetyl transfer mechanism, (b) general base mechanism.



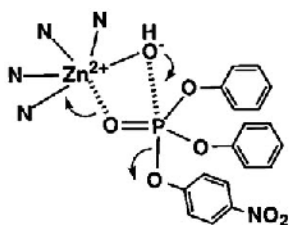


Figure 3 Proposed difunctional or “push–pull” mechanism for diphenyl 4-nitrophenyl phosphate by Breslow’s metallomicelle **5**.

proposed for **5b** (see Figure 3), in which zinc(II)-bound OH^- acts as a nucleophile, whereas the zinc(II) ion acts as an electrophile binding the substrate. However, the catalytic mechanism in micelles was not straightforward, requiring identification of other undefined species such as aggregates, as indicated by the complex kinetic term higher than first order for **5**. At pH 8, Breslow’s lipophilic macrocyclic zinc(II) complex was estimated to hydrolyze DNP about 24 times more slowly than Menger’s lipophilic **3** (at pH 6) [21].

Tagaki et al. [24] and Fornasier et al. [25] reported another type of metallomicelle attached with a metal-bound alkoxide nucleophile. Tagaki’s zinc(II) and copper(II) complexes (with possible structures **6a** and **b**) promoted the hydrolysis of 4-nitrophenyl picolinate in a comicellar system with hexadecyl trimethylammonium bromide. However, no detailed mechanistic study was reported. Scrimin’s zinc(II) and copper(II) complexes (proposed structures **7a** and **b**) also promoted the hydrolysis of 4-nitrophenyl picolinate. A postulated mechanism for the catalytic activity of **7** is shown in Figure 4. An aggregate of **7** more effectively

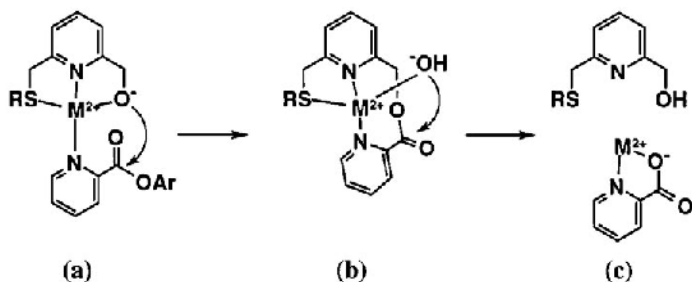
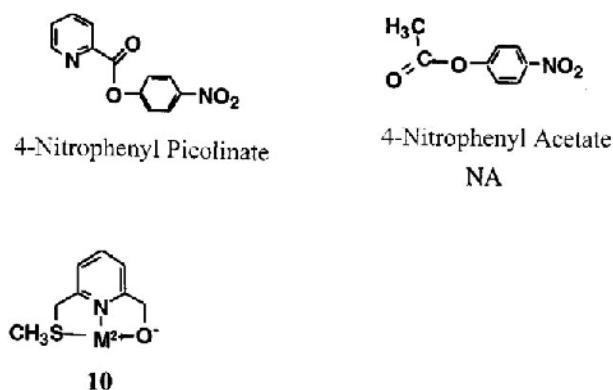


Figure 4 Proposed intramolecular reaction mechanism for 4-nitrophenyl picolinate hydrolysis by Scrimin’s metallomicelle **7**. See text for details.





Scheme 5 Ester substrates and nonsurfactant alkoxide-bound metal complex.

hydrolyzed the substrate than the nonsurfactant homologue **10** (Scheme 5), as a result of the concentration of the lipophilic ester substrate in the micelle. The rate promotion was speculated to be due to the formation of the ternary complex (Figure 4a). Then, an intramolecular attack of the metal-activated hydroxyl group (i.e., the metal-bound alkoxide; see Figure 4b) may yield an acyl transfer intermediate, which is quickly hydrolyzed by a metal-bound OH^- (or water) to form micellar ligand and metal-picolinate (see Figure 4c). A similar mechanism was proposed for the Tagaki hydrolysis system, which, however, had no experimental support at all. Nonligand substrates such as 4-nitrophenyl acetate (NA) were not cleaved by **7**.

Recently, Bunton et al. synthesized the $\text{C}_{16}\text{H}_{33}$ -attached triamine copper(II) complex **8b** [26], which promoted the hydrolysis of diphenyl 4-nitrophenyl phosphate (DNP) at alkaline pH. The catalytic activity was almost the same as that for Menger's previously reported micellar system with **3b**. The active species was proposed to be the hydroxide-bound copper(II) complex **8b**. The pK_a value of the copper(II)-bound water molecule was speculated to be about 8 from the fact that the nonalkylated and tetradecyl homologous copper(II) complexes have a pK_a value of 8 (determined by DNP hydrolysis kinetics). Since the micellar metal complex **8b** was not fully characterized either in the solid state or in a micellar solution, its hydrolysis mechanism remains to be elucidated.

It was disappointing to see that despite the prolonged efforts, these earlier metallomicellar systems failed to give us a clear mechanistic picture that would allow us to give a precedence that may assist us in the design of new synthetic decontaminants.



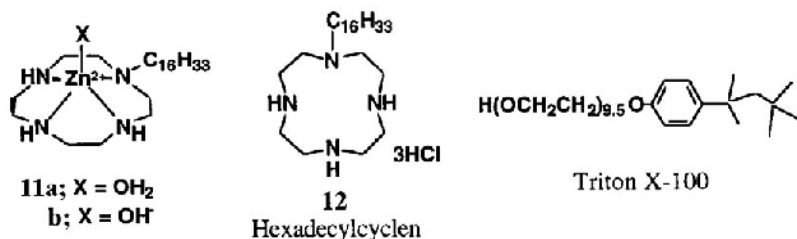
IV. HYDROLYSIS OF ESTERS WITH A NEW LIPOPHILIC MACROCYCLIC TETRAAMINE ZINC(II) COMPLEX

IV.A. A Long Alkyl-Pendant Macrocyclic Tetraamine and Its Zinc(II) Complex

Very recently, a new type of metallomicelle with a zinc(II) cyclen attached to a hexadecyl group, **11**, has been synthesized (Scheme 6). An unprecedented clear picture of its catalytic behavior in a comicellar solution with Triton X-100 has been disclosed [28]. Since the parent Zn^{2+} —cyclen complex **2** showed discrete behavior in aqueous solution (i.e., no dissociation of the zinc(II) ion; stable, discrete monomeric species) [7,13,14], **11** was anticipated to behave in an orderly way, and that turned out to be the case.

The long alkyl chain ($\text{C}_{16}\text{H}_{33}$)-attached cyclen (haxadecylcyclen, **12**) was synthesized and recrystallized as the $\cdot 3\text{HCl}$ salt from aqueous HCl solution. Since the new ligand **12** was not sufficiently soluble (e.g., 1 mM) at neutral pH during the potentiometric pH titration, a neutral detergent Triton X-100 (10 mM) was added to keep the solution (1 mM) homogeneous at 25°C with $I = 0.10$ (NaNO_3). The concentration of Triton X-100 is much higher than its critical micelle concentration of 0.08 mM (determined by the vertical plate method). The mixed hydration constants $\log K_{1-3}$ (a_{H^+} is the activity of H^+) were determined to be 9.95, 8.27, and 2.3. The corresponding $\log K_n$ values for hydrophilic cyclen (1,4,7,10-tetraazacyclododecane) in the absence of Triton X-100 are 11.04, 9.86, and <2 [13]. The lower basicity of **12** in Triton X-100 with respect to that of cyclen may be due to the more feasible neutral (i.e., nonionic) states of amines in the lipophilic environments. This probably is the first report of how the basicity of macrocyclic polyamines changes in lipophilic micelles.

A crystalline zinc(II) complex **11a** (ZnL—OH_2) was obtained by mixing **12** and $\text{Zn}(\text{ClO}_4)_2$ in methanol. The elemental analysis (C, H, N) suggested a $\text{ZnL—OH}_2(\text{ClO}_4)_2$ formula, which represents the first distinct 1:1 metal complex



Scheme 6 Lipophilic cyclen, its zinc(II) complex, and neutral surfactant Triton X-100.



with a lipophilic ligand: compare Breslow's macrocyclic complex **5**, which has the molecular formula $(\text{ZnL})_2 \cdot \text{OH}^- \cdot (\text{ClO}_4)_3$, whose structure was not identified. Compound **11a** is soluble in MeOH and CH_3CN , slightly soluble in CHCl_3 , but hardly soluble ($<0.1 \text{ mM}$) in H_2O . Its critical micelle concentration at 25°C with $I = 0.10$ (NaNO_3) was $0.3 \mu\text{M}$. Triton X-100 (10 mM) was selected as a neutral surfactant that allowed sufficient solubility (e.g., 1 mM) of **11** for all of the following studies. The potentiometric pH titration curve of $\mathbf{12} \cdot 3\text{HCl}$ (1 mM) in the presence of an equimolar amount of ZnSO_4 and 10 mM Triton X-100 revealed stoichiometric zinc(II) complexation at $3.5 < \text{pH} < 5.5$, followed by dissociation of one hydron from the complex at $5 < \text{pH} < 9$. An identical titration curve was obtained with the isolated 1:1 complex **11a** (1 mM) mixed with three equivalents of HClO_4 under the same conditions. The titration data nicely fit with equilibria 1–4 involving a 1:1 ZnL complex ($\text{ZnL}\text{—OH}_2$ **11a**), an OH^- -bridged dinuclear complex ($(\text{ZnL})_2\text{OH}^-$ **13**, and an OH^- -bound mononuclear complex ($\text{ZnL}\text{—OH}^-$, **11b**) (see Figure 5). This is the first specification of mixed species of metallomices in aqueous solution. No further dehydration or precipitation of $\text{Zn}(\text{OH})_2$ was observed up to pH 10, indicating a high stability of those zinc(II) complexes under comicellar conditions.

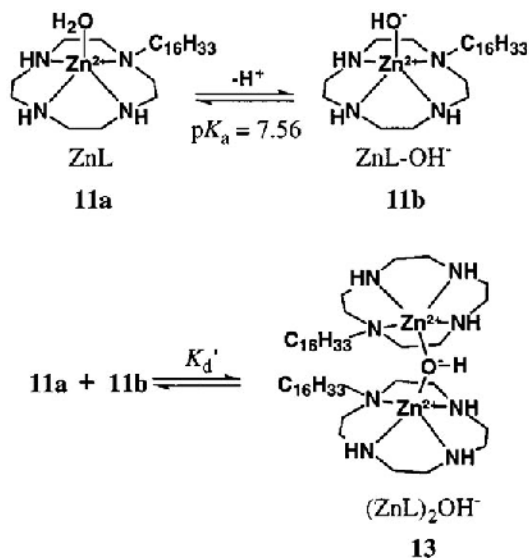
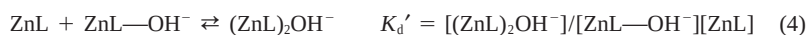
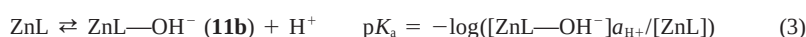
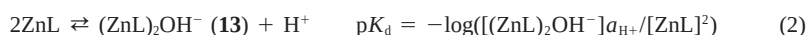


Figure 5 Solution equilibrium for zinc(II)–hexadecylcyclen complexes in the presence of 10 mM Triton X-100.





The obtained $\log K(ZnL)$, pK_d , pK_a , and $\log K_d'$ values are 12.90, 3.92, 7.56, and 3.64, respectively. A distribution diagram for the hexadecylcyclen- Zn^{2+} system as a function of pH at concentrations of [total zinc(II)] = [total L] = 1 mM, [Triton X-100] = 10 mM, and 25°C with $I = 0.10$ (NaNO₃) is displayed in Figure 6. Although the stability of the ZnL complex **11a** is lower than that of Zn^{2+} -cyclen **2a** ($\log K(ZnL') = 15.3$), **11a** is sufficiently stable above pH 6. The pK_a value of 7.56 for the zinc(II)-bound water of **11a** is not greatly changed from those for **2a** in the presence (7.83) and absence of Triton X-100 (10 mM, 7.86) under the same conditions [29]. The similarity in pK_a values with or without Triton X-100 implies little perturbation from the neutral surfactant on Zn^{2+} -cyclen **2**. The occurrence of the hydroxide-bridged dinuclear complex **13** (see Figure 5) at neutral pH is accounted for by postulating the favorable aggregation of the two Zn^{2+} -cyclen units with the lipophilic groups and Triton X-100 micelles (i.e., the local concentration of **11** in the micelles is much higher than the total concentration in the micellar solution). As found in the following kinetic study, the hydroxide-bridged dinuclear complex **13** is kinetically inactive as a nucleophile toward esters. The previously mentioned Breslow's complex $(ZnL)_2 \cdot OH^- \cdot (ClO_4)_3$ may have a structure and properties similar to **13**, which might explain its curious behavior (e.g., low reactivity and less soluble nature) [23].

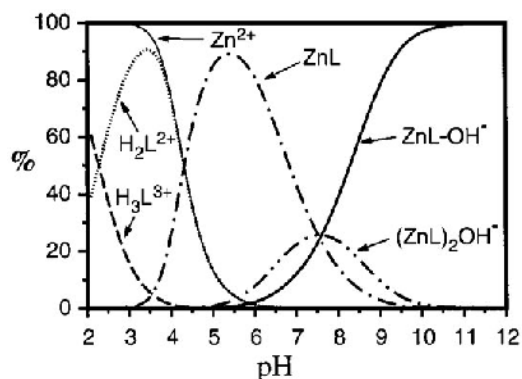


Figure 6 Species distribution resulting from a 1:1 solution (1 mM of both hexadecylcyclen **12** (=L) and Zn^{2+}) in the presence of 10 mM Triton X-100 at 25°C with $I = 0.10$ (NaNO₃).



IV.B. Hydrolysis of 4-Nitrophenyl Acetate by **11**

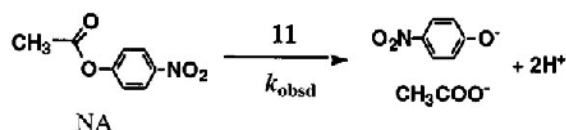
Hydrolysis of 4-nitrophenyl acetate (NA) (0.5–2.0 mM) was catalyzed by **11** in 10% volume/volume (v/v) CH₃CN aqueous solution under comicellar conditions with 10 mM Triton X-100 at pH 9.2 (20 mM CHES buffer) and 25°C (Scheme 7). The second-order dependence of the rate constant, k_{obsd} , on the concentration of NA (10–50 μM) and **11** (0.2–1.0 mM) at pH 10.2 (2 mM CAPS buffer) and 25°C with $I = 0.10$ (NaNO₃) fits the kinetic equation (5). No other reaction such as acetate transfer to Triton X-100 was observed, as confirmed by a ¹H NMR experiment with a 10% D₂O solution of 2.0 mM NA, 0.2 mM **3**, and 10 mM Triton X-100. Since the second-order kinetics held after several catalytic cycles, it was concluded that the NA hydrolysis catalytic. In Equation (5), v_{obsd} is the observed NA hydrolysis rate catalyzed by **3**, as derived by subtraction of the buffer-promoted NA hydrolysis rate from total NA hydrolysis rate.

$$v_{\text{obsd}} = k_{\text{obsd}}[\text{total zinc(II) complex}][\text{NA}] \quad (5)$$

$$= k_{\text{NA}}[\mathbf{11b}][\text{NA}] \quad (6)$$

The effect of the surfactant concentration (0.75–30 mM) on the pseudo-first-order rate constant (k'_{NA}) for the NA (0.1 mM) hydrolysis promoted by 0.2 mM **11** is shown in Figure 7. In the range of $0 \leq [\text{Triton X-100}] \leq 10$ mM, with an increase in the concentration of Triton X-100, the hydrolysis rate increased, probably because the slightly lipophilic NA goes preferentially into the comicelles to react with **11**. Increasing the Triton X-100 concentration above 10 mM results in a gradually decreasing rate, probably because **11** and NA are thinly spread and separated in different comicellar phases. As a control experiment, the hydrolysis of NA (0.1 mM) promoted by Zn²⁺—cyclen **2** (1 mM was used to produce rates comparable to those of 0.2 mM **11**) and [Triton X-100] (0–30 mM) under the same conditions were determined. A plot of k'_{NA} vs. surfactant concentration is shown in Figure 8. In this case, an increase in the concentration of Triton X-100 induces a slower NA hydrolysis, which is again explained by favorable transfer of the lipophilic NA into the micellar phase, and the hydrophilic **2** remains in the aqueous phase.

The observed rate constant k_{obsd} under the conditions employed for the potentiometric pH titrations (i.e., 25°C, $I = 0.10$ NaNO₃, 10 mM Triton X-100, 1



Scheme 7 4-Nitrophenyl acetate hydrolysis by **11**.



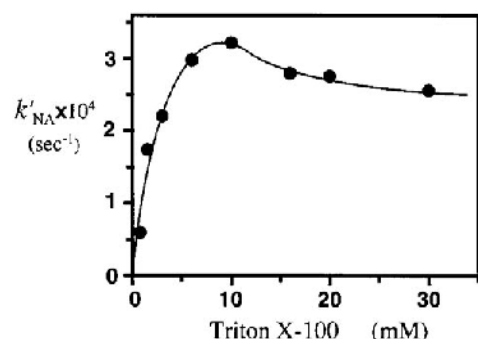


Figure 7 Effect of Triton X-100 concentration on the pseudo-first-order rate constant (k'_{NA}) for the hydrolysis of NA (0.1 mM) catalyzed by **11** (0.2 mM) in 10% (v/v) CH_3CN aqueous solution (pH 9.2 CHES buffer [20 mM]) at 25°C with $I = 0.10$ (NaNO_3).

mM **3**) is plotted as a function of pH (=6.9–10.5) in Figure 9. The resulting sigmoidal curve with an inflection point at pH 8.5 almost overlaps with the sigmoidal distribution curve for the $\text{ZnL}-\text{OH}^-$ species **3b** obtained in the equilibrium studies. This means that among **11a**, **11b**, and **13**, the kinetically reactive species is only the monomeric hydroxide-bound zinc(II) complex **11b**, as defined by the kinetic equation 6. The second-order rate constant k_{NA} was determined to be $5.0 \text{ M}^{-1} \text{ sec}^{-1}$. Unless the OH^- -bridged zinc(II) complex **13** is considered as one of the dehydrated species, the kinetic inflection should appear around pH 7.6, the same value as $\text{p}K_a$ of **11a** (Eq. 3). This fact provides kinetic support for the

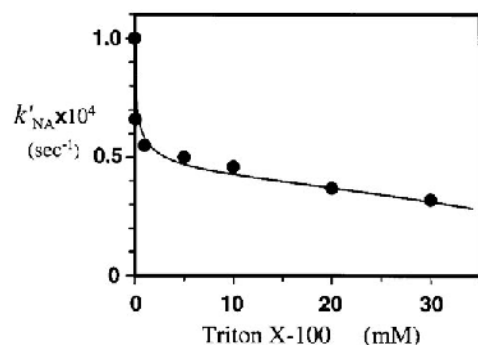


Figure 8 Effect of Triton X-100 concentration on the pseudo-first-order rate constant (k'_{NA}) for the hydrolysis of NA (0.1 mM) catalyzed by **2** (1.0 mM) in 10% (v/v) CH_3CN aqueous solution (pH 9.2 CHES buffer [20 mM]) at 25°C with $I = 0.10$ (NaNO_3).



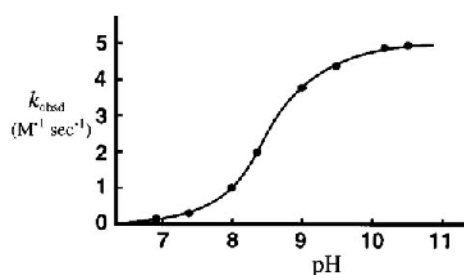


Figure 9 Rate-pH profile for the second-order rate constant (k_{obsd}) in the hydrolysis of NA (50 μM) catalyzed by **11** (1.0 mM) and Triton X-100 (10 mM) in aqueous solution at 25°C with $I = 0.10$ (NaNO_3).

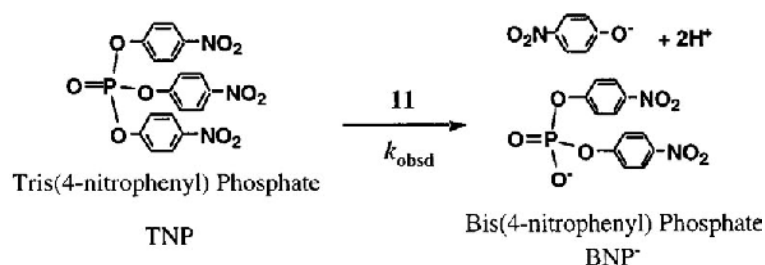
thermodynamic consideration of **13**. It should be recalled that in the hydrolysis of NA with Zn^{2+} —cyclen **2**, the kinetic sigmoidal curve in the rate-pH profile (corresponding to Figure 9) has an inflection at pH 7.9 and almost overlaps with the thermodynamic distribution curve for the monomeric hydroxide-bound species **2b**, where no other species such as the hydroxide-bridged **13** was involved [30].

As a reference, the NA hydrolysis catalyzed by Zn^{2+} —cyclen **2b** has been determined in the presence of 10 mM Triton X-100 by the same method in 10% (v/v) CH_3CN aqueous solution (pH 9.2 with 20 mM CHES buffer) with $I = 0.10$ (NaNO_3) at 25°C. The rate constant of $4.6 \times 10^{-2} \text{ M}^{-1} \text{ sec}^{-1}$ is smaller than that of $0.10 \text{ M}^{-1} \text{ sec}^{-1}$ for the same reaction in the absence of Triton X-100. Provided that only the NA partitioned in aqueous phase was subjected to attack by the hydrophilic **2b**, it can be estimated that approximately 50% of NA is partitioned in the micellar phase, since the k_{NA} value in the presence of Triton X-100 was decreased by half. The comparison of these k_{NA} values (5.0 vs. $4.6 \times 10^{-2} \text{ M}^{-1} \text{ sec}^{-1}$) clearly shows that the lipophilic cyclen complex **11b** is a 100-fold better catalyst in a micellar system. In the micellar phase, the lipophilicity of both the substrate NA and catalyst **11b** would contribute to stabilization of the supramolecular assembly, leading to more frequent collisions between them.

IV.C. Hydrolysis of Tris(4-Nitrophenyl) Phosphate by **11**

Because tris(4-nitrophenyl)phosphate (TNP) [31] has insufficient solubility in only 10 mM Triton X-100 aqueous solution, methanol should be added as a cosolvent. The critical micelle concentration of Triton X-100 was determined to be 0.15 mM in 10% (v/v) MeOH aqueous solution at 25°C with $I = 0.10$ (NaNO_3). The TNP (5–20 μM) was very rapidly and catalytically hydrolyzed by **11** (20–





Scheme 8 Tris(4-nitrophenyl) phosphate hydrolysis by **11**.

200 μM) to yield 4-nitrophenolate and bis(4-nitrophenyl) phosphate (BNP) in the pH range 6.9–10.5 (Scheme 8). The subsequent hydrolysis of BNP to mononitrophenyl phosphate was much slower.

The second-order dependence of the TNP hydrolysis rate catalyzed by **11** fits the kinetic equation 7. A plot of the observed rate constants k_{obsd} as a function of the pH using 0.1 mM **11** and 10 μM TNP gave a similar sigmoidal curve to that found in the earlier NA hydrolysis with an inflection point at pH 8.3 (see Figure 10). Therefore, the same species **11b** is concluded to react with NA and TNP. The second-order rate constant k_{TNP} (see Eq. [8]) was extremely large: $1.1 \times 10^3 \text{ M}^{-1} \text{ sec}^{-1}$ at 25°C and pH 10.2 with $I = 0.10$ (NaNO_3).

$$v_{\text{obsd}} = k_{\text{obsd}}[\text{total zinc(II) complex}][\text{TNP}] \quad (7)$$

$$= k_{\text{TNP}}[\mathbf{11b}][\text{TNP}] \quad (8)$$

Under the same conditions, the TNP hydrolysis rate constants with Zn^{2+} —cyclen **2b** in the absence and presence of 10 mM Triton X-100 were $3.8 \text{ M}^{-1} \text{ sec}^{-1}$ and $9.0 \times 10^{-2} \text{ M}^{-1} \text{ sec}^{-1}$, respectively. The hydrophilic complex **2**,

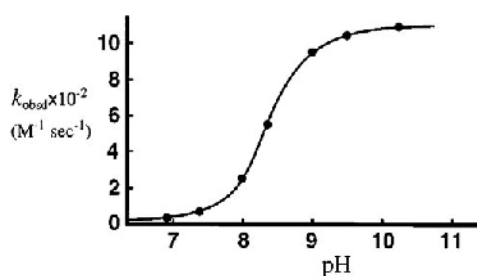


Figure 10 Rate–pH profile for the second-order rate constant (k_{obsd}) in the hydrolysis of TNP (50 μM) catalyzed by **11** (0.1 mM) and Triton X-100 (10 mM) in 10% MeOH aqueous solution at 25°C with $I = 0.10$ (NaNO_3).



most of which remained in aqueous phase, would not attack the TNP partitioned in the comicellar phase. We consider that most of the TNP (>97%) was partitioned in the micelles, since the k_{TNP} decreased to 1/40 of its former value in the presence of micelles. The larger distribution of TNP in the comicellar phase compared to that of 4-nitrophenyl acetate (where the k_{NA} value decreased by a factor of 2 as a result of the presence of Triton X-100 micelles) would be due to the greater lipophilicity of TNP, as suggested by the lower solubility of TNP in 10 mM Triton X-100 aqueous solution. The observed rate enhancement of 290 times by **11b** in the presence of 10 mM Triton X-100 over equimolar **2b** in the absence of Triton X-100 is remarkable. This is translated into a conclusion that TNP (as a model of nerve gases) is much more efficiently destroyed by 1 mM **11b** (in the presence of a surfactant) with a half-life ($t_{1/2}$) of less than 1 sec than by the same concentration of **2b** ($t_{1/2} = 200$ sec). Also, the current experimental finding that the rate decrease of 1/40 in the presence of surfactant by the hydrophilic Zn^{2+} —cyclen **2b** may warn against the conventional wisdom that chemical warfare agent nerve gases [32] would probably be efficiently decontaminated by **2b** in conjunction with surfactant additives. In addition to the supramolecular assembly of **11b** and TNP, the poor solvation of both reactants **11b** and TNP in the comicellar phase would contribute to acceleration of the nucleophilic reaction for the TNP hydrolysis (see Figure 11). Compound **11** in Triton X-100 micelle may be a good mimic of the hydrophobic active center of zinc(II) containing organophosphorus hydrolase.

IV.D. Hydrolysis of Less Lipophilic Bis(4-Nitrophenyl) Phosphate by **11**

The lipophilic zinc(II) complex **11** (0.25–1.0 mM) with 10 mM Triton X-100 also showed phosphodiesterase activity in BNP (5–10 mM) hydrolysis at 35°C with similar kinetic behavior to that exhibited in the NA and TNP hydrolysis. The rate–pH profile curve disclosed an inflection point at pH 8.3 with 1.0 mM **11**, indicating that the kinetically active species was again the zinc(II)-bound OH^- complex **11b** (Scheme 9). The second-order rate constant k_{BNP} (see Eq. [9]) for the hydrolysis of BNP promoted by **11b** was $4.3 \times 10^{-4} \text{ M}^{-1} \text{ sec}^{-1}$ at 35°C and pH 10.2 (20 mM CAPS buffer) with $I = 0.10$ (NaNO_3). The k_{BNP} value for **11b** with 10 mM Triton X-100 is only approximately 20 times larger than the reported k_{BNP} value for **2b** ($2.1 \times 10^{-5} \text{ M}^{-1} \text{ sec}^{-1}$) [9] under the same conditions. The rate enhancement (i.e., 20 times) by the metallomicelles is not as dramatic as that for the more lipophilic TNP (290 times) and NA (50 times), because the less lipophilic nature of BNP relative to that of TNP and NA would result in smaller partition into the metallomicelles.

$$v_{\text{obsd}} = k_{\text{BNP}}[\mathbf{11b}][\text{BNP}] \quad (9)$$



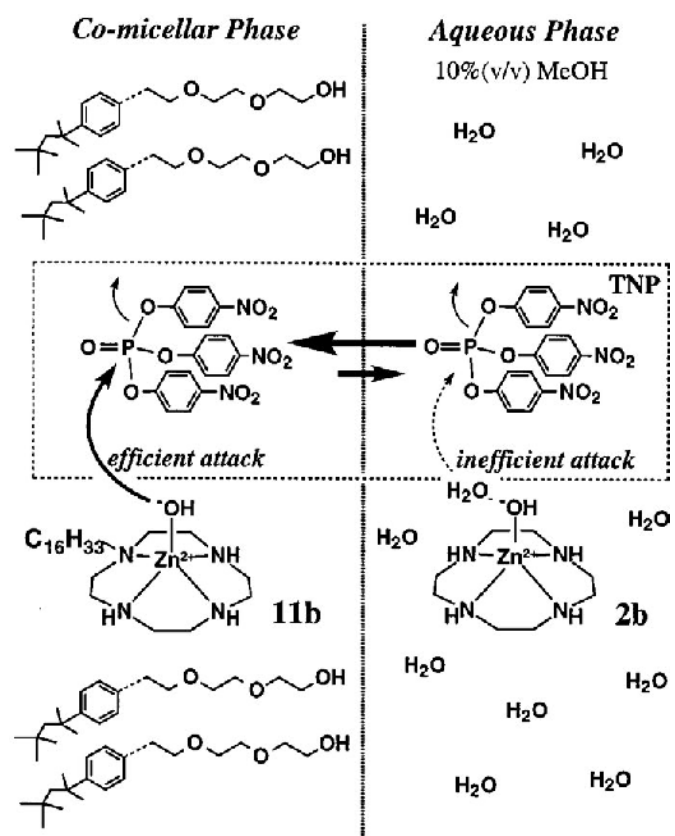
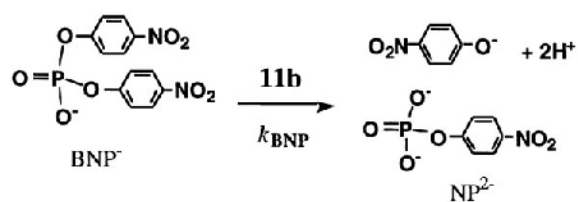


Figure 11 Proposed reaction mechanism for TNP hydrolysis catalyzed by lipophilic and hydrophilic zinc(II)-cyclen complexes, **11b** and **2b**.



Scheme 9 Bis(4-nitrophenyl) phosphate hydrolysis by **11b**.



IV.E. Anion Inhibition of the TNP Hydrolysis

On the basis of the mechanistic study of the well-known inhibition of carbonic anhydrase (a zinc enzyme) by aromatic sulfonamides or anions such as SCN^- [33], we earlier discovered that aromatic sulfonamides, imide-containing derivatives [29,34,35] (thymine, uracil, succinimide, etc.), and inorganic anions are also good inhibitors in our zinc enzyme model study with **1** and **2** [7,8,13]. For instance, succinimide dissociates at physiological pH (despite its $\text{p}K_a$ of about 10) to form a stable $\text{N}^--\text{Zn}^{2+}$ coordination bond to occupy the catalytic site in $\text{Zn}^{2+}-\text{OH}^-$ and thus inhibit the hydrolysis of benzylpenicillin ordinarily catalyzed by **2b** [13]. In the present TNP hydrolysis study, we have also observed strong inhibition by succinimide, phthalimide, and thiocyanate. This fact supports our conclusion that the reactive species is a $\text{Zn}_{2+}-\text{OH}^-$ complex. A special remark concerns phthalimide, which was not soluble enough in an aqueous solution and thus was not studied for its inhibition of ester or β -lactam hydrolysis catalyzed by **2** [13]. In the presence of 10 mM Triton X-100, phthalimide became soluble in 10% MeOH aqueous solution to allow studies of TNP hydrolysis kinetics. The experiments with those inhibitors were conducted with **11** (20 μM) and TNP (10 μM) at 25°C and pH 9.2 (20 mM CHES buffer) with $I = 0.10$ (NaNO_3). The results with various concentrations of these inhibitors are shown in Figure 12. Of succinimide and phthalimide, phthalimide is the stronger inhibitor, as compared by the lower 50% inhibition concentration (ca. 60 vs. < 15 μM).

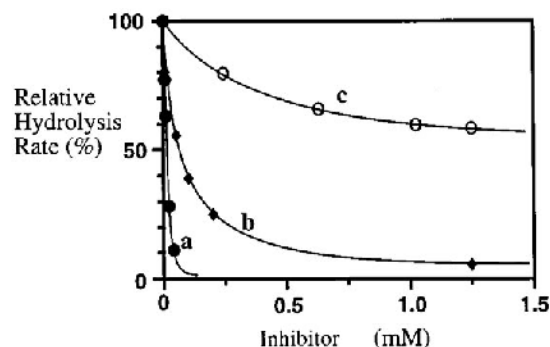


Figure 12 Relative TNP hydrolysis rate catalyzed by **11** (20 μM) and Triton X-100 (10 mM) as a function of the concentration of inhibitor in 10% (v/v) MeOH aqueous solution at 25°C and pH 9.2 (20 mM CHES buffer) with $I = 0.10$ (NaNO_3): (a) for phthalimide, (b) for succinimide, (c) for NaSCN.



V. CONCLUDING REMARKS

Of the reported metallomicellar systems, the Zn^{2+} —hexadecylcyclen complex is the first and most fully characterized lipophilic complex. The isolated species was the crystalline 1:1 ZnL complex **11**. In comicellar solution with the neutral surfactant Triton X-100 (10 mM), complex **11** (1 mM) remains stable up to pH 5 as discrete ZnL—OH_2 **11a** and above neutral pH as discrete ZnL—OH^- **11b** (see the distribution diagram in Figure 6). Near neutral pH partial formation of $(\text{ZnL})_2\text{OH}^-$ **13** had to be taken into consideration on the basis of a potentiometric pH titration and TNP hydrolysis kinetic measurements. The hydroxide-bridged complex **13** is the species that was not observed in the solution of Zn^{2+} —cyclen **2**. Taking into account the equilibria of those three species (see Eqs. [1]–[4]), all the kinetic data for TNP, NA, and BNP hydrolysis were nicely explained. The only reactive species was the Zn^{2+} -hydroxide complex **11b** in the comicelle, which was proved to be catalytically active. The Zn^{2+} — OH^- species is also the sole reactive species with the hydrophilic Zn^{2+} —cyclen complex **2**. Hence, regardless of metallomicelles, the active nucleophile is a Zn^{2+} -bound hydroxide. The lipophilic Zn^{2+} — OH^- species **11b** in micelles is more reactive than the hydrophilic Zn^{2+} — OH^- species **2b** to the lipophilic substrate TNP, as a result of supramolecular assembly in the micelles (i.e., a high local concentration effect) and of more facile reactions between the less solvated reactants in the hydrophobic environment. For a more hydrophilic substrate such as BNP, the lipophilic Zn^{2+} — OH^- species **11b** is not such an outstandingly good catalyst as the hydrophilic Zn^{2+} — OH^- species **2b**. For the first time the kinetic behavior of metallomicelles has been well defined by the new Zn^{2+} —hexadecylcyclen complex **11** in the comicelle, and it deepens our knowledge of the fundamental reaction mechanism of metallomicelles in general. On the basis of the information obtained, metal complexes with even better catalytic properties could be designed. From the practical point of view, although direct comparison is difficult, **11** is presumably one of the best phosphate hydrolyzing catalysts, as well as the best nerve gas detoxifying agent that works at moderate conditions. Since acetylcholinesterase has a hydrophobic entrance for rapid incorporation of acetylcholine into the active center, new lipophilic metal complexes may even efficiently hydrolyze acetylcholine [36]. Other lipophilic phosphodiester and phospholipids may also be hydrolyzed.

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5

Vanadium Haloperoxidases

Alison Butler

University of California, Santa Barbara, California

I. INTRODUCTION

Haloperoxidases (EC group 1.11.1) are enzymes that catalyze the oxidation of a halide (i.e., chloride, bromide, or iodide) by dihydrogen peroxide (generally known as hydrogen peroxide), which results in the halogenation of organic substrates, the production of the corresponding hypohalous acid, or the production of dioxygen by the subsequent reduction of the oxidized halogen species by a second equivalent of dihydrogen peroxide. The nomenclature for the haloperoxidases has historically been based on the most electronegative halide of which the enzyme can catalyze the oxidation by dihydrogen peroxide. Thus chloroperoxidase can catalyze the oxidation of chloride, bromide, and iodide by dihydrogen peroxide; bromoperoxidase can catalyze the oxidation of bromide and iodide by dihydrogen peroxide; and iodoperoxidase can only catalyze the oxidation of iodide by dihydrogen peroxide.

Vanadium bromoperoxidase (V-BrPO) [1,2] has been isolated from many species of marine algae and a terrestrial lichen (see later discussion). Vanadium chloroperoxidase (V-CIPO, EC 1.11.1.10) has recently been discovered in terrestrial fungi [3,4]. In some marine organisms, haloperoxidases are thought to be involved in the biosynthesis of the numerous halogenated marine natural products [5–7]. These compounds include volatile halogenated hydrocarbons [8], chiral halogenated terpenes, and halogenated indoles and phenols, as well as many others [5–9]. In other organisms, e.g., fungi, V-CIPO is thought to function in cell wall degradation, assisting the fungal invasion into the host plant through production of hypochlorous acid [4].

The emphasis of this review chapter will be on the reactivity of the vanadium haloperoxidases. A brief summary of the molecular properties of these en-



zymes will be presented, although, for a more comprehensive discussion of the biophysical properties of these enzymes the reader is referred to other recent reviews [2,9–11] as well as other reviews cited in my chapter in the first edition of this book: Wever and Krenn [12], Butler and Carrano [13], Wever and Kustin [14], and Rehder [15].

II. STRUCTURE OF THE VANADIUM HALOPEROXIDASES

The x-ray structure of vanadium chloroperoxidase (*Curvularia inaequalis*) was solved recently by Messerschmidt and Wever to 2.03 Å resolution [16,17]. Two four-helix bundles form the main structural motif. Vanadate is coordinated at the top of one of these bundles in a broad channel, which is lined on one half with predominantly polar residues and several main-chain carbonyl oxygen atoms (Figure 1). The other half of the channel is hydrophobic, containing Pro47, Pro211, Trp350, Phe393, Pro395, Pro396, and Phe397.

Vanadate is coordinated in a trigonal bipyramidal geometry by hydroxide and His496 in the axial positions and by three nonprotein oxygen atoms in the equatorial plane (Figure 2). Vanadate coordination to the protein is stabilized by multiple hydrogen bonds between the equatorial vanadate oxygen atoms and the

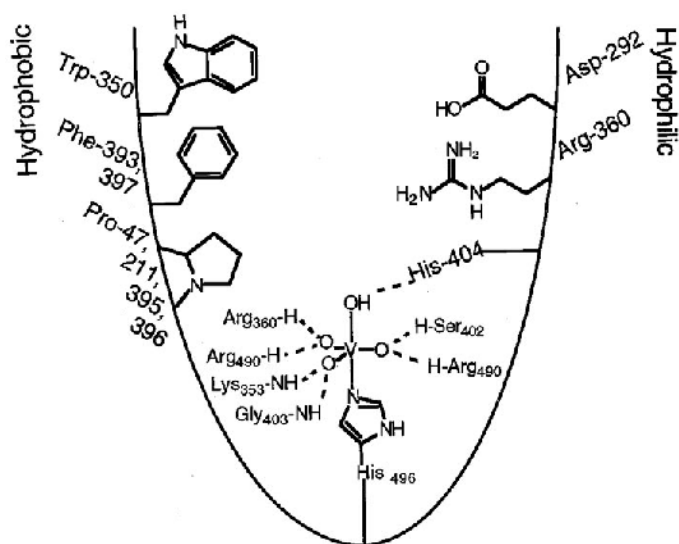


Figure 1 The V-CIPO active site channel.



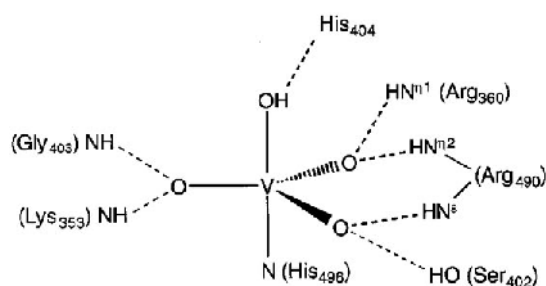


Figure 2 The vanadate site in V-ClPO.

positively charged protein residues Lys353, Arg360, Arg390, and Ser402, as well as the amide nitrogen hydron of Gly403. The apical hydroxide is hydrogen bonded to His404, a residue implicated in catalysis and referred to as the acid/base histidine [16].

The x-ray structure of the peroxide form of V-ClPO reveals a distorted tetragonal pyramid in which vanadium(V) is coordinated by peroxide in an η^2 fashion (1.87 Å V—O bond lengths; 1.47 Å O—O bond length), by His496 (2.19 Å) and an oxygen atom (1.93 Å) in the basal plane, and by an oxo ligand (1.60 Å) in the axial position [16] Figure 3. His404 is no longer hydrogen bonded to the vanadate. One of the peroxide oxygen atoms is hydrogen bonded to Lys353.

Structural features of V-BrPO have not been published, although V-BrPO from *Ascophyllum nodosum* and *Corallina officinalis* has been crystallized [18,19,98]. There is good sequence similarity between V-ClPO (*C. inaequalis*) and V-BrPO (*A. nodosum*), particularly in the active site region [2]. The histidine ligand (His496), the acid–base histidine (His404), and the amino acid residues that hydrogen bond to the equatorial vanadate oxygen atoms are conserved

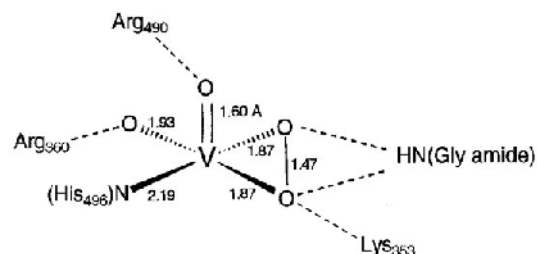


Figure 3 The oxoperoxo-vanadium(V) site in V-ClPO.



[2,17,20]. Extended x-ray absorption fine structure (EXAFS) analysis [21] and bond valence sum analysis [22,23] are consistent with a trigonal bipyramidal vanadium site in V-BrPO (*A. nodosum*). Taken together, these results suggest that the vanadium site in V-BrPO has a trigonal bipyramidal structure like that observed in V-ClPO with coordination to one histidine ligand and multiple hydrogen bonds between the vanadate oxygen atoms and positively charged residues.

The enzyme kinetics (discussed later) show saturation in halide for V-ClPO and V-BrPO, indicating that halide binds to the enzyme. Messerschmidt and Wever [17] propose that the hydrophobic residues Trp350 and Phe397 form a chloride binding site along with His404 in V-ClPO; a hydrophobic binding site for halides is observed in other proteins such as haloalkane dehalogenase [24] and certain amylases [25]. Trp350 is present in V-BrPO (*A. nodosum*), but Phe397 is replaced by a histidine residue in V-BrPO; this suggests that these residues mediate the basis of enzyme's halide specificity.

III. VANADIUM BROMOPEROXIDASE

Vanadium bromoperoxidase (V-BrPO) has been isolated from many species of marine brown algae, including *A. nodosum* [1,26,27], *Laminaria saccharina* [28], *Fucus distichus* [29], and *Macrocystis pyrifera* [29]; the red algae *Ceramium rubrum* [30] and *C. pilulifera* [31]; and a terrestrial lichen, *Xanthoria parietina* [32].

Two vanadium bromoperoxidases that differ in carbohydrate content [26,33] have been isolated from *A. nodosum*. The most abundant bromoperoxidase, V-BrPO-I, was found in the thallus, and the other bromoperoxidase, V-BrPO-II, was reported to be present on the thallus surface [26]. A previous report also concluded that V-BrPO is present in two different locations of *A. nodosum*, one in the cell walls of the transitional region between the cortex and medulla of the thallus and the other in the cell wall of the thallus surface [34]. More recent experiments demonstrate that vanadium-dependent bromoperoxidase activity is present in both the cortical and surface protoplasts of *M. pyrifera* [35], *L. saccharina*, and *L. digitata* [36]. The biosynthesis of V-BrPO in the protoplasts of *L. saccharina* has been shown using [³⁵S]-methionine [36]. The vanadium bromoperoxidases are all acidic proteins [26] with very similar amino acid compositions [37]. V-BrPO (*A. nodosum*) has been crystallized, although refined structural data have not been reported yet [38]. A different isolation procedure, based primarily on a two-phase extraction system, has been described [39,40]. This procedure works well for certain types of algae (e.g., *Laminaria*) but not for the isolation of V-BrPO from *A. nodosum*, the principal source of V-BrPO for the mechanistic studies.

As isolated V-BrPO contains only about 0.4 vanadium atom per subunit;



however, 1 gram-atom of vanadium per subunit can be achieved by addition of excess vanadate and subsequent removal of adventitiously bound vanadium(V) [26,29,41]. A nonheme iron bromoperoxidase was reported to be isolated from certain species of Rhodophyta (marine red algae) [31,42]; however, it has now been shown that a small amount of vanadium contained in these enzymes was responsible for the observed activity [43,44]. In these enzymes, the metal ions can be completely removed and the apoenzyme can be fully reconstituted by addition of vanadate [44].

The standard procedure for removal of active-site vanadium(V) has been to incubate V-BrPO in 0.1 M phosphate-citrate buffer pH 3.8, containing 10 mM ethylenediaminetetra-acetic acid (edta). These conditions remove over 95% of the vanadium, which produces the inactive apo-BrPO derivative [1,45]. The essential component of the apoprotein preparation is the phosphate, without which vanadium is not completely removed and the enzyme is not completely inactivated [46]. In fact phosphate in the absence of edta is sufficient for preparation of apo-BrPO [46]; inactivation by phosphate is much faster at low pH (pH $\sim$ 4) than at neutral or higher pH. However, phosphate inactivation does not occur in the presence of dihydrogen peroxide [46].

The activity of the apoderivative can be fully restored by addition of vanadate [45]. Addition of other metal ions did not restore bromoperoxidase activity [1,2]. Vanadate is only fully incorporated in the absence of phosphate [45]. Like phosphate, molybdate, arsenate, tetrafluoroaluminate (AlF_4^-), and tetrafluoroberyllate (BeF_4^{2-}) are also reported to be competitive inhibitors of bromoperoxidase reactivation by vanadate [47].

III.A. Reactivity of Vanadium Bromoperoxidase with Bromide

The standard assay for haloperoxidase activity is the halogenation of monochlorodimedone (mcd) (2-chloro-5,5-dimethyl-1,3-dimedone) using dihydrogen peroxide as the oxidant of the halide (Figure 4) [48].

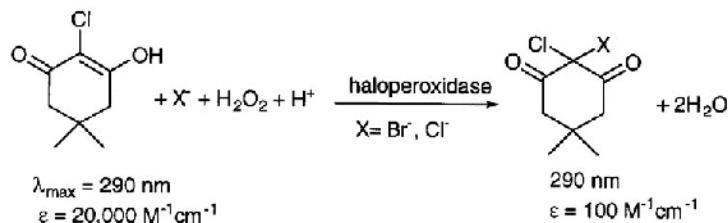


Figure 4 The monochlorodimedone (mcd) reaction for measurement of haloperoxidase activity.



The halogenation of mcd is followed spectrophotometrically at 290 nm. Bromoperoxidase activity is expressed as micromoles of mcd brominated per minute per milligram of enzyme (U/mg). The early work on V-BrPO employed the oxidation of iodide by dihydrogen peroxide [1], forming triiodide (I_3^-), which was followed spectrophotometrically at 353 nm ($\epsilon = 26,400 M^{-1}cm^{-1}$). However, this reaction is less desirable for quantitation of haloperoxidase activity because of competing side reactions, such as the nonenzymatic oxidation of iodide by dihydrogen peroxide and reduction of triiodide by dihydrogen peroxide (discussed later). The specific activity of mcd bromination for V-BrPO isolated from *A. nodosum* is 170 U/mg (at pH 6.5, 2 mM H_2O_2 , 0.1 M Br^- , 50 μM mcd, 0.2 M Na_2SO_4). The specific activities for the *M. pyrifer* and *F. distichus* enzymes are 1730 U/mg (pH 6, other conditions same as specified) and 1580 U/mg (pH 6.5, other conditions same as specified), respectively [28].

III.A.1. The Selectivity of Bromination and the Bromide-Assisted Disproportionation of Dihydrogen Peroxide

The stoichiometry of the V-BrPO-catalyzed bromination reaction is the oxidation of one equivalent of bromide by one equivalent of dihydrogen peroxide, producing one equivalent of brominated organic substrate:



Monochlorodimedone, which is the classic substrate for the characterization of haloperoxidase activity [48], has been used widely in the kinetic and mechanistic investigations of V-BrPO [e.g., 33,49,50]. The extent of mcd bromination by V-BrPO in the presence of an equimolar competing substrate is given in Table 1 [51]. Most of the indoles examined were brominated preferentially over mcd, with the exception of indole-3-acrylic acid, 5-amino-indole, and indoxyl- β -D-glucoside. Cytosine, *trans*-cinnamic acid, and phenol red were found to be very poor substrates when compared to mcd, as supported by the observation of concomitant formation of O_2 in the case of cytosine and phenol red (Soedjak and Butler, unpublished results). Thus, although V-BrPO can effect the bromination of a variety of organic substrates, many are not halogenated efficiently since more dihydrogen peroxide is consumed than halogenated product produced.

In the absence of an organic halogen acceptor, the oxidized bromine intermediate is reduced by a second equivalent of dihydrogen peroxide, producing bromide and dioxygen [52]. The net reaction is the disproportionation of dihydrogen peroxide to dioxygen and water



although the efficient catalytic reaction requires the presence of a halide (particularly bromide and chloride; discussed later) to catalyze this process. The stoichi-



Table 1 Substrate Specificity of V-BrPO^a

Competing substrate ^b	mcd reacted (%)	Competing substrate	mcd reacted (%)
Cytosine	99.0	Indole-3-acetic acid	47.2
<i>Trans</i> -cinnamic acid	98.9	Farnesol	46.6
Phenol red	97.4	3-Methylindole	32.9
Indole-3-acrylic acid	62.0	5-Hydroxyindole	23.8
5-Aminoindole	60.1	2-Phenylindole	18.9
Indoxyl- β -D-glucoside	59.8	2- <i>tert</i> -Butylindole	5.8
1,3,5-trimethoxybenzene	57.0	2-Methylindole	0.5
Indole-3-methanol	47.6		

^a Reaction conditions: 970 μ M H₂O₂, 50 mM KBr, 50 μ M mcd, 50 μ M competing substrate, and 2 nM V-BrPO in 100 mM sodium phosphate buffer, pH 6.5, with 10% ethanol.

^b The products of the V-BrPO catalyzed reaction of 2-phenylindole, 2-methylindole, and 3-methylindole are 3-bromo-2-phenylindole, 3-bromo-2-methylindole, and 3-methyl-2-oxindole, respectively [57]. Cytosine and 1,3,5-trimethoxybenzene are brominated to 5-bromocytosine and 2-bromo-1,3,5-trimethoxybenzene [29]. Farnesol is converted to the terminal bromohydrin (Butler and McAdara, unpublished results). The products of 2-*tert*-butylindole, *trans*-cinnamic acid, indole-3-acrylic acid, 5-aminoindole, indoxyl- β -D-glucoside, indole-3-methanol, and indole-3-acetic acid have not been characterized yet.

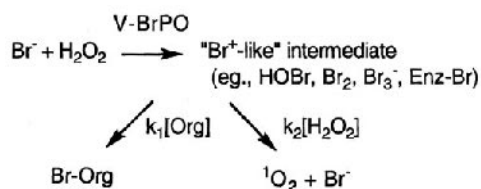
ometry of the V-BrPO-catalyzed bromide-assisted disproportionation of dihydrogen peroxide has been shown to be one equivalent of dioxygen produced per two equivalents of dihydrogen peroxide reacted [53]. Bromide-selective electrode measurements demonstrate that bromide is a catalyst in the bromide-assisted disproportionation of dihydrogen peroxide; the bromide concentration at the end of the reaction is the same as its initial concentration, although during turnover the bromide concentration is reduced [52].

Nature of the Dioxygen and Origin of the Oxygen Atoms. The dioxygen produced in the bromide-assisted disproportionation of dihydrogen peroxide is in the singlet excited state (¹O₂; ¹ Δ_g), as established by analysis of the near-infrared emission spectrum (i.e., λ_{max} 1268 nm), the stoichiometric yield of ¹O₂, the solvent isotope effect on the lifetime of ¹O₂ (H₂O vs. D₂O), and the reduced emission intensity in the presence of specific ¹O₂ quenchers (i.e., azide, histidine) [33]. Singlet oxygen is often detected by chemical trapping means (for a review see Ref. [54]). Use of these traps with haloperoxidases does not provide proof of singlet oxygen production since oxidized halide species (e.g., HOCl, HOBr) can oxidize the traps, particularly the furan derivatives, producing the same product as singlet oxygen oxidation. Also consistent with singlet oxygen formation is

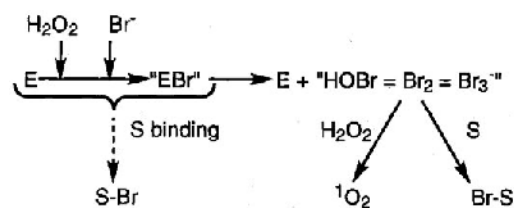


The Minimal Reaction Scheme. A minimal scheme that describes the overall reactivity of V-BrPO is shown in Scheme 1 [50,52]. The $k_1[\text{mcd}]$ reaction is competitive with the $k_2[\text{H}_2\text{O}_2]$ reaction [50], since the sum of the rate of dioxygen formation during mcd bromination and the rate of mcd bromination is equal to the rate of dioxygen formation in the absence of mcd [55]. The reaction of dihydrogen peroxide with any of the proposed intermediates in Scheme 1 is consistent with dioxygen formation in the singlet excited state. Singlet oxygen is produced from the reduction of aqueous solutions of bromine (i.e., an equilibrium mixture of $\text{HOBr}/\text{Br}_2/\text{Br}_3^-$ in aqueous solution) and bromamine species by dihydrogen peroxide [56].

Nature of the Halogenating Intermediate. The selectivity observed in Table 1 and the overall reactivity presented in Scheme 1 raise several questions. For example, is the oxidized halogen intermediate enzyme bound, or is it released from the enzyme active site? Does the organic substrate bind to V-BrPO? The selectivity may be achieved by substrate binding to V-BrPO, which could arise from an enzyme-trapped oxidized halogen species. The oxidized halogen species has not been detected under optimal turnover conditions; it does not build up in solution because of its fast reaction with organic substrates or with excess H_2O_2 producing O_2 . In fact, competitive kinetic studies that compare the reactivity of the V-BrPO/ H_2O_2 /KBr system with that of HOBr demonstrate that the nature of the halogenating species produced by V-BrPO depends on the nature of the organic substrate



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Scheme 2 The modified scheme for V-BrPO reactivity: substrate binding.

[57]. For example, V-BrPO does not release an oxidized bromine species (e.g., HOBr, Br_2 , Br_3^-) in the presence of certain indole derivatives, because these indoles bind to V-BrPO. (Halogenated indoles are marine natural products.) Fluorescence quenching results further confirm that substituted indoles bind to V-BrPO [57]. The Stern–Volmer analysis revealed a binding constant of $1.1 \times 10^5 M^{-1}$ for 2-phenylindole to V-BrPO (*A. nodosum*) (Tschirret-Guth and Butler, unpublished results). A mechanistic scheme involving substrate binding is shown in Scheme 2 [57]; V-BrPO binds H_2O_2 and Br^- , leading to a putative “enzyme-bound” or “active-site trapped” brominating moiety, “E-Br,” which in the absence of an indole may release HOBr (or other bromine species, e.g., Br_2 , Br_3^-). When the indole is present, it binds to V-BrPO, preventing release of an oxidized bromine species and leading to indole bromination.

III.A.2. Steady-State V-BrPO Enzyme Kinetics

Because mcd is the usual substrate used to evaluate haloperoxidase activity, it was used as the halogen acceptor in steady-state enzyme kinetic analyses. Detailed steady-state analyses of the rate of mcd bromination [49,50] and dioxygen formation [29,50,58] catalyzed by V-BrPO from *A. nodosum*, *M. pyrifera*, and *F. distichus* fit a substrate-inhibited bi–bi Ping-Pong kinetic mechanism, in which the substrates bromide and dihydrogen peroxide can also act as inhibitors at certain pH's. Bromide is a noncompetitive inhibitor of dihydrogen peroxide binding to V-BrPO at pH 4–5.5 [49,50,58]. The kinetic parameters (K_m^{Br} , $K_m^{H_2O_2}$, K_{ii}^{Br} , K_{is}^{Br}) obtained in the dioxygen formation reaction and the mcd bromination reaction agree within a factor of ca. 2, providing further evidence that the rate limiting steps in both reactions are the same [50]. Bromide inhibition is strongest about pH 5–5.5 for V-BrPO from the three sources examined [50,58]. The inhibition constants for dihydrogen peroxide could only be determined for the bromide-assisted dioxygen formation reaction since high concentrations of dihydrogen peroxide were required. Under these conditions the kinetics of mcd bromination were complicated by the competing dioxygen formation reaction. Dihydrogen peroxide inhibition is strongest at pH 7–8. H_2O_2 inhibition is noncompetitive at



all pH's, and $K_{ii}^{H_2O_2}$ is equal to $K_{is}^{H_2O_2}$, showing that bromide binding does not affect the inhibition by H_2O_2 [53]. Fluoride is also a competitive inhibitor of H_2O_2 binding in both the mcd bromination reaction and the bromide assisted disproportionation of dihydrogen peroxide [50]. Fluoride inhibition is uncompetitive with respect to bromide binding [50].

The steady-state kinetic studies of both V-BrPO and V-ClPO (discussed later) suggest that a residue with pK_a of 5.7–6.5 is important for reactivity [49,50,55,59]. Under peroxide inhibition conditions of V-BrPO (*A. nodosum*), the steady-state kinetic constants indicate that an ionizable group with a pK_a between 6.5 and 7 is involved in the inhibition [55]. This residue must be dehydrated prior to binding of dihydrogen peroxide. It seems reasonable to assume, given these pK_a values, that this residue is histidine, i.e., specifically the analogous histidine in V-BrPO to His404 in V-ClPO.

III.A.3. Inactivation of V-BrPO by H_2O_2 and Formation of 2-Oxohistidine

As discussed, H_2O_2 is a fully reversible, noncompetitive inhibitor of V-BrPO when the steady-state kinetics are observed during the initial portion of the reaction and when the pH is >5.5 [55]. If the reaction proceeds for longer times (e.g., >10 min), the specific activity of V-BrPO decreases. The inactivation that occurs on prolonged turnover between pH 6 and 8 can be fully reversed by the addition of vanadate, suggesting that protein-bound vanadium is released under turnover at high concentrations of dihydrogen peroxide, forming the apoenzyme derivative. Vanadium release was confirmed by atomic absorption analysis. At pH 4 and 5, inactivation also occurs under turnover, accompanied by the release of vanadium, but this turnover is not reversed on addition of vanadate.

The irreversible inactivation of V-BrPO that occurs at low pH [55] was found to produce 2-oxohistidine as identified by high-performance liquid chromatography (HPLC) using electrochemical detection [60]. Inactivation of V-BrPO and formation of 2-oxohistidine require all the components of turnover (i.e., bromide, dihydrogen peroxide, and V-BrPO) as well as low pH (Figure 5). Neither

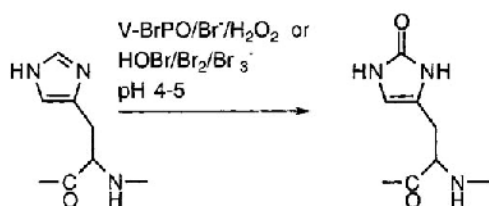


Figure 5 Formation of 2-oxohistidine by V-BrPO and aqueous bromine.



inactivation of V-BrPO nor 2-oxohistidine formation is observed in the presence of dihydrogen peroxide alone. Moreover, the inactivation and 2-oxohistidine formation are not the result of oxidation by singlet oxygen [60]. The oxidation of histidine to 2-oxohistidine turns out to be mediated by aqueous bromine species, as confirmed by the formation of *N*^α-benzoyl-2-oxohistidine upon addition of aqueous bromine to *N*-benzoylhistidine at low pH (Figure 5). When hypobromite is added to *N*^α-benzoylhistidine in the presence of dihydrogen peroxide at neutral pH, conditions under which HOBr would react with H₂O₂ to form ¹O₂, the formation of *N*^α-benzoyl-2-oxohistidine is not observed. In addition, several functional mimics of V-BrPO (*cis*-VO₂⁺ in strong acid, MoO[O₂]₂ [oxalato]²⁻ at pH 5; discussed later) catalyze the bromide and dihydrogen peroxide mediated oxidation of *N*^α-benzoylhistidine to *N*^α-benzoyl-2-oxohistidine [60].

III.A.4. Peroxide Selectivity

In addition to dihydrogen peroxide, V-BrPO is able to use peracids (i.e., peracetic acid, *m*-chloroperoxybenzoic acid, *p*-nitroperoxybenzoic acid, phenyl peracetic acid) to catalyze the bromination of organic substrates (i.e., cytosine, mcd, amines) [29]. Unlike in the reactions using dihydrogen peroxide, the uncatalyzed rate of bromide oxidation by peracetic acid at pH ≤ 6.5, i.e., the pH range used for V-BrPO studies, is appreciable; however, the V-BrPO-catalyzed reactions are much faster, particularly in the higher pH range [29]. Dioxygen is not formed from peracids because peracids do not readily reduce the oxidized bromine species (e.g., HOBr, Br₂, Br₃⁻, BrNHR) in the time frame of the enzymatic reactions (i.e., several minutes). It has been established that peracid reactivity actually arises from direct use of peracid and not from dihydrogen peroxide, which could be formed by the hydrolysis of the peracids [29].

The interesting feature of the V-BrPO reactions with peracetic acid in the presence of amines (i.e., added amines or amine-containing buffers) is the formation of bromamines (BrNHR) [29]. Bromamines have been proposed as possible intermediates in haloperoxidase reactions [61,62]; however, their direct detection had not been reported previously. Bromamine formation is not observed when the peroxide source is dihydrogen peroxide because the bromamine is rapidly reduced by dihydrogen peroxide, forming dioxygen and bromide.

It is not possible to establish whether amines are preferentially brominated over organic substrates in the V-BrPO reactions. Bromamine buildup is not observed under V-BrPO-catalyzed conditions when an appropriate halogen acceptor is present, either because the organic substrate is brominated before bromamine formation or because the bromamine concentration never builds up to detectable levels because of very rapid substrate bromination by the bromamine. If bromination of mcd is carried out with peracetic acid as the source of peroxide and in the presence of 3 mM tris buffer, Br-tris is only observed after the mcd has been



completely brominated. This does not mean that mcd is brominated preferentially, because bromamine bromination is a fast reaction. *N*-bromo-tris brominates mcd within the time of rapid hand mixing. Moreover, the rates of mcd bromination by peracetic acid and bromide in various buffers (e.g., tris, hydrogen carbonate, phosphate, Capso) at pH 8.5 are identical. Bromocapso, bromotaurine, and *N*-bromosuccinimide, like *N*-bromo-tris, are efficient brominating agents. Thus, the mechanisms of bromination via the intermediate pathway of amine-buffer bromination versus direct bromination of mcd cannot be distinguished.

Alkyl hydroperoxides, including ethyl hydroperoxide, cuminyl hydroperoxide, and *tert*-butyl hydroperoxide, are not used by V-BrPO to catalyze bromination reactions [29]. These alkyl hydroperoxides have the thermodynamic driving force to oxidize bromide; however, they are kinetically slow. Several examples of vanadium(V) alkyl peroxide complexes have been well characterized [63], including $[V^{(V)}O(OOR)(\text{oxo-2-oxidophenyl})\text{ salicylidenaminato}]$ ($R = t\text{-Bu}$, CMe_2Ph), which has been used in the selective oxidation of olefins to epoxides. The synthesis of these compounds seems to require elevated temperatures, and their oxidation under catalytic conditions has not been reported. We have found that alkyl hydroperoxides do not coordinate to vanadate in aqueous solution at neutral pH, conditions under which dihydrogen peroxide readily coordinates to vanadate and vanadium(V) complexes (de la Rosa and Butler, unpublished observations). Thus, the lack of bromoperoxidase reactivity with the alkyl hydroperoxides may arise from slow binding of the alkyl hydroperoxides to V-BrPO.

III.B. Reactivity of V-BrPO with Other Halides and Pseudohalides

III.B.1. Chlorination and Chloride-Assisted Disproportionation of Dihydrogen Peroxide

In addition to bromide and iodide, V-BrPO can catalyze the oxidation of chloride [64]. As mentioned previously and discussed more fully later, a distinct enzyme, vanadium chloroperoxidase, has also been discovered. Originally it was thought that V-BrPO could only catalyze the oxidation of bromide and iodide by dihydrogen peroxide. In fact, under the standard mcd bromoperoxidase assay conditions, in which the V-BrPO concentration is ca. nanomolar, very little, if any, chlorination of mcd is observed. However, it seemed very unusual that V-BrPO could be inhibited by fluoride and bromide, but apparently not by chloride [27]. In reinvestigating the halide specificity of V-BrPO, it was discovered that when the enzyme concentration is increased 100-fold to $0.1\ \mu\text{M}$, chlorination is observed at an appreciable rate [64]. The specific chloroperoxidase activity is $0.76\ \text{U/mg}$ (under conditions of $1\ \text{M}$ certified 100% bromide-free KCl, $2\ \text{mM}\ \text{H}_2\text{O}_2$, $50\ \mu\text{M}$



mcd in 0.1 M citrate buffer, pH 4.5), which is ca. 200 times less than the maximum specific bromoperoxidase activity.

In addition to mcd, V-BrPO catalyzes the chlorination of phenol red to tetrachlorophenol blue [64]. Chlorination of amines (e.g., taurine, ammonia, valine, serine, leucine) is also catalyzed by V-BrPO forming the stable chloramine derivative, even in the presence of dihydrogen peroxide. Unlike bromamines, the chloramine is not reduced by dihydrogen peroxide, or is reduced only very slowly [53].

In the absence of an organic substrate, V-BrPO catalyzes the chloride-assisted disproportionation of dihydrogen peroxide, forming dioxygen. The rate of dioxygen formation is equal to the rate of mcd chlorination, suggesting that the rate-limiting step for both reactions is the formation of a common intermediate (e.g., the oxidized chloride species) analogous to the reactions with bromide (see Scheme 1). The rate of the chloride-assisted dioxygen formation is inhibited by amines (e.g., taurine), as is consistent with chloramine formation, since dihydrogen peroxide cannot reduce chloramine to form dioxygen and chloride [53], although taurine does not inhibit mcd chlorination. By contrast, the bromide-assisted disproportionation of dihydrogen peroxide is not inhibited by amines, because dihydrogen peroxide rapidly reduces bromamine compounds, forming singlet oxygen [29,53]. Consequently, the rates of mcd bromination and bromide-assisted disproportionation of dihydrogen peroxide (in the absence of mcd) are equal, independently of the presence of amine.

Previously proposed mechanisms of the biosynthesis of certain chlorinated compounds have invoked electrophilic bromination of alkenes followed by passive chloride attack [62]. Although this mechanism could explain the origin of adjacent brominated and chlorinated carbons, it does not readily account for compounds containing chlorine only. Thus, with the discovery of chloroperoxidase activity of the vanadium enzyme, the origin of specific chlorinated marine natural products can now be addressed.

III.B.2. Oxidation of Pseudohalides Catalyzed by V-BrPO

Thiocyanato [65–67] and isothiocyanato [68,69] organic compounds are a newly discovered class of marine natural products with potent antifungal, antibacterial, ichthyotoxic, and other biological activities. It has been proposed that the thiocyanate anion is directly involved in the biosynthesis of some of these compounds [65]. Indeed, V-BrPO catalyzes the thiocyanation of 1,2-dimethylindole and 1,3,5-trimethoxybenzene to 1,2-dimethyl-3-thiocyanato-indole and 1-thiocyanato-2,4,6-trimethoxybenzene, respectively, in a process in which V-BrPO first catalyzes the oxidation of NCS^- by H_2O_2 [70]. ^{13}C nuclear magnetic resonance (NMR) of the oxidation of KS^{13}CN (133.4 ppm vs. TMS) by H_2O_2 catalyzed by V-BrPO shows the production of several oxidized thiocyanate species, including



hypothiocyanate (OSCN⁻; 128.6 ppm), thiooxime (SCNO⁻ or SCNOH; 156.5 ppm), and the putative dithiocyanate ether anion (NCS-O-SCN⁻; 127.6 ppm), which is unstable, as well as hydrogen carbonate (HCO₃⁻; 160.2 ppm) [70]. These species, with the exception of hydrogen carbonate, could mediate the electrophilic thiocyanation reactions.

IV. VANADIUM CHLOROPEROXIDASE

IV.A. Haloperoxidase Activity of V-ClPO

Since the publication of the first volume of *Bioinorganic Catalysis*, a new enzyme, vanadium chloroperoxidase (V-ClPO), has been found to be produced by certain terrestrial fungi (e.g., *Curvularia inaequalis* and other dematiaceous hyphomycetes) [4]. Because halogenated natural products have not been identified in the fungi that produce V-ClPO, and because this enzyme is secreted from the fungus and produces hypochlorous acid (HOCl), it is speculated that the physiological function of V-ClPO is to aid the invasion of the fungus into the plant cell wall [4].

Vanadium chloroperoxidase (*C. inaequalis*) is a 67,488 Da protein that comprises 609 amino acid residues, as determined from deoxyribonucleic acid (DNA) sequence analysis [20]. As isolated, V-ClPO may contain a variable content of vanadium, depending on the concentration of vanadate in the growth medium; however, one vanadium(V) per subunit can be achieved by addition of excess vanadate to the growth medium or to the purified protein [3,71]. Like V-BrPO, V-ClPO is stable in the presence of organic substrates, to elevated temperatures, and to the presence of high concentrations of strong oxidants (e.g., HOCl) [59].

The reactivity of V-ClPO (*C. inaequalis*) has not been investigated as extensively as has that of V-BrPO (*A. nodosum*). However, the oxidized halogen intermediate for V-ClPO (*C. inaequalis*) has been identified because it builds up in solution under its turnover conditions (e.g., 0.2 mM H₂O₂, 1 mM Cl⁻, 64 nM V-ClPO in 0.1 M phosphate buffer, pH 4.5) [59]. V-ClPO generates HOCl, which can be detected spectrophotometrically. In addition, HOCl can be separated from the reaction solution by ultrafiltration. Since only ca. 50 mM HOCl was detected when starting with an initial concentration of 200 mM H₂O₂, it was inferred that some reduction of HOCl by H₂O₂ could have occurred. Thus the overall reactivity in Scheme 1 likely holds for V-ClPO with chloride.

The mcd chlorination kinetics for V-ClPO (*C. inaequalis*) also fit a substrate inhibited bi-bi Ping-Pong mechanism. The kinetic constant for chloride, $K_m^{Cl^-}$, is reported to be 0.25 mM at pH 4.5 [3]. The kinetic constant for dihydrogen peroxide, $K_m^{H_2O_2}$, varies as a function of pH: 0.5 mM at pH 3.2 to 0.01 mM at pH 5 [59]. As with V-BrPO (*A. nodosum*), chloride is both a substrate for and



an inhibitor of V-ClPO [3,59]. At pH 3.1, chloride inhibition is competitive: K_i^{Cl} is 6 mM; at pH 4.1 chloride inhibition becomes noncompetitive. Nitrate also inhibits V-ClPO, competitively with respect to chloride and uncompetitively with respect to dihydrogen peroxide at pH 5.5. The K_i^{nitrate} is 2 mM at pH 5.5.

IV.B. Phosphatase Activity of V-ClPO

Recently the amino acid sequence of vanadium chloroperoxidase was determined to have similar stretches with three families of acid phosphatases, which were previously considered unrelated [72]. This sequence raises questions about the phosphatase activity of apo-V-ClPO and whether the acid phosphatases can coordinate vanadate and carry out peroxidative halogenation chemistry. In fact, apo-V-ClPO does have phosphatase activity, catalyzing the hydrolysis of *p*-nitrophenol phosphate (*p*-NPP). In addition, *p*-NPP displaces vanadate from V-ClPO. At this point, the haloperoxidase activity of the acid phosphatases containing coordinated vanadium(V) has not been reported.

V. FUNCTIONAL MIMICS OF THE VANADIUM HALOPEROXIDASES

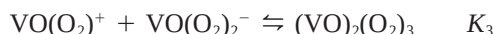
Initial studies on functional mimics of V-BrPO were driven by the lack of spectroscopic techniques capable of observation of the vanadium(V) site in the enzyme. Early on it was found that acidic solutions of *cis*-dioxovanadium(V) (*cis*-VO₂⁺) catalyzed the oxidation of halides by dihydrogen peroxide, resulting in halogenation of an organic substrate *and* halide-assisted disproportionation of dihydrogen peroxide [73].

When ⁵¹V nuclear magnetic resonance (NMR) was used to follow the catalysis of trimethoxybenzene (tmb) bromination, the only vanadium species that were observed under conditions of 0.5 mM total vanadium(V) were oxodiperoxovanadium(V) (VO(O₂)₂⁻, -688 ppm), oxoperoxovanadium(V) (VO(O₂)⁺, -529 ppm), and *cis*-dioxovanadium(V) (-540 ppm). Within the experimental error of the integration, all of the vanadium was detected in the vanadium(V) oxidation state under turnover conditions, since the integrated signal intensity at various times throughout the reaction was equivalent to that of an equimolar solution of *cis*-VO₂⁺.

In this biomimetic system, *cis*-VO₂⁺ coordinates dihydrogen peroxide, forming the monoperoxo or diperoxo species, in ratios dependent on the dihydrogen peroxide and acid concentrations [74].



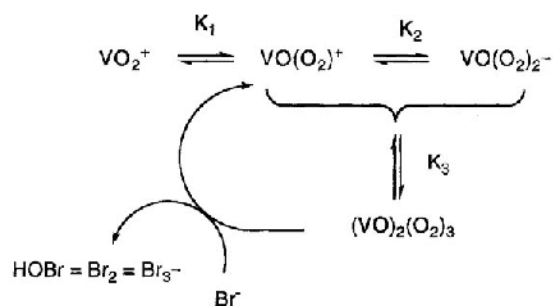
The equilibrium constant for coordination of the first equivalent of H_2O_2 to VO_2^+ is $3.7 \times 10^4 \text{ M}^{-1}$ and is independent of pH [75]. The binding constant for the second equivalent of H_2O_2 is 0.6 M and depends on pH with significantly less peroxide coordination at higher acid concentration [63]. Since the publication of the first edition of *Bioinorganic Catalysis*, the specific oxidant of bromide has been identified:



Neither $\text{VO}(\text{O}_2)^+$ nor $\text{VO}(\text{O}_2)_2^-$ oxidizes bromide; rather they associate to form the dinuclear $(\text{VO})_2(\text{O}_2)_3$, which oxidizes bromide (Scheme 3) [76]. The association constant for the dinuclear compound is rather small ($K_3 = 9 \text{ M}^{-1}$ at pH 0–2); this explains why it is not observed by ^{51}V NMR at low concentrations of vanadium [73,76]. The dinuclear compound has now been observed by ^{51}V NMR at very high concentrations of vanadium [76]. A complex of $\text{V}_2\text{O}_2(\text{O}_2)_3(\text{HGly})_2(\text{H}_2\text{O})_2$ has been isolated in which a μ -peroxo coordination of one peroxide is proposed [76].

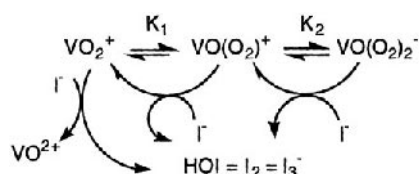
Other groups have also examined vanadium(V) catalyzed oxidation of bromide by dihydrogen peroxide in acidic aqueous or aqueous/organic mixtures, although without examining the detailed speciation of the vanadium peroxo compounds in solution [77–82]. These reports have focused more on the nature of the substrate brominated and the product distribution under different conditions.

Analogous to the preceding *cis*-dioxovanadium(V)-catalyzed system of bromide oxidation by dihydrogen peroxide, Secco carried out a detailed kinetic analysis of vanadate-catalyzed oxidation of iodide by dihydrogen peroxide [75]. Peroxovanadate species (i.e., $\text{VO}[\text{O}_2]^+$ and $\text{VO}[\text{O}_2]_2^-$) or their hydronated forms oxidize iodide in acidic aqueous solution, forming VO_2^+ and $\text{VO}(\text{O}_2)^+$, respectively; however, once $\text{VO}(\text{O}_2)^+$ and $\text{VO}(\text{O}_2)_2^-$ are consumed, iodide reduces



Scheme 3 The catalytic cycle for peroxidative bromination catalyzed by *cis*- VO_2^+ .

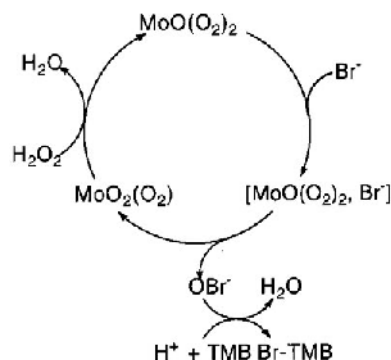




Scheme 4 The catalytic cycle for peroxidative iodination catalyzed by *cis*-VO₂⁺.

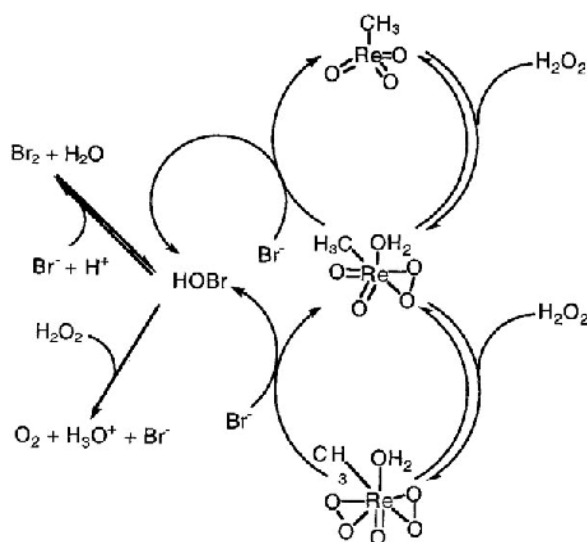
VO₂⁺, forming the vanadyl (VO²⁺) species (Scheme 4). The results of Secco's spectrophotometric investigation were largely confirmed by ⁵¹V NMR followed under similar catalytic conditions [83].

As a result of the simple vanadate site in V-ClPO, it is relevant to examine the peroxidative halogenation reactions of other simple transition metal complexes, such as MoO₃ (Scheme 5) [84], WO₃ [84], and MeReO₃ (Scheme 6) [85,86]. In all of these systems, a mononuclear metal peroxo species oxidizes the halide, in acidic aqueous solution. Recently we discovered that transition-metal-ion-grafted mesoporous silicate materials catalyze peroxidative halogenation reactions at neutral pH [87]. These materials include Mⁿ⁺/MCM-48 and Mⁿ⁺/MCM-41, where the transition metal ion can be Ti(IV), W(VI), Mo(VI), or V(V), among others. Ti/MCM-48 contains a three-dimensional bicontinuous cubic pore array [88–90] within the silicate structure onto which Ti(IV) has been grafted. Ti/MCM-41 contains a one-dimensional hexagonal pore array onto which Ti(IV) has been grafted [91]. These silicate materials have large surface areas (1000–1600 m²/g) and variable pore diameters (20–150 Å), which make them attractive in chemical sensor and catalytic applications. Particularly attractive is their activ-



Scheme 5 The catalytic cycle for peroxidative bromination catalyzed by MoO₃.





Scheme 6 The catalytic cycle for peroxidative halogenation catalyzed by MeReO_3 .

ity in aqueous solution at neutral pH and in organic solvents. The reactivity at neutral pH suggests that bound M^{n+} -peroxide must be activated by hydrogen bonding interactions from a neighboring silanol hydroxyl group or the titanol (in the case of the Ti/MCM materials) hydroxyl group.

Coordination compounds of vanadium(V) also catalyze peroxidative halogenation reactions where the reactive oxidant is a monoperoxo complex of a mononuclear vanadium compound (Figure 6) [11,22,92–95,99].

The second-order rate constants for the oxidation of bromide by various peroxo metal species are summarized in Table 2. The peroxo-V-BrPO complex has the largest rate constant for the oxidation of bromide, most likely reflecting the influence of the “acid/base” histidine (i.e., His404 in V-ClPO from *C. inaequalis*). His404 lies in hydrogen bonding distance of vanadium(V). It may assist the reductive cleavage of coordinated peroxide upon nucleophilic attack by bromide. None of the model complexes has this feature built in. The relatively large rate constants for the oxidation of bromide by $\text{VO}(\text{O}_2)(\text{Hheida})$ and $\text{VO}(\text{O}_2)(\text{bpg})$ that were observed in acetonitrile, but not water, may be a result of the hydration of the side-on bound peroxide that occurs in acetonitrile, but not water [95]. The relatively large rate constant for rhenium over vanadium and molybdenum may reflect the greater oxophilicity of high-valent methylrhenium than of vanadium or molybdenum.

A vanadium system that catalyzes the oxidation of hydrogen bromide by



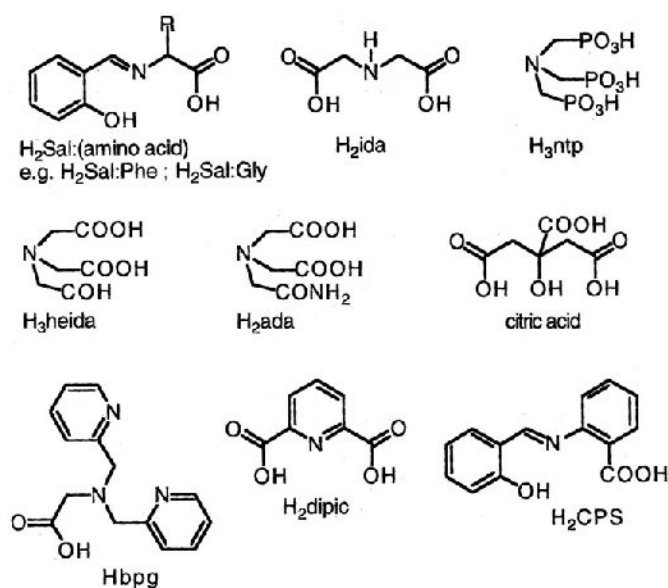


Figure 6 Ligands of the vanadium(V) complexes.

Table 2 Second-Order Rate Constant for the Oxidation of Bromide by Peroxo Metal Species

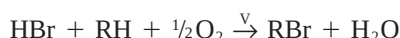
Oxidant	Conditions	Rate constant $M^{-1}\text{sec}^{-1}$	Reference
V-BrPO-(O ₂)	pH 7.9 ^a	2.78×10^3	49
	pH 4.0 ^a	1.75×10^5	49
MeReO ₂ (O ₂)	pH 0	350	85
MeReO(O ₂) ₂	pH 0	190	85
VO(O ₂)(Hheida)	CH ₃ CN ^b	280	95
VO(O ₂)(bpg)	CH ₃ CN ^b	21	95
(VO) ₂ (O ₂) ₃	pH 0.7–2.0	4.1	76
MoO(O ₂) ₂ (H ₂ O) ₂	pH 1.0–5.1	1.5×10^{-2}	84
MoO(O ₂) ₂ (H ₂ O)(OH) ⁻	pH 1.0–5.1	2.4×10^{-3}	84
MoO(O ₂) ₂ (C ₂ O ₄) ²⁻	pH 5.1 with 20% MeOH	9.2×10^{-3}	84
	pH 5.0	4.9×10^{-3}	99

^a Lower limit as calculated by $k_{\text{cat}}/K_{\text{m}}^{\text{H}_2\text{O}_2}$.

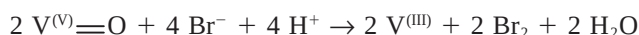
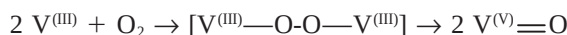
^b Bromide oxidation did not occur in water.



dioxygen forming molecular bromine (Br_2) is sodium vanadate and tetraglyme in 1,2-dichloroethane [96]. Vanadium functions as an electron transfer catalyst, cycling between V(V) and V(III) with a turnover rate for the bromination of various organic substrates (1-octene, styrene, anisole, ethoxybenzene) of ca. 100 mol Br_2 /mol V hr^{-1} . The stoichiometry of the reaction is



The electron transfer role of vanadium has possible relevance to vanadium bromoperoxidase, although this system and V-BrPO differ in that V-BrPO requires dihydrogen peroxide for catalytic activity. A speculative catalytic cycle has been proposed to be



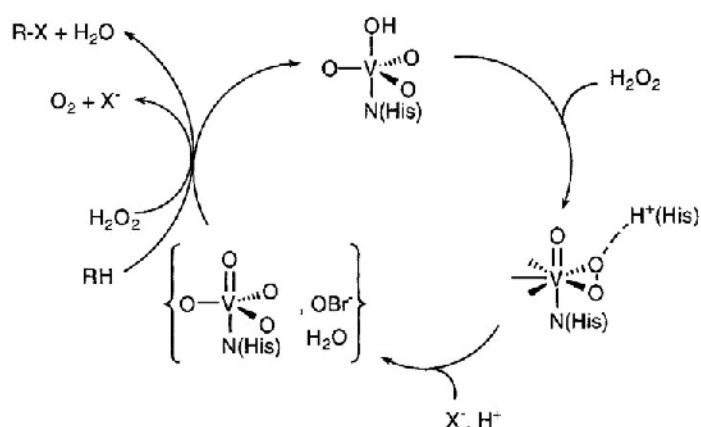
The reaction solution is electron paramagnetic resonance-(EPR) silent during turnover, indicating the absence of an appreciable buildup of V(IV)O, although the authors conclude that a V(IV) species cannot be ruled out with certainty. In fact, V(III) is not observed (i.e., identified spectrophotometrically) under turnover conditions, which consist of bubbling air and gaseous hydrogen bromide through a solution of vanadate and tetraglyme in 1,2-dichloroethane. The pentagonal bipyramidal complex, $[\text{V}^{\text{(III)}}(\text{teg})(\text{Br})_2]\text{Br}$ (teg = tetraethyleneglycol), has been identified as the reaction product of V(V)-tetraglyme and HBr, in the absence of dioxygen.

VI. MECHANISTIC CONSIDERATIONS OF THE HALOPEROXIDASES

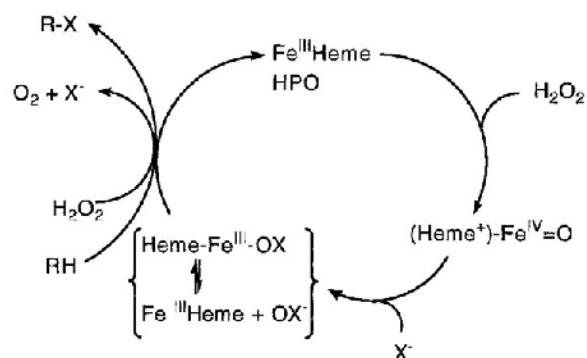
The vanadium haloperoxidases function first by coordination of dihydrogen peroxide followed by oxidation of the halide (Scheme 7). The consensus seems to be that the vanadium center functions as a Lewis acid, remaining in the 5+ oxidation state, as opposed to functioning as an electron transfer catalyst, although it should be pointed out that the reduction potential of the vanadate center has not been measured.

Most other peroxidases are Fe-heme-containing systems, which function as two-electron redox catalysts (Scheme 8). Dihydrogen peroxide oxidizes the Fe-heme moiety by two electrons, forming Compound 1 (a heme⁺ $\text{Fe}^{\text{IV}}\text{=O}$ species) [97]. Compound 1 oxidizes the halide ion, forming the active halogenating species. This mechanism cannot be operative in V-BrPO because the vanadium is already in its highest accessible oxidation state. Moreover, native V-BrPO does not oxidize bromide without an acceptable peroxide source. However, it should





Scheme 7 Summary of V-BrPO catalysis.



Scheme 8 Summary of FeHeme ClPO catalysis.

be realized that redox cycling between vanadium(III) and vanadium(V) is possible, as observed in the vanadium-tetraglyme system in 1,2-dichloroethane (discussed earlier) [96].

VII. CONCLUSION

One of the most interesting, yet unsolved questions about vanadium bromoperoxidase is still the biogenesis of the chiral halogenated marine natural products. With



the discovery of the selectivity of V-BrPO resulting from the binding of organic substrates to V-BrPO and the trapping of an oxidized bromine moiety in the enzyme active site [51,57], regioselective and enantioselective halogenation catalyzed by V-BrPO (and possibly V-ClPO) is an attractive goal to pursue. In addition to the reactivity of the V-haloperoxidases, their stability in the presence of high concentrations of strong oxidants or organic solvents and at elevated temperatures adds to their attractiveness as industrial biocatalysts.

Equally intriguing is the design of new functional mimics of the V-haloperoxidases to test our understanding of the important structural features of these enzymes and the important catalytic residues in these enzymes. Catalytic halogenation has many useful applications in synthetic organic chemistry, both as end products and as synthons. In this regard, the transition-metal-ion-grafted MCM silicate materials, which catalyze peroxidative halogenation reactions, offer a simple, useful, and direct approach to catalytic halogenation [87].

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6

Molybdenum and Tungsten Enzymes

Robert S. Pilato

University of Maryland, College Park, Maryland

Edward I. Stiefel

Exxon Research and Engineering Company, Annandale, New Jersey

I. INTRODUCTION: THE MOLYBDENUM AND TUNGSTEN ENZYMES, A BIOLOGICAL PERSPECTIVE

Molybdenum and tungsten are the only elements of the second and third row transition series to have known biological functions. Molybdenum has been recognized since the 1930s [1] for its role in nitrogen fixing enzyme systems (see Chapter 7). However, from 1953 [2], it was realized that molybdenum is essential for diverse aspects of metabolism in a wide range of organisms. Tungsten was first identified in 1973 [3] in an enzyme from a thermophilic organism and since 1990 has been extensively studied [4,5] in the hyperthermophilic archaea (formally termed archaeobacteria).

In the first edition of *Bioinorganic Catalysis* [6], Chapter 6 was entitled “Catalysis by Molybdenum-Cofactor Enzymes.” In this complementary chapter in the second edition, we include not only bioinorganic catalysis by molybdenum but also that by tungsten enzymes. The expanded scope is appropriate, given the structural similarities of the molybdenum and tungsten cofactors and the similar reactions catalyzed by these enzyme families. There is not a single ubiquitous chemically distinct “cofactor” for either molybdenum or tungsten enzymes. Rather, a class of cofactors containing either molybdenum or tungsten and one or two metal-bound pterin-substituted-1,2-enedithiolate ligands is present at the



active sites. Insight into the biosynthesis of these cofactors is now available [7,8]. In the last 5 years, 20 new enzymes have been confirmed to contain either molybdenum or tungsten, and in the last 3 years representative members of both molybdenum and tungsten enzyme families have been crystallographically characterized.

There were 37 distinct enzymes that contain molybdenum or tungsten known by the end of 1997. The enzymes are diverse in function, broadly distributed, and include oxidases, reductases, dehydrogenases, a transhydroxylase, and a hydratase. The Mo enzymes are found in eubacteria, archae, protista, fungi, plants, and animals (including humans) and are essential for respiration and carbon and nitrogen assimilation. Several of the enzymatic substrates and products are key components in the nitrogen, sulfur, selenium, carbon, and arsenic cycles and have major biological and environmental impact.

In bacteria, molybdenum enzymes are essential for anaerobic metabolism, allowing the use of nitrate, dimethyl sulfoxide (dmsO), amine oxides, sulfur, formate, carboxylates, and carbon dioxide as terminal electron acceptors. The assimilation of nitrogen by plants, algae, fungi, and bacteria is, in part, facilitated by the molybdenum enzyme nitrate reductase and a host of enzymes that oxidize one- and two-ring N-heterocyclic compounds. In plants, abscissic acid, a hormone that controls leaf loss, seed germination, and other plant functions, is produced from the corresponding aldehyde by a molybdenum-containing aldehyde oxidase [9]. The molybdenum enzymes sulfite oxidase, dmsO reductase, and polysulfide reductase are catalysts in the sulfur cycle. The reduction of dmsO yields volatile dimethyl sulfide, which is photo-oxidized in the atmosphere to methanesulfonic acid (MSA), a compound important in cloud nucleation. Eventually, MSA is converted by soil bacteria to sulfite, which is in turn oxidized to sulfate by the molybdenum enzyme sulfite oxidase.

In the methanogenic bacteria [10], the first step in the reduction of CO_2 is catalyzed by formyl methanofuran dehydrogenase, an enzyme that contains either molybdenum or tungsten. In the carboxydobacteria, the oxidation of carbon monoxide (CO dehydrogenase) and, in a variety of organisms, the oxidation of formate (formate dehydrogenase), both producing CO_2 , is catalyzed by molybdenum or tungsten enzymes. The molybdenum enzyme arsenite oxidase catalyzes the oxidation of arsenite (As^{3+}) to arsenate (As^{5+}), the levels of which can be determining factors in marine algal blooms [11,12].

In higher animals, the production of antioxidants (such as uric acid) [13,14], the oxidation of toxins such as bisulfite [15], the production of retinoic acid (from retinal) [16], and the metabolism of purines [17] are all catalyzed by molybdoenzymes. Although these processes are important to normal metabolism and general health, in certain pathological states, the molybdoenzymes may contribute to tissue damage. For example, xanthine oxidase initiates the free radical tissue damage (by generating $\text{O}_2^{\cdot-}$ and H_2O_2) that occurs upon reperfusion after cardiac failure in humans and other mammals [18–20]. The inability of a significant



portion of the population to metabolize sulfite (bisulfite) properly has warranted its removal from certain foods and the addition of warning labels to those products in which sulfites are used as a preservative or freshener. Other disorders in humans such as gout [21], xanthinuria [22], and oxidase deficiency [23–25] are associated with the dysfunction of molybdenum enzymes. Oxidase deficiency is a fatal genetic disorder that manifests itself in a lack of enzymatic activity of sulfite, xanthine, and aldehyde oxidases. Sulfite buildup leads to severe mental retardation, separation of the optic lens, and, inevitably, premature death.

The tungsten enzymes differ from those of molybdenum in that they have been generally found in extreme thermophiles, where they perform key steps in carbon metabolism and energy generation [5]. The utilization of tungsten by thermophiles may be attributed to its biological availability in these extreme environments or to a chemical advantage that tungsten displays under thermophilic conditions. Given their thermal stability and the high temperature reactions they catalyze, the tungsten enzymes are of industrial interest as potential new biocatalysts.

There are several recent reviews of the molybdenum and tungsten enzymes [4–6,23,26–36]. In this chapter, we first define the metallocofactors and offer a compilation of the enzymes and their diverse activities. We then focus on the active-site structures, highlighting the confluence of crystallographic and spectroscopic studies. This is followed by a discussion of pertinent spectroscopic, structural, reactivity, and theoretical model studies. We then turn our attention to the mechanisms of catalytic activity of the molybdenum and tungsten enzymes.

II. THE MOLYBDENUM AND TUNGSTEN COFACTORS

II.A. Nomenclature and General Features

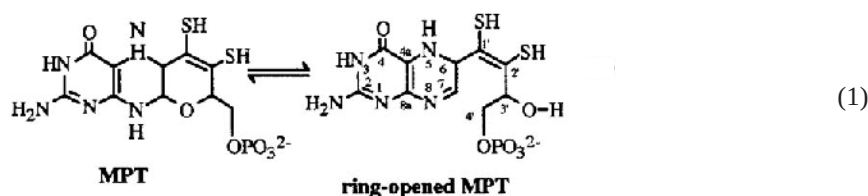
With the exception of nitrogenase, all known molybdenum and tungsten enzymes require a pterin-substituted 1,2-enedithiolate ligand. The ligand was initially called “molybdopterin” and later abbreviated as MPT [37,38]. Molybdopterin is a ligand in the coordination sphere of both the molybdenum and tungsten cofactors but contains neither metal, leading to confusion among nonspecialists. Moreover, it seems inappropriate to maintain the “molybdopterin” designation for a moiety common to both molybdenum and tungsten cofactors. Therefore, to conserve continuity with the earlier literature, we maintain the MPT usage with the understanding that it is the *metal*-binding pterin dithiolate, i.e., the pterin-substituted 1,2-enedithiolate ligand bound to either molybdenum or tungsten in its respective cofactor.

Although IUPAC recommendations have been followed throughout the book for the use of “hydron” to indicate H^+ in natural abundance, in this chapter,

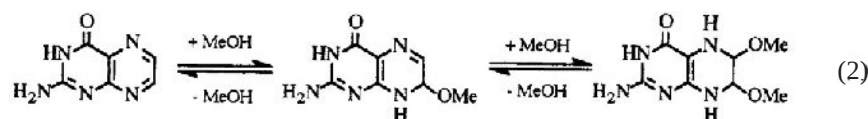


“proton” has been retained to avoid possible confusion with other terms, such as CEPT (coupled electron proton transfer).

From recent x-ray crystallographic studies of both molybdenum and tungsten enzymes, MPT is now known to be a C(6)-substituted 5,6-dihydropterin that is covalently alkoxyated at the C(7) position by the alcohol of the 1,2-enedithiolate side chain (Eq. 1) [39].



Alkoxylation of the pterin results in the formation of a fused organic tricyclic ring containing pyrimidine, reduced pyrazine, and dihydropyran rings. The presence of the dihydropyran ring has led to the MPT covalent alkoxyate being referred to as the “pyranopterin” in the recent literature. Pterin alkoxylation is well established and is generally reversible, as shown in Eq. 2 [40–42] for the parent pterin heterocycle. Crystallographic results to date show that all active cofactors clearly contain the dihydropyran ring.



The ring-opened form of MPT can be unambiguously numbered as a dihydropterin (Eq. 1). However, several numbering schemes have been proposed for the ring-closed form of MPT, including schemes used to number flavins, another pterinoid-containing organic tricyclic compound. Since a numbering scheme for MPT has yet to be adopted by the bioinorganic community, we will use the numbering scheme for the ring-opened form of the cofactor throughout this chapter.

Prior to 1993, it was thought that the pterin was either a nonalkoxylated dihydropterin or a tetrahydropterin. Such structures for MPT (and the various cofactors that contain MPT) permeate the earlier literature. Prior to 1990, it was thought that there was a single cofactor with a single type of pterin ligand, which was termed the *molybdenum cofactor*, *Moco*. However, it is now clear that the molybdenum and tungsten cofactors are a diverse class, members of which all contain either MPT or one of its variants. Hence, the molybdenum cofactor and tungsten cofactor enzymes form a family, much like heme or Fe-S proteins, in which the detailed structure of the prosthetic group varies.

There are at least five distinct MPT containing ligands (Figure 1) [31]. In



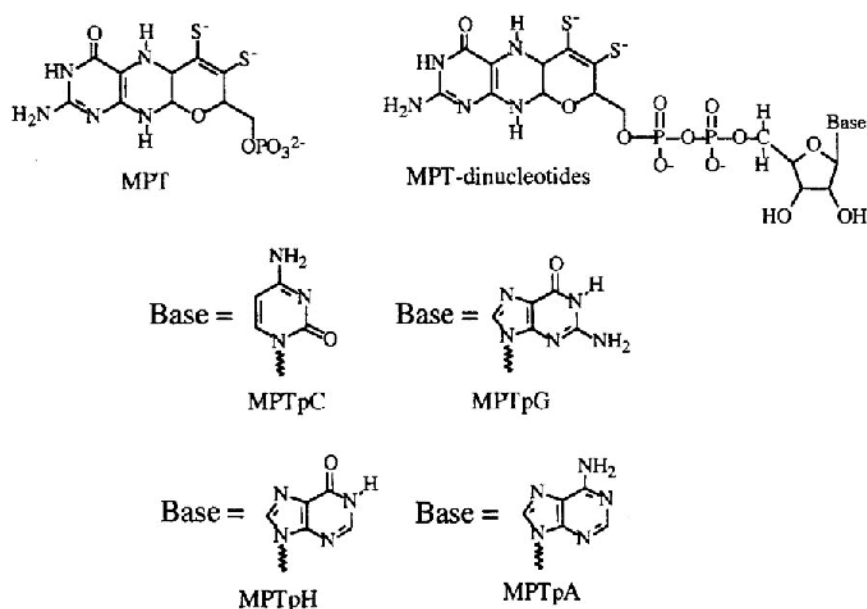


Figure 1 MPT and the dinucleotide MPT variants: MPTpC, MPT-cytosine dinucleotide; MPTpG, MPT-guanosine dinucleotide; MPTpA, MPT-adenosine dinucleotide; MPTpH, MPT-inosine dinucleotide.

addition to MPT itself, there are currently four known dinucleotides that contain MPT. The second nucleotide is bound via a pyrophosphate linkage to MPT [43–46]. Consistent with the nomenclature for pyrophosphates, the various MPT derivatives are designated MPTpC, MPTpG, MPTpA, and MPTpH, containing cytosine, guanine, adenine, and hypoxanthine, respectively. As in flavin and nicotinamide dinucleotides, the nucleotides of the MPT derivatives have been shown by x-ray crystallography to aid in cofactor binding to the proteins [27,39,47].

In addition to the presence of one of five MPT derivatives, cofactor variants differ in metal–ligand stoichiometry, coordination number, and coordination geometry. The cofactor variations include (1) the numbers of bound MPT derivatives, either one or two; (2) the presence or absence of oxido and/or sulfido coordination; (3) the presence or absence of bound protein ligands (either serine, cysteine, or selenocysteine); and (4) the presence or absence of coordinated water, hydroxido, or hydrosulfido ligands. These distinctions are discussed in detail in Section IV.

The molybdenum and tungsten centers are classified by the coordination of their M^{VI} states and sorted into enzyme groups based on protein sequence homology. There are four distinct subfamilies of enzymes, which are based on



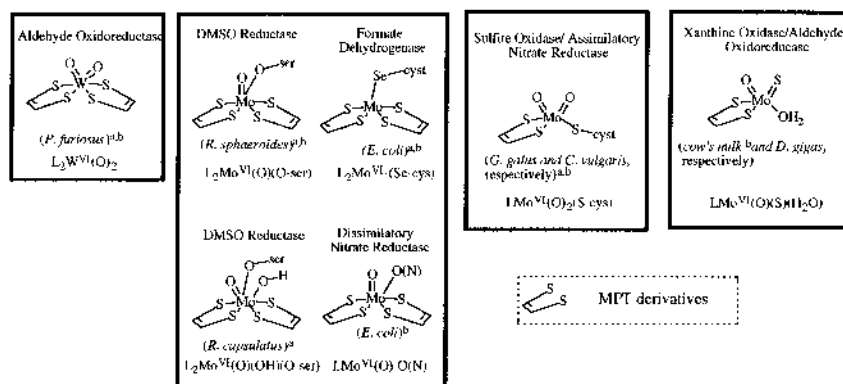


Figure 2 The structures of the MPT cofactor subfamilies. The boxes represent subfamilies of molybdenum and tungsten enzymes with high sequence homology. Cofactor structure type determined: ^aby x-ray crystallography from the listed source; ^bby EXAFS.

sequence homology (Figure 2) [31,35]. Subfamily members have similar metal–MPT stoichiometries and metal coordination but can differ in protein and oxido coordination.

The tungsten enzymes, with a cofactor containing two MPT ligands, lack a coordinated amino acid side chain [4,5] and comprise a unique class. A second protein sequence–based subfamily comprises a large group of bacterial enzymes, including dmsO reductase, formate dehydrogenase, and dissimilatory nitrate reductase [31,35]. The proteins of this subfamily allow considerable variation in metal coordination [29], since there are bis(oxido), mono(oxido), and des(oxido) (i.e., nonoxido) metal sites. The cofactors in this subfamily all appear to contain two bound MPT dinucleotides and a bound amino acid side chain (from serine, cysteine, or selenocysteine) [31,35]. The eukaryotic sulfite oxidases and assimilatory nitrate reductases comprise the third subfamily of molybdenum and tungsten enzymes. The cofactor of this subfamily contains a single MPT ligand, bis(oxido) coordination in the Mo^{VI} state, and a bound cysteine thiolate [31,35]. The fourth genetic subfamily constitutes a large group of C–H hydroxylating enzymes that includes xanthine oxidase and molybdenum aldehyde oxidoreductases. These enzymes have a cofactor variant with one bound MPT and oxido–sulfido coordination of the $Mo(VI)$ state [31,35].

II.B. A Historical Perspective on the Molybdenum and Tungsten Enzymes

The first evidence for the presence of a common cofactor component in the molybdenum enzymes came from genetic studies. Pateman and coworkers [48],



studying the fungus *Aspergillus nidulans* in the early 1960s, suggested that a common genetic determinant for nitrate reductase and xanthine dehydrogenase might involve a cofactor that contained molybdenum. This common cofactor was referred to as *Moco* and was later shown to contain a pterin by Rajagopalan and coworkers [49]. In contrast, the molybdenum nitrogenases possess a homocitrate-containing iron–molybdenum sulfide cluster referred to as *FeMoco* [50]. The distinction between *Moco* (MPT containing cofactors) and *FeMoco* was initially based on the observed noncomplementary biological activity of nitrogenase and other molybdoenzymes [51]. The *FeMoco*-deficient UW45 mutant of *Azotobacter vinelandii* [50] and the *Moco*-deficient Nit-1 mutant of *Neurospora crassa* [52,53] provide apoproteins commonly used to assay for *FeMoco* and *Moco*, respectively. As shown schematically (Figure 3), extracts from both molybdenum and tungsten enzymes, in the presence of molybdate, reconstitute the apoprotein of nitrate reductase from Nit-1. These same extracts will not reconstitute the apoproteins from UW45 (*A. vinelandii*) and therefore have no *FeMoco* (nitrogenase) activity. Moreover, extracts from nitrogenases will activate UW45, but not Nit-1. The distinction between the molybdenum cofactors and *FeMoco* has been amply reinforced by chemical, biochemical, and genetic studies [54].

It is the (MPT)Mo(O)₂ cofactor variant that likely reconstitutes the nitrate reductase activity in the Nit-1 mutant [29,31]. Although the (MPT)Mo(O)₂ unit could be provided directly by sulfite oxidase, nitrate reductase, and even xanthine

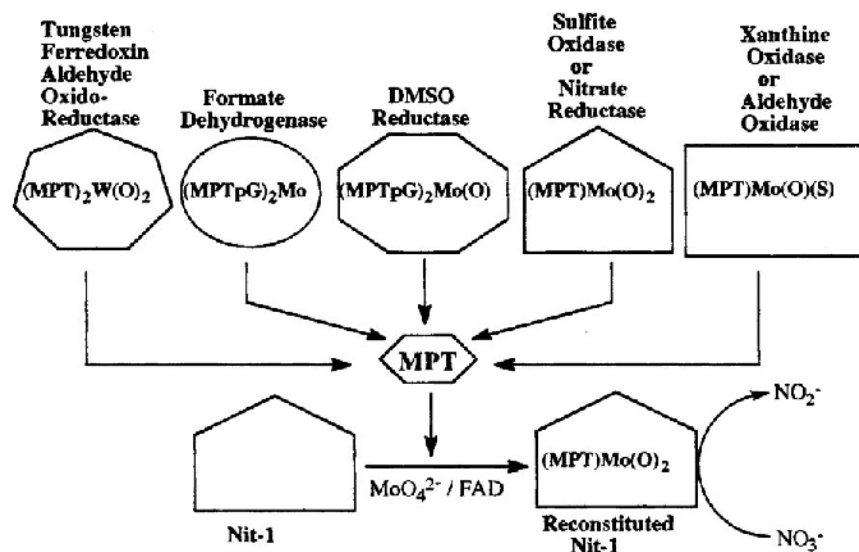


Figure 3 The reconstitution of aponitrate reductase from the Nit-1 *Neurospora crassa* mutant by MPT-containing extracts from the molybdenum and tungsten enzymes.



oxidase (upon hydrolysis of its $\text{Mo}=\text{S}$ bond), reconstitution of Nit-1 is possible with extracts from a host of molybdenum and tungsten enzymes. Many of these enzymes have cofactor variants with two bound MPT ligands, contain a second nucleotide, and/or lack the oxido coordination found in the nitrate reductase cofactor. The ability of all of these extracts to activate Nit-1 suggests that this assay does not require an intact nitrate reductase cofactor. Rather, some “stabilized” version of MPT, which can be converted to the required $(\text{MPT})\text{Mo}(\text{O})_2$ cofactor variant in the presence of molybdate, is needed.

III.C. Characterization of the Cofactor

The evidence for a pterin-substituted 1,2-enedithiolate was first reported by Rajagopalan, Johnson, and coworkers, who isolated pterins from the oxidative decomposition of molybdenum-bound MPT, Figure 4 [7,49,55,56]. In complementary work, Taylor and coworkers confirmed the structure of several of the pterin decomposition products by direct synthesis (see Section V. A) [30,57–59]. Urothione, first isolated in 1940 from human urine [60], was shown to be a metabolic degradation product of MPT [37]. Other isolated pterin-containing decomposition and/or derivatized products from molybdenum enzymes include Form A, Form B (a urothione-like product), and camMPT (Figure 4) [7]. Two other pterins, Form Z and the MPT precursor, can be obtained from molybdenum deprived organisms, *N. crassa* Nit-1, and oxidase-deficient children, neither of which pro-

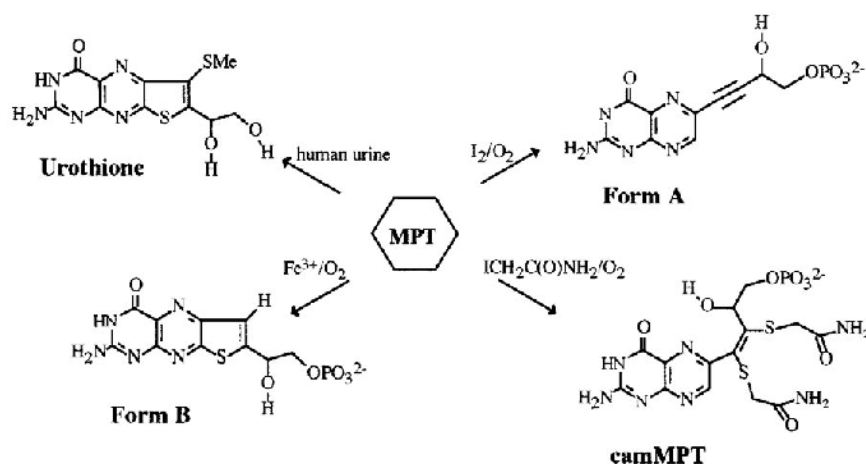
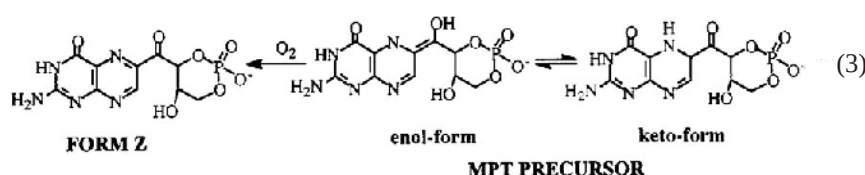


Figure 4 MPT derived products, including urothione, Form A, Form B, and camMPT.



duces active molybdenum cofactor [23,25,55]. Form Z (Eq. 3) is the oxidation product of the precursor to MPT [25,55].



All of the isolated pterin derivatives have a four-carbon side chain attached to the C(6) position of the pterin with unsaturation at the 1' and 2' carbons of the side chain (consider the enol forms of the MPT precursor and Form Z). The sulfur atoms at the 1' and 2' positions of urothione and camMPT are derived from the 1,2-enedithiolate sulfur atoms of the cofactor. All of the dinucleotide MPT derivatives have been isolated and characterized as their carboxymethylamide (cam) derivatives camMPTpA, camMPTpC, camMPTpG, and camMPTpH, respectively [45,61].

With the exception of the MPT precursor, all of the isolated pterins (Eq. 3) are fully oxidized and all lack the dihydropyran ring found crystallographically in the active cofactor. This observation is not surprising, given the reversible nature of pterin alkoxylation and the oxygen sensitivity of reduced pterins.

II.D. MPT Biosynthesis

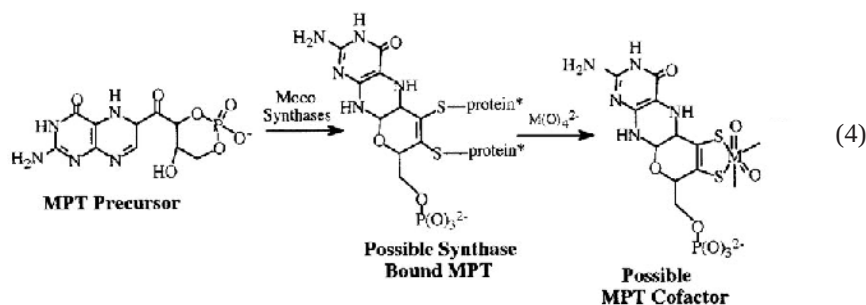
What is known about the biosynthesis of MPT and the MPT dinucleotides has been reviewed elsewhere from both a biochemical and a genetic perspective [7,8,62–64]. Here we only discuss the final steps in MPT biosynthesis, which involve the enzyme molybdopterin synthase [65,66].

Molybdopterin synthase is a 27 kDa protein (in its inactive resting state) that is comprised of 16 and 10 kDa subunits [67]. The protein has a tendency to dissociate and the exact stoichiometry of the active synthase remains uncertain. Activation of the protein requires that the smaller subunit undergo a single sulfur-for-oxygen exchange, a finding that has been confirmed by mass spectral analysis of both active and inactive 10 kDa subunits. Since conversion of the precursor to MPT requires two sulfur atoms, it has been postulated that the active form of the synthase contains two 10 kDa subunits [67]. The synthase not only provides sulfur atoms for MPT precursor conversion but also transports MPT to the apomolybdenum (or presumably apotungsten) enzyme.

The precursor to the cofactor is an α -phosphorylated ketone (commonly drawn in its enol form). It has been demonstrated that α -substituted ketones (including α -phosphorylated ketones) are viable precursors to 1,4-dithiines [68,69] and metallo-1,2-enedithiolates [70–73]. These chemical conversions require



acidic-thiol functional groups and are assisted by Lewis acids and dehydrating conditions. It has been proposed that Moco synthase has thiolaspartic or thiolglutamic acid residues, which are involved in MPT biosynthesis (Eq. 4). Although this proposal is consistent with chemical precedent, the assertion has yet to be confirmed [67].



If this conjecture is correct, the intermediate form of MPT would be bound to the synthase protein via thioester linkages. Given the hydrolytic instability of thioesters, the synthase bound MPT derivative should serve as an ideal precursor to metal-containing MPT derivatives that could be generated either by hydrolysis of the thioester followed by complexation or by direct reaction with molybdate, tungstate, or a simple derivative of these oxyanions.

III. MOLYBDENUM AND TUNGSTEN ENZYMES

III.A. The Source and Properties of the Enzymes

Information on the sources, subunits, cofactors, protein ligands, and prosthetic groups for the molybdenum and tungsten enzymes is given in Tables 1a–e. The biologically relevant reactions catalyzed are shown in Tables 2a–e, where they have been classified as oxidations of one-ring nitrogen heterocycles; oxidations of two-ring nitrogen heterocycles; oxidations and reductions of oxygen-bearing carbon atoms; oxidations and reductions of N, S, Se, As, and Cl centers; and non-redox reactions. With the exception of the hydration of acetylene by acetylene hydratase [74] (a non-redox reaction), the enzymes are involved in either formal two-electron reduction or two-electron oxidation of substrates.

III.B. Molybdenum and Tungsten Catalyzed Substrate Oxidations

The enzymes that catalyze substrate oxidation are oxidases, hydroxylases, dehydrogenases, and oxidoreductases. Most of the substrate oxidations catalyzed by molybdenum and tungsten enzymes involve the net transfer of an oxygen atom



Table 1a Molybdenum Enzyme Catalyzed Oxidations of One-Ring N Heterocycles

Enzymes	Source	Subunits	Cofactor ^a	Bound AA res.	Prosthetic groups	Ref.
iso-Nicotinic acid hydroxylase	Bacterial	$\alpha_2\beta_2\gamma_2$	(MPTpC)Mo(O)(S) ^{b,c}	Unknown	2 Fe ₂ S ₂ , 2 FAD	246
Nicotinic acid hydroxylase	Bacterial	$\alpha_2, \alpha\beta\gamma$	LMo(O)(S) ^{b,c}	Unknown	Fe ₂ S ₂ , FAD, (Se)	247–250
Nicotine hydroxylase	Bacterial	$\alpha_2\beta_2\gamma_2$	LM(O)(S) ^{b,c,g}	Unknown	Unknown	251
Picolinic acid dehydrogenase	Bacterial	$\alpha\beta\gamma$	(MPTpC)Mo(O)(S) ^{b,c}	Unknown	2 Fe ₂ S ₂	252–254
Pyrimidine oxidase	Mammalian liver	$\alpha_2\beta_2\gamma_2$	(MPT)Mo(O)(S)	Unknown	Unknown	255,256

^a MPT, molybdopterin mononucleotide; MPTpG, molybdopterin guanine dinucleotide; MPTpC, molybdopterin cytosine dinucleotide.

^b Assignment based on the abstraction of S by CN⁻.

^c Assignment based on sequence similarities to aldehyde oxidoreductase [31].

^d Assignment based on EXAFS studies.

^e Assignment based on single crystal x-ray studies.

^f Assignment based on sequence similarity to aldehyde ferredoxin oxidoreductase [31].

^g L is used when the exact MPT derivative present is unknown.

^h (?) is used in cases where the exact metal coordination geometry is unknown.



Table 1b Molybdenum Enzyme Catalyzed Oxidations of Two-Ring N Heterocycles

Enzymes	Source	Subunits	Cofactor	Bound AA Res.	Prosthetic groups	Ref.
Isoquinoline oxidoreductase	Bacterial	$\alpha\beta$	(MPTpC)Mo(O)(S) ^{b,c}	Unknown	2 Fe ₂ S ₂	257
Quinaldic acid 4-oxidoreductase	Bacterial	$\alpha\beta$	(MPTpC)Mo(O)(S) ^{b,c}	Unknown	2 Fe ₂ S ₂ , 2 FAD	258,259
Quinaldine 4-oxidoreductase	Bacterial	$\alpha_2\beta_2\gamma_2$	(MPTpC)Mo(O)(S) ^{b,c}	Unknown	4 Fe ₂ S ₂ , 2 FAD	260
Quinoline oxidoreductase	Bacterial	$\alpha_2\beta_2\gamma_2$	(MPTpC)Mo(O)(S) ^{b,c}	Unknown	2 Fe ₂ S ₂ , 2 FAD	261
Xanthine dehydrogenase	Bacterial, mam- malian, fowl	α_2	(MPT)Mo(O)(S) ^d	Unknown	4 Fe ₂ S ₂ , 2 FAD	262
Xanthine oxidase	Bacterial, mam- malian, fowl	α_2	(MPT)Mo(O)(S) ^d	Unknown	4 Fe ₂ S ₂ , 2 FAD	262

See Table 1a for footnotes.



Table 1c Molybdenum and Tungsten Enzyme Catalyzed Oxidations and Reductions of Oxygen-Bearing Carbon Centers

Enzymes	Source	Subunits	Cofactor	Bound AA res.	Prosthetic groups	Ref.
Aldehyde dehydrogenase	Mammalian liver	α_2	(MPT)Mo(O)(S) ^{b,c}	Unknown	4 Fe ₂ S ₂ , 2 FAD	16,93,263,264
Aldehyde oxidoreductase	Bacterial	α_2	(MPTpC)Mo(O)(S) ^{b,c}	None	2 Fe ₂ S ₂	109
Aldehyde ferredoxin oxidoreductase	Bacterial	α_2	(MPT) ₂ W(O) ₂ ^e	Unknown	2 Fe ₂ S ₂ , Fe?	110,265
Carbon monoxide oxidoreductase	Bacterial	$\alpha_2\beta_2\gamma_2$	(MPTpC)Mo(?)	Unknown	4 Fe ₂ S ₂ , 2 FAD, Se	43,266
Carboxylic acid reductase	Bacterial	$\alpha\beta$, $\alpha_3\beta_3\gamma$	(MPT)W(O) ^f	Unknown	Fe ₂ S ₂ , 2 FAD	267
Formaldehyde ferredoxin oxidoreductase	Bacterial	α_4	(MPT) ₂ W(O) ^f	Unknown	4 Fe ₄ S ₄	268–270
Formate dehydrogenase	Bacterial	$\alpha\beta\gamma$, $\alpha_2\beta_2$	(MPTpG)Mo(X) ^e (MPTpG)W(X) X = S or O	None	Unknown	3,111,122, 123,271–273
Formylmethanofuran dehydrogenase	Bacterial	$\alpha\beta\gamma\delta\epsilon$	(MPTpG)Mo(S), (MPTpA)Mo(S), (MPTpA)Mo(S) (MPTpG)W(S) ⁱ	Unknown	Fe ₂ S ₂	46,274–279
2-Furoyl-coenzyme A dehydrogenase	Bacterial	$\alpha_4\beta_4$	LMo(?) ^{g,h}	Unknown	Fe ₂ S ₂ , cytochrome <i>b</i>	280
Glyceraldehyde-3-phosphate ferredoxin oxidoreductase	Bacterial	α	(MPT)W(?) ^h	Unknown	6 Fe	281
Pyridoxal oxidase	Bacterial	α_2	LM(?) ^{g,h}	Unknown		282

See Table 1a for footnotes.



Table 1d Molybdenum Enzyme Catalyzed Oxidations and Reductions of As, Cl, N, and S Centers

Enzymes	Source	Subunits	Cofactor	Bound AA Res.	Prosthetic groups	Ref.
Arsinite oxidase	Bacterial, algae		(MPTpC)Mo(?) ^h	Unknown	Fe ₃ S _y	283,284
Biotin-S-oxide reductase	Bacterial		LMo(?) ^{g,h}	Unknown	Unknown	285,286
Chlorate reductase	Bacterial		LMo(?) ^{g,h}	Unknown	2 Cytochrome <i>b</i> , 2 FAD	78,287–289
dmso reductase	Bacterial	α , $\alpha\beta\gamma$	(MPTpG) ₂ Mo(O), (MPTpG) ₂ Mo(O) ₂	Serine		112,113,115, 229
Nitrate reductase (assimilatory)	Plants	α_2	(MPT)Mo(O) ₂ ^d	Cysteine	2 Cytochrome <i>b</i> , 2 FAD	105,106
	Fungal	α_2				
	Bacterial	α_4				
Nitrate reductase (dissimilatory)	Bacterial	α_2 , $\alpha\beta$, $\alpha\beta\gamma$, $\alpha\beta\gamma\delta$	(MPTpG)Mo(O)	Unknown	4 Fe ₄ S ₄	107
Nitrate reductase (periplasmic)	Bacterial					
Nitrite oxidase	Bacterial	unknown	LMo(?) ^h	Unknown	Unknown	290,291
Polysulfide reductase	Bacterial	$\alpha\beta\gamma$	(MPTpG)Mo(?) ^h	Unknown	Unknown	292,293
Sulfite oxidase	Mammalian, liver	α_2	(MPT)Mo(O) ₂ ^d	Cysteine	4 Fe ₂ S ₂	228,294
	Bacterial	α			2 Cytochrome <i>b</i>	101,102,116, 295
Selenate reductase	Bacterial				Fe ₂ S ₂	296
Tetrathionite reductase	Bacterial		LMo(?) ^{g,h}	Unknown	Unknown	78
Trimethylamine-N-oxide reductase	Bacterial	α_2	LMo(?) ^{g,h}	Unknown	Unknown	82,297

See Table 1a for footnotes.

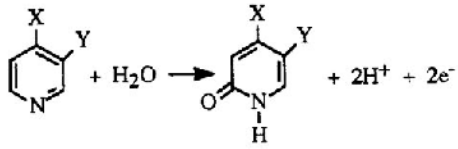
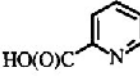
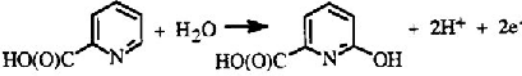
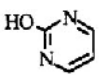
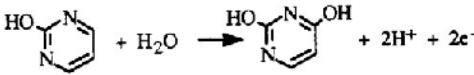


Table 1e Molybdenum and Tungsten Enzyme Catalyzed Nonredox Processes

Enzymes	Source	Subunits	Cofactor	Bound AA res.	Prosthetic groups	Ref.
Acetylene hydratase	Bacterial	α	LW(?) ^{g,h}	Unknown	2 Fe ₂ S ₂	74
Pyrogallol transhydroxylase	Bacterial		(MPTpG)Mo(?) ^h	Unknown	6 Fe ₂ S ₂ , FAD	77,80,81

See Table 1a for footnotes.

Table 2a Molybdenum Enzyme Catalyzed Oxidations of One-Ring N Heterocycles

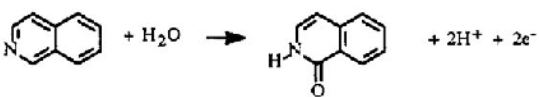
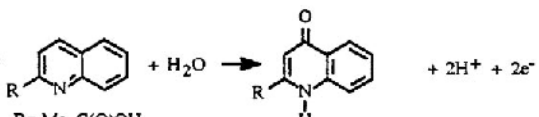
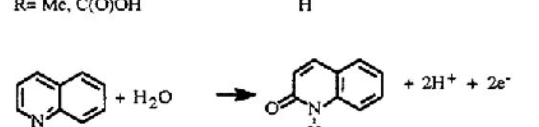
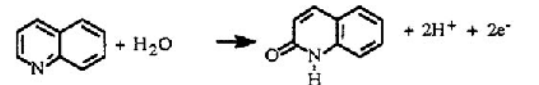
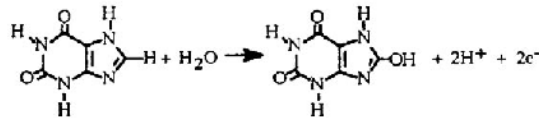
Enzymes	Reactions at the active site
nicotinic acid hydroxylase {X= H, Y= C(O)OH} iso-nicotinic acid hydroxylase {X= C(O)OH and Y= H} nicotine hydroxylase  X= H, Y=	 $\text{Pyridine-X-Y} + \text{H}_2\text{O} \rightarrow \text{3-Hydroxy-Pyridine-X-Y} + 2\text{H}^+ + 2\text{e}^-$
picolinic acid dehydrogenase 	 $\text{Picolinic acid} + \text{H}_2\text{O} \rightarrow \text{3-Hydroxypicolinic acid} + 2\text{H}^+ + 2\text{e}^-$
pyrimidine oxidase 	 $\text{Pyrimidine} + \text{H}_2\text{O} \rightarrow \text{2,4-Dihydroxypyrimidine} + 2\text{H}^+ + 2\text{e}^-$

to substrate from H_2O . However, there is now a significant number of enzymatic oxidation and reduction reactions catalyzed by these enzymes that do not involve such “stoichiometric” oxo transfer. For example, formate dehydrogenase converts HCO_2^- to CO_2 , liberating a proton (hydron) and two electrons [75,76], which obviously does not require an oxygen source. Pyrogallol transhydroxylase (see Table 2e), though catalyzing a process with no net redox, does oxidize one of its substrates, 1,2,3-benzenetriol (pyrogallol), while reducing the other substrate 1,2,3,5-benzenetetrol (phloroglucinol) [77]. The oxidation regenerates phloroglucinol, and the reduction generates 1,3,5-benzenetriol, which is subsequently catabolized. The pyrogallol transhydroxylase enzyme is unique among the known molybdenum and tungsten enzymes insofar as the oxygen atom transferred to pyrogallol is presumed to not originate from water. The electrons generated from this oxidation are not transferred to dioxygen or to another protein through an additional cofactor.

Although dioxygen is the terminal acceptor of both electrons and protons (and H^+) in the molybdenum and tungsten oxidases, O_2 is not *directly* involved in substrate oxidation. The activation of molecular oxygen is effected by (non-Mo- and non-W-containing) prosthetic groups such as flavin-adenine dinucleo-



Table 2b Molybdenum Enzyme Catalyzed Oxidations of Two-Ring N Heterocycles

Enzymes	Reactions at the active site
isoquinoline oxidoreductase	
quinaldic acid 4-oxidoreductase (R = COOH)	
quinaldine 4-oxidoreductase (R = Me)	
quinoline oxidoreductase	
xanthine oxidase xanthine dehydrogenase	

tide (FAD), which are present in the enzymes but not intimately involved in substrate oxidation. The molybdenum and tungsten dehydrogenases and several of the oxidoreductases do not use dioxygen as an oxidant but rather are coupled to other terminal acceptors by electron transfer processes. For the dehydrogenases, the terminal oxidizing equivalents are accommodated by electron-accepting prosthetic groups from which the electrons are shuttled to other processes including oxidative phosphorylation. Although sulfite oxidase and other molybdoenzymes have been historically designated oxidases, many of these enzymes actually function as dehydrogenases under physiological conditions as they do not directly use dioxygen as their electron acceptor.

III.C. Molybdenum and Tungsten Catalyzed Substrate Reductions

With the exception of tetrathionate reductase [78] and polysulfide reductase [79], the reducing molybdoenzymes are involved in the removal of an oxygen atom from either an arsenic-, sulfur-, selenium-, nitrogen-, carbon-, or chlorine-centered

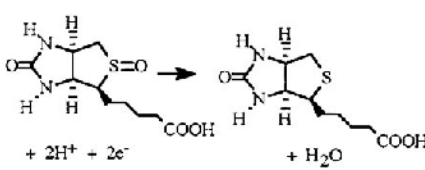
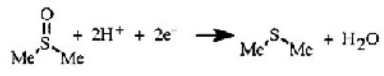


Table 2c Molybdenum and Tungsten Enzyme Catalyzed Oxidations and Reductions of Oxygen-Bearing Carbon Centers

Enzymes	Reactions at the active site	Mo or W
aldehyde oxidoreductase (dehydrogenase)	$\text{RC(O)H} + \text{H}_2\text{O} \longrightarrow \text{RC(O)OH} + 2\text{H}^+ + 2\text{e}^-$	both
glyceraldehyde-3-phosphate ferredoxin oxidoreductase	$\text{HC(O)CH(OH)CH}_2\text{OPO}_3^{2-} + \text{H}_2\text{O} \longrightarrow \text{HOC(O)CH(OH)CH}_2\text{OPO}_3^{2-} + 2\text{H}^+ + 2\text{e}^-$	W
formaldehyde ferredoxin oxidoreductase	$\text{HC(O)H} + \text{H}_2\text{O} \longrightarrow \text{HC(O)OH} + 2\text{H}^+ + 2\text{e}^-$	both
carbon monoxide oxidoreductase	$\text{CO} + \text{H}_2\text{O} \longrightarrow \text{CO}_2 + 2\text{H}^+ + 2\text{e}^-$	Mo
formate dehydrogenase	$\text{HC(O)OH} \longrightarrow \text{CO}_2 + 2\text{H} + 2\text{e}^-$	both
formylmethanofuran dehydrogenase	$ \begin{array}{c} \text{O}=\text{C}=\text{O} \\ + \\ 2\text{H}^+ + 2\text{e}^- \longrightarrow \end{array} \begin{array}{c} \text{R} \\ \\ \text{C}_5\text{H}_3\text{O} \\ \\ \text{CH}_2\text{NH} \\ \\ \text{C}=\text{O} \\ \\ \text{H} \end{array} + \text{H}_2\text{O} $ $\text{R} + \text{C}_5\text{H}_3\text{O} \longrightarrow \text{C}_5\text{H}_3\text{O} + \text{R}^+$	both
2-furoyl-coenzyme A dehydrogenase	$ \text{CoA-S-C(=O)-C}_5\text{H}_3\text{O} + \text{H}_2\text{O} \longrightarrow \text{CoA-S-C(=O)-C}_5\text{H}_3\text{O} + 2\text{H}^+ + 2\text{e}^- $	Mo
pyridoxal oxidase	$ \begin{array}{c} \text{CHO} \\ \\ \text{HOH}_2\text{C}-\text{C}_5\text{H}_3\text{N} + \text{H}_2\text{O} \longrightarrow \text{COOH} \\ \\ \text{Me} \end{array} + 2\text{H}^+ + 2\text{e}^- $	Mo
pyruvate ferredoxin oxidoreductase	$\text{CH}_3\text{C(O)COO}^- + \text{H}_2\text{O} \longrightarrow \text{CH}_3\text{C(O)O}^- + \text{CO}_2 + 2\text{H}^+ + 2\text{e}^-$	W



Table 2d Molybdenum Enzyme Catalyzed Oxidations and Reductions of Sulfur, Nitrogen, Arsenic, and Chlorine Centers

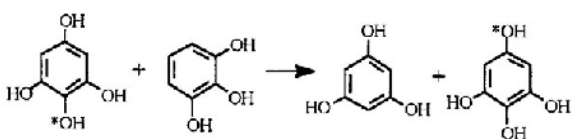
Enzymes	Reactions at the active site
arsenite oxidase	$\text{AsO}_3^{3-} + \text{H}_2\text{O} \longrightarrow \text{AsO}_4^{3-} + 2\text{H}^+ + 2\text{e}^-$
biotin-S-oxide reductase	 $+ 2\text{H}^+ + 2\text{e}^- \longrightarrow \text{biotin} + \text{H}_2\text{O}$
chlorate reductase	$\text{ClO}_3^- + 2\text{H}^+ + 2\text{e}^- \longrightarrow \text{ClO}_2^- + \text{H}_2\text{O}$
dmsO reductase	 $+ 2\text{H}^+ + 2\text{e}^- \longrightarrow \text{DMS} + \text{H}_2\text{O}$
nitrate reductase (→)	
nitrite oxidase (←)	$\text{NO}_3^{2-} + 2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{NO}_2^{2-} + \text{H}_2\text{O}$
polysulfide reductase	$\text{S}_n^{2-} + \text{H}^+ + 2\text{e}^- \longrightarrow \text{SH}^- \quad (n=3-8)$
sulfite oxidase	$\text{SO}_3^{2-} + \text{H}_2\text{O} \longrightarrow \text{SO}_4^{2-} + 2\text{H}^+ + 2\text{e}^-$
selenate reductase	$\text{SeO}_4^{2-} + 2\text{H}^+ + 2\text{e}^- \longrightarrow \text{SeO}_3^{2-} + \text{H}_2\text{O}$
tetrathionate reductase	$\text{S}_4\text{O}_6^{2-} + 2\text{e}^- \longrightarrow 2\text{S}_2\text{O}_3^{2-}$
trimethylamine-N-oxide reductase	$\text{Me}_3\text{NO} + 2\text{H}^+ + 2\text{e}^- \longrightarrow \text{Me}_3\text{N} + \text{H}_2\text{O}$

substrate. Except for pyrogallol transhydroxylase (discussed earlier), the oxygen atom eventually ends up in H_2O [77,80,81].

Tetrathionate reductase converts tetrathionate to two equivalents of thio-sulfate, with no change in the number of oxygen atoms. Polysulfide reductase catalyzes the conversion of sulfur (in the form of a polysulfide) to sulfide (HS^-), a two-electron transfer process, through which a sulfur atom is reduc-



Table 2e Non-redox Processes Catalyzed by Molybdenum and Tungsten Enzymes

Enzymes	Reactions at the active site	Mo or W
acetylene hydratase	$\text{H}-\text{C}\equiv\text{C}-\text{H} + \text{H}_2\text{O} \rightarrow \text{H}_3\text{C}-\overset{\text{O}}{\parallel}{\text{C}}-\text{H}$	W
pyrogallol transhydroxylase		Mo

tively removed from polysulfide [79]. Each of these enzymes catalyze an electron-transfer process that is not coupled to the removal of an oxygen atom from substrate.

Although molybdenum and tungsten enzymes carry the name of a single substrate, they are often not as selective as this nomenclature suggests. Many of the enzymes process more than one substrate, both in vivo and in vitro. Several enzymes can function as both oxidases and reductases, for example, xanthine oxidases not only oxidize purines but can deoxygenate amine N-oxides [82]. There are also sets of enzymes that catalyze the same reaction but in opposite directions. These enzymes include aldehyde and formate oxidases/carboxylic acid reductase [31,75] and nitrate reductase/nitrite oxidase [83–87]. These complementary enzymes have considerable sequence homology, and the direction of the preferred catalytic reaction depends on the electrochemical reduction potentials of the redox partners that have evolved to couple the reactions to cellular redox systems and metabolic requirements.

III.D. Electrochemical Potentials

Redox potentials of the molybdenum centers in several of the enzymes have been obtained by potentiometric titration (Table 3a). Although the substrate reaction chemistry requires the metal center to participate in net two-electron redox reactions, the simple electron-transfer reactions of the active sites occur in one-electron steps involving the $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ and $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ couples. Several of the molybdenum enzymes studied have $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ and $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ couples that differ by less than 40 mV. However, in sulfite oxidase the $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ (38 mV) and $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ (–239 mV) couples are separated by roughly 275 mV [88]. In formate dehydrogenase (*D. desulfuricans*) the $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ (–160 mV) and $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ (–330 mV) couples are separated by 170 mV [89]. Both the $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ and



Table 3a Substrate Reduction Potentials

Redox reaction	$E^{\circ'}$ (pH 7) (V) ^a	Ref.
$\text{NO}_3^- + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NO}_2^- + \text{H}_2\text{O}$	+0.42	299,300
$\text{dmsO} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{dms} + \text{H}_2\text{O}$	+0.16	299,300
$\text{uric acid} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{xanthine} + \text{H}_2\text{O}$	−0.36	299,300
$\text{SO}_4^{2-} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{SO}_3^{2-} + \text{H}_2\text{O}$	−0.38	299,300
$\text{CO}_2 + \text{H}^+ + 2\text{e}^- \rightarrow \text{HC(O)O}^-$	−0.429	299,200
$\text{CO}_2 + \text{H}^+ + 2\text{e}^- + \text{methanofuran} \rightarrow$ $\text{formylmethanofuran} + \text{H}_2\text{O}$	−0.497	10

^a Redox potentials are reported relative to the normal hydrogen electrode (NHE) at pH 7. All other activities are equal to 1.

Table 3b Reduction Potentials for the Molybdenum Sites in Several Enzymes

Enzymes	$\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ (V) ^a	$\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ (V) ^a	Ref.
Assimilatory nitrate reductase (<i>C. vulgaris</i>)	+0.011	−0.019	96
Respiratory nitrate reductase (<i>E. coli</i>)	+0.220	+0.180	98
dmsO reductase (<i>E. coli</i>)	−0.075	−0.090	229
Xanthine oxidase (<i>milk</i>)	−0.355	−0.355	298
Sulfite oxidase (<i>Gallus gallus</i>)	+0.030	−0.239	88
Formate dehydrogenase (<i>Methanobacterium formicicum</i>)	−0.400		271

^a Redox potentials are reported relative to the normal hydrogen electrode (NHE).



$\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ redox potentials for a large majority of hydroxylases appear at approximately -350 mV [90–95].

The potentials found for nitrate reductases [96] vary with the role of the particular enzyme. Assimilatory nitrate reductase, found in plants, algae, and fungi, is involved in the first step in nitrogen assimilation and has a molybdenum center that operates at around 0 mV. Respiratory (dissimilatory) nitrate reductase, utilized by bacteria in energy yielding processes, has a molybdenum center that operates at around $+200$ mV [97,98].

As can be seen in Table 3, the reduction potentials of the molybdenum centers are sufficient to effect the desired enzymatic reactions, a seemingly simple finding that underlies the role of molybdenum and tungsten in the direct oxidation or reduction of substrate.

The cofactors of both xanthine and aldehyde oxidases belong to the $\text{LMo}^{\text{VI}}(\text{S})(\text{O})$ subfamily (see Section IV). However, inactive dioxo forms, $\text{LMo}^{\text{VI}}(\text{O})_2$, of both xanthine and aldehyde oxidase are known. These dioxo forms do not catalyze oxidation of the respective substrates of these enzymes. The $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ redox potential for the inactive bis(oxido) form of xanthine oxidase differs from the oxido–sulfido form by -30 mV (bovine xanthine oxidase) and -100 mV (chicken liver xanthine oxidase) [91]. Although the difference is small, given the xanthine/uric acid reduction potential (-360 mV), it is possible that the $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ couple (-433 mV) of the chicken-liver xanthine oxidase bis(oxido) form impedes the effective oxidation of xanthine for redox reasons alone. However, the bis(oxido) form of bovine xanthine oxidase (with a reduction potential of -386 mV) should be able to oxidize xanthine, since the redox potential, and hence the thermodynamic driving force, is sufficient for activity [91,92,99]. As substrate oxidation does not occur, the “chemical” differences between the bis(oxido) and oxido–sulfido (Mo^{VI}) forms must be critical to the dramatic difference in activity (see Section VI.E.1).

III.E. Prosthetic Groups and Modus Operandi

Although the potential of the metal site is an important determinant of the reactions catalyzed by the molybdenum and tungsten enzymes, the thermodynamic driving force for the reactions is provided by the overall redox process. Completion of the reaction and regeneration of a catalytically competent Mo site is generally dependent upon the ability of the prosthetic groups in the enzyme to deliver either oxidizing or reducing equivalents, which are required for catalytic turnover.

The half reactions for the oxidation of formate, sulfate, and xanthine; the reduction of nitrate; and the reduction of CO_2 via the carbamate of methanofuran are listed in Table 3b. The potentials for several relevant redox partners are listed in Table 4. On the basis of the respective $E^{\circ'}$ values for the substrate reactions and their physiological redox partners, it is clear that most (if not all) reactions



Table 4 Relevant Redox Potentials^a

Reactions	$E^{\circ'}(\text{pH } 7) \text{ V}^a [299,300]$
$\text{O}_2 + 4\text{H}^+ + 4\text{e}^- \rightarrow 2\text{H}_2\text{O}$	0.815
$\text{O}_2 + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{O}_2$	0.295
Cytochrome <i>b</i> (Fe^{3+}) + $\text{e}^- \rightarrow$ cytochrome <i>b</i> (Fe^{2+})	0.075
Riboflavin + $2\text{H}^+ + 2\text{e}^- \rightarrow$ dihydroriboflavin	-0.238
$\text{S} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{H}_2\text{S}$	-0.29
$\text{NAD}^+ + \text{H}^+ + 2\text{e}^- \rightarrow \text{NADH}$	-0.32
Ferredoxin (ox) + $\text{e}^- \rightarrow$ Ferredoxin (red)	-0.35 to -0.5

^a Redox potentials are reported relative to the normal hydrogen electrode (NHE) at pH 7. All other activities are equal to 1.

of the molybdenum enzyme are highly favorable thermodynamically. Interestingly, the reduction of carbon dioxide in methanofuran dehydrogenase requires nearly -500 mV, and in this case the source of the reducing equivalents is unknown. Ferredoxins are present in organisms containing methanofuran dehydrogenase, and it is likely that either a ferredoxin or a flavodoxin (the strongest known physiological reductants) is required to achieve this potential [10].

Shown in Figures 5–7 are the redox pathways for xanthine oxidase, sulfite oxidase, and nitrate reductase (assimilatory and respiratory), respectively. These schemes address the electron and proton (hydron) flows. The action of the molybdoenzymes is conceptually similar to that of electrochemical cells in which half reactions occur at different electrodes. In the enzymes, the half reactions occur at different prosthetic groups and intraprotein (internal) electron transfer allows the reactions to be coupled (i.e., the circuit to be completed). In essence, this is the “modus operandi” of these enzymes, which must be determined before intimate mechanistic considerations are seriously addressed.

In addition to its unique molybdenum center, xanthine oxidase (Figure 5) contains two-iron, two-sulfur (Fe_2S_2) clusters and FAD. In general, most oxidases contain a flavin as the reaction site for the terminal oxidant, O_2 [95,100]. The electrical conduit between the molybdenum center and the flavin is provided by the Fe_2S_2 clusters. The reduction of FAD to FADH_2 followed by reoxidation by molecular oxygen results in the formation of H_2O_2 . In xanthine dehydrogenase, the FAD is a one-electron acceptor reduced preferentially to $\text{FADH}^\bullet$ [95] and thus limiting the enzyme’s activation of dioxygen.

In sulfite oxidase, cytochrome b_5 heme is the intermediate provider of oxidizing equivalents, Figure 6 [101,102]. The oxidizing equivalents are obtained from the cytochrome *c*/cytochrome oxidase shuttle, which occurs outside the confines of sulfite oxidase.

In the reductases, heme or reduced nicotinamide-adenine dinucleotide



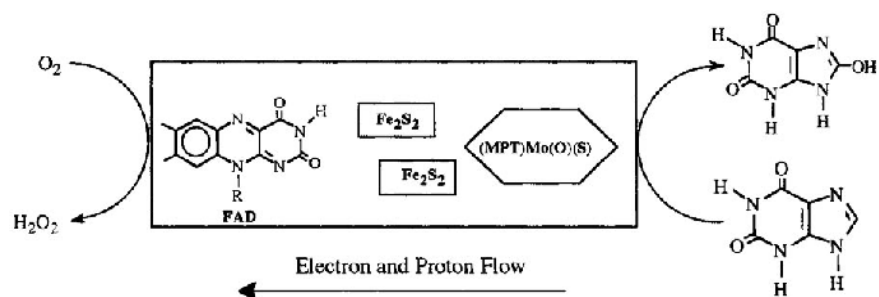


Figure 5 The modus operandi of xanthine oxidase (one subunit) showing the sites of substrate oxidation and oxygen reduction and the flow of electrons and protons (H^+).

(NADH) serves as the common terminal reducing agent, and iron-sulfur clusters again provide the redox conduit [87,103,104]. In assimilatory nitrate reductase (Figure 7), nitrate is reduced at the molybdenum site with reducing equivalents ultimately provided by NADH. However, both FAD and heme *b* are part of the active enzyme and function as the intermediate reducing agents to bring the necessary redox equivalents to the Mo site [105,106]. Respiratory nitrate reductases allow for utilization of nitrate as a source of oxidizing equivalents. Here again the reduction of nitrate occurs at the molybdenum center, but, unlike in assimilatory nitrate reductase, a membrane-bound electron transfer chain provides reducing equivalents. A number of four-iron, four-sulfur (Fe_4S_4) clusters, and/or hemes, may be present in the various isolates of respiratory nitrate reductase, Figure 7 [107].

Although all of the substrate reactions of the molybdenum enzymes are thermodynamically favorable (with the possible exception of methanofuran dehy-

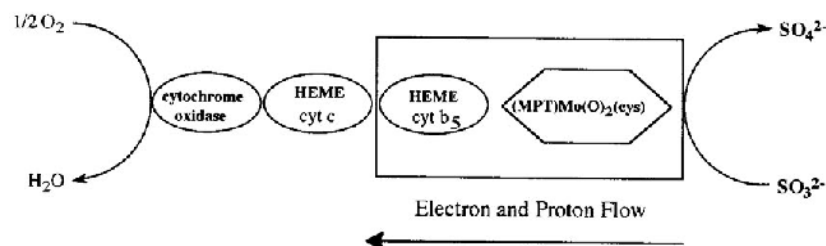
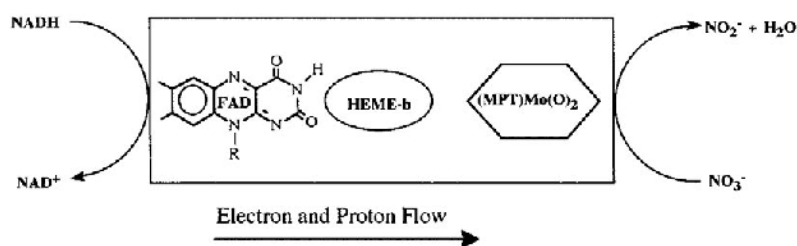


Figure 6 The modus operandi of sulfite oxidase (one subunit) showing the site of sulfite oxidation and the electron and proton (H^+) flows, including the link to cytochrome *c* oxidase, and the activation of dioxygen, the terminal electron acceptor.



(a)



(b)

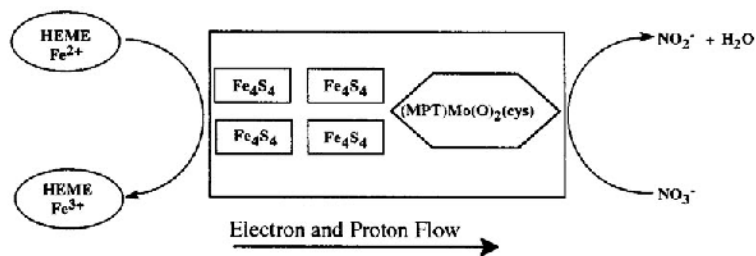


Figure 7 The modus operandi of nitrate reductase: (a) assimilatory nitrate reductase (plants, fungi, algae); (b) respiratory (dissimilatory) nitrate reductase (*Escherichia coli*, *Pseudomonas*).

drogenase) [10], in each reaction a catalyst is required to achieve a significant rate. Several organisms are able to capitalize on the thermodynamically favorable nature of the reactions and utilize molybdenum and tungsten enzymes not only as catalysts for the particular chemical reactions, but also in the capture of chemical energy and the supply of either oxidizing or reducing equivalents for biosynthesis. Unfortunately, in certain cases the accumulation of excess redox capacity can also be harmful. For example, the storage of reducing equivalent by xanthine oxidase contributes to the free radical damage of heart muscle during reperfusion of cardiac failure victims [19,20,95,108]. It has been suggested that H₂O₂, O₂⁻, and, ultimately, hydroxyl radicals are rapidly formed upon reperfusion after a heart attack. This phenomenon has been intensively studied [20]. The most recent measurements of the quantity of xanthine oxidase in heart tissue demonstrate that there is sufficient enzyme to initiate the observed radical damage [20]. In addition, the chemical basis for the conversion of xanthine dehydrogenase (the form of



the enzyme generally found in mammals) to xanthine oxidase (the form that leads to tissue damage) has now been elucidated [95].

IV. THE COORDINATION SPHERES OF MOLYBDENUM AND TUNGSTEN

IV.A. The Contribution of X-Ray Crystallography

The recent x-ray crystallographic studies [27,39,109] of the tungsten enzyme aldehyde oxidoreductase (*Pyrococcus furiosus*) [110]; the molybdoenzymes formate dehydrogenase (*Escherichia coli*) [111], dmsO reductase (*Rhodobacter capsulatus*, and *Rhodobacter sphaeroides*) [112–115]; sulfite oxidase (chicken liver, *Gallus* sp.) [116]; and aldehyde oxidoreductase (*Desulfovibrio gigas*) [109,117] have confirmed that both molybdenum and tungsten are bound to the 1,2-enedithiolate sulfur atoms of a unique pterin-containing organic ligand (i.e., one of the MPT variants). It had long been assumed that all of the molybdenum and tungsten cofactors possess one MPT ligand per metal. However, the first crystallographic study [110], that of tungsten aldehyde oxidoreductase, revealed a cofactor containing two MPT ligands per metal center. In addition to the tungsten-containing aldehyde oxidoreductase (*P. furiosus*) [110], the 2:1 ligand–metal stoichiometry has now been established for dmsO reductase and formate dehydrogenase (*E. coli*) [36,39,111]. On the basis of protein sequence homology, it is thought that this 2:1 ligand–metal stoichiometry is common to the tungsten enzymes and to one of the three subfamilies of molybdenum enzymes (see Figure 2).

A second unexpected result, found in all of the crystallographic studies, is that MPT is a C(7) alkoxylation-5,6-dihydropterin. The hydroxyl group of the four-carbon (1,2-enedithiolate) side chain has added to the pterin to form a dihydropyran ring (Section II.A, Figure 1) [27,39]. The presence of the tricyclic alkoxylation pterin was surprising insofar as none of the isolated MPT derivatives contains this moiety (Figure 4). However, given the known reactivity of pterins with alcohols (Eq. 2) [40–42] and the reversible nature of the alkoxylation reactions, the x-ray findings are not inconsistent with the ring-opened MPT derivatives isolated by Rajagopalan and coworkers [7].

Our understanding of many metalloenzymes has increased enormously with the successful application of protein crystallography. However, the x-ray work itself is not sufficient to define the coordination geometry of either molybdenum or tungsten with full accuracy. Therefore, in the following sections the x-ray crystallography results have been augmented with results from extended x-ray absorption fine structure (EXAFS) [118], resonance Raman (RR) [119], and electron paramagnetic resonance (EPR) spectroscopy [120] to provide the basis for our current structural understanding of the molybdenum and tungsten cofactors.



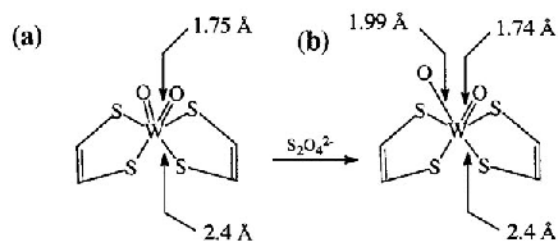


Figure 8 The coordination geometry around tungsten as suggested from the x-ray crystallographic studies and EXAFS of aldehyde oxidoreductase (*P. furiosus*): (a) oxidized form; (b) reduced form [110].

IV.B. The Tungsten Cofactor of Aldehyde Oxidoreductase (*P. furiosus*): a L_2W^V Site

From the x-ray structure of aldehyde oxidoreductase from *P. furiosus*, it is clear that tungsten is coordinated by four sulfur atoms of two MPT ligands [110]. EXAFS places the W—S bonds at an average distance of 2.40 Å for the substrate-reduced enzyme [121]. The dihedral angle between the planes of the 1,2-enedithiolates is 97° and the arrangement of the W and 4 S atoms is best described as distorted square pyramidal. The two MPT ligands of the L_2W site are arranged so that their terminal alkyl phosphates are linked by Mg^{2+} below the basal plane of the square pyramid. Using current x-ray crystallographic methods, it is not possible to define the remaining coordination sphere of tungsten. However, it is clear that there are no protein ligands to tungsten, although histidine and glutamate side-chain residues are present in the region of the active site. From EXAFS [121], the reduced form of tungsten appears to have a single oxido ligand with a W=O bond distance of 1.74 Å. The substrate-reduced site also appears to contain an additional W—O bond at 1.99 Å. Oxidation of the enzyme leads to the generation of a second oxido ligand where the average W=O bond distance is 1.75 Å. The most reasonable structural assignment for the oxidized tungsten cofactor is $L_2W^V(O)_2$ (Figure 8).

IV.C. Members of the L_2Mo^V Family of Cofactors

IV.C.1. The Molybdenum Cofactor of Formate Dehydrogenase (*Escherichia coli*), L_2Mo -SeCys

From an analysis of their protein sequences, dmsO reductase, respiratory nitrate reductase, and formate dehydrogenase (FDH) are assigned as members of a large



class of prokaryotic molybdenum enzymes [31]. It has been proposed that this sequence homology exists in part to accommodate a structurally similar bis(enedithiolate) molybdenum-containing cofactor.

The (MPTpG)₂Mo containing cofactor of FDH (*E. coli*) lies at the interfaces of four domains and, in addition to a single bound protein residue (selenocysteine), is held in place through an extensive hydrogen-bonding network [111,122]. Although not in direct contact with the cofactor, an arginine and a histidine are in the vicinity of the active site. The catalytic necessity of the selenocysteine and histidine is established [123].

Although there are similarities between the FDH active site and that of dmsO reductase (see Section IV.C.2), the molybdenum of the FDH cofactor lacks oxido coordination (Figure 9). The coordination geometry of the Mo^{VI} state is completed by the selenocysteine with a Mo—Se bond distance of 2.7 Å and what has been assigned as a hydroxido ligand at 2.2 Å. The Mo—S bond distances of 2.3–2.6 Å are consistent with thiolate coordination from the two MPTpG ligands. The overall geometry of the six-coordinate molybdenum center is best described as a distorted trigonal prism. Reduction of the enzyme (presumably to Mo^{IV}) results in loss of the hydroxido ligand, which is accompanied by a change in the coordination geometry to pseudo square pyramidal, where the sulfur atoms of the MPTpG are the basal ligands and the selenocysteine is the axial ligand. Molybdenum is ≈0.4 Å above the basal plane of the four sulfur atoms.

Recent EXAFS [124] studies are in agreement with the crystallography but suggest that the selenocysteine and one of the sulfur atoms of the MPTpG ligand are in bonding contact, with a Se—S bond distance of 2.19 Å.

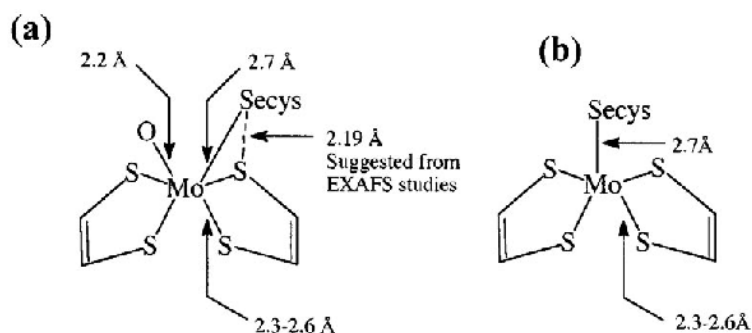


Figure 9 The coordination geometry around molybdenum as suggested from the x-ray crystallographic studies of formate dehydrogenase (*Escherichia coli*): (a) oxidized form; (b) reduced form. The dotted line and Se—S bond distance are suggested from EXAFS [124] but are not seen in the x-ray structure [111,124].



IV.C.2. The Active Site of Dimethyl Sulfoxide Reductase (*R. sphaeroides* and *R. capsulatus*)

Dimethyl sulfoxide reductases from both *R. sphaeroides* and *R. capsulatus* have been the subject of several x-ray studies [112–115]. In addition, extensive resonance Raman (RR) [119,125] and EXAFS studies [126,127] of both oxidized and reduced enzymes have been reported. Despite these studies, there is no single fully accepted picture for the active site of dmsO reductase and its transformations during turnover. The following discussion outlines the structural features of the active site and the cofactor that are common to the various analyses as well as the current points of contention.

The dmsO reductase active site lies at the base of a large depression in the protein surface with the molybdenum and three sulfur atoms of the (MPTpG)₂Mo(O-ser) site exposed. All x-ray studies show a molybdenum-bound serine with a Mo—O distance from 1.7 to 1.9 Å, which EXAFS places at ≈1.9 Å (see Figure 11A). There is also a tryptophan in the vicinity of the active site [112–115].

One point of contention is the nature of the bonding between molybdenum and the two sulfur-donating MPTpG ligands. One of the x-ray studies (*R. capsulatus*) [113,114] and all of the EXAFS studies [126,127] suggest that the two MPTpG ligands are bound to the metal with nearly equivalent Mo—S bonds in the reduced (both dms and dithionite) and oxidized forms (Figure 10). The reported Mo—S bond distances range from 2.3 to 2.5 Å and are within experimen-

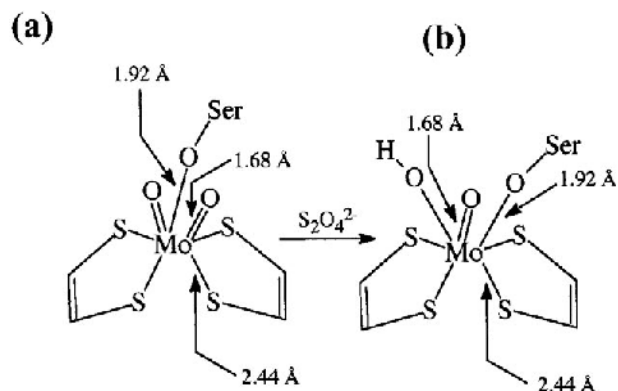


Figure 10 The coordination geometry around molybdenum from x-ray crystallographic studies of dmsO reductase from *R. capsulatus* that suggest similar Mo—S bond lengths for the two bound MPTpG ligands: (a) oxidized form with bis(oxido); (b) reduced form with mono(oxido) [113,114].



tal error of those found in 1,2-enedithiolate molybdenum complexes. However, two x-ray studies suggest that the two MPTpG ligands are very different in their bonding to molybdenum [112,115]. These distinct ligands have been designated as the "P-pterin" and the "Q-pterin." For *R. sphaeroides* dmsO reductase in its oxidized form, Rees and coworkers report [115] that the Mo—S bonds of the P pterin and one of the two Mo—S bonds of the Q-pterin are at the expected Mo—S bond distance of 2.4 Å, whereas the sulfur trans to the pterin of Q-MPTpG has the unusually long Mo—S distance of 3.1 Å. At this distance the sulfur must either bear considerable negative charge, be protonated (hydronated), or be oxidized to the thione (Figure 11). The crystallographic results do not allow a distinction among these alternatives. Upon reduction (of *R. sphaeroides* dmsO reductase) both Mo—S distances to the Q-MPTpG lengthen and the sulfur trans to the pterin is thought to be fully dissociated from the metal with a Mo—S distance of 3.7 Å [115]. A similar finding is reported in the study of dmsO reductase *R. capsulatus* (in its oxidized form). In this study, the Mo—S distances for the Q-MPTpG are reported at 3.5 and 3.9 Å, this suggests that the sulfurs of the Q-MPTpG are essentially not metal bound and that the dithiolate may have been oxidized to a dithietene containing a S—S bond [112] (Figure 12).

In the RR (*R. sphaeroides*), two C=C and two C—S stretching modes are observed [119], clearly indicating that the two enedithiolates of the (MPTpG)₂Mo(O-ser) moiety are inequivalent (consistent with two of the x-ray studies). However, given the energies of these bands, it is unlikely that either 1,2-enedithiolate is dissociated from the metal center, an observation consistent with the results of EXAFS and one of the x-ray studies (*R. capsulatus*). One of the ligands possesses a low-energy C=C stretch and a high-energy C—S stretch,

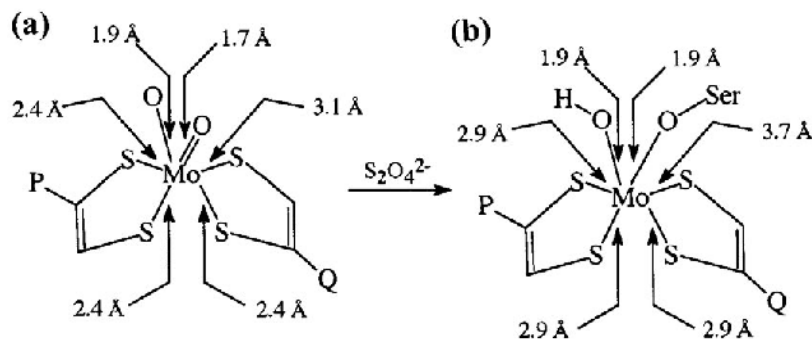


Figure 11 The coordination geometry around molybdenum from x-ray crystallographic studies of dmsO reductase from *R. sphaeroides* that suggest inequivalent Mo—S bonds for the two bound MPTpG ligands: (a) oxidized form with mono(oxido); (b) reduced form with des(oxido) [115].



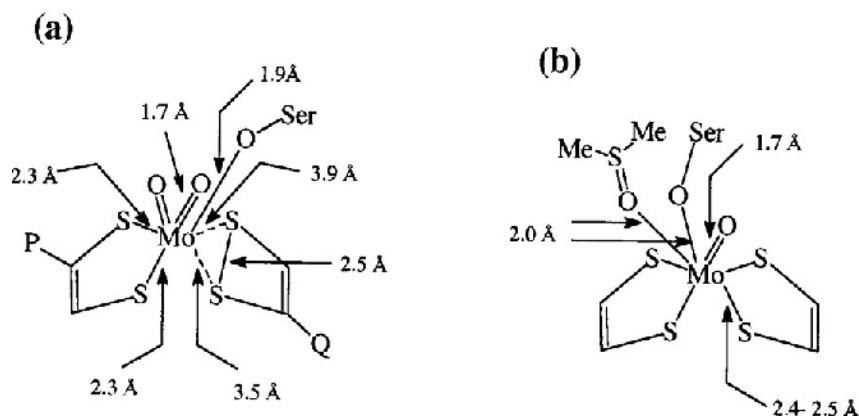


Figure 12 The coordination geometry around molybdenum: (a) from x-ray crystallographic studies of dmsoreductase from *R. capsulatus* that suggest inequivalent Mo—S bond lengths for the two bound MPTpG ligands with a bis(oxido) Mo(VI) center [112]. (b) As suggested from x-ray crystallographic and EXAFS studies of dmsoreductase from *R. capsulatus* treated with dimethyl sulfide [126].

suggesting considerable π delocalization within the 1,2-enedithiolate ligand. A contribution from the thione resonance form generally leads to such observed π delocalization within the 1,2-enedithiolate ligand. Given the C=C and C—S stretching modes of the other MPTpG ligand, this ligand has been described as a dithiolate with little contribution from the thione resonance form. The bands assigned to the C=C and C—S stretches do not show gross changes with oxidation state of the molybdenum. As such, neither EXAFS nor RR substantiates the changes in the Mo—S bonding modes with oxidation state that are suggested by two of the x-ray studies of dmsoreductase [112,115].

The second major point of contention is the number of oxido ligands bound to molybdenum in both oxidized and reduced forms of the cofactor. The x-ray crystallography of dmsoreductase (*R. sphaeroides*) [115], the EXAFS [127], and RR studies [119] each conclude that the oxidized (presumably Mo^{VI}) cofactor has mono(oxido) coordination and that the dithionite-reduced cofactor (presumably Mo^{IV}) lacks oxido coordination. From the x-ray studies of the *R. sphaeroides* protein it was concluded that the cofactor, (MPTpG)₂M(O)(O-ser), has a geometry that is best described as a distorted trigonal prism. The single oxido ligand has a Mo=O bond distance of 1.68 Å [115], as defined by EXAFS (1.7 Å x-ray) [127]. The RR active Mo=O stretching mode was found at 862 cm⁻¹ (819 cm⁻¹ when ¹⁸O-labeled) [119]. The observed ¹⁶O/¹⁸O energy shift is consistent with the single oxido ligand. Independently, x-ray, EXAFS, and RR strongly



support the loss of the oxido ligand upon reduction of the enzyme with dithionite. These studies suggest that the cofactor cycles between $(\text{MPTpG})_2\text{Mo}^{\text{VI}}(\text{O})(\text{O-ser})$ and $(\text{MPTpG})_2\text{Mo}^{\text{IV}}(\text{O-ser})$ states.

However, two x-ray studies of dmsO reductase from *R. capsulatus* [112–114] complicate the picture by reporting that Mo^{VI} is coordinated by two oxido ligands with an average $\text{Mo}=\text{O}$ bond distance of 1.7 Å. One study suggests that Mo^{VI} is seven-coordinate with two oxido, four thiolato, and one serine ligand. A recent EXAFS study supports the notion of a seven-coordinate Mo^{VI} center but with monoxido monohydroxido coordination, $(\text{MPTpG})_2\text{Mo}(\text{O})(\text{OH})(\text{O-ser})$ [126]. In either case, this is a very congested coordination sphere for Mo^{VI} . Indeed, an examination of the crystallographic bond distances shows that these “seven-coordinate” molybdenum centers may have short S—O distances that could indicate the presence of an S—O bond. Such an S—O bond, rather than an Mo—O bond, may be responsible for the extra oxygen atom observed. The S—O form could be an inactive form of the protein related to the “unready” form of the Ni hydrogenases, which are also postulated to have an S—O bond [128–130].

The most recent x-ray and EXAFS studies of dmsO reductase (*R. sphaeroides*) used enzyme crystals grown in the presence of excess dimethylsulfide [126]. The reduced molybdenum site is apparently seven-coordinate with a bound dmsO (Figure 12). The $\text{Mo}=\text{O}$ bond length is 1.7 Å (1.6 Å x-ray); the Mo—O bond distances of the dmsO and serine are each ≈ 2.0 Å. The Mo—S bond distances were 2.4–2.5 Å.

Given the sequence homology and overall structural similarity of proteins of dmsO reductases from *R. sphaeroides* and *R. capsulatus*, a common cofactor and active-site structures would be expected. Discrepancies in the cofactor structure may result from three factors: (1) poor resolution, crystallographic disorder, and/or series termination effects (which can be prejudiced by restraints used in the crystallographic refinement) leading to the inability of x-ray crystallography to define the geometry of the metal center; (2) existence of active and inactive cofactor forms that differ in geometry and coordination number; and (3) the possibility that different polymorphs of dmsO reductase exist with different and discrete cofactors. An interesting possibility is that the enzyme cycles between a mono(oxido) and a des(oxido) form, but there is a bis(oxido) or S—O form that is more stable to oxidative degradation and that has been crystallographically captured in the *R. capsulatus* x-ray structure [128–130].

IV.C.3. The Molybdenum Site of Respiratory Nitrate Reductase (*Escherichia coli*)

Respiratory nitrate reductase has considerable protein sequence homology with dmsO reductases and formate dehydrogenases. Both preliminary crystallographic



reports and genetic studies suggest that this enzyme contains a bis(enedithiolate) coordinated molybdenum center. In accord with dmsO reductase (*R. sphaeroides*) and in contrast to the assimilatory enzyme (see Section IV.D.2), EXAFS supports monoxido coordination of the Mo^{VI} state and the lack of oxido coordination in the Mo^{IV} state (Figure 13) [131]. The reduced form of dissimilatory nitrate reductase appears to have either two $\text{Mo}-\text{O}$ or two $\text{Mo}-\text{N}$ ligands with a distance of 2.10 Å and $\text{Mo}-\text{S}$ bonds at an average distance of 2.35 Å, which have been assigned to the sulfur atoms of the MPTpG, 1,2-enedithiolate ligand. Upon reoxidation with nitrate, an oxido ligand is generated with a $\text{Mo}=\text{O}$ distance of a 1.66 Å. The Mo^{VI} state is also coordinated by either two oxygen or nitrogen ligands with $\text{M}-\text{O}$ or $\text{M}-\text{N}$ distances of 2.10 Å (Figure 13).

IV.D. The $\text{LMo}^{\text{VI}}(\text{O})_2(\text{S-cys})$ Site: The Cofactor of Sulfite Oxidase and Assimilatory Nitrate Reductase

Protein sequence homology suggests that sulfite oxidase and assimilatory nitrate reductase are members of the same molybdenum enzyme subfamily [31]. Consistent with this classification, the cofactors of sulfite oxidase and assimilatory nitrate reductase differ significantly from those in dmsO reductase, aldehyde oxido-reductase, xanthine oxidase (see Section IV.E.), and even respiratory nitrate reductase (Section IV.D). The EXAFS of both sulfite oxidase [132–136] and assimilatory nitrate reductase [131,137,138] and x-ray studies of sulfite oxidase (chicken liver) [116] confirm that the molybdenum center is coordinated by two sulfur atoms from a single MPT ligand and by the sulfur atom of a cysteine side chain. The Mo^{VI} state is bis(oxido) coordinated (Figure 14).

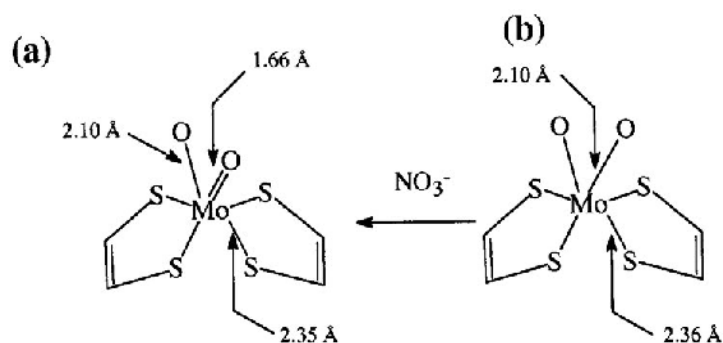


Figure 13 The coordination geometry around molybdenum as suggested from EXAFS and genetic studies of respiratory nitrate reductase (*E. coli*): (a) oxidized form; (b) reduced form [131].



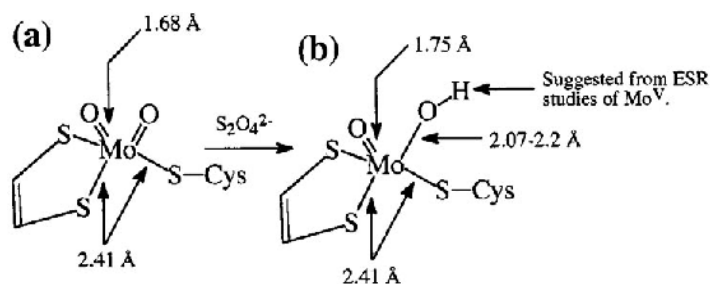


Figure 14 The coordination geometry around molybdenum as suggested from the x-ray crystallographic and EXAFS studies of sulfite oxidase (*Gallus gallus*): (a) oxidized bis(oxido) form; (b) reduced mon(oxido) form [116,132–136].

IV.D.1. The Sulfite Oxidase Molybdenum Center

The average $\text{Mo}=\text{O}$ bond distance of the $(\text{MPT})\text{Mo}(\text{O})_2(\text{S-cys})$ cofactor of sulfite oxidase is 1.68 Å by EXAFS (Figure 14). The RR results are consistent with bis(oxido) coordination of Mo^{VI} and the two expected $\text{Mo}=\text{O}$ stretching modes are found at 903 and 881 cm^{-1} [119,139]. Upon reduction and reoxidation in the presence of H_2^{18}O the $\text{Mo}=\text{O}$ bands shift to 890 and 848 cm^{-1} , respectively [119,139]. The difference in the ^{18}O isotopic shifts for the symmetric and asymmetric bands is consistent with labeling of only one of the oxido ligands. This observation has precedent in the labeling of bis(oxido) model complexes and is supported by normal coordinate analysis [140].

From EXAFS studies it has been concluded that there are at least two sulfur atoms at an average $\text{Mo}-\text{S}$ distance of 2.41 Å. Recent x-ray results of sulfite oxidase show that molybdenum is coordinated by two sulfur atoms of one MPT ligand and one sulfur from a cysteine thiolate. As deduced from the energy of the Raman active $\text{C}=\text{C}$ and $\text{C}-\text{S}$ stretching modes, there is considerable π delocalization in the 1,2-enedithiolate [139].

Upon reduction of sulfite oxidase, one oxido ligand appears to be replaced by an oxygen at a distance of 2.07 Å. The EPR studies of the Mo^{V} state suggest that the oxido is replaced by a hydroxido ligand [141,142]. RR confirms the loss of one oxido ligand upon reduction and suggests that the 1,2-enedithiolate is not grossly affected by changes in the metal oxidation state [139]. The x-ray crystallographic results corroborate those of the EXAFS studies, suggesting that the reduced cofactor has a single oxido ligand with a $\text{Mo}=\text{O}$ distance of 1.75 Å and a hydroxido ligand or bound water with a $\text{Mo}-\text{O}$ distance of 2.2 Å. From x-ray crystallography [116] the reduced molybdenum center is coordinated by five ligands and has approximately square-pyramidal geometry. The basal plane of the pyramid is formed by the two sulfur atoms of MPT, the sulfur of the



cysteine thiolate, and the oxygen of the hydroxido/water ligand. The remaining oxido ligand occupies the apical position. This apical oxido appears to act as a spectator ligand since it is structurally conserved in the Mo(IV) and Mo(VI) states and since only one oxido ligand (the nonspectator) is labeled upon reoxidation of the reduced enzyme in the presence of H_2^{18}O [119,139].

The x-ray studies of sulfite oxidase were done on enzyme crystals grown in the presence of sulfate and glycerol. Under these conditions an oxyanion (believed to be either sulfite or sulfate) is incorporated into the active-site pocket and the molybdenum center is reduced to either Mo(V) or Mo(IV). Five residues (three arginines, tryptophan, and tyrosine) have been shown to form hydrogen bonds to three oxygens of the oxyanion, which has been modeled as sulfite. These residues would serve both to polarize the substrate (sulfite) and to hold it near the active site. Interestingly, the metal center is not directly bound to the oxyanion and the Mo—S bond distance is 5.2 Å. For a bond to form between molybdenum and sulfite, assuming an $\eta^2\text{-SO}_3^{2-}$ bonding mode, sulfite would have to move 1.5–2.0 Å closer to the metal. Such movement might require conformational changes around the active site.

IV.D.2. The Assimilatory Nitrate Reductase Molybdenum Center

Structural studies of assimilatory nitrate reductase have been limited to EXAFS [131,137,138]. These results clearly support the conclusion from protein sequence analysis that nitrate reductase and sulfite oxidase are members of the same subfamily of molybdenum enzymes. From EXAFS the Mo(VI) center of assimilatory nitrate reductase has two oxido ligands with an average Mo=O bond distance of 1.72 Å. There are also at least two (and possibly three) Mo—S distances at 2.44 Å, consistent with thiolate coordination by MPT and cysteine sulfur atoms. Upon the reduction of the enzyme (generating Mo^{IV}) one of the oxido ligands is replaced by either a Mo—O or Mo—N with a bond distance of 2.04 Å. As with sulfite oxidase [143], EPR studies of the Mo^V form suggest that the oxido ligand is replaced by a hydroxido ligand upon reduction [144,145].

IV.E. Members of the LM^{VI}(O)(S) Family of Cofactors: The Hydroxylases

IV.E.1. Xanthine Oxidase and Dehydrogenase

Of all of the molybdenum enzymes, mammalian xanthine oxidase/dehydrogenase has been the most studied (Figure 15). These studies, along with those of other members of this relatively large class of hydroxylases (Table 1a–c), suggest that all molybdenum enzymes that catalyze hydroxylation of C—H bonds contain a common structural motif. This motif is unique in high-valent molybdenum chem-



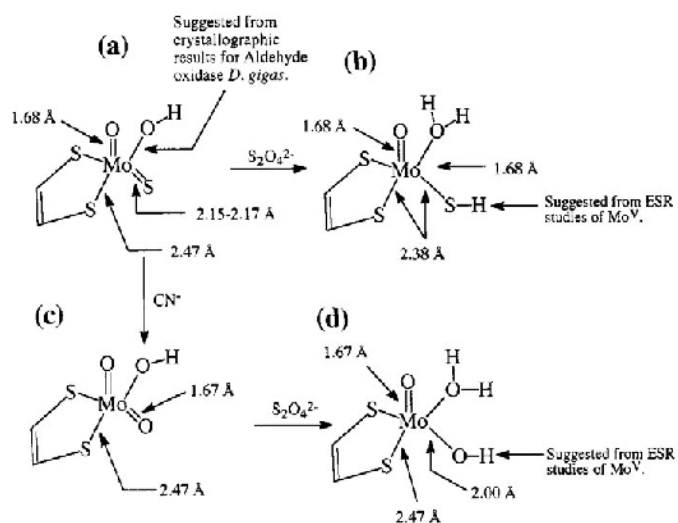


Figure 15 The coordination geometry around molybdenum as suggested from the EXAFS of xanthine oxidase (*mammalian*): (a) oxidized form; (b) reduced form; (c) desulfo oxidized form; (d) desulfo reduced form [133,135,146–151].

istry since the Mo(VI) coordination includes an oxido and a sulfido ligand, for which there are very few well-defined model complexes. Numerous EXAFS studies have shown that the active oxidized cofactor of this family has a terminal sulfido ligand with a Mo=S bond distance of between 2.15 and 2.17 Å [133,135,146–151]. This ligand is accompanied by an oxido with a Mo=O bond distance of 1.7 Å and two Mo—S bonds at 2.38–2.42 Å assigned to the MPT 1,2-enedithiolate sulfur atoms.

Upon cofactor reduction (substrate oxidation), the terminal Mo=S is lost and is replaced by a sulfur with a Mo—S distance of 2.38 Å. Complementary EPR studies of the Mo^V state suggest that, upon reduction, a hydrosulfido ligand (SH[−]) is coordinated to the metal [152–158]. The oxido remains in all oxidation states and likely acts as a spectator ligand.

“Cyanolyzed” xanthine oxidase and xanthine dehydrogenase are inactive for the oxidation of xanthine to uric acid [159]. Cyanide abstracts a sulfur atom from the cofactor generating Mo^{IV}. Upon reoxidation a bis(oxido) molybdenum (VI) with an average Mo=O bond distance of 1.67 Å is generated (Eq. 5). Upon reduction of cyanolyzed xanthine oxidase with dithionite one oxido ligand is



replaced by a Mo—O(N) bond at 2.00 Å [133]. The EPR data support the assignment of this ligand as hydroxido (OH^-) [152–158]. The bis(oxido) form of xanthine oxidase clearly undergoes electron and proton (hydron) transfer reactions that parallel the sulfido form, but nevertheless lacks the ability to oxidize xanthine.

IV.E.2. Aldehyde Oxidoreductase (*D. gigas*)

The x-ray crystallographic results for aldehyde oxidoreductase from the bacterial source *D. gigas* [109,117] clearly support the assertion that the molybdenum enzymes involved in C—H hydroxylation are a unique subfamily utilizing the $\text{LM}^{\text{IV}}(\text{O})(\text{S})$ structural motif (Figure 16). In addition to two Mo—S bonds at a distance of 2.4 Å, attributed to the 1,2-enedithiolate of MPTpC, a Mo=S bond at 2.2 Å, a Mo=O bond at 1.7 Å, and a Mo—O bond at 2.2 Å complete the molybdenum coordination sphere. With the exception of the long Mo—O bond these results are consistent with EXAFS studies [149]. The long Mo—O bond has been assigned to a coordinated water, but, given the resolution of the x-ray study, it is hard to rule out a hydroxido ligand (which is expected at ≈ 1.9 Å). Residues in the active-site pocket should be capable of activating the bound water and/or polarizing the incoming aldehydic substrate [109,117,160].

As with xanthine oxidase, the sulfido ligand of the active form of aldehyde oxidoreductase is readily replaced by an oxido ligand to yield a cofactor with a structure that resembles that of oxidized sulfite oxidase and assimilatory nitrate reductase. Both x-ray and EXAFS data are available for the bis(oxido) form, and, with the exception of the oxido replaced sulfido ligand, few changes are obvious in the overall structure of the oxidized form of the desulfo cofactor. Upon reduction of the enzyme the oxido ligand is presumably reduced to hydroxido, an observation that is supported by EPR data for the Mo^{V} state.

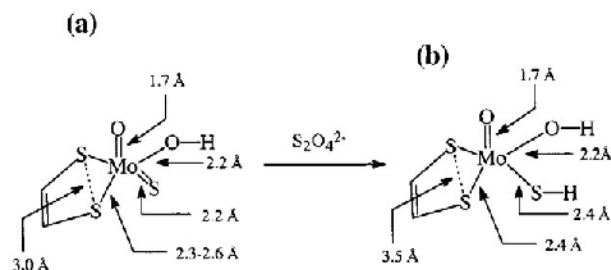


Figure 16 The coordination geometry around molybdenum as suggested from the x-ray crystallography of aldehyde oxidoreductase (*D. gigas*): (a) oxidized form; (b) reduced form [109,117].



V. SYNTHETIC APPROACHES, CHEMICAL MODELS, AND THEORETICAL STUDIES PERTINENT TO THE MOLYBDENUM AND TUNGSTEN ENZYMES

Chemical systems of relevance to the molybdenum and tungsten enzymes include synthetic pterins, α -phosphorylated ketones (as precursor models), and a variety of molybdenum and tungsten oxido, sulfido, and 1,2-enedithiolate complexes. These compounds have been used to (1) confirm the identity of MPT derivatives; (2) define steps in MPT biosynthesis; (3) calibrate spectroscopic observations; (4) give precise geometries and reactivities that can be used as input for theoretical studies; and (5) provide options for mechanistic consideration.

V.A. Synthesis of Pterins

The organic synthetic work of Taylor [57–59] and of Joule and coworkers [161,162] led to the confirmation of the structures of Form A, Form B, and urothione (see Figure 4) [30,57–59]. Dephospho-Form A has been prepared in both possible absolute configurations (R and S at the 3'-carbon), Figure 17. By comparing the circular dichroism (CD) spectra of these isomers with that of authentic dephospho-Form A, the absolute configuration of Form A at C3' was determined

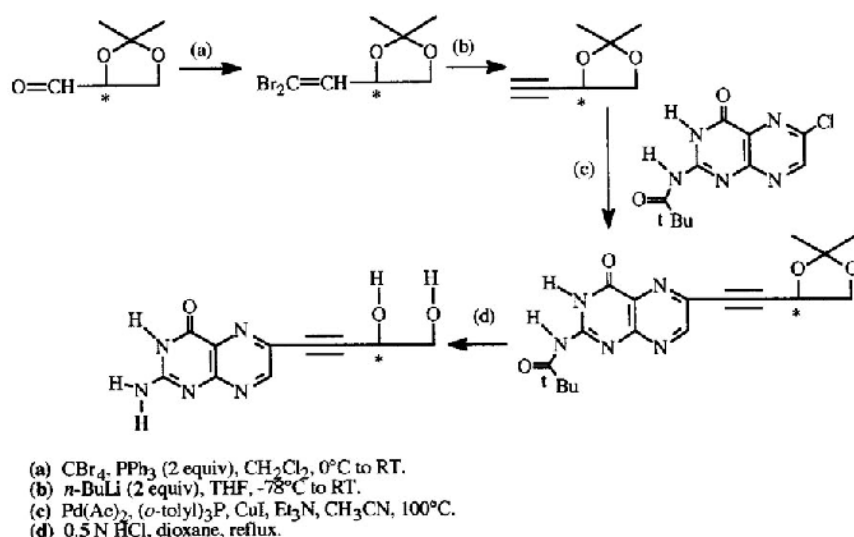


Figure 17 The enantioselective synthesis of dephospho Form A. *Denotes asymmetric carbon atom [57–59].



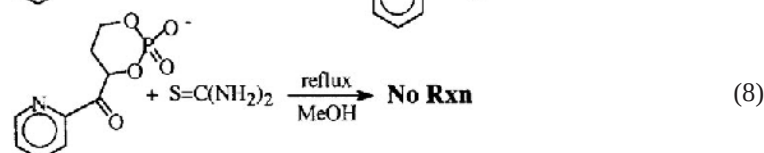
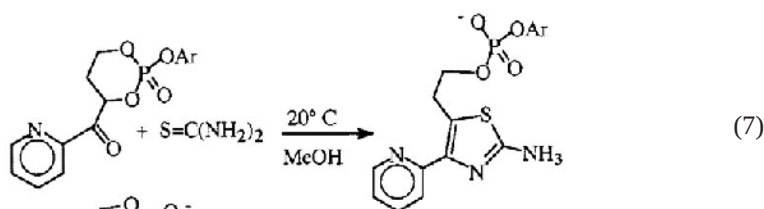
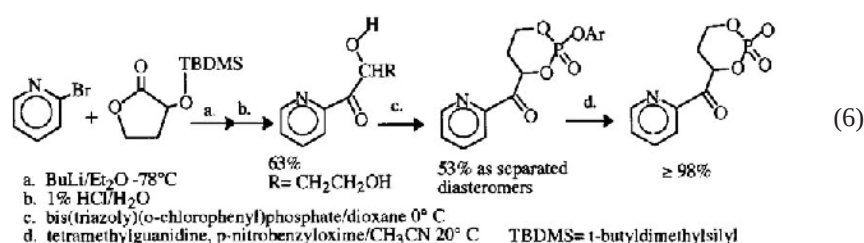
to be S [58]. This absolute configuration is consistent with that found in MPT, which from x-ray crystallography is R at C3'. The stereochemical assignments of MPT and Form A differ as a result of a priority change in designating absolute configuration and not because of a configurational change at C3' [110].

V.B. Precursor Models and 1,2-Enedithiolate Synthesis

As discussed in Section II.D, the precursor to MPT is an α -phosphorylated ketone, commonly drawn in its enol form. The dialkyl phosphate of the precursor is contained in a six-membered ring. Such compounds are absent in the synthetic literature and only recently have simple analogs been prepared (Eq. 6) [163].

The phosphate triester reacts with a range of sulfur nucleophiles that add to the carbon α to the ketone and open the six-membered phosphate ring. Thio-urea adds not only to the α -carbon, but also to the ketone carbon, resulting in elimination of the ketone oxygen as water, a reaction sequence that mimics the conversion of the Moco precursor to a 1,2-enedithiolate (Eq. 7).

Whereas the triester reacts with a range of sulfur nucleophiles, the corresponding diester does not react with thionucleophiles under ambient conditions (Eq. 8), suggesting that the precursor, which is a diester, must be activated for conversion to MPT. This activation could involve the C3' hydroxyl group of the precursor, which is missing in the model, or Lewis acidic groups present in molybdopterin synthase.



Given that the MPT precursor is an α -substituted ketone, it is not surprising that 1,2-enedithiolates can be prepared from this moiety. Biologically, this conversion likely involves the thiol carboxylate residues of MPT synthase and, presumably, a molybdenum or tungsten oxoanion. Chemically, several reagents have been used to facilitate a similar conversion (Figure 18) [70–72,164–166].

V.C. Molybdenum and Tungsten 1,2-Enedithiolates, Complexes

V.C.1. Synthesis

Dithiolene complexes were extensively studied prior to the realization that this ligand type is present in molybdenum and tungsten enzymes. The early studies were driven by the remarkable redox activity of the complexes, which can be ligand and/or metal centered. Early work on metallo-1,2-enedithiolate complexes involved homoleptic bis(coordinated) complexes of the late transition metals and tris(coordinated) complexes of early transition metals, including molybdenum and tungsten. Much of this work concentrated on complexes of $(\text{CN})_2\text{C}_2\text{S}_2^{2-}$ (mnt^{2-}), $(\text{CF}_3)_2\text{C}_2\text{S}_2^{2-}$, $\text{C}_6\text{H}_4\text{S}_2^{2-}$ (bdt^{2-}), and $(\text{CH}_3)_2\text{C}_6\text{H}_3\text{S}_2^{2-}$ (tdt^{2-}) ligands [167–169].

Newer synthetic methods have allowed pterin and quinoxaline substituted 1,2-enedithiolate complexes to be prepared. The complexes $\text{Cp}_2\text{Mo}\{\text{S}_2\text{C}_2(6\text{-N}(2)\text{-pivaloylpterin})(\text{C}(\text{O})\text{Me})\}$ and $\text{Cp}_2\text{Mo}\{\text{S}_2\text{C}_2(2\text{-quinoxaline})(\text{C}(\text{O})\text{Me})\}$ have been prepared with both natural abundance and $>80\%$ ^{34}S enrichment (Eq. 9 and 10) [170,171].

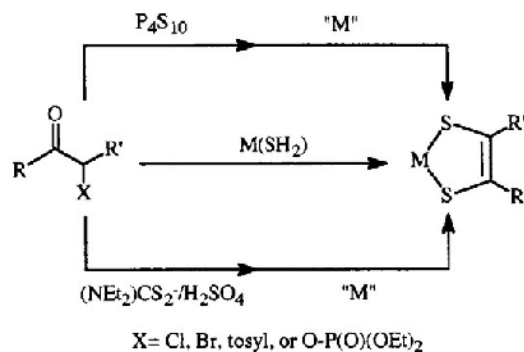
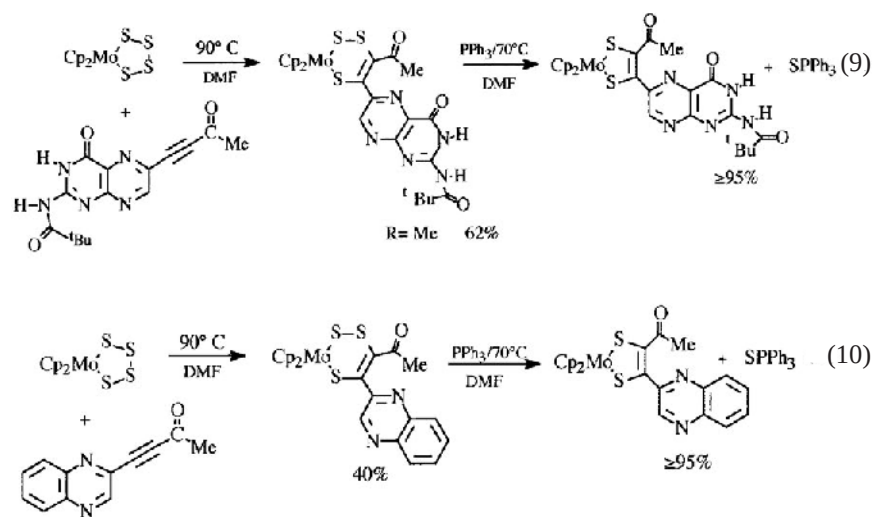


Figure 18 Synthetic routes to metallo-1,2-enedithiolates from α -substituted ketones [70–72,164–166].





These complexes were used as spectroscopic models in resonance Raman studies where the major sulfur-sensitive band at 349 cm^{-1} shifts to 341 cm^{-1} upon ^{34}S enrichment. The energy and shift compare well with results on dmsO reductase from *Rhodobacter sphaeroides*, where a band at 350 shifts to 341 cm^{-1} upon ^{34}S enrichment [125].

As shown in Eq. 7 and 8, 1-persulfido-2-enethiolate (trithiolene) complexes $\text{Cp}_2\text{Mo}\{\text{S}_3\text{C}_2(2\text{-quinoxaline})(\text{C}(\text{O})\text{Me})\}$ and $\text{Cp}_2\text{Mo}\{\text{S}_3\text{C}_2(6\text{-N}(2)\text{-pivoylpterin})(\text{C}(\text{O})\text{Me})\}$ are generated as precursors to the corresponding 1,2-enedithiolate complexes. The conversion of the trithiolene to the corresponding 1,2-enedithiolate complex may be relevant to the reactivity of (MPT)Mo(S)(O) cofactor variants [36].

V.C.2. Reactions of Heterocyclic Substituted 1,2-Enedithiolate

In addition to providing spectroscopic models, the oxidation of quinoxaline-substituted molybdenum and tungsten 1,2-enedithiolates has yielded the corresponding 3-sulfido-thienoquinoxaline (thiophene-containing) derivatives (Figure 19) [172,173]. This transformation models the oxidative conversion of MPT to form B and the metabolic conversion of MPT to urothione [37,49].

V.D. Molybdenum and Tungsten Oxido 1,2-Enedithiolate Complexes

The realization that many of the Mo and W cofactors are 1,2-enedithiolate complexes with oxido or sulfido coordination has encouraged studies of complexes



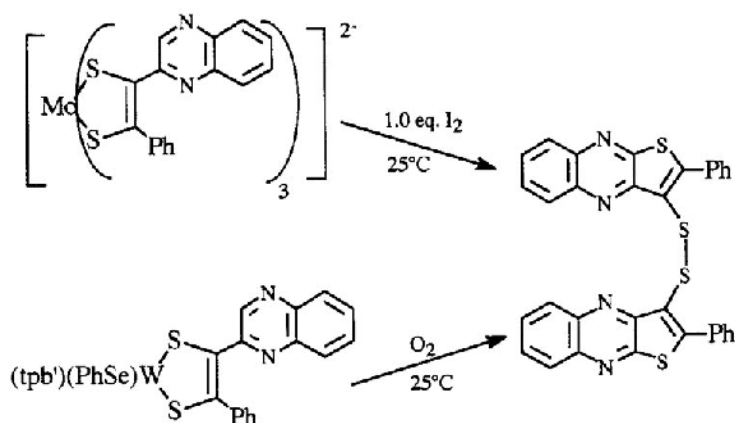


Figure 19 Conversions of a quinoxaline-substituted metallo-1,2-enedithiolate to a sulfido-thienoquinoxaline, analogous to the conversion of MPT to urothione and Form B [37,49].

containing these ligand combinations. Oxido 1,2-enedithiolate complexes of molybdenum were initially generated as side products in the synthesis of the tris(1,2-enedithiolate) complexes. $\text{MoO}(\text{mnt})_2^{2-}$ was initially reported in 1969–1970 [174,175] and its Ph_4P^+ salt has a Mo–O stretching frequency at 930 cm^{-1} .

In 1986, it was shown that $\text{Mo}^{\text{IV}}\text{O}(\text{bdt})_2^{2-}$ could be prepared from $\text{Mo}^{\text{IV}}\text{O}_2(\text{CN})_4^{4-}$, and $\text{Mo}^{\text{V}}\text{O}(\text{bdt})_2^-$ could be prepared from $\text{Mo}^{\text{V}}\text{O}(\text{SPh})_4^-$ [176]. The oxido bis(1,2-enedithiolate) Mo(V) and Mo(IV) complexes are readily interconverted. Both complexes are square-pyramidal with an oxido as the apical ligand. The respective Mo–O stretching frequencies are 941 and 905 cm^{-1} .

V.E. Reactivity of Molybdenum and Tungsten Oxido Bis(1,2-Enedithiolate) Complexes

Recent work by Nakamura, Ueyama [177–185], Sarkar [186–191], and co-workers has led to the preparation and interconversion of several oxido bis(1,2-enedithiolate) complexes of Mo and W. These complexes take the form $\text{Mo}^{\text{VI}}\text{O}_2\text{L}_2$ and $\text{M}^{\text{IV}}\text{OL}_2$ where $\text{M} = \text{Mo}$ or W and L is a 1,2-enedithiolate ligand, either $(\text{CN})_2\text{C}_2\text{S}_2^{2-}$ (mnt^{2-}) or $\text{C}_6\text{H}_4\text{S}_2^{2-}$ (bdt^{2-}).

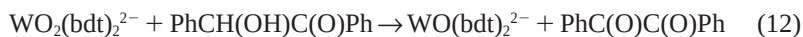
$\text{Mo}^{\text{IV}}\text{O}(\text{bdt})_2^{2-}$ can be converted to the dioxo molybdenum(VI) complex in good yield by the following reaction:



This conversion is a clean reactivity model for the Mo enzyme trimethylamine *N*-oxide reductase. The molybdenum(VI) bis(oxido) complex has a distorted octahedral geometry [181].

$[\text{PPh}_4]_2[\text{W}^{\text{VO}}(\text{bdt})_2]$ was first prepared by the reaction of $\text{WO}(\text{SPh})_4^-$ with the acid form of the free ligand. The reversible W(IV)/W(V) redox couple allows preparation of the W(IV) complex, $\text{WO}(\text{bdt})_2^{2-}$, by borohydride reduction of the W(V) complex. The W(IV) complex reacts with trimethylamine *N*-oxide in the same manner as the corresponding molybdenum complex (Eq. 11) [178]. The formal oxygen atom transfer is reminiscent of the oxygen transfer processes characteristic of tungsten enzymes.

The structures of all three oxidation states in the tungsten bdt series have been reported. The W(IV) and W(V) states are similar to the Mo analogs [178]. The bis(oxido) W(VI) complex is a distorted octahedron. The W—S bonds *trans* to the oxido ligand are lengthened with W—S distances *cis* to the oxido at 2.425(4) Å and *trans* to the oxido at 2.597(4) Å, respectively. The W(VI) complex reacts with benzoin to give benzil (Eq. 12), but only reacts slowly with PPh_3 [183].



This reactivity suggests that although these complexes effectively catalyze a reaction that likely proceeds by a coupled electron proton transfer, they appear to be relatively poor oxygen atom donors.

The corresponding mnt and naphthalene 2,3-dithiolato complexes were prepared and structurally characterized [192,193]. The complex $\text{Mo}^{\text{IVO}}[\text{S}_2\text{-C}_2(\text{COOCH}_3)_2]_2^{2-}$ has also been briefly reported [180]. A useful route to $\text{WO}(\text{mnt})_2^{2-}$ involves the reaction of WO_4^{2-} , Na_2mnt , and $\text{Na}_2\text{S}_2\text{O}_4$ at pH 5.5 [188].

Bis(dithiolate) coordination appears to stabilize mononuclear Mo(IV,VI) and W(IV,VI) sites. There seems to be little tendency to form oxido-bridged M(V) complexes by comproportionation of the M(VI) and M(IV) complexes, which is quite prevalent in the chemistry of molybdenum and tungsten with other ligands such as dithiocarbamates. An attempt to use the sterically hindered 1,2-enedithiolate, Ph_3Sibdt , yields the blue mononuclear Mo(V) complex, $\text{MoO}(\text{Ph}_3\text{Sibdt})_2$. Upon exposure of this complex to air, the oxido-bridged Mo(VI) dimer $(\text{Ph}_3\text{Sibdt})\text{MoO}_2(\mu\text{-O})\text{MoO}_2(\text{Ph}_3\text{Sibdt})^{2-}$ is formed [182]. Apparently, if only one dithiolene is present the tendency to form an oxido-bridged dimer is quite strong. As such, it may prove difficult to prepare mono(1,2-enedithiolate) complexes as reactivity models.

Reactions of trimethylamine *N*-oxide have been mentioned as synthetically useful and relevant to the reaction of trimethylamine *N*-oxide reductase [82]. Sarkar and coworkers have also reported the reactions with CO_2 , HSO_3^- [189], and acetylenes [186]. Sarkar and Das reported [190] that $\text{WO}(\text{mnt})_2^{2-}$ reacts with $\text{CO}_2/\text{HCO}_3^-$ to form HCOO^- and $\text{WO}_2(\text{mnt})_2^{2-}$. This formal reduction of CO_2



mimics the reaction of formate dehydrogenase, albeit in the reverse direction. $\text{WO}_2(\text{mnt})_2^{2-}$ also oxidizes HSO_3^- to HSO_4^- in a reaction that displays saturation kinetics. Such kinetic behavior suggests that there is a slow preequilibrium, which may involve bound reactant. The reaction is inhibited by a variety of oxyanions, including the reaction product, supporting this mechanistic suggestion. This oxidation of sulfite clearly resembles the sulfite oxidase reaction (see Table 2d). However, in neither case (enzyme or model) has the requisite ^{18}O labeling experiment been performed to show whether direct oxo transfer reactions are occurring.

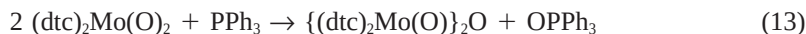
The one non-redox reaction catalyzed by a tungsten enzyme involves the hydration of acetylene to acetaldehyde [5]. This same reaction is catalyzed by $\text{WO}(\text{mnt})_2^{2-}$ but not by $\text{WO}_2(\text{mnt})_2^{2-}$ [186]. The five-coordinate W(IV) complex should have an open site that may be available to coordinate and activate the acetylene substrate [194].

The bis(1,2-enedithiolate) complexes discussed closely resemble the metal centers found in the dmsoreductase family of Mo enzymes and in the tungsten enzymes. The reactivity of mono(1,2-enedithiolate) complexes remains a continuing challenge as synthetic chemists pursue accurate models for the xanthine oxidase and sulfite oxidase families of metal sites. New 1,2-dithiolate ligands [70,71] and complexes are needed to demonstrate ligand effects to help elucidation reaction mechanism.

V.F. Other Model Complexes and Oxygen Atom Transfer

A significant number of molybdenum oxido complexes either oxidize or reduce enzymatic substrates. Formally, many of these processes involve the inner sphere transfer of a neutral oxygen atom (O). This process is equivalent to the coupled transfer of 2e^- and O^{2-} in opposite direction [195].

Of the complexes studied, those with ancillary ligands that contain sulfur atoms have received the most attention. Among the most studied are the bis(dialkylthiocarbamate) molybdenum oxido complexes [196,197]. Unfortunately, although these complexes react with certain enzyme substrates, their common problem as models for Moco active sites involves the propensity of the Mo^{VI} dioxo and Mo^{IV} monoxo complexes to form a comproportionated Mo^{V} μ -oxido dimer (Eq. 13; dtc = diethyldithiocarbamate).



One approach to limit dimer formation in model complexes involves the use of bulky ligands and weakly coordinating solvents. Holm and coworkers [196,198–201] have studied oxygen atom transfer reactions of 2,6-bis(2,2-diphenyl-2-thioethyl)pyridinate [2] molybdenum oxo complexes. In contrast to the structurally similar dithiocarbamate molybdenum complexes, the tendency of the 2,6-bis(2,2-diphenyl-2-thioethyl)pyridinate Mo^{IV} monoxo complex to undergo di-



merization with the Mo^{VI} dioxo complex is greatly reduced by the bulk of the ligand [198–201].

Molybdenum complexes without sulfur coordination can also undergo oxygen atom transfer reactions. An example is the interconversion by oxygen atom transfer of the $(\text{tpb}')\text{Mo}^{\text{VI}}\text{bis}(\text{oxido})$ and $(\text{tpb}')\text{Mo}^{\text{IV}}(\text{oxido})(\text{X})$ complexes, where $\text{tpb}' = \text{hydro tris}(3,5 \text{ dimethyl-1-pyrazolyl}) \text{ borate}$ with $\text{X} = \text{F}^-, \text{Cl}^-, \text{Br}^-, \text{NCS}^-, \text{OPh}^-, \text{OMe}^-, \text{SPh}^-$ [202,203]. As with other molybdenum systems, these complexes can be incorporated into a catalytic cycle for the deoxygenation of dmsu utilizing PPh_3 as the oxygen atom acceptor. The rate of oxidation of PPh_3 increases in going from $\text{X} = \text{OPh}^- < \text{SPh}^- < \text{Cl}^-$, revealing a significant electronic effect on the reactivity of the Mo site.

The tpb' family generally requires coordinating solvent to limit oxido-bridged dimer formation. However, $(\text{tpb}')\text{Mo}^{\text{VI}}(\text{O})_2\{\eta^1\text{-S-P(S)(OEt)}_2\}$ complex can be deoxygenated to generate $(\text{tpb}')\text{Mo}^{\text{IV}}(\text{O})\{\eta^2\text{-S}_2\text{P(OEt)}_2\}$ [202]. The flexible coordinating ability of the thiophosphanate limits the comproportionation reaction in the absence of a coordinating solvent and allows expansion of the scope of studies on this family of complexes.

V.G. Models and Electron Paramagnetic Resonance

The reduction of $(\text{tpb}')\text{Mo}^{\text{VI}}(\text{O})_2\text{SPh}$ has been shown first to generate $[(\text{tpb}')\text{Mo}^{\text{V}}(\text{O})_2\text{SPh}]^-$, which is subsequently protonated to $(\text{tpb}')\text{Mo}^{\text{V}}(\text{O})(\text{OH})\text{SPh}$. Although the source of the proton in these studies is unclear, the observed $A(^1\text{H}) = 13.1 \times 10^{-4} \text{ cm}^{-1}$ is similar to that observed in desulfo xanthine oxidase [204–206].

In related work, spectroscopic analysis was performed on a series of $\text{Mo}(\text{V})$ complexes, $[\text{MoOXL}]$, where L is the tetradentate ligand *N,N'*-dimethyl-*N,N'*-bis(2-mercaptophenyl)ethylenediamine and X is an oxido, hydroxido, sulfido, hydrosulfido, or chloro ligand [207–210]. The reaction chemistry for this series is shown in Figure 20. The EPR data were analyzed and compared with the results from the molybdenum enzymes. Extensive isotopic labeling with $^{95,97,98}\text{Mo}$, ^{17}O , and ^{33}S was utilized. A comparison of the data (Table 5) with those of the various signals of xanthine oxidase reveals a close relationship between the spectra obtained for $[\text{MoOSL}]^-$ and the “very rapid” signal, as is evident from the highly anisotropic ^{33}S coupling. The spectrum collapses to form the “rapid”-type signal upon protonation of the sulfido ligand to form $[\text{MoO}(\text{SH})\text{L}]$.

In the rapid and slow signals of xanthine oxidase [211], as well as in $[\text{MoO}(\text{OH})\text{L}]$ and $[\text{MoO}(\text{SH})\text{L}]$, ^1H hyperfine splitting that is consistent with the proposed hydroxyl or hydrosulfido ligands (Table 5) is observed. Although this ^1H hyperfine splitting is assigned to either HO^- and HS^- groups in these model complexes, previous work had shown relatively large ^1H hyperfine splitting due to coordinated $\text{N}=\text{H}$ containing ligands, for example, in $\text{Mo}(\text{S}_2\text{CNEt}_2)(\text{HNSC}_6\text{H}_4)_2$, where $A(^1\text{H}) = 7.4\text{G}$ [212,213]. Hyperfine splitting from ^{17}O for $[\text{MoO}(\text{SH})\text{L}]$



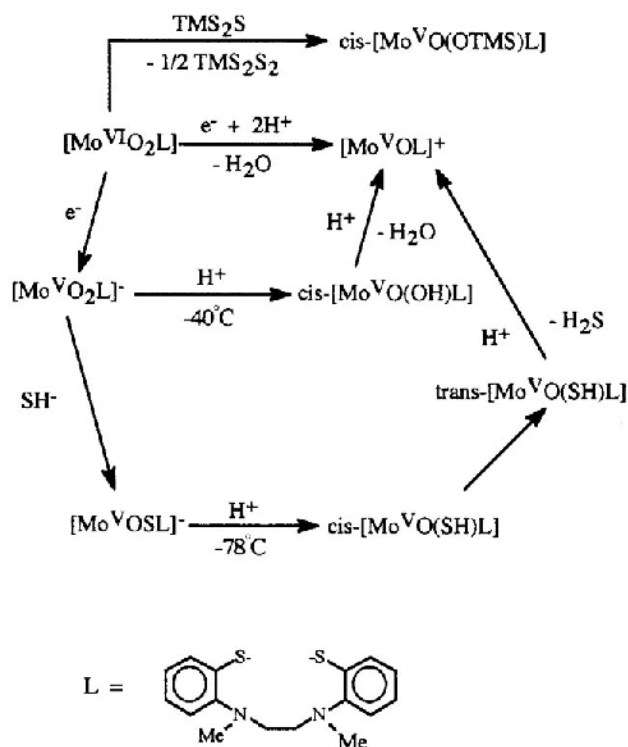


Figure 20 The series of Mo(V) complexes, $[MoOXL]$, where L is the tetradentate ligand *N,N'*-dimethyl-*N,N'*-bis(2-mercaptophenyl) prepared and studied by EPR. Hyperfine (superhyperfine) coupling constants and *g* values are listed in Table 5 [207–210].

and $[MoO(OH)L]$ is also observed, with the latter having two different couplings, consistent with the presence of both hydroxyl and oxo ligands.

V.H. Model for the Sulfido Site of the Molybdenum Hydroxylases

It has long been a synthetic challenge to prepare mono(oxido) mono(sulfido) molybdenum centers that are analogous to the Mo(VI) state of enzymes in the xanthine oxidase family. Although studies in this area have met with limited success, it seems possible that the sulfido linkage of these centers could be stabilized by the 1,2-enedithiolate or by a molybdenum-bound thiolate ligand.

Ethylene sulfide reacts with $\{(tpb')Mo^{IV}(O)(\eta^2-S_2P(i\text{-propyl})_2)\}$ to generate

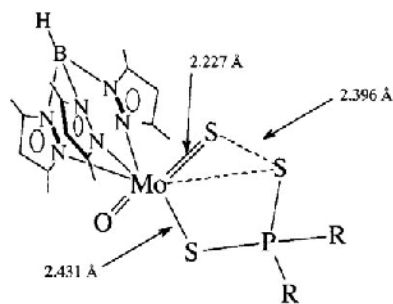


Table 5 A Comparison of ^1H , ^{17}O , and ^{33}S EPR Superhyperfine Splittings in the Xanthine Oxidase Very Rapid, Rapid, and Slow Forms with Molybdenum Model Complexes^a

Source	$a^1\text{H}$	$a^{17}\text{O}$	$a_1^{33}\text{S}$	$a_2^{33}\text{S}$	$a_3^{33}\text{S}$
Xanthine oxidase rapid	11.7–12.8	6.5	3.2	3.3	3.3
[Mo(O)(SH)L]	9.7	2.0	2.8	<3.2	<3.2
Xanthine oxidase slow	13.7–14.9	≈9			
[Mo(O)(SH)L]	14.8	2.3, 7.5			
Xanthine oxidase very rapid			<3.4	22	<4.1
[Mo(O)(S)L]			2.8	25.6	6.4

^a Splittings expressed as $\text{cm}^{-1} \times 10^{-4}$. L = *N, N'*-bis(2-mercaptophenyl)ethylenediamine [207–211].

a species that in the solid state is best described as intermediate between $(\text{tpb}')\text{Mo}^{\text{VI}}(\text{O})(\text{S})\{\eta^2\text{-S}_2\text{P}(\text{i-propyl})_2\}$ and $(\text{tpb}')\text{Mo}^{\text{IV}}(\text{O})\{\eta^2\text{-S}_3\text{P}(\text{i-propyl})_2\}$ [26,214]. The Mo—S bond at 2.227(2) Å is substantially longer than that expected for a Mo=S but still reflects some double-bond character (Figure 21). The S—S bond distance of 2.396(3) Å suggests partial bond formation and is longer than the S—S distance in polysulfido complexes and sulfur, which is generally in the 2.0–2.1 Å range. The results from this study remind us of the redox fluidity of molybdenum thiolato complexes. The sulfur atoms of $(\text{tpb}')\text{Mo}^{\text{VI}}(\text{O})(\text{S})\{\eta^2\text{-S}_2\text{P}(\text{i-propyl})_2\}$ are all formally S^{2-} and two of the sulfur atoms of $(\text{tpb}')\text{Mo}^{\text{IV}}(\text{O})\{\eta^2\text{-S}_3\text{P}(\text{i-propyl})_2\}$ are both formally S^{1-} . An interconversion between these two

**Figure 21** X-ray structure of $(\text{tpb}')\text{Mo}^{\text{VI}}(\text{O})(\text{S})\{\eta^2\text{-S}_2\text{P}(\text{i-propyl})_2\}$ showing the contribution of $(\text{tpb}')\text{Mo}^{\text{IV}}(\text{O})\{\eta^2\text{-S}_3\text{P}(\text{i-propyl})_2\}$ to the solid-state structure [26,214].

forms simply requires a formal two-electron redox process between molybdenum and the sulfur atoms. Other examples of partially oxidized S—S systems have been discussed [36]. This type of internal redox process, as described by Stiefel and coworkers, is commonly observed in the chemistry of molybdenum thiolato and sulfido complexes [26,36,215].

The isolation of 1-persulfido-2-enethiolate (trithiolene) complexes and their conversion to the corresponding 1,2-enedithiolate complexes (Eq. 7 and 8, see page 121) serve to demonstrate that MPT could stabilize the sulfido ligand of the molybdenum hydroxylases [170,171]. The Mo—S(1) bond length at 2.510(2) Å is rather long, but the Mo—S(2) bond length at 2.451(2) Å is not unlike the Mo—S distances found in molybdenum 1,2-enedithiolate and in di- or polysulfido complexes. The S(2)—S(3) bond length at 2.074(2) Å is best described as a single S—S bond, and S(3) is not within bonding distance of the metal center (Mo—S(3) = 3.704(2) Å). This complex is related to the sulfido dithiolate structure of the xanthine oxidase Mo(VI) site by an internal electron transfer where the metal is formally reduced and the sulfido oxidized [36].

V.I. Theoretical Models

Theoretical studies have been increasingly used to aid the elucidation of metallo-enzyme active site structures and mechanisms. In Moco enzymes, several groups have been active [216–221]. Greatbanks et al. have done *ab initio* calculations of a wide variety of dihydropterin structures and conclude that 7,8-, 5,7- and 5,8-dihydropterin structures are energetically quite similar, and, therefore, each represents a potential candidate for the open-chain form of MPT [219].

Pietsch and Hall use Møller/Plesset perturbation theory to do calculations aimed at discerning the energetics of conversion of an $\text{Mo}^{\text{VI}}\text{O}_2^{2+}$ site to a $\text{Mo}^{\text{IV}}\text{O}^+$ site using a phosphane, PMe_3 , as the oxo acceptor [221]. Alternative mechanisms to simple oxo transfer do not appear to have been considered. The calculations reveal a simple pathway for oxo transfer in which the phosphane ligand perpendicularly approaches the O-Mo-O plane, eventually (with rotation) forming an $\text{OMo}(\text{OPMe}_3)$ product. This calculation used a simplified model coordination sphere, $\text{MoO}_2(\text{SH})_2(\text{NH}_3)_2$, to simulate the bis(oxido)-mono(oxido) interconversion in enzyme systems. It is instructive to view this simple reaction pathway for oxo transfer and see that it does not present any energetically unreasonable intermediates. Particularly interesting is the second (nonreacting) oxygen, which plays a “spectator” role [222]. The presence of this oxido ligand in the final Mo product orients the *d*-orbital distribution, allows tracking of the electron flow during the oxo transfer reaction, and contributes strongly to reaction energetics.

Calculations on xanthine oxidase-like active sites have been carried out by Bray and Deeth and by Voityuk et al. using density functional (DF) approaches [216–218,220]. Bray and Deeth show that the $\text{LMoOS}(\text{OH})$ structure is quite favorable for the Mo(VI) state of xanthine oxidase. Five-coordination in the



Mo(VI) state seems preferred to either four- or six-coordinate alternatives. Moreover, the fifth and least definitively identified enzymatic ligand is clearly assigned as a hydroxyl group by the calculational protocol. Studies of reaction energetics favor a transfer of this hydroxyl group to the substrate during turnover. Interestingly, the calculation also appears to support the type of carbon–metal interaction favored by Lowe et al. for the activation of the C—H bond of xanthine [238].

The DF calculations of Voityuk et al. [220] find that, in addition to the LMoOS(OH) structure, $\text{LMoOS(OH}_2\text{)}$, LMoO(SH)(OH) , and LMoS(OH)_2 structures are also energetically reasonable. The lack of planarity of the dithiolene chelate ring is noted, producing conformational options with the dithiolene $\text{C}=\text{C}$ and the oxido groups on the same or opposite sides of the MoS_2 plane. The degree of structural and protonation (hydronation) flexibility shows that many different conformations are accessible to the active sites in the xanthine oxidase family. Therefore, although theoretical studies can be used to rule out unreasonable structures, the choice between the remaining reasonable structures requires an integrative approach involving theoretical, structural, and spectroscopic techniques.

VI. MECHANISMS OF ACTION

Recent x-ray crystallographic and spectroscopic studies provide an unprecedented view of the molybdenum and tungsten active sites (see Section IV). These studies, coupled with work on model systems (see Section V), now provide boundaries for the discussion of possible reaction mechanisms. In this section, we review the catalytic role of the molybdenum and tungsten sites, including both the processing of substrates and the reactivation of the active centers. The following topics are considered: (1) Lewis acid assisted coordination catalysis, (2) coupled electron proton transfer (CEPT), (3) oxygen atom transfer, and (4) CEPT with concomitant water activation. Possible mechanisms for acetylene hydratase, formate dehydrogenase, polysulfide reductase, dmsO reductase, sulfite oxidase, and xanthine oxidase are discussed.

VI.A. Lewis Acid Assisted Coordination Catalysis

The divalent ions Mg^{2+} , Ca^{2+} , and Zn^{2+} have long been known to serve as Lewis acids in important biological processes [223,224]. As Lewis acids, these metals either activate substrate toward hydrolysis or activate a nucleophile such as water or serine. The high-valent tungsten site in acetylene hydratase may play one (or both) of these roles.

Acetylene hydratase [74] is unique among the molybdenum and tungsten enzymes. This enzyme catalyzes the hydration of an unsaturated organic substrate, acetylene, a reaction that is neither an oxidation nor a reduction. Although tungsten may assume different oxidation states, the non-redox nature of the sub-



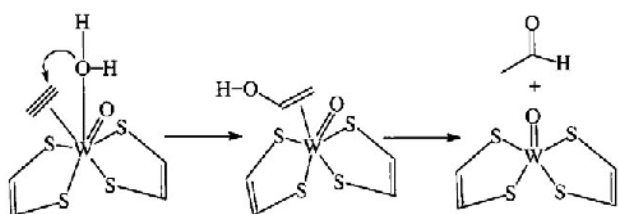


Figure 22 Possible mechanism for the W(IV)-catalyzed hydration of acetylene to acetaldehyde.

strate reaction implies that any role for oxidation state change lies in activation and/or deactivation of the enzyme. Interestingly, studies of oxido-tungsten-1,2-enedithiolate complexes (see Section V.E) clearly demonstrate that tungsten oxidation state changes modulate catalytic activity of the metal complex. Specifically, whereas $[(mnt)_2W^{IV}O]^{2-}$ is an active catalyst for acetylene hydration, $[(mnt)_2W^{VI}O_2]^{2-}$ is not [186].

Hydration of alkynes is generally Lewis acid assisted [225]. In acetylene hydratase, acetylene could bind to the W(IV) center and be activated toward nucleophilic attack by water. To increase its nucleophilicity, water could either coordinate to the metal center (with deprotonation) or be activated through contact with basic amino acid residues or other hydrogen bonding acceptors. Both methods are known for biological water activation [223,224]. The active site of acetylene hydratase has not yet been crystallographically characterized, and site-directed mutagenesis, producing enzymes of altered activity, has yet to be described. It is not presently known whether the metal or the protein is involved in water activation. Regardless, the initial product in the hydration would likely be the enol (vinyl alcohol), which, upon tautomerization, yields acetaldehyde (Figure 22).

VI.B. Coupled Electron Proton Transfer (CEPT)

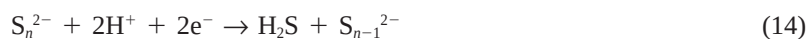
VI.B.1. Substrate Reactions

There are a large number of biological reactions that formally require hydrogen atoms or hydrides to be removed from or added to a substrate. Chemically, hydride, H^- , is a common reagent, but because of its large negative redox potential, it is biologically far less prevalent. In biological systems, “hydride” transfer often corresponds to the transfer of a proton (hydron) and two electrons [197, 226]. Related hydrogen atom transfer reactions describe the transfer of a proton and a single electron.



Oxidation state changes at molybdenum or tungsten, $M(IV) \rightleftharpoons M(V) \rightleftharpoons M(VI)$, are generally coupled to gross changes in the pK_a of the metal-bound ligands, which include OH^- and SH^- . The low pK_a of these ligands when bound to the $M(VI)$ state generally leads to their being fully deprotonated to O^{2-} and S^{2-} , respectively. In contrast, the much higher pK_a of the OH and SH moieties when bound to either the $M(V)$ or $M(IV)$ states leads to these ligands being protonated (OH , OH_2 , SH , SH_2) under physiological conditions. Therefore, proton transfer reactions on molybdenum and tungsten centers are generally coupled to electron transfer reactions and oxidation state changes of the metal, a process referred to as *coupled electron proton transfer* (CEPT) [197,226,227].

Most of the substrate reactions catalyzed by the molybdenum and tungsten enzymes involve either incorporation or removal of an oxygen atom. For a CEPT process to apply to these reactions, transfer of protons and electrons must occur concomitantly with either the addition or elimination of water (see Section VI.D). However, the substrate reactions of polysulfide reductase and formate dehydrogenase [122,228] (Eq. 14 and 15)



are unique among those catalyzed by molybdenum and tungsten in that oxygen atoms are not removed from or incorporated into the substrate as part of the reductive or oxidative process, respectively. Therefore, a proton coupled two-electron reduction or oxidation of substrate is sufficient to effect the reaction. The electron transfers may be either inner sphere or outer sphere, so it is not a prerequisite that substrate bind to the metal center. Although outer-sphere electron transfer cannot be ruled out, it is considered less likely since both polysulfides and formate are good ligands for high oxidation state molybdenum and tungsten centers. To our knowledge, no attempts to inhibit either polysulfide reductase or formate dehydrogenase with substrate mimics while monitoring the metal coordination sphere (by EXAFS or EPR) have been reported.

The x-ray crystallographic results for formate dehydrogenase [111,122] show that the cofactor is des(oxido) but coordinated by selenocysteine in both the Mo^{VI} and Mo^{IV} oxidation states. The lack of an oxido is consistent with the CEPT role for the cofactor with the selenocysteine [3,111,122] serving as the proton acceptor while modulating the redox behavior of the molybdenum center (Figure 23).

VI.B.2. CEPT for Active Site Reactivation

In addition to simple CEPT being a favored mechanistic possibility for polysulfide reduction and formate oxidation, CEPT is almost certainly involved, in *all cases*, in the reactivation of the molybdenum and tungsten cofactor sites



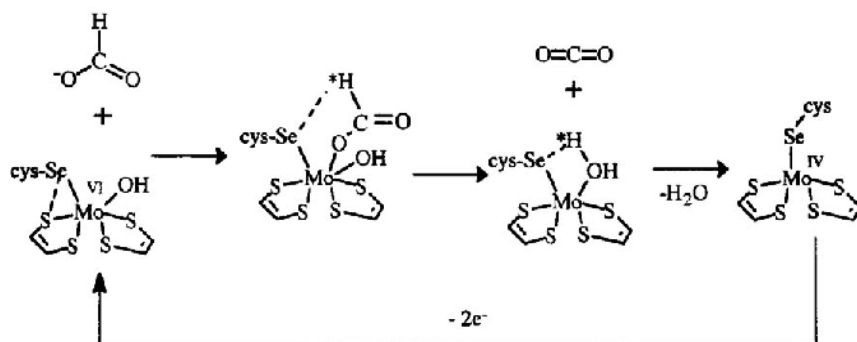
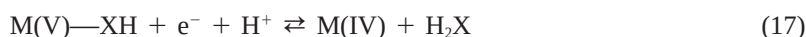
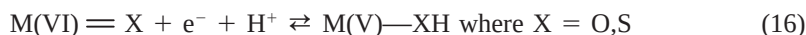


Figure 23 Possible Se-cys assisted oxidation of formate by the (MPTpG)₂Mo(Se-cys)(OH) site of formate dehydrogenase.

[197,226]. As such, CEPT is involved in the critical step of preparing the molybdenum and tungsten centers for substrate reaction. The active state for oxidation is generally thought to be a M(VI) state, whereas that for reduction is a M(IV). In most of the enzymes, M(VI)-bound oxido and sulfido ligands are transformed by protonation to hydroxido and hydrosulfido, respectively, in the lower oxidation states. This change reflects the increased basicity of the M(V) and M(IV) states. The following conversions



involve CEPT and represent the oxidative and reductive activations of the cofactor sites. The proton transfer step may be assisted by protein residues, whereas electron transfer usually involves other prosthetic groups within the protein.

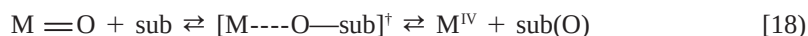
With the exception of some of the dmsO reductases [229,230] (and possibly acetylene hydratase [74]) the molybdenum and tungsten enzymes all contain other redox-active prosthetic groups. The formate dehydrogenase, sulfite oxidase, and aldehyde oxidoreductase crystallographically studied [109,111,116,117] indicate that the most likely path for electron transfer is through the protein backbone to the MPT ligands. The pterin and/or the nucleotide of the cofactors is generally closer to the other redox active prosthetic groups than is the molybdenum or tungsten atom.

VI.C. Oxo Transfer

The large majority of substrate reactions catalyzed by molybdenum and tungsten enzymes deal with a two-electron oxidation or reduction and are associated with



the addition or removal of an oxygen atom, respectively. Studies of molybdenum mono(oxido) and bis(oxido) models (see Section V.D.3 and V.F) have shown that a number of substrates can be oxidized or reduced in reactions that are best described as oxo-transfer processes [196,198,231]. These reactions entail the formal transfer of an oxygen atom. A major determinant of the feasibility of oxygen atom transfer are the relative $M=O$ and $sub(O)$ bond strengths. The reactions are thought to involve innersphere oxygen atom transfer, where, in the activated complex or intermediate, an oxidized substrate is bound to the metal (Eq. 18).



Substrate binding is supported by activation entropies ($\Delta S^{\ddagger}$) that are generally in the range of -20 to -35 eu for both oxidative and reductive processes [232].

Model studies clearly demonstrate that oxo transfer is a viable mechanism for many of the enzyme reactions shown in Table 2d. However, primarily because of difficulties in labeling studies, it has not yet proved possible to validate oxo transfer as a physiologically relevant enzymatic mechanism. Although it has been possible to oxidize and reduce molybdenum centers using certain oxygen atom donors or acceptors, these experiments serve only to demonstrate that such processes are possible and not that they are part of the physiologically relevant pathway [231,233].

VI.D. Coupled Electron Proton Transfer with Concomitant Water Activation or Elimination

Although oxo transfer allows the oxidation of enzyme substrates in biological model systems [196,198,231], the ultimate source of the oxygen incorporated by molybdenum or tungsten enzymes is water (Eq. 19). As such, in the absence of an oxygen atom donor,



these oxidative enzymes are stoichiometrically satisfied by a combination of CEPT and water activation. As microscopic reversibility suggests, the reductive enzymes may combine CEPT in the opposite direction with the elimination of water.

Formate dehydrogenase and polysulfide reductase function by what could be called "simple" CEPT (see Section VI.B.2). However, if CEPT is an important component of substrate reactivity in most of the molybdenum and tungsten enzymes, it must involve not only substrate activation but also water addition or elimination. Involvement of the metal in these processes could rely on oxidation state-coupled pK_a changes of aqua, hydroxido, or hydrosulfido ligands. Whether the metal is directly involved in substrate binding and water activation or whether



it is the protein that activates water and the metal only serves as the conduit for CEPT is not discernible in most cases given our current structural, spectroscopic, and kinetic understanding.

VI.E. Possible Mechanisms

Mechanisms of action for the metal centers in acetylene hydratase, polysulfide reductase, and formate dehydrogenase have been briefly described in Sections VI.A and VI.B. The discussion, in each case, was relatively straightforward insofar as the natures of these reactions lend themselves to simple mechanistic proposals. The mechanism by which the metal centers function in most of the other Mo and W enzymes is not as obvious. We elect to discuss mechanistic roles for the molybdenum centers in xanthine oxidase, sulfite oxidase, and dmsO reductase. These enzymes are representative members of each large class of molybdenum enzymes, and the large body of literature on each enzyme makes detailed discussion possible.

VI.E.1. A Possible Mechanism for Xanthine Oxidase

Over the course of study of xanthine oxidase, proposals for the mechanism by which the metal functions have ranged from hydride transfer to oxo transfer to CEPT linked to water activation [226,233–235]. The latter proposal is presently favored, given what is known about (1) the source of the oxygen atom incorporated into product, (2) the fate of the substrate C(8)—H bond, (3) the role of the sulfido ligand, and (4) substrate binding.

The Source of the Oxygen Atom. Although H_2O is the ultimate source of the oxygen atom incorporated into the urate product of xanthine oxidase, whether the immediate source of the oxygen atom is a molybdenum bound aqua, hydroxido, or oxido ligand or a protein associated water has been the subject of considerable controversy. Increasing evidence suggests that an oxygen atom bound to the molybdenum center as either an aqua or a hydroxido ligand is transferred to substrate and that the single oxido ligand is not the source of the oxygen atom incorporated into the urate product [236].

The x-ray crystallographic work on aldehyde oxidoreductase suggests (see Section IV.E) that the cofactor, $(\text{MPT})\text{Mo}^{\text{VI}}(\text{O})(\text{S})(\text{L})$ where $\text{L} = \text{OH}$ or H_2O , possesses a bound aqua/hydroxido ligand (L) [109,117,160]. From early EPR studies, where the $\text{Mo}(\text{V})$ center of xanthine oxidase was generated with substrate in the presence of H_2^{17}O , a “very rapid signal” was found to be coupled to two ^{17}O atoms. The stronger ^{17}O coupling is assigned to a metal-bound product and the weak ^{17}O coupling is assigned to the oxido ligand [153,154,156,157,237]. Subsequent EPR (and ENDOR) studies under single turnover conditions in H_2^{17}O gave a $\text{Mo}(\text{V})$ center that exhibit only strong ^{17}O coupling and lacks the weak



^{17}O coupling assigned to the oxido ligand [234,238]. Assuming the correctness of the assignment, the oxido ligand is not labeled in the single turnover and can therefore be eliminated as a source of the oxygen atom transferred to the product. Enrichment of the aqua/hydroxido ligand in the single turnover experiment makes it the likely source of the oxygen atom incorporated into the product. This conclusion is consistent with x-ray [47,109,160], EXAFS [135,150,151,213], and theoretical results [216–218,220] that suggest that the oxido ligand is maintained in both the oxidized and reduced metal centers and appears to act as a spectator ligand [222] in the catalytic cycle (Figure 24).

Since the oxido ligand is not the source of the oxygen atom incorporated into product, simple oxo transfer does not appear to be viable mechanism. Rather, some form of CEPT seems more likely and, as discussed in the next section, appears to involve the sulfido ligand of the $(\text{MPT})\text{Mo}^{\text{VI}}(\text{O})(\text{S})(\text{L})$ cofactor where $\text{L} = \text{OH}$ or H_2O .

The Role of the Sulfido Ligand. The inactivity of desulfo-bis(oxido)xanthine oxidase suggests a unique role for the sulfido ligand of the $(\text{MPT})\text{Mo}(\text{O})(\text{S})(\text{L})$ xanthine oxidase cofactor. The $\text{Mo}^{\text{VI}}/\text{Mo}^{\text{V}}$ and $\text{Mo}^{\text{V}}/\text{Mo}^{\text{IV}}$ redox potentials for desulfo xanthine oxidase are sufficient to oxidase xanthine to uric acid and therefore the role of the sulfido ligand is not simply to modulate the reduction potential of the metal [91,92,99]. The EPR studies, using C(8) deuterium labeled xanthine as substrate, show that ^1H hyperfine observed in the rapid signal is due to a proton that originated on the C(8) position of substrate [239]. This result, combined with EXAFS, which shows a lengthening of the $\text{M}-\text{S}$ bond, suggests that $(\text{MPT})\text{Mo}^{\text{V}}(\text{O})(\text{SH})$ is an intermediate in the catalytic cycle [152–158]. In this scheme, the sulfido ligand of the $\text{Mo}(\text{VI})$ state functions as the proton acceptor while the metal accepts two electrons in this CEPT from

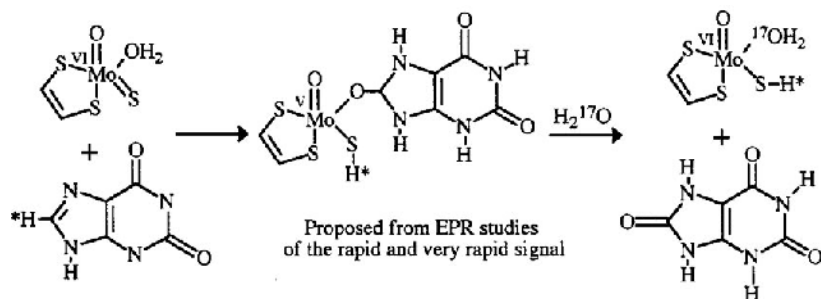


Figure 24 Incorporation of an oxygen label into xanthine oxidase during single turnover and the intermediate resting site of the C(8) proton as determined from EPR studies (see text).



substrate to cofactor. One electron oxidation yields the Mo(V) intermediate (Figure 24).

Interestingly, the desulfo cofactor can also undergo CEPT reactions as seen in the dithionite reduction of xanthine oxidase, where an oxido ligand is converted to a hydroxido ligand. Since the oxo can function as a proton acceptor, a unique feature of the sulfido must be invoked to explain the inactivity of the desulfo enzyme. The formation and stabilization of the hydrosulfido ligand must be essential to the oxidation of purines, facilitating the forward reaction in a way that the oxido/hydroxido conversion does not. Given our current understanding, it is not possible to place accurate values on either the $\text{Mo}=\text{S}/\text{Mo}-\text{SH}$ or $\text{Mo}=\text{O}/\text{Mo}-\text{OH}$ bond strengths or the relative pK_a values of the $\text{Mo}-\text{SH}/\text{Mo}-\text{OH}$ ligands. If the $\text{Mo}=\text{S}/\text{Mo}-\text{SH}$ conversion is thermodynamically more favorable, or if the sulfido is a better base, it could explain the observed reactivities. Since sulfur is significantly larger than oxygen, and its orbitals more diffuse, the sulfido is more accessible to substrate than is the oxido ligand and this could also explain the inactivity of the desulfo enzymes. It is unlikely that the stabilization of the hydrosulfido is due to the protein, since protein hydrogen bonding would stabilize both the hydrosulfido and hydroxido ligands. Ligand field strength could contribute to differences in reactivity since both oxido and hydroxido are stronger field ligands than either sulfido and hydrosulfido [240]. In addition, the enhanced redox reactivity of the sulfur based ligands (sulfido and hydrosulfido) relative to the oxygen base ligands (oxido and hydroxido) may also play a role, although such suggestions are, at this time, conjecture [36].

Substrate Binding. Xanthine oxidase is inhibited by the substrate mimic allopurinol (a drug used in the treatment of gout), which, from EPR studies, appears to bind to the metal center. This finding suggests that purines have direct access to the molybdenum active site in xanthine oxidase and could be activated toward nucleophilic attack by direct binding of the metal center to N(7). Structural considerations and EPR studies suggest that the tight binding of allopurinol could be assisted by a $\text{S}-\text{H}\cdots\text{N}$ hydrogen bond (Figure 25) [241].

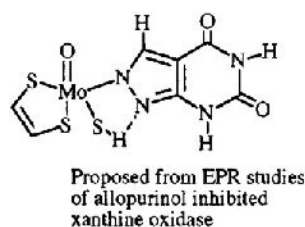


Figure 25 Suggested Mo(V) site of allopurinol inhibited xanthine oxidase.



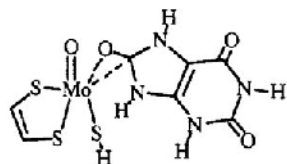
The decrease in rate of reaction of xanthine oxidase with the size of purine substrate is also consistent with a size-selective active site pocket and possible metal binding of substrate [242,243]. A strongly coupled nitrogen is not observed in the very rapid signal, which is thought to include bound product, so it would appear that the urate is not N bound to the molybdenum center [152–158].

From recent ENDOR studies [120,218,238] of the very rapid signal of xanthine oxidase generated using $^{13}\text{C}(8)$ labeled xanthine, it has been suggested that the urate product is bound to molybdenum via an $\eta^2\text{-C=O}$. This interpretation is based upon the observed $^{13}\text{C}(8)$ superhyperfine coupling to molybdenum, which allows approximation of the Mo—C distance at 2.2 Å. Such a Mo—C distance is shorter than expected for an alkoxylate (3.2 Å) or four-membered Mo, O, C, N ring (2.6 Å) and has led to the $\eta^2\text{-C=O}$ proposal shown in Figure 26. However, the data suggesting a direct Mo—C bond are not yet fully compelling [233,234].

Possible Xanthine Oxidase Mechanism. The proposed reaction mechanism, (Figure 27), which must still be regarded as a working hypothesis, entails metal binding of substrate, metal-assisted activation of water, CEPT, and stabilization of the hydrosulfido ligand.

VI.E.2. Possible Mechanisms for Sulfite Oxidase

Given the notion of microscopic reversibility, and the similarity in the active sites of sulfite oxidase and nitrate reductase (assimilatory), determining the mechanism of action of sulfite oxidase impacts upon our understanding of reductases in the (MPT)Mo(O)₂ family. A key issue in the mechanism of sulfite oxidase is whether substrate binds to the metal center during the catalytic cycle. Substrate (or product) binding to the molybdenum center, as proposed for the catalytic



Proposed from ENDOR studies
using $^{13}\text{C}(8)$ labeled xanthine

Figure 26 Mo^V bound $\eta^2\text{-O-C}(8)$ uric acid proposed from ENDOR studies of xanthine oxidase using ^{13}C labeled xanthine [120,218,238].



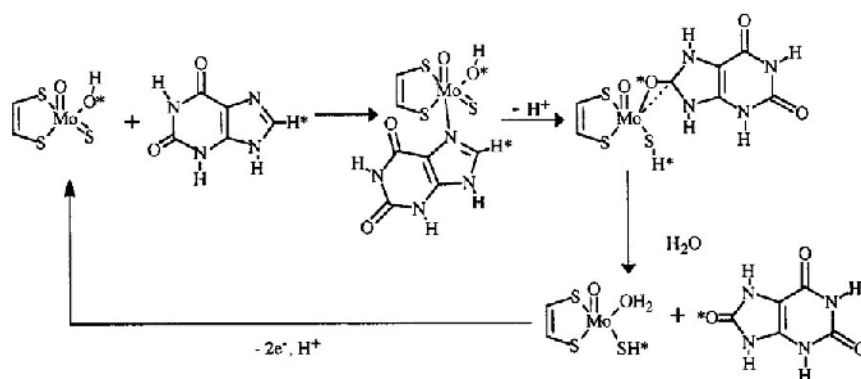


Figure 27 CEPT-H₂O addition mechanism for the oxidation of xanthine to uric acid by the (MPT)Mo^{VI}(O)(S)(OH) site of xanthine oxidase.

activity of a number of (oxido) molybdenum complexes [198,232], is necessary for oxo transfer. However, substrate (or product) binding does not exclude a CEPT process.

EPR studies suggest that oxyanions have access to the sulfite oxidase metal site. ¹⁷O coupling in EPR studies of sulfite oxidase in the presence of ¹⁷O labeled phosphate show that phosphate (and by inference sulfite) can bind to the molybdenum center [244]. If sulfite approaches the (MPT)Mo(O)₂ site, the sulfur lone pair of sulfite could attack the oxido ligand, initiating oxo transfer and generating bound sulfate as the initial product. Comparative kinetic studies of sulfite oxidase using sulfite and dimethyl sulfite can be interpreted as favoring oxo transfer. Although sulfite binds more strongly than dimethyl sulfite (*K_m* = 28 μM for sulfite and 6.3 mM for dimethyl sulfite), the limiting rate is nearly identical for the two substrates [245]. This observation suggests that the rate of oxidation is not dependent upon the charge or the reduction potential of the substrate, but rather on the nature of the new bond formed, an observation consistent with oxo transfer (Figure 28) [196,198].

However, in light of the recent x-ray crystallographic results for sulfate-bound sulfite oxidase, alternative interpretations remain viable [116]. In the crystal, the oxyanion (sulfite or sulfate) is bound to the protein with a Mo—S distance >5 Å. The negative charge of sulfite is substantially diffused by contacts with an extensive hydrogen-bonding network involving five-hydrogen bond donors. Metal binding of sulfite would require considerable movement of the anion, presumably accompanied by considerable reorganization of the active site pocket. On the other hand, a water or hydroxide hydrogen bound to an oxido ligand could



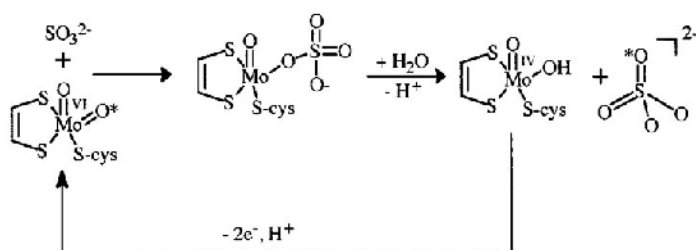


Figure 28 Oxo transfer mechanism for the oxidation of SO_3^{2-} to SO_4^{2-} by (MPT)Mo-(S-cys)(O)₂ site of sulfite oxidase.

be the source of the oxygen atom incorporated into sulfate and a CEPT process linked to water activation could be the mechanism of action in sulfite oxidation [116] (Figure 29). Such a process would represent an outer-sphere example of a CEPT–water activation process.

More studies of the sulfite oxidase/nitrate reductase catalytic cycles, especially those using carefully isotopically labeled enzyme and substrate, are required before a clear mechanistic picture for (MPT)Mo(O)₂(S-cys) family of enzymes will be available.

VI.E.3. Possible Mechanisms for Dimethyl Sulfoxide Reductase

As with the mechanism of sulfite oxidase, a key issue is whether substrate (dmso) binds to the metal center during turnover. Substrate binding to the dmso reductase

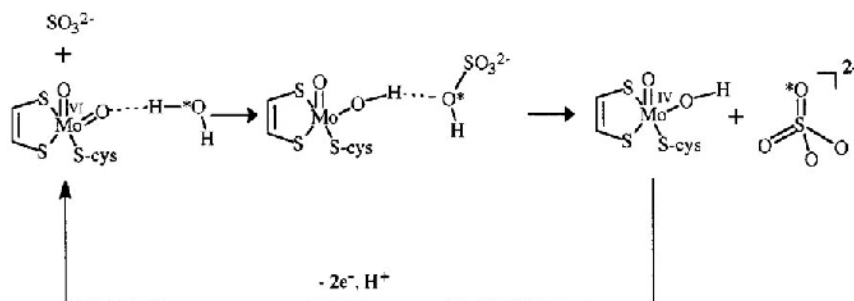


Figure 29 CEPT-H₂O addition mechanism for the oxidation of SO_3^{2-} to SO_4^{2-} by the (MPT)Mo(S-cys)(O)₂ site of sulfite oxidase.



molybdenum center is necessary for (but not exclusive to) an oxo transfer mechanism. In model studies, the binding of dmsO is associated with a large negative entropy. However, such effects are easily accommodated in enzymes by conformational changes in the protein. The most recent x-ray and EXAFS studies of dmsO reductase (*R. capsulatus*) used enzyme crystals grown in the presence of excess dimethylsulfide [126]. The reduced molybdenum site apparently contained a dmsO bound to the molybdenum with a Mo—O bond distance ≈ 2.0 Å. If this structure reflects an intermediate or inhibited form of the enzyme, it nicely supports an oxo transfer mechanism (Figure 30).

However, other studies show that reduction of dmsO reductase in the absence of excess product yields what appears to be a des(oxido) molybdenum with a metal-bound hydroxido ligand [115]. If dmsO were to hydrogen bond to this ligand, CEPT transfer linked to water loss could also generate the mono(oxido) oxidized form of the enzyme (Figure 31).

The oxo transfer mechanism is supported by studies of dmsO reductase using labeled dmsO and a water-soluble phosphane as the terminal oxygen atom acceptor [231]. In these studies, an ^{17}O label from dmsO is transferred to the phosphane acceptor with high efficiency ($>80\%$ under single turnover conditions) [231]. Although results of these studies suggest that an oxo-molybdenum linkage is likely, it can only be said the oxygen atom is abstracted from dmsO, and subsequently transferred to a phosphane acceptor. The importance of a molybdenum oxido species in this process must be spectroscopically confirmed (e.g., Raman spectroscopy of dmsO labeled molybdenum oxido site) to establish the location of the labeled oxygen [231].

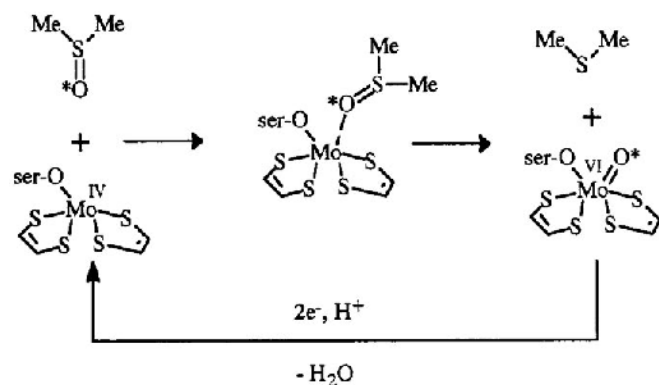


Figure 30 Oxo transfer mechanism for the reduction of dmsO to dms by the (MPTpG)₂ Mo^{IV}(O-Ser)(OH) site of dmsO reductase.



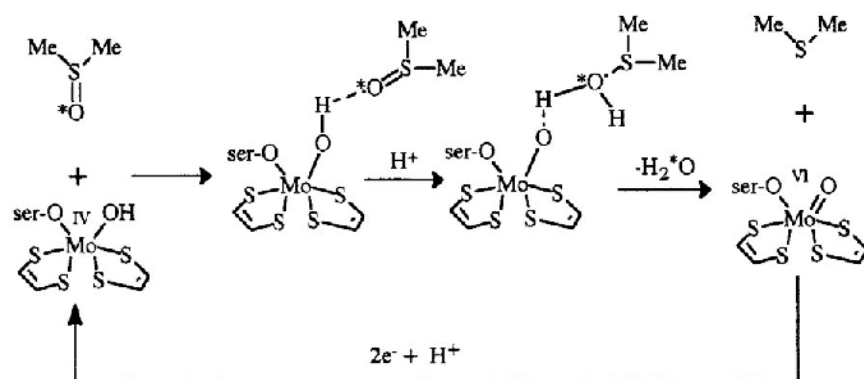


Figure 31 CEPT-H₂O elimination mechanism for the reduction of dmsO to dms by (MPTpG)₂Mo^{IV}(O-Ser)(OH) site of dmsO reductase.

VII. CONCLUDING REMARKS

The molybdenum and tungsten enzymes now represent a broad and important family of almost 40 proteins. The family has been broken down into four genetic/structural subfamilies, each containing either molybdenum or tungsten with one of the MPT variants. Our knowledge of these enzymes is expanding rapidly, as evidenced by the number of new enzymes discovered and crystallographically characterized since the publication of the first edition of *Bioinorganic Catalysis* (1993). We are now at the level of understanding wherein intimate questions of mechanism can be formulated and addressed. The next few years should see the continued integration of work from x-ray, models, spectroscopy (EXAFS, EPR/ENDOR, Raman), and kinetics giving us strong insight into the functioning of the remarkable family of Mo and W enzymes.

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7

Catalysis by Nitrogenases and Synthetic Analogs

David J. Evans, Richard A. Henderson, and Barry E. Smith

John Innes Centre, Norwich, England

I. INTRODUCTION

Nitrogenases (EC 1.18.6.1) are the enzymes responsible for the biological fixation of atmospheric dinitrogen to ammonia. These enzymes are found only in bacteria, although some of the bacteria form symbioses with plants.

Three types of nitrogenase are now known [1–3]. Each consists of two essential, readily separable, dioxygen-sensitive metalloproteins, viz, a small iron protein (Fe protein), which acts as a very specific, magnesium adenosine triphosphate-activated electron donor to the second, larger protein ($M_r \sim 220,000$), which differs for each nitrogenase. All of the large proteins contain iron and acid-labile sulfur, one also contains molybdenum (MoFe protein), one contains vanadium (VFe protein), but the third apparently contains only iron (FeFe protein). The MoFe protein is an $\alpha_2\beta_2$ tetramer. The VFe and FeFe proteins are $\alpha_2\beta_2\gamma_2$ hexamers with polypeptides essentially homologous to the α and β subunits of the MoFe protein plus an additional small subunit, γ ($M_r \sim 13,000$). The larger proteins contain the sites at which substrates bind and are transformed into products [4,5].

The Fe proteins of all three nitrogenases are very similar [1]. The Fe protein of Mo nitrogenase is a dimer ($M_r \sim 65,000$) of equivalent subunits with a single Fe_4S_4 cluster bound between the subunits by bonds between the cluster Fe atoms and the S atoms of two cysteine residues from each subunit [2]. The x-ray crystallographic structure at 0.29 nm resolution of the Fe protein from *Azotobacter vinelandii* (Av2) has been described (Figure 1) [6]. This shows that the Fe_4S_4



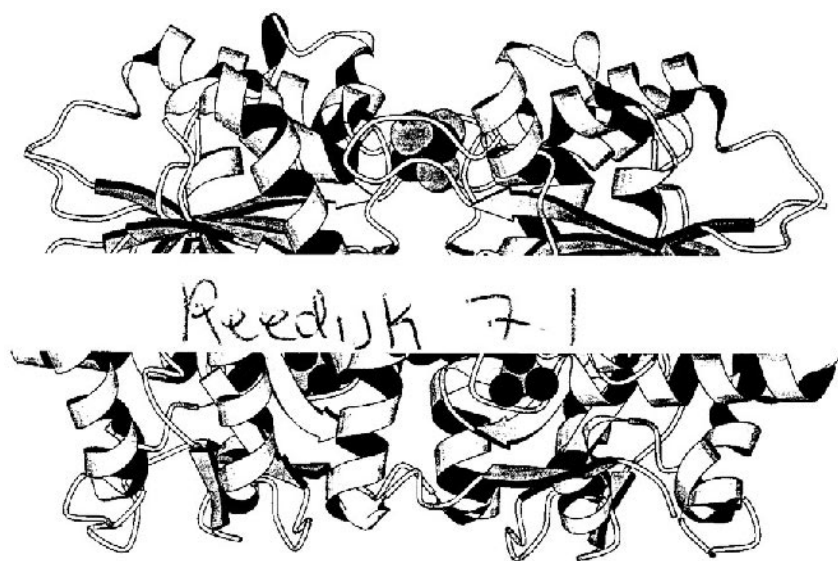


Figure 1 The structure of the Fe protein (Av2) from *Azotobacter vinelandii* (after Ref. 6). The dimeric polypeptide is depicted by a ribbons diagram and the Fe_4S_4 cluster and ADP by space filling models (MOLSCRIPT [132]). The Fe_4S_4 cluster is at the top of the molecule, bound equally to the two identical subunits, and the ADP molecule spans the interface between the subunits.

cluster is bound between the two subunits at one end of the dimer. Each subunit has an eight-stranded, predominantly parallel β sheet that is flanked by nine α helices, and the subunits are related by a molecular twofold rotation axis passing through the cluster. The polypeptide fold observed in the Fe protein is a motif commonly found in nucleotide-binding proteins. This motif, initially recognized by sequence analysis [7], includes, between residues 9 and 16 of Av2, a characteristic sequence pattern GXXXXGKS known as Walker's motif A [8], where X represents any amino acid residue. The crystallographic structure [6] revealed a site for binding molybdate ions (added to the crystallization solution) on the residues of the Walker A motif and also a region partially occupied by an adenosine diphosphate (ADP) molecule with its terminal phosphate close to the bound molybdate. The adenine part of the ADP is bound to the other subunit across the subunit interface. These data were interpreted in terms of adenine nucleotide binding to both subunits with the molybdate representing the third phosphate of ATP. The position of the nucleotide was consistent with two nucleotide binding sites on the Fe protein with one bound with its adenine group in one subunit



and the other in the other subunit. These binding sites on each subunit were approximately 2 nm from the Fe_4S_4 cluster, indicating that the nucleotide does not interact directly with the cluster although the binding of both MgATP and MgADP caused characteristic changes in the spectroscopy and properties of the Fe_4S_4 cluster. These observations imply that the subunit interface provides a coupling mechanism between the nucleotide binding site and the Fe_4S_4 cluster through conformational changes. This hypothesis has been strongly supported by the observation [9] that deleting one residue (Leu127) from between Asp125 in the ATP binding site and Cys132, a ligand to the Fe_4S_4 cluster, causes conformational changes in the Fe_4S_4 cluster similar to those observed on MgATP binding and allows electron transfer from the Fe protein to the MoFe protein without MgATP binding or hydrolysis. Nucleotide binding and/or hydrolysis causing the switching between alternative conformational states of proteins is a general transducing mechanism for coupling the mechanism of this hydrolysis to a variety of biochemical processes [10,11].

The MoFe proteins from a number of bacterial sources have been isolated. Their polypeptide structures are highly conserved and their inorganic components (2 Mo; ~ 30 Fe; $\sim 32 \text{ S}^{2-}$ per molecule) are all very similar [3]. The MoFe proteins from *A. vinelandii*, *Clostridium pasteurianum*, and *Klebsiella pneumoniae* are denoted by Av1, Cp1, and Kp1, respectively. Early Mössbauer spectroscopy demonstrated [12] that the Fe atoms were probably present as clusters, and a wide range of spectroscopic techniques have been used in attempts to understand the structures of these clusters.

X-ray crystallography has revealed that each $\alpha_2\beta_2$ tetrameric MoFe protein contains two each of two types of cluster [13–16]. These are the iron molybdenum cofactor (FeMoco) centers and the Fe_8S_7 P clusters. The two FeMoco centers are bound within the α subunits about 1 nm below the surface of the protein and are separated by about 7 nm. The P clusters are situated at the interface of the α and β subunits, and each is approximately 1.9 nm from one of the FeMoco centers.

The tetrameric MoFe protein is made up of two $\alpha\beta$ dimers with interactions between the helices of the β subunits dominating the interactions at the tetramer interface [17]. A rather open channel of 0.8–1.0 nm diameter and 3.5 nm length extends through the center of the tetramer (Figure 2).

FeMoco (Figure 3) can be considered as being made up of two clusters of fragment composition Fe_4S_3 and MoFe_3S_3 bridged by three nonprotein sulfur ligands (see Section II). In the original description [13] of the Av1 structure one of these ligands was designated Y since it had a somewhat lower electron density than the other two ligands. However, more recent data on Av1 [14] and those available on Cp1 [15] and Kp1 (see later discussion) are consistent with all three bridging ligands being sulfide. The homocitrate, an essential component of FeMoco [18–20], binds to the molybdenum atom through hydroxyl and carboxyl-





Figure 2 The tetrameric structure of the MoFe protein (Av1) from *Azotobacter vinelandii* (after Kim and Rees [17]). The two FeMoco clusters (top left and bottom right) and the P clusters (top right and bottom left) are depicted by space filling models and the $\alpha_2\beta_2$ polypeptides by ribbons diagrams (MOLSCRIPT [132]). The FeMoco clusters are bound only to the α subunits, whereas the P clusters span the interface of the α and β subunits.

ate oxygen atoms. The FeMoco is bound to the protein by Cys275 and His442. The cysteinate ligand binds to the tetrahedral Fe atom at one end, and the histidine binds to the Mo atom at the other end of the molecule. Both of these residues had earlier been identified as potential cluster ligands from molecular biological studies [21,22].

It has proved difficult to define the structure of the P clusters precisely; however, a consensus is now emerging. The initial description [13] indicated that the P clusters consisted of two Fe_4S_4 clusters, each bound either to the α or to the β subunit by two cysteinate ligands and both sharing bridging cysteinate ligands from the α and β subunits (Figure 4a). Later analysis at higher (0.22 nm) resolution [14] indicated that the two Fe_4S_4 clusters might also be bridged by a sulfur-sulfur link between their corners (Figure 4b). Both of these analyses were on crystals of Av1. However, data from Cp1 indicated that the two corner sulfur atoms could not be more than 0.1 nm apart [15]. This bond length is clearly impossible and led to the suggestion that the two Fe_4S_4 clusters shared a corner sulfur atom, i.e., that the P clusters were in fact Fe_8S_7 clusters (Figure 4c). High-



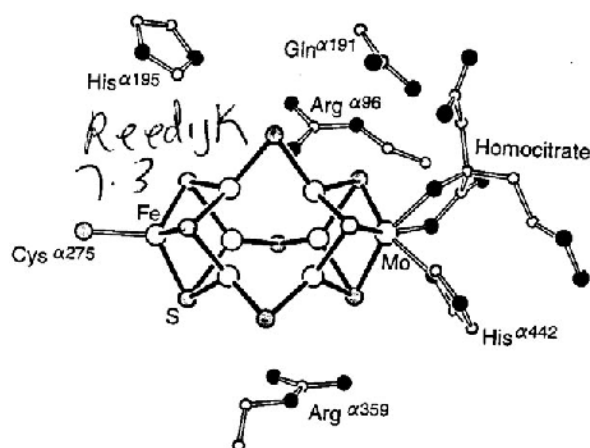


Figure 3 Structure of the iron molybdenum cofactor, FeMoco (after Refs. 14 and 15). The FeMoco is coordinated, within the α subunits of the $\alpha_2\beta_2$ tetrameric structure, by residues His α 442 and Cys α 275. However, Gln α 191 and His α 195 are important in defining reactivity.

resolution 0.16 nm data obtained with Kp1 (D. M. Lawson, S. M. Roe, S. Mayer, C. Gormal, and B. E. Smith, unpublished results) are consistent with this last formulation and have also indicated that the structure of the P clusters changes upon oxidation. This observation may explain the earlier uncertainties in determining the P cluster structure since it is difficult to maintain a totally anaerobic environment for the protein over several days as it is crystallized.

Rees's group have recently revised their analysis of the Av1 structure by using data obtained on the protein in two oxidation states at 0.2 nm resolution [16]. They now accept the Fe_8S_7 formulation for the P clusters and find that the reduced structure is that given in Figure 4c. On oxidation of both the FeMoco and P clusters the former is essentially unchanged, but the P cluster structure is modified to that shown in Figure 4d. This can be described as containing bridged Fe_4S_4 and Fe_4S_3 mainly bound, respectively, to the α and β subunits. Two of the bonds in the reduced cluster, from Fe atoms to the central S atom, have been broken. The backbone amide nitrogen and the sulfur of Cys α 88 coordinate one of these Fe atoms; the other is bound by the O_δ of Ser β 188 and the sulfur of Cys β 153.

FeMoco can be extracted intact from the MoFe proteins and can be combined, to form active protein, with the MoFe protein polypeptides from mutants (e.g., *nifB* mutants) unable to synthesize FeMoco [23]. The function of FeMoco



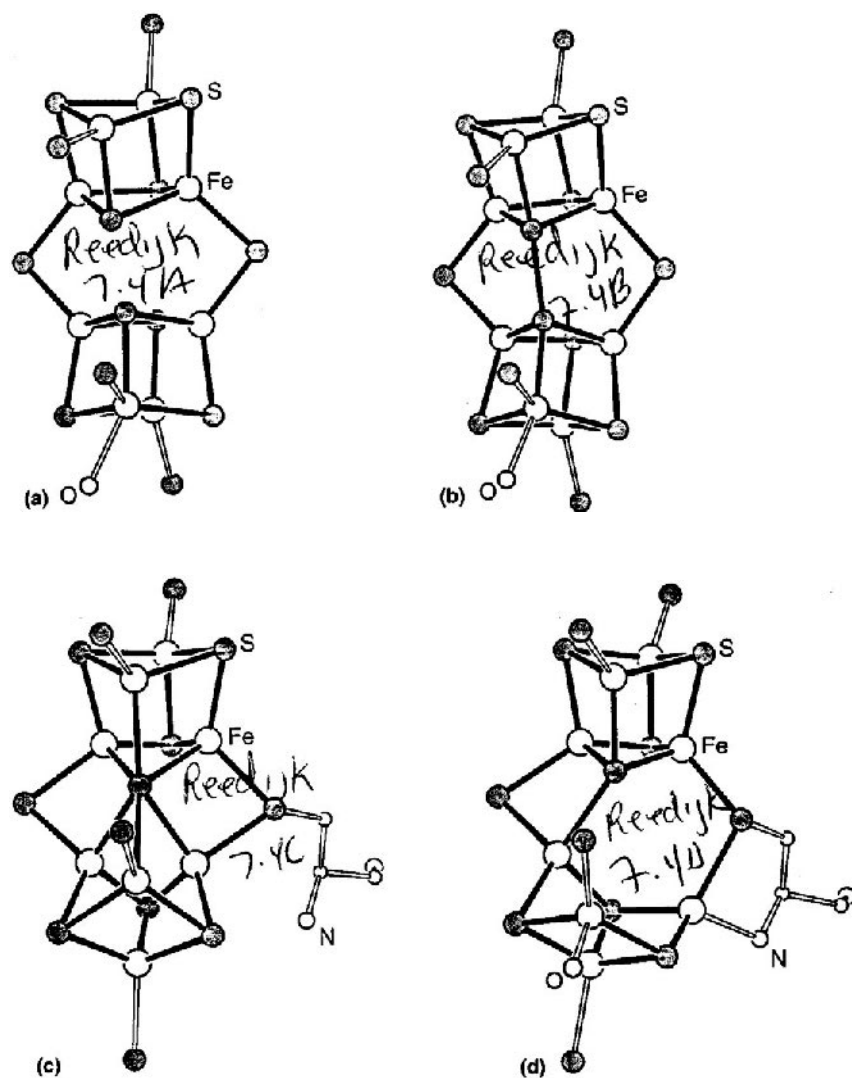


Figure 4 Structure of the P clusters: (a) Fe₈S₈ as described by Kim and Rees [13]; (b) Fe₈S₈ as described by Chan, Kim, and Rees [14]; (c) reduced Fe₈S₇ as proposed by Bolin et al. [15] and confirmed by Peters et al. [16]; (d) oxidized Fe₈S₇ as described by Peters et al. [16].



is almost certainly to act as (at least part of) the enzyme's substrate binding and reducing site. The best evidence for this assignment comes from a class of mutants (*nifV* mutants) that have altered substrate specificity. They are poor at nitrogen fixation, and (in contrast to the wild-type enzyme) H_2 evolution from their nitrogenases is inhibited by CO. The MoFe protein from a *nifV* mutant was purified, then FeMoco was extracted from it and used to activate the MoFe protein polypeptides from a *nifB* mutant. The protein formed in this experiment had the same substrate-reducing properties as the MoFe protein from the *nifV* mutant: i.e., the substrate-reducing properties had transferred with the FeMoco (4). It is probable that *nifV* encodes a homocitrate synthase (EC 4.1.3.21) and nitrogenase from *nifV* mutants has citrate rather than homocitrate bound to FeMoco [18–20].

A large number of synthetic metal–sulfur cluster analogs have been prepared and some proposed as suitable models for FeMoco (see Section II). Some of the analogs exhibit spectroscopic properties very similar to those of the cofactors, but none shows nitrogen fixing ability.

There is limited evidence on the role of the P clusters in nitrogenase function, although EPR and UV/visible spectroscopic studies have indicated that the P clusters become oxidized when nitrogenase is reducing N_2 or hydrons but not ethyne [24]. Mutagenesis (to Ala) of individual bridging Cys ligands of the P clusters in Kp1 prevented assembly of the subunits into a tetramer [25], indicating that the P clusters might have a structural role. However, this is unlikely to be their only function, particularly since mutagenesis of both bridging Cys ligands to Ala yields a functional protein [26a].

Recently, two groups [26b,26c] have published procedures for forming a putative “transition state” complex between the Fe protein and the MoFe protein, stabilized by MgADP and AlF_4^- , as previously done with nucleotide-switch proteins. The complex formed has a stoichiometry of MoFe protein to 2 Fe proteins, i.e., $(\alpha\beta\gamma_2)_2$. The solution structure of this complex has been shown by small-angle x-ray scattering studies [26d] to be consistent with that proposed by Howard and Rees [2] on the basis of cross-linking and site-directed mutagenesis studies. This proposal placed the FeS cluster of the Fe protein and the P clusters of the MoFe protein 1.8 nm apart, on the pseudo-twofold axis relating the α and β subunits of the MoFe protein. The crystallographic structure of the complex has now been solved at 0.3 nm resolution [26e] and confirms this overall structure, but it also shows substantial conformational changes in the Fe protein structure with relatively modest changes in that of the MoFe protein. Specifically, each monomer of the Fe protein rotates about 13° toward the subunit interface to generate a more compact form. This movement shifts the Fe_4S_4 cluster about 0.4 nm toward the surface, closing the distance between this cluster and the P cluster to approximately 1.4 nm. Two $MgADP.AlF_4^-$ are bound per Fe protein dimer, with each nucleotide primarily associated with a single monomer and oriented approximately parallel to the dimer interface. This protein–nucleotide interaction differs



from that observed with the isolated Fe protein (see earlier discussion and Figure 1) and renders the overall nucleotide–Fe protein structure much more comparable with that deduced for G proteins. This structure firmly places the P clusters on the electron transfer path from the Fe protein to FeMoco, implying that this is at least one of their roles, although it does not fully explain why such a novel structure is needed.

Our current understanding of the mechanism by which the nitrogenases convert N_2 into NH_3 is heavily dependent on the interplay between enzymatic studies and complementary investigations on simpler synthetic model compounds. This is discussed in detail in Section III. The active-site chemistry of the nitrogenases involves the binding of N_2 and its conversion to NH_3 by a sequence of electron- and hydron-transfer reactions. The structure and reactivity of the various putative nitrogenous intermediates involved at each step in the pathway have been proposed in the light of the established chemistry of the model compounds. Simple mononuclear chemical models with end-on coordinated N_2 are the best understood, but various other models of how the enzyme binds N_2 , including the bridging and side-on coordinated modes, have also been proposed (Section III).

In addition to converting N_2 to NH_3 the nitrogenases can reduce hydrons to H_2 and transform a number of small, unsaturated molecules such as unsaturated hydrocarbons, cyanide ion (CN^-), and azide ion (N_3^-). Our understanding of the enzymatic reactions is again underpinned by studies on chemical models. It has been possible to reduce a number of nitrogenase substrates on Fe-S cluster compounds, and so the possibility that P clusters are involved in the reduction of at least some substrates cannot be totally discounted. Against this hypothesis is the observation that the FeMoco-deficient MoFe protein polypeptides from *nifB* mutants, although they contain P clusters, cannot reduce any nitrogenase substrate under normal assay conditions [27].

The VFe proteins have been shown to contain both the spectroscopic equivalent of the P clusters and an analog of FeMoco called the iron vanadium cofactor (FeVaco). EXAFS spectroscopy has indicated that FeVaco is structurally very similar to FeMoco, with the only real difference being that Mo is replaced by V [1].

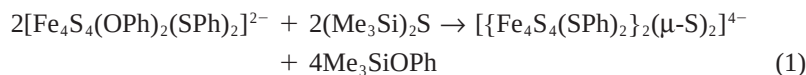
Early preparations of the third, iron-only, nitrogenase had very low nitrogen-reducing activity, which was not commensurate with the enzyme's *in vivo* activity. However, more recently preparations with good nitrogen-reducing activity have been described [28], thus demonstrating that this is a true analog of the Mo and V enzymes. Structural information is limited, but it is clear that activity requires homologues of the genes required for FeMoco or FeVaco biosynthesis, and thus it is probable that the FeFe protein contains an analogous cofactor (FeFeco) [1].



II. MODELS FOR NITROGENASE P CLUSTER AND COFACTORS

II.A. P Cluster Analogs

The function of the P clusters is unclear but is most likely as a component of the electron transfer pathway. Although they are probably not sites of catalytic activity in themselves, it is appropriate to describe attempts to prepare their chemical analogs here. As described, there has been some confusion as to the structure of the P clusters of nitrogenase. No chemical analogs with structures the same as any of those proposed for the P clusters have been isolated. Several monosulfido- or thiolato-bridged Fe_4S_4 dimers have been prepared, such as sulfido- or thiolato-bridged 3:1 site-differentiated clusters [29–31] and the sulfido-bridged cluster $[\{\text{Fe}_4\text{S}_4\text{Cl}_3\}_2(\mu\text{-S})]^{4-}$ [32]. A monocysteinate bridged dimer consisting of two Fe_4S_4 clusters $[\{\text{Fe}_4\text{S}_4(\text{L})\}_2(\mu\text{-Cys-OMe})]^{3-}$ (Figure 5), where H_3L is 1,4,7-tris(4-sulfanylbzoyl)-1,4,7-triazacyclononane (Figure 6) and HCys-OMe is L-cysteine methyl ester, has been reported [33]. The only example of a bis(sulfido-bridged) dimeric Fe_4S_4 cluster is the spectroscopically characterized $[\{\text{Fe}_4\text{S}_4(\text{SPh})_2\}_2(\mu\text{-S})_2]^{4-}$ cluster (Figure 5) isolated from the reaction of the 2:2 site-differentiated cluster $[\text{Fe}_4\text{S}_4(\text{OPh})_2(\text{SPh})_2]^{2-}$ with hexamethyldisilathiane (Eq. 1) [34].



No other bis(sulfido-bridged) or bis(thiolato-bridged) dimeric Fe_4S_4 clusters have been produced. The linear $[\text{Fe}_4\text{S}_6(\text{SEt})_4]^{4-}$ cluster can be described

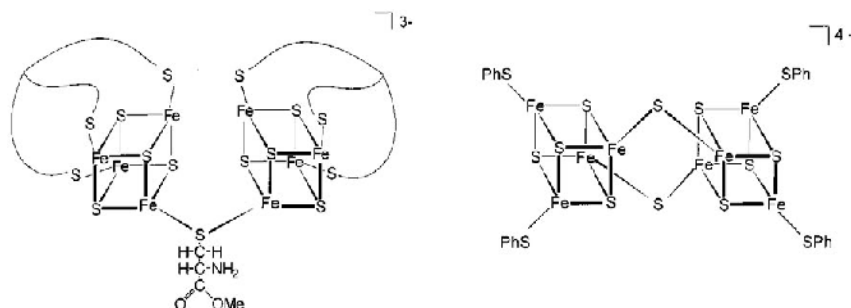


Figure 5 Diagrammatic representation of the monocysteinate bridged cluster $[\{\text{Fe}_4\text{S}_4(\text{L})\}_2(\mu\text{-Cys-OMe})]^{3-}$ where H_3L is 1,4,7-tris(4-sulfanylbzoyl)-1,4,7-triazacyclononane [33], with a depiction of the bis(sulfido-bridged) dimeric Fe_4S_4 cluster $[\{\text{Fe}_4\text{S}_4(\text{SPh})_2\}_2(\mu\text{-S})_2]^{4-}$ [34].



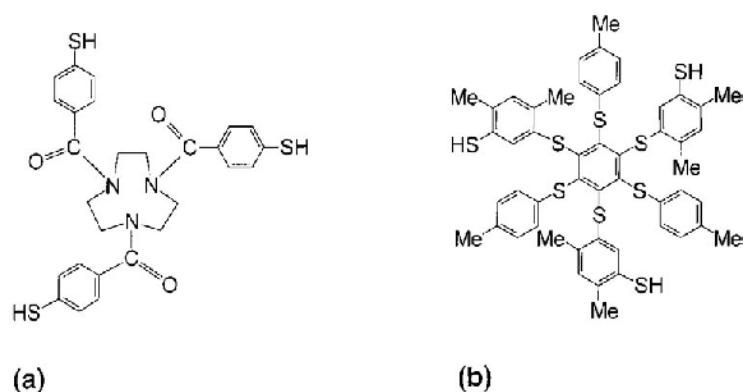


Figure 6 The trithiol proligands (a) 1,4,7-tris(4-sulfanylbenzoyl)-1,4,7-triazacyclononane, H_3L and (b) 1,3,5-tris((4,6-dimethyl-3-mercaptophenyl)thio)-2,4,6-tris(4-tolylthio)benzene, H_3LS_3 .

as comprising two Fe_2S_2 fragments bridged by two μ -S atoms [35], and in the tetranuclear $[Fe_4S_4(CO)_{12}]^{2-}$ ion an intermolecular disulfide bridge joins two Fe_2S_2 cluster fragments [36]. The octanuclear cluster $[Fe_8S_{12}(CNBu^i)_{12}]$ contains two Fe_4S_5 subunits coupled through two sulfido-bridges [37]. Another mode of coupling has been observed in the octanuclear cluster $[Fe_8S_8(PCy_3)_6]$ ($Cy = C_6H_{11}$); here two Fe_4S_4 clusters are directly joined through two $Fe-S$ bonds with no intermediate bridging atoms [37,38]. As the uncertainty of the P cluster structure is resolved, and as more insight is gained into its function, it is likely that directed attempts at chemical synthesis of their analogs will continue.

II.B. Chemical Analogs of the Nitrogenase Cofactors

The FeMoco of the Mo nitrogenases from *A. vinelandii* [13,14], *C. pasteurianum* [15], and *K. pneumoniae* is a heterometallic cluster of stoichiometry $MoFe_7S_9$ (homocitrate), and the structure as determined by X-ray crystallography (Figure 3) shows that the cluster comprises an Fe_4S_3 unit linked by three bridging sulfides to an Fe_3S_3Mo (homocitrate) unit. The six central Fe atoms exhibit a remarkably unusual distorted trigonal iron-sulfido coordination.

Cluster models of the polymetallic aggregates in nitrogenase should (1) contain structural features akin to those defined by the crystal structure; (2) possess or approximate the observed stoichiometry; (3) provide coordination sites at which substrate binding and activation may occur. A model need not mimic protein-bound cofactor reactivity, since isolated cofactors do not reduce sub-



strates as they do when in the protein environment. However, when introduced into an appropriate protein environment, model clusters should exhibit the expected substrate binding and reactivity.

The unique structure of the FeMoco has provided a challenge for synthetic chemists. Indeed it is difficult to envisage how such a molecular assembly can be constructed in situ. The current approach is to prepare synthetic analogs of fragments of the cofactor structure and to explore their reactivities.

II.B.1. The Fe_4S_3 Unit

The Fe_4S_3 unit contains a single tetrahedral Fe through which one end of FeMoco is tethered to the protein by a cysteinate sulfur. This bonding mode is mimicked in the cuboidal 3:1 site-differentiated cluster $[\text{Fe}_4\text{S}_4(\text{L})(\text{Cys-OMe})]^{2-}$ [5]. There are only a limited number of structurally defined Fe_4S_3 clusters. These include Roussin's black anion $[\text{Fe}_4\text{S}_3(\text{NO})_7]^-$ [39–41] (Figure 7), its dianion [41], and the related clusters $[\text{Fe}_4\text{S}_3(\text{NO})_4(\text{PPh}_3)_3]^{n+}$ ($n = 0$ or 1) [42]. In each case there is metal–metal bonding between the Fe atoms. Dimensionally there are some similarities to the Fe_4S_3 unit of FeMoco. Although providing a route to the chemistry of Fe_4S_3 , the required nitrosyl coordination makes their application as synthetic building blocks for cofactor limited. A more significant, structurally defined Fe_4S_3 fragment is found in the polymetallic cluster $[\text{MFe}_4\text{S}_6(\text{PEt}_3)_4\text{X}]$ ($M = \text{Mo}$ or V ; $X = \text{Cl}$ or SPh), described later [43,44].

II.B.2. The Central, Trigonal Iron Atoms

Examination of the cofactor structure shows that the six central, 3-coordinate, Fe centers are best described as trigonal pyramidal rather than trigonal planar,

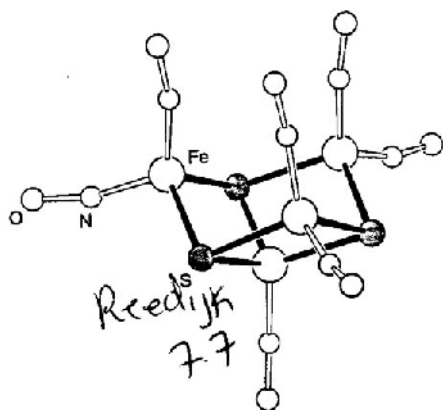


Figure 7 The structure of Roussin's black anion, $[\text{Fe}_4\text{S}_3(\text{NO})_7]^-$ [39].



as each of the Fe atoms is positioned below the S_3 plane, toward the center of the cluster, by a mean distance of ca. 0.051 nm [38]. There are no known examples of sulfido-coordinated 3-coordinate Fe complexes. Bulky thiolate ligands have been employed to stabilize low-coordinate high-spin Fe^{II} complexes [45–47], and some of these have been described as containing 3-coordinate, trigonal planar Fe. However, on reexamination of the crystallographic data for $[Fe(SR)_2]_2$ ($R = C_6H_2Bu^t_{3-2,4,6}$ or $C_6H_2Ph_{3-2,4,6}$) [45,46] and by Mössbauer criteria it has been argued that the geometry about Fe in these complexes is not 3- but distorted tetrahedral 4-coordinate [48]. The fourth coordination “bond” arises because of agostic interactions between Fe and hydrogens on aryl substituents or Fe and the π -electron cloud of phenyl rings of the ligand. There is one unambiguous example of a homoleptic 3-coordinate thiolato-Fe complex, and this is the $[Fe(SC_6H_2Bu^t_{3-2,4,6})_3]^-$ anion (Figure 8), which shows a slight deviation from planar coordination about Fe [47].

Holm and coworkers have adopted the approach of employing bulky trialkylphosphane ligands to generate Fe complexes with a coordination geometry intermediate between that of the predominant tetrahedral organization for Fe and that found for the central Fe atoms of the cofactor. This intermediate geometry still has 4-coordinate Fe, but the Fe has moved down into the S_3 plane. Structurally confirmed examples of such behavior have been observed for $[Fe_4S_3(NO)_4(PR_3)_3]$ ($R = Et, Ph$) [38,42], $[MFe_4S_6(PEt_3)_4X]$ ($M = Mo, V$; $X = Cl, SPh$) [43,44], $[Fe_6S_6(PBu^t_3)_4Cl_2]$ [49,50], $[Fe_6S_6(PEt_3)_4(SC_6H_4-4-Br)_2]$ [51], $[Fe_6S_6(PEt_3)_6]^+$ [52], and $[Fe_7S_6(PEt_3)_4Cl_3]$ [53]. As a target it is hoped that clusters with labile phosphane ligands may be employed in generating Fe centers with planar sulfur coordination that then may collapse, with loss of phosphane, to give novel trigonal pyramidal geometries for Fe that approximate those found in the cofactor.

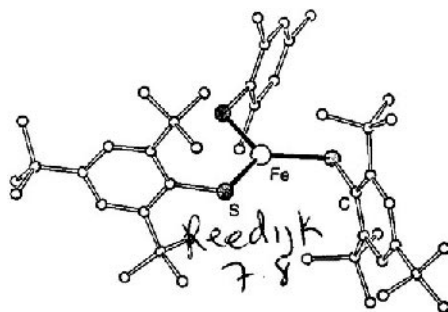


Figure 8 The structure of the homoleptic 3-coordinate iron complex $[Fe(SC_6H_2Bu^t_{3-2,4,6})_3]^-$ anion; terminal methyl groups of one thiolate omitted for clarity [47].



II.B.3. The $\text{Fe}_3\text{S}_3\text{Mo}(\text{Homocitrate})$ Unit

Before structures of the Mo nitrogenases were reported, the stoichiometries of FeMoco and FeVaco were thought to be $\text{MoFe}_{6-8}\text{S}_{8-10}$ and $\text{VFe}_{6-7}\text{S}_{4-6}$, and each was also thought to contain a molecule of homocitrate. X-ray absorption spectroscopic analysis (XANES and EXAFS) had established various interatomic distances within the cofactors. Many Mo-Fe-S clusters had been synthesized and characterized [54–57]. At that time the best models of the local environment about the Mo or V of the cofactors were clusters that contained the $\{\text{MoFe}_3\text{S}_4\}^{3+}$ [57] or the isoelectronic $\{\text{VFe}_3\text{S}_4\}^{2+}$ core [58–60]. These cluster cores partially mimic the first and second coordination spheres about the Mo or V in the MFe_3S_4 fragment of the cofactors but do not mimic the long-range structure of the cofactors. In some instances molecules that are substrates or pseudosubstrates for cofactor have been bound to the heterometal in the model clusters as illustrated in the following examples: (1) Spectroscopy has shown that CN^- , N_3^- , N_2H_4 , PhNHNH_2 , and NH_3 bind to Mo of monocubanes of the type $[\text{MoFe}_3\text{S}_4(\text{SR})_3(\text{cat})(\text{solv})]^{3-}$ (cat = substituted catecholate) [61,62]; $[\text{NEt}_4]_3[\text{MoFe}_3\text{S}_4(\text{SEt})_3(\text{tccat})(\text{CN})]$ (H_2tccat = 3,4,5,6-tetrachlorocatechol) and $[\text{NEt}_4]_3[\text{MoFe}_3\text{S}_4(\text{SC}_6\text{H}_4\text{-4-Cl})_3(\text{tccat})(\text{N}_3)]$ have been characterized crystallographically [62]. (2) Both CN^- and N_2H_4 can act as intercluster bridging units between MoFe_3S_4 cubanes to give the double-bridged double cubanes $\{[\text{MoFe}_3\text{S}_4\text{Cl}_2(\text{tccat})]_2(\mu\text{-S})(\mu\text{-L}')\}^{n-}$ ($\text{L}' = \text{N}_2\text{H}_4$, $n = 4$; $\text{L}' = \text{CN}^-$, $n = 5$) [63]; N_2H_4 also acts as the bridge in the monobridged cluster $[\text{NEt}_4]_4\{[\text{MoFe}_3\text{S}_4\text{Cl}_3(\text{tccat})]_2(\mu\text{-N}_2\text{H}_4)\}$ [64]. (3) When a V monocubane inserted into a semirigid trithiolate cavitand ligand is reacted with an excess of CN^- , three cyanides can bind to the V (Eq. 2: $\text{H}_3\text{LS}_3 = 1,3,5\text{-tris}((4,6\text{-dimethyl-3-mercaptophenyl})\text{thio})\text{-2,4,6-tris}(4\text{-tolylthio})\text{benzene}$, Figure 6) [65]. (4) When the V-containing cluster anion $[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{bpy})(\text{dmf})]^-$ is reacted with a slight excess of PhNHNH_2 or NH_3 in acetonitrile solution, the cluster anions $[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{bpy})(\text{PhNHNH}_2)]^-$ and $[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{bpy})(\text{NH}_3)]^-$ are readily prepared [66].



More recently, and since publication of the structure of FeMoco, molecules have been synthesized that contain Mo in a coordination environment, NO_2S_3 , more akin to that established for the Mo environment in FeMoco. Clusters have been prepared from the reaction of $[\text{NEt}_4]_4\{[\text{MoFe}_3\text{S}_4(\text{SEt})_2(\text{tccat})]_2(\mu\text{-SEt})_2\}$ or $[\text{NEt}_4]_2[\text{MoFe}_3\text{S}_4(\text{L})(\text{tccat})(\text{dmsO})]$ with imidazole or histidine methyl ester, where the Mo atom is coordinated by two catecholate oxygens and N(3) of imidazole (Figure 9), or histidine, binding as neutral molecules [67]. Coucouvanis has developed the chemistry of related clusters in which the MoFe_3S_4 cubane contains Mo-coordinated polycarboxylate. Some of these molecules are catalytically active (see Section IV). The clusters $[\text{NEt}_4]_3[\text{MoFe}_3\text{S}_4\text{Cl}_4(\text{C}_2\text{O}_4)]$,



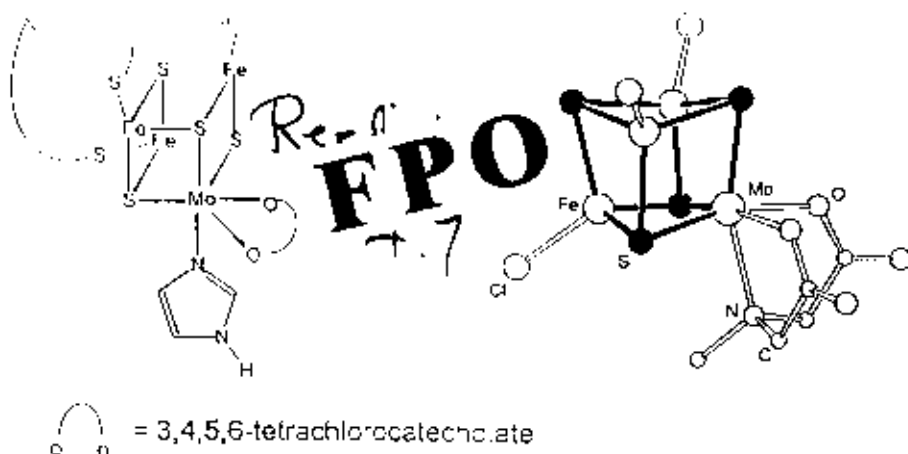


Figure 9 Diagrammatic representation of an $\{\text{MoFe}_3\text{S}_4\}^{3+}$ core-containing cluster with the molybdenum atom in an NO_2S_3 coordination environment [67] and the structure of $[\text{MoFe}_3\text{S}_4\text{Cl}_3(\text{mide})]^{2-}$, where mide is (methylimino)diethanoate [68].

$[\text{NEt}_4]_3[\text{MoFe}_3\text{S}_4\text{Cl}_3(\text{C}_2\text{O}_4)(\text{CN})]$, $[\text{NEt}_4]_5[\{\text{MoFe}_3\text{S}_4\text{Cl}_2(\text{C}_2\text{O}_4)\}_2(\mu\text{-CN})(\mu\text{-S})]$, and $[\text{NEt}_4]_2[\text{MoFe}_3\text{S}_4\text{Cl}_3(\text{mide})]$ (H_2mide = (methylimino)diethanoic acid) (Figure 9) have been crystallographically characterized [68,69]. In the latter complex the coordination environment about the Mo comprises two carboxylate oxygens and a nitrogen atom from the tridentate chelate, (methylimino)diethanoate, and is again similar to that found for the Mo atom of FeMoco. The target cluster molecule that remains elusive is that in which both homocitrate and an N-donor ligand (preferably imidazole) are bound to the Mo atom of the $\{\text{MoFe}_3\text{S}_4\}^{3+}$ core.

II.B.4. Other Polymetallic Aggregates

Two homometallic octanuclear clusters, $[\text{Fe}_8\text{S}_{12}(\text{CNBu}^t)_{12}]$ [37] and $[\text{Fe}_8\text{S}_8(\text{PCy}_3)_6]$ [37,38], have been isolated (see earlier discussion), as has the related heterometallic cluster $[\text{Mo}_2\text{Fe}_6\text{S}_8(\text{PET}_3)_6(\text{tccat})_2]$ [70]. The latter cluster is the first example of an Fe—S edge-linked, reduced MoFe_3S_4 double-cubane with Mo atoms at the periphery of each cuboid subunit. The reduced double-cubane core is stabilized by triethylphosphane coordinated to the Fe atoms. The utility of such materials as precursors to potential cofactor models, both structural and functional, has yet to be explored.

Heterometal cuboidal clusters $[\text{MFe}_4\text{S}_6(\text{PET}_3)_4\text{Cl}]$ ($M = \text{Mo}$ or V) [43,44]



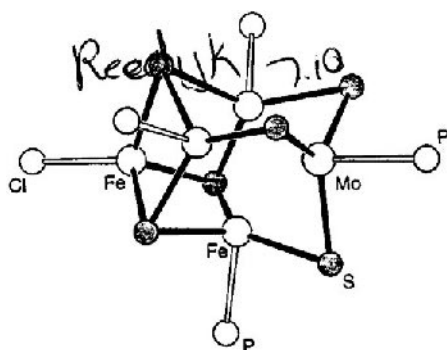


Figure 10 The core structure of the polymetallic cluster $[\text{MoFe}_4\text{S}_6(\text{PEt}_3)_4\text{Cl}]$; ethyl groups on phosphorus atoms omitted for clarity [43,44].

have been prepared by spontaneous self-assembly from $[\text{MCl}_3(\text{thf})_3]$, hexamethyldisilathiane, and $[\text{FeCl}_2(\text{PEt}_3)_2]$ in tetrahydrofuran (thf). When reacted with the sodium thiolates NaSR ($\text{R} = \text{Et}$ or Ph), these clusters give the thiolate derivative $[\text{MFe}_4\text{S}_6(\text{PEt}_3)_4(\text{SR})]$. The crystallographic structures for $[\text{MoFe}_4\text{S}_6(\text{PEt}_3)_4\text{X}]$ ($\text{X} = \text{Cl}, \text{SPh}$) both exhibit the same $\{\text{Fe}_4(\mu_3\text{-S})_3(\mu\text{-S})_3\text{M}\}^+$ core structure (Figure 10), which is composed of a cuboidal Fe_4S_3 cluster, where the apical-cuboid iron atom is tetrahedral and linked through $\mu_3\text{-S}$ to three trigonal pyramidal iron atoms, which are in each case themselves linked through three $\mu\text{-S}$ atoms to the heterometal.

In the related V clusters a similar core structure is seen but with shorter Fe_4S_3 bond distances. The ten atom $\{\text{Fe}_4(\mu_3\text{-S})_3(\mu\text{-S})_3\}$ core is contained in the FeMoco structure, Figure 3, with the same bond connectivity and similar spatial arrangement and, as such, probably provides the best starting point for development of higher nuclearity clusters related to the nitrogenase cofactors.

III. THE MECHANISMS OF THE NITROGENASES

III.A. Introduction

The mechanism of any process can be discussed at several levels of sophistication. This is especially pertinent to the definition of the mechanism of action of enzymes and, in particular, those that require several components to accomplish the catalysis, such as the nitrogenases. In such systems, we can define a mechanism merely as that order in which the various components associate and dissociate. However, this in no way addresses the problems of the underlying chemistry of the enzyme's action. In the following discussion of the mechanism of action



of the nitrogenases, we will show how a multidisciplinary approach, by chemists, biochemists, and geneticists, is essential in order to obtain mechanistic detail down to the atomic level. The following discussion is largely devoted to the Mo enzyme. Studies on the V nitrogenases and their chemical models are in their infancy, but where data are available, comparisons will be made.

III.B. The Electron-Transfer Scheme

The simplest level of mechanistic information for the nitrogenases is the electron-transfer sequence shown in Figure 11. This scheme has been discussed in several previous reviews [71,72], and herein we shall outline only the basic pattern as a foundation for the later discussion.

The electrons are transferred into the nitrogen-fixing system through a ferredoxin or flavodoxin (or in vitro sodium dithionite), mediating the transfer of a single electron to the Fe protein. In turn, the Fe protein transfers electrons (one at a time) onto the MoFe protein, where N_2 is transformed into NH_3 . The conversion of N_2 into NH_3 by nitrogenase thus occurs by a sequence of simple electron- and hydron-transfer reactions: the electrons are mediated through the electron-transfer system (Figure 11) and the hydrons ultimately are supplied from the aqueous solvent.

III.B.1. The Iron Protein–Nucleotide Interaction

The elementary reactions by which the Fe protein is cycled between the oxidized and reduced forms are shown in Figure 12. The Fe protein (Figure 1) binds two molecules of MgATP or MgADP, resulting in two effects: (1) the presence of MgATP changes the midpoint potential of the Fe protein (from *K. pneumoniae*) from $E_m = -200$ mV to -320 mV vs. NHE, whereas the presence of MgADP changes it to $E_m = -350$ mV vs. NHE, thus rendering the Fe protein a thermodynamically stronger reductant; (2) the presence of the nucleotide seems to induce a rapid conformational change in the Fe protein [11,71].

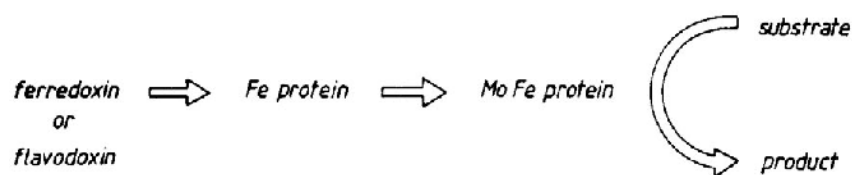


Figure 11 The electron-transfer sequence for the nitrogenases.



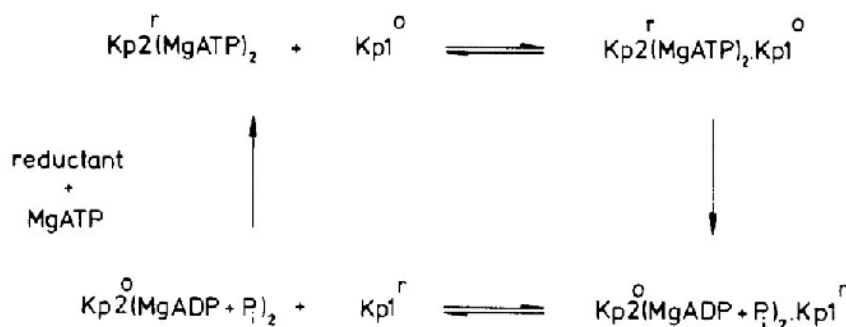


Figure 12 The Fe protein cycle for *Klebsiella pneumoniae*; Kp1 is the MoFe protein; Kp2 is the Fe protein.

III.B.2. The Interaction Between the Fe Protein and the MoFe (or VFe) Protein

The electrons are subsequently transferred to the MoFe or VFe protein one at a time. The rate of binding of the Fe protein to the MoFe protein has been estimated to occur with a rate constant, $k > 5 \times 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ sec}^{-1}$, which is close to the diffusion-controlled limit (72). The Fe protein–MoFe protein electron transfer is followed, when $\text{S}_2\text{O}_4^{2-}$ is the reductant, by the rate-determining dissociation of the two proteins.

The complete sequence of reactions established for the Mo enzyme is shown in Figure 13. This scheme, in combination with that for the Fe protein (in Figure 12), represents the most complete mechanistic picture available [72].

In this scheme, the species designated by E_n represent one-half of the functioning tetrameric MoFe protein (that is, a single FeMoco center), with the subscript showing the number of times the Fe protein cycle has been completed. The single arrow shown between the various states of the MoFe protein corresponds to the three elementary reactions of Figure 12. This sequence of three elementary reactions is repeated a total of eight times around the cycle to accomplish the limiting stoichiometry observed with the enzyme (Eq. 3).



The rate-limiting step of nitrogenase is the dissociation of the reduced MoFe protein from the oxidized Fe protein and has a rate constant $k = 6.4 \pm 0.8 \text{ sec}^{-1}$. An implicit assumption in the multistep mechanism shown in Figure 13 is that the three elementary reactions coupling each state of the MoFe protein are unperturbed by the reduction level of the large protein. This assumption has been verified for the species E_0 and E_1 [73].



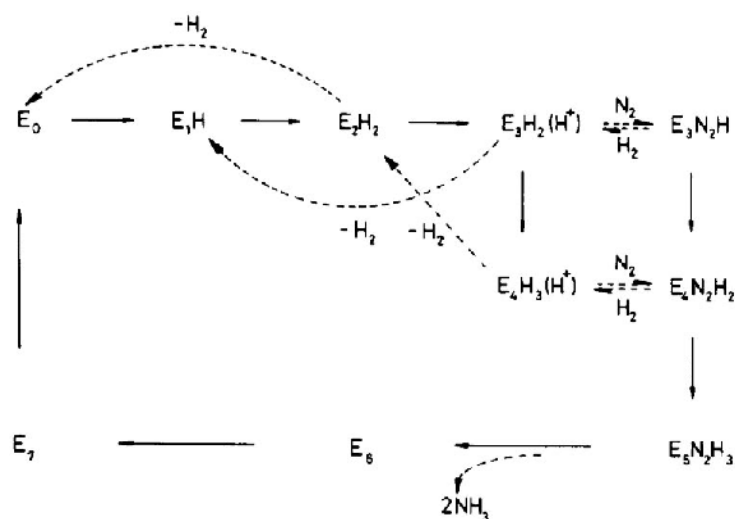


Figure 13 The MoFe protein cycle. Solid arrows connecting E_n and E_{n+1} correspond to the three elementary reactions in Figure 12. The dashed arrows correspond to the binding of N_2 or release of H_2 or NH_3 .

There are two general mechanistic points to be made about this scheme [72]. First, the implicit feature of the scheme is that the Fe protein cannot be reduced by the flavodoxin (or dithionite in vitro) while it is bound to the large protein but only after dissociation of the two proteins. This would indicate that there may be a common site for the electron to enter into and depart from the Fe protein. Second, from simulation of the H_2 evolution kinetics, there is strong evidence that the MoFe protein can only bind N_2 or release NH_3 or H_2 when it is not complexed to the smaller protein.

The only kinetic study on a V nitrogenase [74] reports that the rates of binding of the reduced Fe protein with the VFe protein and the subsequent electron-transfer rates are very similar to those for Mo nitrogenase, as the components of the V nitrogenase form a slightly weaker electron-transfer complex.

We must now consider questions at a higher level of resolution, such as, At what state, E_n , does the N_2 bind to the enzyme, and at what states is H_2 or NH_3 released?

In Figure 13, the state E_0 represents the MoFe protein as isolated in the presence of sodium dithionite, $Na_2S_2O_4$. In the species E_1H and E_2H_2 it is proposed that the hydrons are binding directly to the substrate binding site, preparing the site for the binding of the N_2 [72]. The state E_2H_2 is the first species that is capable of evolving H_2 . The addition of the third electron gives the species E_3H_2 ,



and it is this state together with its successor, E_4H_3 , at which N_2 binds by displacing H_2 (see later discussion). Subsequent species, E_3N_2H , $E_4N_2H_2$ and $E_5N_2H_3$, represents states in which the N_2 ligand becomes successively more hydronated. Between species $E_5N_2H_3$ and E_7 both molecules of NH_3 are released from the enzyme.

III.C. The Substrate Binding Site

Spectroscopic evidence has been widely employed to gain information about substrate binding sites on the MoFe protein. In the presence of the inhibitor CO two EPR signals are observed, and recent ENDOR experiments have demonstrated that these high-CO and low-CO EPR signals arise from FeMoco with one or two CO molecules bound [75]. Several years ago it was proposed, from the variety of different types of inhibitory behavior observed with the alternative substrates, that there were five sites in nitrogenase that were capable of binding substrate or inhibitor molecules. More recently, after a reinvestigation of some of the original studies, this number has been rationalized to two sites [76]. It is important to understand what is meant by "site" in this context. It does not necessarily mean different clusters or even different metals on the same cluster; it can be as simple as the same metal but in a different oxidation state. Thus, we could be observing all substrates binding to the Mo atom but in E_0 , E_1H , E_2H_2 , etc. There is, however, good evidence that in at least two cases more than one substrate/inhibitor can be bound to the enzyme simultaneously: CN^- in the presence of N_2O or C_2H_2 , and CO in the presence of C_2H_2 [77]. Furthermore, some substrates yield more than one type of reduction product. Does this mean that each reduction product is associated with a different site?

The observation, described in Section I, that FeMoco from a *nifV* mutant strain carries the mutant phenotype represents one of the firmest lines of evidence for the involvement of FeMoco as the N_2 binding site [4]. The bound N_2 cannot bridge the two FeMoco centers in the MoFe protein since the x-ray crystallographic data clearly show that the FeMoco centers are some 7 nm apart. The *nifV* product is almost certainly a homocitrate synthase. A system for the in vitro synthesis of FeMoco has been developed [78]. This requires the mixing of extracts of an *A. vinelandii* mutant strain with an extract from a strain containing a mutation in *nifN* or *nifE* in the presence of molybdate, homocitrate, and MgATP. By replacing the homocitrate with one of more than thirty analogs it has been shown [79] that catalytically competent FeMoco requires an organic component with the following features: (1) 1- and 2-carboxyl groups, (2) a hydroxyl group, (3) the R configuration of the chiral center, and (4) a four to six carbon chain length with two terminal carboxyl groups. Analogs with alternative geometries and structures resulted in enzymes with dramatically different catalytic proper-



ties. These data show clearly that homocitrate is intimately involved in substrate reduction.

Site-directed mutagenesis experiments have demonstrated the importance for substrate reduction of other parts of the FeMoco environment (see Figure 3) within the protein. In particular, Glu191 and His195 in the α subunit are critical. Substitution of Glu191 by Lys eliminates N_2 reduction and also affects the ethyne reduction activity [80]. Most interestingly, when His195 is mutated to Glu, N_2 reduction is eliminated but N_2 can still inhibit hydron and ethyne reduction [81]. These data indicate that with this mutant the N_2 can still bind to the active site although it is not reduced to NH_3 . Thus His195 in addition to the carboxyl groups of homocitrate seems to play a critical role in N_2 reduction possibly by acting as a source of hydrons or by stabilizing intermediates.

Reactions of isolated FeMoco have also been used in attempts to identify substrate binding sites. Interpretation of these studies is complicated by the existence of vacant protein ligand binding sites on FeMoco, i.e., the sites where cysteine and histidine bind FeMoco to the protein. It is assumed that in extracted cofactor these sites are occupied by the solvent *N*-methylformamide. ^{19}F NMR and x-ray absorption experiments [82] and EPR data [83] have demonstrated that CN^- and methylisocyanide bind to isolated FeMoco. The EPR data indicated that there may be more than one site for CN^- binding but that one of these may be the tetrahedral iron atom that binds cysteine in the protein.

More recently, following studies on synthetic Fe-S clusters (Section IV.F), a kinetic approach has been used to identify substrate binding sites on isolated FeMoco. In these experiments perturbation of the rate of binding of thiophenolate, PhS^- , to FeMoco by a range of nitrogenase substrates has been studied. The data indicate that CN^- prefers to bind to the tetrahedral Fe, but in the presence of an excess of CN^- another molecule binds at or close to Mo. No evidence for binding of CO and C_2H_2 was obtained. Imidazole, N_3^- , and Bu^tNC bind exclusively at or close to the Mo atom, whereas hydrons probably bind at a bridging sulfur close to the tetrahedral Fe atom [84].

III.D. The Binding of Dinitrogen

In order to understand the binding and activation of N_2 it is necessary to move away from studies on the enzyme. The level of mechanistic information available from studies on any catalyst as it turns over is limited for two reasons. First, we are perforce looking at a large number of steps all together, and so the detail we can get about any one step is severely limited; for instance, we cannot look at any one of the steps shown in Figure 13, in isolation from the others. Second, the nature of the rate-limiting step means that any reaction that occurs after this process is kinetically hidden. Some of the questions that we wish to address about the nitrogenases are as follows: What is the geometry of N_2 when it binds to a



metal site? What characteristics of the binding site cause it to have a high affinity for N_2 ? How are hydrogen ions involved in the binding of N_2 , in the hydronation of the substrate, and in the release of the products? The answers to these questions (and more) can be provided only by the investigation of relatively simple transition metal complexes that aim to mimic the functional group chemistry performed by the nitrogenases.

It is important to stress from the outset that these metal complexes do not aim to model the complicated polypeptide environment of the active site in the enzyme but are designed to keep the system as simple as possible so that the chemistry associated with binding and activation of N_2 can be defined in detail. The study on N_2 complexes does not elaborate just the pathway that the enzyme adopts to convert N_2 to NH_3 , but a wide variety of conceivable routes that are available. The role of the chemist is in part to delineate all of the possible pathways by which N_2 can be converted through to NH_3 . It is just as important to understand the pathways that the enzyme does not use as it is to know the route it does adopt if we are to appreciate fully the mechanism of the nitrogenases.

III.D.1. The Coordination of Dinitrogen

Inspection of the large number of N_2 complexes that have been prepared and structurally characterized by x-ray crystallography reveals that to date there are seven ways in which the ligand binds to the metal (Figure 14) [85–88]. When bound to a single metal or two metals, N_2 can bind in two possible orientations: either end-on or side-on. The remaining examples of N_2 coordination to three or four metal centers are based on only one example of each. This is disappointing since the cluster environment of all metals in nitrogenase makes it likely that there are, at the very least, secondary interactions between the N_2 ligand and several metals at the active site.

In very general terms, two factors make a site particularly good for binding N_2 : (1) the metal must be electron-rich and be associated with a relatively low formal oxidation state and with electron-releasing ligands, and (2) the site must be polarizable, again associated with the same features as in (1), and coordinated by soft donor atoms such as sulfur [89].

III.D.2. Models for Binding Dinitrogen by the Enzyme

The variety of coordination modes for N_2 defined in the simple metal complexes has given rise to various proposals of how the nitrogenases bind and activate N_2 . There are three models extant: mononuclear end-on, mononuclear side-on, and bridging N_2 species. In order to maximize the useful information from model systems it is imperative that the studies have a firm structural basis. In this context, the most well-defined model is that based on the mononuclear end-on coordination, and it is on this system that we will concentrate first.



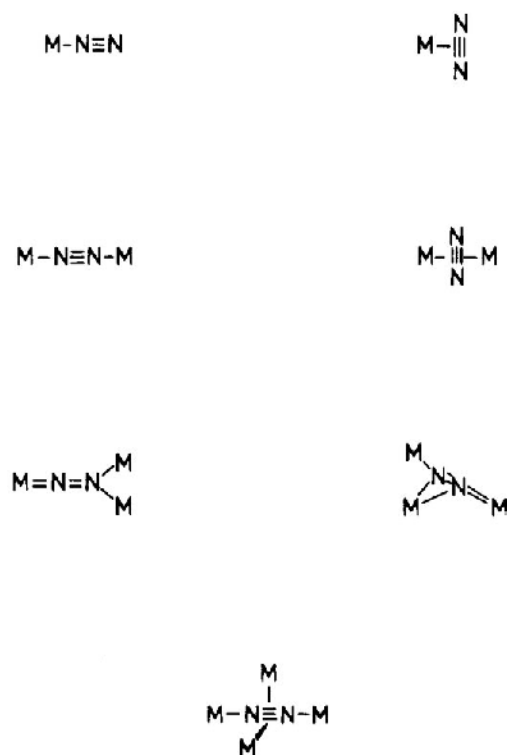


Figure 14 The binding of dinitrogen found in chemical complexes.

The mononuclear system has been developed largely by using the “Mo(diphosphane)₂” [90] core. This site has the advantages that it is inert to protic attack and that it causes the metal to have a high affinity to N_2 ; furthermore, the phosphane chelates remain bound to the metal during the reaction, and they occupy four of the six available coordination sites on the metal, leaving only two free positions where binding and activation of substrates can occur.

III.D.3. Binding of Dinitrogen at a Metal-Hydride Site

There are several circumstantial lines of evidence that indicate that N_2 binds to the enzyme by displacing H_2 (i.e., that the binding site of the enzyme is a metal hydride). The main evidence for a hydride site is (1) that H_2 is a specific, competitive inhibitor for the reduction of N_2 ; (2) the limiting stoichiometry of the MoFe nitrogenase in which we observe the apparent obligatory evolution of H_2 during



the reduction of N_2 ; and (3) the observation that in the pre-steady state H_2 is the first reduction product.

However, there are severe constraints imposed on the nature of this hydride site [72]: (1) nitrogenase catalyzes the formation of HD from D_2 ; (2) when operating under an atmosphere of T_2 less than 2.4% of T^+ is incorporated into the solvent; and (3) when operating under an atmosphere of HD no D_2 can be detected. On these grounds, it has been proposed that N_2 binds to the metal hydride site by the mechanism shown in Figure 15. In this mechanism, the metal site is a dihydride, but most importantly there is a role for the hydron in this binding action. The hydron interacts with the hydride ligands directly, in effect abstracting H^- from the metal and generating a vacant site at which N_2 can bind.

If we consider that the enzyme reached the dihydridic state by binding two electrons and two hydrons, on purely energetic grounds it is surprising that the enzyme should adopt a hydridic N_2 -binding site, since it wastes two electrons. Having consumed these two electrons (and the coupled four ATP molecules), it now seems prepared to waste them by evolving H_2 . This is particularly surprising since it is known from studies on simple chemical systems that N_2 is equally capable of displacing halide, H_2O , or even NH_3 [86] and that these simple substitution processes would not involve the waste of any electrons.

Studies on the binding of a variety of small molecules (including N_2) by $[MoH_4(dppe)_2]$ (1,2-bis(diphenylphosphino)ethane [$dppe$] = $Ph_2PCH_2CH_2PPh_2$) in the presence of acid [91] illustrate a role for the hydron, which is to increase the lability of the hydride ligands (by as much as 1×10^5), as shown in a much simplified form in Figure 16. In this mechanism, it can be seen that the dihydridic state has already been attained, but the studies on the chemical system show that this state can be stable and may have no propensity either to evolve H_2 or to bind a hydron. However, if this dihydridic state involves the metal in a sufficiently high oxidation state, then coupling of the hydride ligands to form a coordinated H_2 molecule can occur [92,93]. The coupling of the hydrogen atoms effectively reduces the oxidation state of the metal and thus increases its affinity for a hydron. When the hydron binds, it labilizes the system to the loss of H_2 , thus generating a vacant site at which either N_2 or H_2 can bind (thus rationalizing the competitive inhibition of these two substrates).

No model for the interaction of N_2 , H_2 , and hydrons has yet explained all

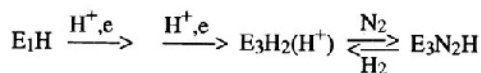


Figure 15 Simplified illustration of the binding of dinitrogen at the enzyme-based hydridic site.



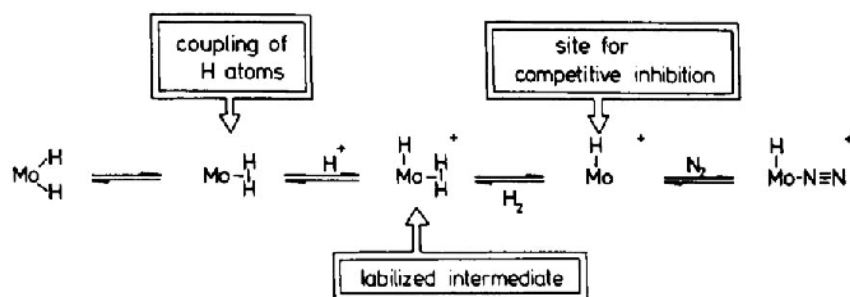


Figure 16 Proposed involvement of hydron (H^+) and dihydrogen ligand in the binding of dinitrogen.

the facets observed with the enzyme. However, if we assume that the lifetime of the hydronated H_2 species is insufficiently long to allow scrambling of all the hydride ligands, then the model shown in Figure 16 is able to rationalize most of the observations (1) to (3) listed previously. What is clear, however, is that there is more than one H_2 problem associated with nitrogen fixation. For instance, if the binding site becomes "overreduced," then the interaction with hydrogen ions results not in activation of the substrate but in preferential reduction of H^+ to H_2 [94].

It is interesting that the intimate mechanisms shown in Figures 15 and 16 introduce a role for the hydron in the binding of the substrate, besides its more familiar role of hydronating the N_2 molecule on the pathway to NH_3 . Clearly it is economical for the enzyme to maximize the use of an essential reagent.

With the identification of a 6-coordinate Mo center in FeMoco concerted effort has gone into identifying which Fe atoms bind N_2 both from a theoretical standpoint [95] and with synthetic iron-sulfur-based clusters [96]. However, there is no a priori reason why N_2 cannot bind to Mo, to make a seven-coordinate species or, indeed, why a Mo ligand cannot dissociate to give access to the substrate. Just such a type of reaction has been observed in $[\text{MoH}_2(\eta^2\text{-O}_2\text{CMe})(\text{dppe})_2]^+$, showing that a carboxylate ligand (for instance, homocitrate in the enzyme) can act as a leaving group at a reduced Mo center, which then provides a site at which N_2 or other substrates can bind [97]. The reactions are summarized in Figure 17. With an excess of acid, electrochemical reduction of $[\text{MoH}_2(\eta^2\text{-O}_2\text{CMe})(\text{dppe})_2]^+$ yields H_2 catalytically via the detected, but short-lived $[\text{MoH}_3(\eta^1\text{-O}_2\text{CMe})(\text{dppe})_2]$, as shown in the top line of Figure 17.

Parenthetically, it is worth noting that the corresponding W analog of this intermediate, $[\text{WH}_3(\eta^1\text{-O}_2\text{CMe})(\text{dppe})_2]$, is sufficiently stable to be isolated and has been structurally characterized by x-ray crystallography [98]. It contains the novel hydrogen bond between W-H and a carboxylate oxygen and upon hydrona-



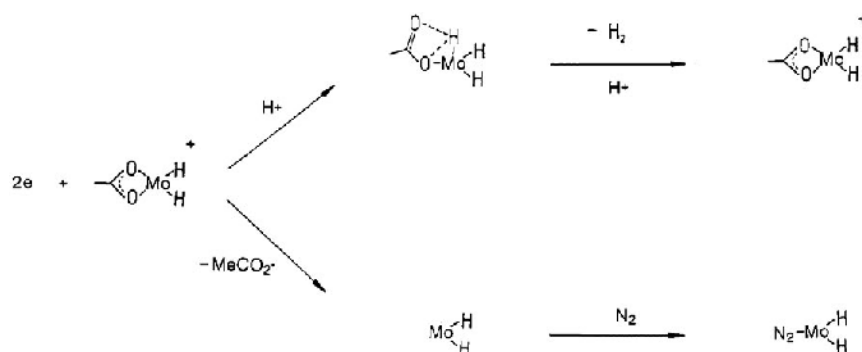


Figure 17 Dihydrogen production and dinitrogen binding in the reduction of $[\text{MoH}_2(\eta^2\text{-O}_2\text{CMe})(\text{dppe})_2]^+$.

tion gives the dihydride, $[\text{WH}_2(\eta^2\text{-O}_2\text{CMe})(\text{dppe})_2]^+$. Most unexpectedly, it shows an unprecedented selectivity since the H_2 is derived exclusively from the hydride ligands in the parent complex and not from the acid [99].

At low concentrations of acid, the reduction of $[\text{MoH}_2(\eta^2\text{-O}_2\text{CMe})(\text{dppe})_2]^+$ follows a different course, and dissociation of the carboxylate occurs to expose a site on the Mo at which N_2 , CO, or ethyne can bind. In the presence of N_2 $[\text{Mo}(\text{N}_2)\text{H}_2(\text{dppe})_2]$ is formed, and subsequent dissociation of H_2 and binding of a further molecule of N_2 give the *trans*- $[\text{Mo}(\text{N}_2)_2(\text{dppe})_2]$ product.

It is not difficult to see how this same chemistry could operate on the cofactor. Three electron reduction of cofactor could cause the carboxylate residue of the homocitrate ligand to dissociate from Mo and open up a site where N_2 can bind [100]. This proposal is consistent with what we know about nitrogenase. The x-ray crystal structure of the MoFe protein is that of the E_0 state. Only upon the addition of three electrons will N_2 bind. In addition, obligatory H_2 evolution of the enzyme may be a consequence of needing to hydronate the metal center to facilitate reduction of the site in the E_0 , E_1 , and E_2 states prior to binding N_2 . It also shows that H_2 loss from a Mo-H precursor is not essential for the generation of a N_2 -binding site. Rather H_2 release can occur after N_2 binding.

However, the role of homocitrate is still not entirely clear. It is a very exotic molecule to use if its only role is as a leaving group after electron transfer and before N_2 binding. It is not clear, at this stage, why a simpler carboxylate would not be just as suitable.

III.E. Conversion to Ammonia: Sites of Hydronation

The most obvious role for the hydron is its involvement, when coupled to electron transfer, in the conversion of N_2 to NH_3 . It has been established that in simple



mononuclear complexes a single N_2 ligand can be transformed into two NH_3 molecules via the nitrogenous species shown in Figure 18. It is this chemistry that forms the basis of the catalytic formation of NH_3 at the "Mo(diphosphane)₂" core [101]. The most important points to be made about this picture are (1) that it represents the only model for the action of nitrogenase based on well-defined chemical precedent, and (2) that all of the nitrogenous species shown have been structurally authenticated, often by x-ray crystallography. The simplicity of this representation belies the more complicated mechanistic picture (Figure 19).

The parallels between Figures 18 and 19 are obvious and the same species clearly occur in both. The complete mechanistic picture is a summary of several studies each complete in itself wherein the simple stoichiometric conversion of one of the species shown in Figure 18 into its successor has been monitored. The simplicity of such an approach means that the electronic and steric factors that influence the reaction rate and product distribution can be defined in great detail.

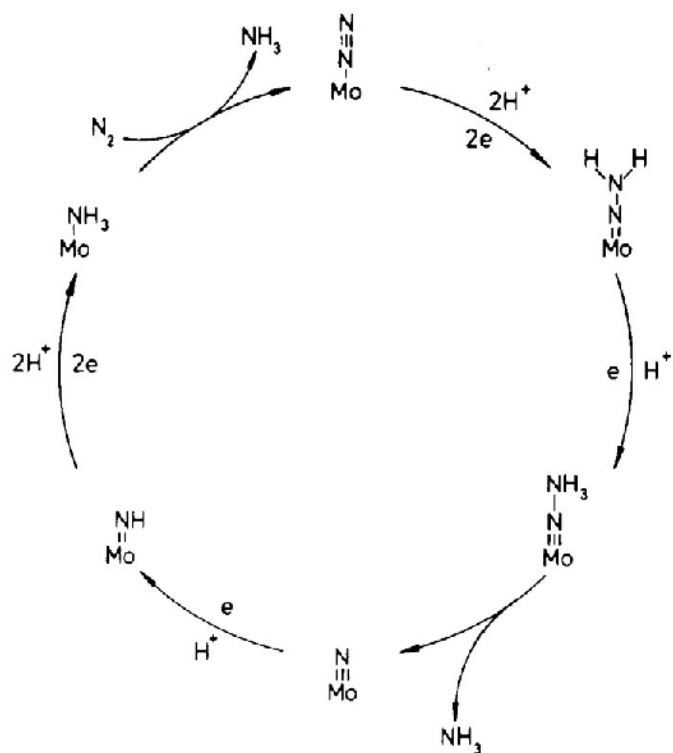


Figure 18 Proposed conversion of dinitrogen to ammonia at a mononuclear site, based on structurally characterized complexes.



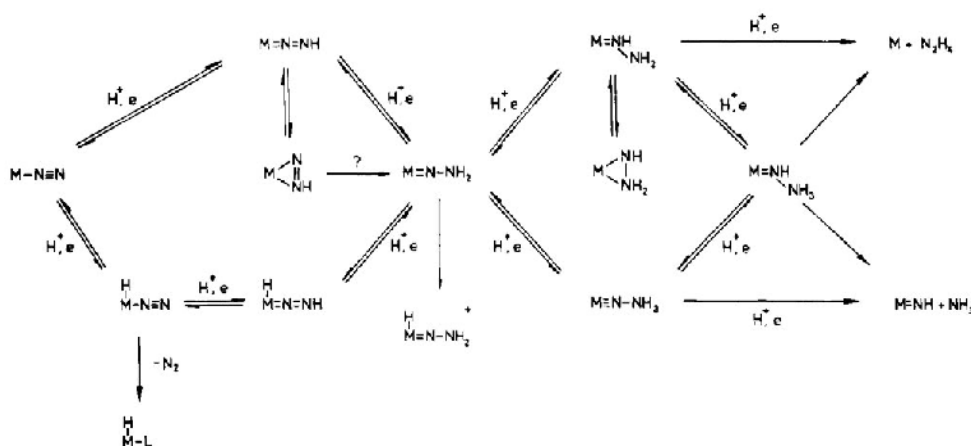


Figure 19 Pathways for the conversion of dinitrogen to ammonia or hydrazine at a mononuclear site, established from a series of mechanistic studies on simple metal complexes.

The basis of the transformations of the nitrogenous residues in the metal complexes is identical to that operating in the enzyme: the successive transfer of electrons and hydrons to the nitrogenous ligand. The major difference is that there is no external source of electrons; consequently the electrons have to be supplied by the metal. Thus as the nitrogenous ligand becomes progressively more hydronated; the metal center becomes correspondingly more oxidized, as illustrated in the example shown in (Eq. 4), in which dihydronation of the N_2 complex results in a hydrazido(2-)-complex; and, as a consequence, the formal oxidation state of the metal increases from 0 to 4.



This reaction is one of those that form the basis of the overall scheme shown in Figure 19. Inspection of this stoichiometric equation reveals that Figure 19 is simplified in the sense that it only addresses those transformations occurring at the nitrogenous residue; any other changes in the metal's coordination sphere have been ignored. We shall return to these ancillary reactions later.

Discussion of the full mechanistic picture of Figure 19 falls naturally into two sections: the formation of the hydrazido(2-) state and its subsequent decomposition to give NH_3 or N_2H_4 .

III.E.1. Formation of the Hydrazido(2-) Species

Thus far, three different routes have been established by which the N_2 ligand is converted into the hydrazide state. In the top pathways of Figure 19, the hydron



directly attacks the lone pair of electrons on the remote nitrogen atom to give a diazenide. The dominant pathway then leads to the hydrazide species via a second direct hydronation on the remote nitrogen atom [102,103]. In addition, there is some evidence that under certain conditions another pathway can operate [104] in which a side-on coordinated diazenide can also hydronate through to hydrazide; however, now the addition of the second hydron occurs at the unhydronated atom. The resulting diazene species then undergoes a hydron-catalyzed rearrangement to give the hydrazide product. The influences of the metal and the coligands on the acidity and hydron-transfer rates of nitrogenous residues have also been studied [105,106].

The only other established route to hydrazide species is that shown in the lower, left-hand portion of the scheme. Under conditions where hydronation at the N_2 ligand is slow or thermodynamically unfavorable, hydronation of the metal can occur [107]. Often hydronation at the metal is deactivating, and relatively rapid loss of N_2 ensues. However, if the N_2 ligand is still bound reasonably tightly and is sufficiently basic to bind a hydron, then the reaction can proceed through to the hydrazide state as shown.

Studies on the reduction of N_2 by the enzyme have only detected one nitrogenous intermediate [72,76,108]. This intermediate is characterized by its ability to produce N_2H_4 upon acid or alkali quench. It was suggested that this N_2H_4 was derived from a hydrazido(2-) species on the basis of the observation in the chemical systems that this state represents a potential well in the formation of NH_3 and because some isolated hydrazido(2-) complexes treated with either acid or alkali can produce N_2H_4 .

This enzyme intermediate is formed at the E_4 stage [72], a species that has consumed four electrons relative to E_0 . However, two of these electrons have been consumed in the evolution of H_2 ; therefore, the E_4 state is effectively only two electrons reduced relative to E_0 . Thus in order to liberate N_2H_4 (a four-electron reduction product), at least two electrons must be supplied from the metal clusters in the enzyme. The identification of the detected intermediate as a hydrazide has been criticized [76], and certainly the derived N_2H_4 could result from a wide variety of species including coordinated N_2 or diazene. The major sources of ambiguity are that the number of electrons present in the enzyme's reservoir is unknown and that the number of hydrons present on the intermediate cannot necessarily be equated to the number of electrons transferred. A simple example illustrates the problems in assigning reduction levels to proposed intermediates on the basis of the number of electrons available. Thus, in $Mo=N=NH_2$ the ligand could be a hydrazido(2-) species with the metal formally in the +2 oxidation state or it could be an isodiazene with the metal now having a 0 oxidation state.

This type of dichotomy can occur in several areas depicted in Figure 19, and where the electrons actually reside can only be resolved by accurate bond



length determinations using x-ray crystallography. All that can be said with certainty about the nature of the detected enzyme intermediate is that at least four electrons are available on quenching the enzyme system in order that N_2H_4 is liberated. The firm identification of this intermediate can result only from spectroscopic analysis.

III.E.2. The Decomposition of the Hydrazide State

In modeling the formation of NH_3 from the hydrazide state there is a complication since in some simple chemical systems N_2H_4 can be the nitrogenous product. This variation in the nitrogenous product can be turned to advantage, since by establishing the pathways that lead to N_2H_4 formation on the one hand and NH_3 on the other, the factors that favor metal–nitrogen cleavage and nitrogen–nitrogen cleavage, respectively, can be delineated. Another major problem in trying to look at the later stages of the fixation of N_2 is to find suitable, analytically clean systems. All the studies that describe the pathways shown on the right-hand side of Figure 19 are on alkyl- and/or aryl-substituted hydrazides. This use of substituents on the remote nitrogen atom has the advantage that the electronic influences of the substituents act as a probe for the site of hydronation. Direct hydronation at the remote nitrogen atom will reflect the relative electron-releasing or electron-withdrawing capabilities of the alkyl and aryl groups, whereas attack at the nitrogen atom adjacent to the metal, or the metal itself, is much less sensitive to the nature of these substituents.

Starting from the hydrazide state (shown in the center of the scheme), hydronation can occur initially at either nitrogen atom, depending on the system being studied [109–111]; attack at the remote nitrogen atom results in a hydrazidium ligand, and attack at the adjacent nitrogen atom yields an end-on hydrazido(1-) species. When the hydrazido(1-) ligand is formed, subsequent hydronation at the nitrogen atom adjacent to the metal can result in the direct cleavage of the metal–nitrogen bond and formation of N_2H_4 . In a similar fashion, further hydronation of the hydrazidium ligand can result in the cleavage of the nitrogen–nitrogen bond, liberation of one molecule of NH_3 , and formation of an imido species. If these were the only hydronation pathways available to the hydrazide species, the distinction between the NH_3 - and N_2H_4 -forming characteristics would be straightforward. However, both the hydrazidium and hydrazido(1-) ligands can undergo an alternative hydronation to form the common $MNHNH_3$ species. This species has been identified in both N_2H_4 - and NH_3 -forming pathways. This last observation appears to indicate that provided sufficient electrons are available, what discriminates between metal–nitrogen cleavage and nitrogen–nitrogen cleavage (and hence between N_2H_4 and NH_3 formation) is not primarily the site and extent of hydronation of the intermediates; rather, it is the nature of the metal site to which the nitrogenous residue is coordinated. Specifically, if the site forms strong metal–nitrogen multiple bonds then NH_3 formation will be favored.



One final pathway is worthy of comment, and that is the route involving the side-on hydrazido(1-) ligand. Studies on structurally well-defined complexes containing this residue [109,112] show that the side-on species is insufficiently basic to bind a hydron directly but must ring-open before it becomes susceptible to attack. Furthermore, the ring-opening step is invariably so slow that it is rate-limiting. It is worth noting that dehydronation of the side-on η^2 -hydrazido(1-) ligand in $[\text{Mo}(\eta^2\text{-MePhNNH})(\text{S}_2\text{CNEt}_2)_2]^+$ [112] generates a transient η^2 -hydrazido(2-) species before rearrangement to $[\text{Mo}(\text{NNMePh})(\text{S}_2\text{CNEt}_2)_2]$. In another study [113], dehydronation of the η^2 -hydrazido(1-) ligand in $[\text{W}(\eta^5\text{-C}_5\text{Me}_5)(\text{NHNH}_2)]^+$ to give the η^1 -hydrazido(2-) species $[\text{W}(\eta^5\text{-C}_5\text{Me}_5)(\text{NNH}_2)]$ has been shown to occur by two pathways. With strong bases, rapid dehydronation gives the transient η^2 -hydrazido(2-) species, which subsequently slowly ring-opens to give the product. However, with weak bases, ring opening of the hydrazido(1-) ligand is followed by dehydronation.

The only step remaining to complete the stoichiometric evolution of two NH_3 molecules is the hydronation of the imido ligand. This has been accomplished at the “ $\text{Mo}(\text{dppe})_2$ ” core both chemically (but with destruction of the integrity of the complex) [114,115] and electrochemically (with retention of the basic site and subsequent binding of N_2) [116]. In both cases amido species are proposed intermediates and such a species has been detected in the electrochemical study.

III.E.3. General Principles from the Chemical Studies

Several general points emerge from these studies on the model systems, which may pertain to the action of the enzyme.

1. In all cases, the hydronation of nitrogenous ligands containing lone pairs of electrons is rapid and probably diffusion-controlled.
2. Generally, although hydronation occurs at the remote nitrogen atom in the initial stages and at the adjacent nitrogen atom in the later stages, throughout the scheme there are stages at which hydronation at the metal can successfully compete with hydronation of the substrate. This seems to be particularly prevalent at the N_2 and the hydrazido(2-) stages [117].
3. Intramolecular rearrangement mechanisms, involving the migration of a hydride ligand onto a nitrogen residue, have not been observed in the presence of hydrons [118,119]. It seems that acid-catalyzed rearrangements have a lower activation energy than intramolecular routes.
4. A point, alluded to earlier, concerns what happens to the rest of the coordination sphere of the metal during the hydronation reactions. In the model systems, it is frequently observed that in order to stabilize



the increasingly more hydronated N_2 residue, the metal sheds electron-withdrawing ligands and replaces them with more electron-releasing groups. Although this characteristic may only be due to the shortcomings of the model systems, it is tempting to speculate that the active site of nitrogenase may also operate a similar strategy: one site where the nitrogenous residue is undergoing transformations and another, in close proximity, where anionic groups (such as halide or hydroxide) can bind rapidly and thus facilitate the hydronation of the substrate.

5. Finally, it is important to reemphasize that all the nitrogenous residues shown in Figure 19 have been structurally characterized in simple complexes.

III.F. Other Models for Dinitrogen Activation

The preceding discussion refers only to end-on, mononuclear N_2 species. Two other proposals have been put forward for the N_2 binding mode in the enzyme based on a dinuclear coordination and side-on coordination. Both of these proposals have been reviewed extensively before, and we will concentrate here only on those aspects pertaining to the enzyme's action.

III.F.1. Bridged Dinitrogen Systems

Since chemists became interested in the nitrogen fixation problem it has been advocated that the enzyme operates by using two metals to bind N_2 , in either a homodinuclear (two Fe centers or two Mo centers) or a heterodinuclear (one Fe and one Mo center) sense [120]. The main advocates of this proposal argue that there are two advantages to a dinuclear site [121,122]. First, since the N_2 ligand has to be reduced in order to be converted to NH_3 , two metals can mediate this reduction better than one, since each has to undergo a less drastic redox change than would one metal. Second, since the N_2 ligand has to be sufficiently basic to be hydronated to give NH_3 two metals can impart a greater electron density onto the ligand than can one metal. Although many nonbiological nitrogen-fixing systems are argued to operate via dinuclear species, none of them is sufficiently simple to yield the level of mechanistic detail that characterizes the mononuclear chemistry. Many of the systems studied are very good nitrogen fixers, but little is known about the nature and structure of the species that bind N_2 . These problems are compounded by the multicomponent nature of the reaction mixtures.

In an attempt to understand the fundamental hydronation chemistry of bridged N_2 complexes, we developed the system based on $[M(S_2CNEt_2)_3]_2(\mu-N_2)$ ($M = Nb$ or Ta , the same group of the Periodic Table of the Elements as V). These systems have the advantages that they react with an excess of acid to give stoichiometric yields of N_2H_4 , and the reactants and products have been



structurally characterized by x-ray crystallography. The established mechanism is shown in Figure 20, where the spectroscopically detected intermediates are indicated [122–124].

The two major features of this scheme are as follows: In the majority of systems studied, the slowest step in the reaction manifold is the addition of the first hydron to the bridging ligand. This contrasts with the behavior observed in the mononuclear systems, where the initial hydronation of a N_2 residue is never rate-limiting. We believe this large difference in the rate constants for hydronation (at least 1×10^3) has its origins in the availability of lone pairs of electrons. When a hydron approaches a mononuclear, end-on N_2 , there is a lone pair of electrons available on the remote nitrogen atom and so rapid hydron binding can ensue. In contrast, when a hydron approaches a bridged N_2 residue, there is no lone pair of electrons and, in order to bind, the hydron must disrupt the multiple bonding between the two metal centers, including the bridging ligand; thus the reaction is correspondingly slower. The other feature of the bridged N_2 mechanism to note is that the N_2 ligand in these systems is sufficiently basic to bind three hydrons before the bridge cleaves.

As a result of this mechanistic study, we can compare the reactivity of mononuclear and bridging N_2 species in very general terms. The proposed advantages of operating via a bridged species that were outlined previously must now be balanced against the disadvantages. One disadvantage of the enzyme binding substrate's using two metals rather than one is that it has twice as many bonds to make and break compared to a single metal site. This is both thermodynamically and kinetically disadvantageous. Furthermore, if the enzyme operates by using a single metal, the addition of the first hydron is very facile, whereas in using a bridging N_2 mode the addition of the first hydron can be slow.

On a related theme, it has been shown that N_2 coordinated to a Mo center can be transferred to Fe center presumably involving a transient heterodinuclear species, as shown in outline in Eq. 5 [125].

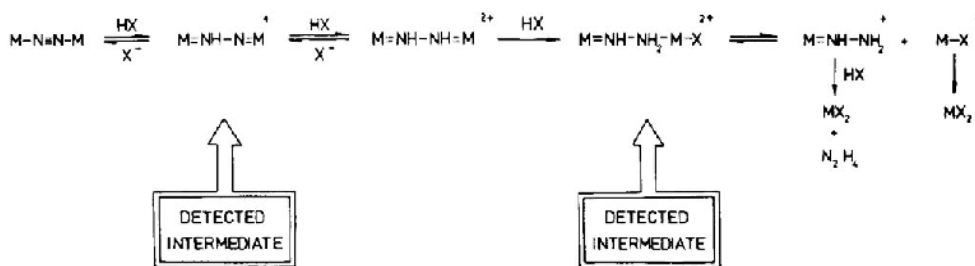


Figure 20 The formation of hydrazine from the bridged dinitrogen complex, $[M(S_2CNEt_2)_3]_2(\mu-N_2)$ ($M = Nb$ or Ta).





However, it is not clear what advantages there would be to the enzyme to operate such a system, unless one metal could rapidly scavenge N_2 from solution and then can pass it on to a metal center capable of activating the N_2 toward hydronation.

III.F.2. Side-on Dinitrogen System

The final proposal of how the nitrogenases bind N_2 we shall discuss is that in which the ligand is coordinated in a side-on fashion to a single metal [126]. This type of coordination of the enzyme was advocated by comparison with the established behavior of C_2H_2 with nitrogenase. It was argued that because C_2H_2 is stereospecifically converted to *cis*- $\text{C}_2\text{H}_2\text{D}_2$ in the presence of deuterons, this indicated that C_2H_2 coordinated to the active site in a side-on fashion, and hence so would the isoelectronic N_2 . By further analogy with the C_2H_2 case, it was argued that side-on N_2 hydronated to give N_2H_2 , which was liberated from the active site and disproportionated to N_2H_4 and N_2 or decomposed to N_2 and H_2 . In this way, the obligatory evolution of H_2 (discussed earlier) and the observation that only the reduction of N_2 involves obligatory H_2 evolution are both natural consequences of the involvement of free N_2H_2 . The subsequent pathway from N_2H_2 to NH_3 involves the reduction of free N_2H_4 by the enzyme. Many studies on chemical systems have been interpreted in terms of this type of behavior. However, there are several lines of evidence that indicate that this is not a pathway employed by the enzyme: (1) C_2H_2 has little choice in the way it binds to metals: it must bind in a side-on fashion. In contrast, N_2 has a lone pair of electrons at each end of the molecule available to coordinate to metals and it does just that in the vast majority of its complexes. (2) Only a few complexes containing a side-on coordinated N_2 are known and the mechanism of hydronation of this residue is unexplored. (3) N_2H_4 is a poor substrate for the Mo nitrogenase because of its weak binding to the enzyme ($K_m = 20\text{--}30 \text{ mmol dm}^{-3}$). (4) The involvement of N_2H_2 cannot explain why H_2 is the first reduction product. (5) Use of fumarate or allyl alcohol did not detect free N_2H_2 during the reduction of N_2 by Mo nitrogenase [76].

III.G. Other Substrates

Understanding of how the nitrogenases work can be derived not only from studies on the reaction with N_2 but also from investigation of the wide variety of other small, unsaturated molecules that the enzyme is capable of transforming, shown in Table 1. We will not discuss all the “alternative substrates” here, but restrict our attention to those areas investigated by using the complementary approach of studies on the enzyme and in simple chemical models. Although the reduction



Table 1 Nitrogenase Substrates and Their Reduction Products

Substrate	No. electrons/substrate	Products
$\text{N}\equiv\text{N}$	6e	NH_3
$\text{RC}\equiv\text{CH}$	2e	$\text{RCH}=\text{CH}_2$ (RCH_2CH_3)
$\text{C}\equiv\text{N}^-$	6e	$\text{CH}_4 + \text{NH}_3$ (CH_3NH_2)
$\text{RC}\equiv\text{N}$	6e	$\text{RCH}_3 + \text{NH}_3$
$\text{N}=\text{N}=\text{O}$	2e	$\text{N}_2 + \text{H}_2\text{O}$
$\text{N}=\text{N}=\text{N}^-$	^a	$\text{N}_2 + \text{NH}_3 + \text{N}_2\text{H}_4$
<i>cyclo</i> - C_3H_4	2e	$\text{CH}_3\text{CH}=\text{CH}_2 + \text{cyclo-}\text{C}_3\text{H}_6$
2H^+	2e	H_2
NO_2^-	6e	NH_3
$\text{MeN}=\text{NMe}$	^a	$\text{CH}_4 + \text{NH}_3 + \text{CH}_3\text{NH}_2$

^a Variable function of electrons.

of N_3^- ion has been studied both in the enzyme and in chemical models, this work is now over 15 years old and will not be addressed here [114,127].

III.G.1. Cyanides and Isocyanides

Careful analysis of the kinetic data for the reduction of CN^- by Mo nitrogenase from *A. vinelandii* shows that at high concentrations of CN^- a constant rate of reduction is observed, that rate depends only on the pH of the solution [128]. This observation is inconsistent with the earlier proposal that CN^- and HCN bind independently, and the revised model is one in which the CN^- binds to a single site (Figure 21).

The most important observation in the pre-steady-state kinetics of the CN^- system is that after a short lag (100 msec) there is a phase (lasting about 3 sec) where the evolution of H_2 is linear and only after these 3 sec does CN^- reduction occur. This long lag prior to CN^- reduction would correspond to 18 to 20 electron transfer steps from the Fe protein. More realistically this delay probably involves a CN^- -induced modification of the enzyme, such as a ligand substitution reaction (this modified state of the enzyme is designated as *E in Figure 21). However, this modification step is too slow to be part of the steady-state cycle for CN^- reduction. Also, it cannot be a slow activation of the enzyme prior to turnover, since the onset of H_2 evolution is the same in both the presence and the absence of CN^- . Steady-state observations indicate that cyanide binds to a more oxidized form of the MoFe protein than binds N_2 , but that state cannot be defined because of the long lag phase.

It is not possible to distinguish between HCN or CN^- as the species that binds to the enzyme, nor is it possible to define whether the substrate binds



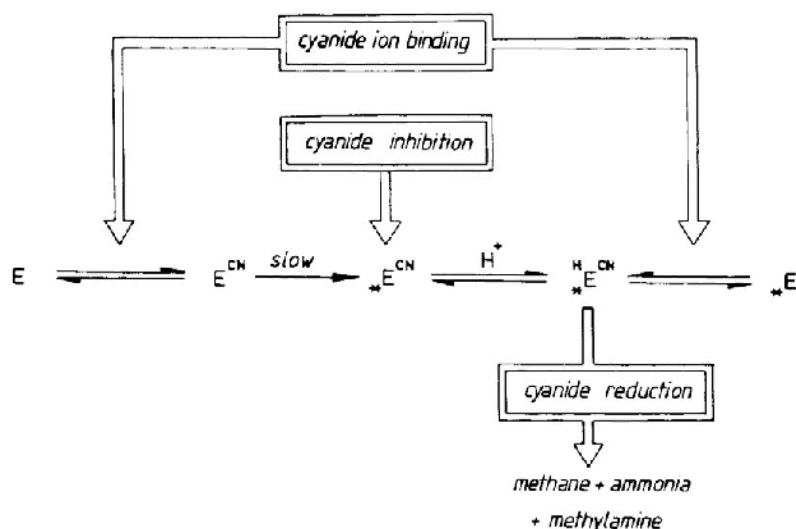


Figure 21 The binding of cyanide at the Mo-based nitrogenase.

through the carbon or the nitrogen atom. In a model system in which the CN^- is bound to the metal through the carbon atom, investigation of the electroreduction of $trans-[Mo(CN)Cl(dppe)_2]$ has shown that activation of the coordinated CN^- can occur in the presence of a hydron source (Eq. 6) [129].



This supports the idea that in order to be readily activated to hydronation the substrate must bind to the enzyme through the carbon atom. This proposal is also consistent with the extensive chemical studies on the hydronation of simple RNC complexes ($R = \text{alkyl or aryl}$) [130], where carbon coordination is enforced. In a related argument, it has now been shown that the Mo-based nitrogenase [131] is not capable of reducing simple organic cyanides such as $MeCN$ or $PhCN$, as was originally proposed, since the RCN molecule is forced to bind to the enzyme through a nitrogen atom.

III.G.2. Ethyne, Ethene, and Ethane

The ability of *K. pneumoniae* Mo nitrogenase to convert ethyne to ethene has been described [77] in terms of the now familiar scheme (Figure 13). C_2H_2 can



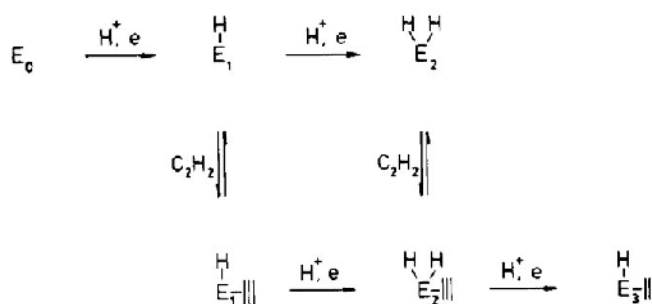


Figure 22 The binding of ethyne and evolution of ethene by the Mo-based nitrogenase.

bind to E_1H or E_2H_2 and after transformation C_2H_4 is released from E_3 or E_4 (Figure 22).

Thus, although the reduction of C_2H_2 to C_2H_4 only requires two electrons, the length of the pre-steady-state lag phase indicates that the product is not released until three electrons have been transferred. The analysis of the kinetic data for the reactions with C_2H_2 showed that the association rate between the MoFe and Fe proteins is enhanced (over the rate established for N_2) by a factor of 6 in the presence of C_2H_2 . Any explanation of how C_2H_2 is converted to C_2H_4 by the enzyme is subject to the stereochemical restriction that in the presence of deuterons, *cis*- $C_2H_2D_2$ is formed; however, this stereospecificity is not rigidly adhered to with other alkynes. On the basis of studies of simple chemical systems [77,133], it has been proposed that hydronation of the C_2H_2 proceeds through the pathway shown in Figure 23. In this pathway, C_2H_2 is progressively hydronated to

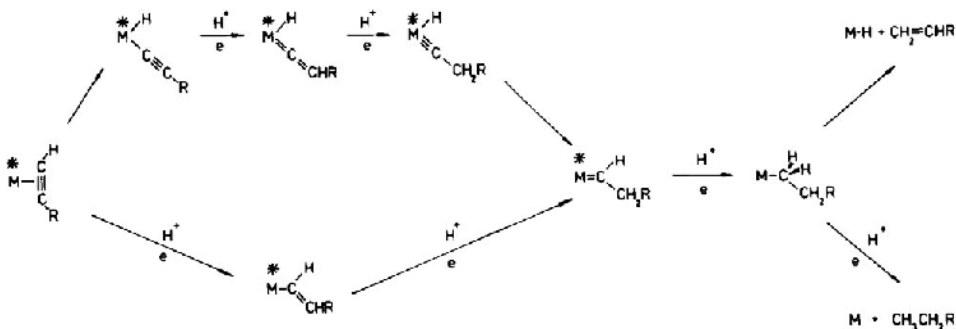


Figure 23 The formation of ethene from ethyne involving the intermediacy of an ethyl species. (Species with asterisk have been isolated in simple complexes.)



the ethyl species, which then undergoes the β -hydrogen atom transfer reaction to produce C_2H_4 . Although this proposal rationalizes why three electrons have to be transferred to the large protein before the release of C_2H_4 , it has difficulty in rationalizing the stereospecificity of the reaction: the derived ethyl species in this scheme could undergo rapid rotation and lose any stereochemical integrity. It has been proposed that there is restricted rotation of the ethyl group within the binding site cavity. Such arguments seem a little contrived and as it turns out they are also unnecessary. Studies on simple chemical systems show that stereospecific formation of *cis*-alkenes can be the natural consequence of the hydronation of alkyne complexes.

Studies on the hydronation of alkynes bound to symmetrical metal sites show two pathways by which stereospecific alkenes can be formed: direct hydronation of the bound alkyne and hydronation of the metal followed by intramolecular hydrogen atom migration to the alkyne. The stereochemistry of the derived alkene can be defined by the rates of hydronation at these two different sites. This is best illustrated by the reaction of anhydrous HCl with $[V(\eta^5-C_5H_5)_2(\eta^2-PhCCPh)]$ [134,135]. The predominant products are $[V(\eta^5-C_5H_5)_2Cl_2]$ and *cis*-PhCHCHPh. This stereoselective formation of the *cis*-alkene is a consequence of initial hydronation of the metal followed by intramolecular hydrogen atom transfer from the V to the face of the alkyne adjacent to the metal to give the *cis*-vinyl species. Provided the subsequent hydronation of $[V(\eta^5-C_5H_5)_2(CPhCHPh)]^+$ occurs at the carbon-metal bond, the stereochemistry of the product is controlled by the site of the initial hydronation and the *cis*-alkene results. A small amount of *trans*-PhCHCHPh is produced, and this isomer is formed by a pathway involving initial, direct hydronation of the coordinated alkyne to give the *trans*-vinyl species. Subsequent hydronation of this vinyl complex at the carbon atom bound to V results in the *trans*-alkene.

The important feature is that no exceptional, contrived, or unprecedented chemistry has to be invoked to rationalize the stereoselective formation of the *cis*-alkene: preferential rapid hydronation of the metal (or binding of an alkyne to a preformed hydrido species) naturally results in the *cis*-alkene.

A pathway that can lead to loss of specificity involves hydronation of the vinyl ligand at the carbon atom remote from the metal. This gives rise to an alkylidene species and consequent formation of a carbon-carbon single bond. Of course, dehydronation can occur to regenerate the vinyl species, but free rotation about this carbon-carbon single bond in the alkylidene would be expected to lead to a mixture of *cis*- and *trans*-vinyl species. Surprisingly, this need not be the case.

Stereoselective formation of a *cis*-vinyl species is observed in the hydronation of *trans*- $[Mo(\eta^2-MeCCH)_2(dppe)_2]$ [136]. Kinetic studies show that the reaction proceeds through two pathways: one involving monohydronation and another involving dihydronation of the complex, probably directly at the alkyne, to give



the corresponding alkylidene. At high concentrations of acid, where the dihydrogenation pathway dominates, the *cis*-vinyl product is the dominant product (formed after a change in the coordination sphere of the Mo and subsequent dehydrogenation of the alkylidene ligand). This must reflect a significant difference in thermodynamic stability between the *cis*- and *trans*-vinyl species, possibly imposed by the ability of the solvent preferentially to solvate (and hence stabilize) one geometry. Of course, this need not always be the *cis*-isomer, and in certain circumstances dehydrogenation of the alkylidene ligand at the carbon atom bound to the metal can occur to produce an alkylidyne.

The observation that the V enzyme produces some ethane (about 3% of the electron flux) from the reduction of C_2H_2 [137] and that, in the absence of C_2H_2 , under an atmosphere of C_2H_4 the Mo enzyme also yielded some C_2H_6 [138] (<1% of the electron flux) led to an investigation of the factors that discriminate between evolution of C_2H_4 and formation of C_2H_6 in simple chemical systems. The salient features of this study, as it relates to the action of the enzyme, are shown in Figure 24 [139].

Here the transformation of the C_2H_2 ligand to the coordinated C_2H_4 has already occurred, and the active species is a hydrido-ethylene species consistent with the consumption of three electrons before the evolution of C_2H_4 observed in the enzymatic reaction. The intramolecular migration of the hydrido ligand onto the C_2H_4 ligand can result in the ethyl species, which is the precursor to C_2H_6 formation. In competition with this reaction is the further hydronation (without electron transfer from the Fe protein) of the metal center, which labilizes the system to loss of C_2H_4 . This model is able to rationalize why under an atmosphere of C_2H_2 , V nitrogenase gives rise to some C_2H_6 whereas Mo nitrogenase only gives C_2H_2 . All else being the same, it may be that the V center (a group 5, first-row transition metal) is less basic and less electron-releasing than a Mo center (a group 6, second-row transition metal). Hence, it may be that the Mo enzyme is more susceptible to hydronation, resulting in the evolution of C_2H_4 , whereas the V site has a greater opportunity to proceed unhindered through the pathway yielding C_2H_6 .

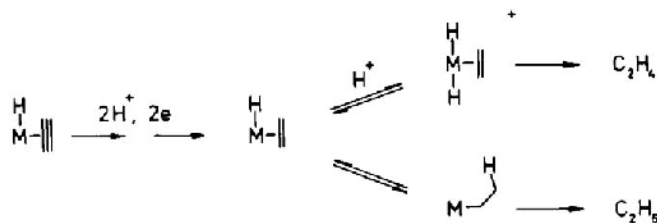


Figure 24 The pathways discriminating between formation of ethane and evolution of ethene.



This mechanism illustrates another putative role for the hydron in nitrogenase—as a catalyst for product release. It further illustrates the restrictions in defining a mechanism for substrate conversion by investigating only the enzyme reaction. The involvement of hydrons in the reactions of the nitrogenases can only be inferred from the consumption of electrons, and key catalytic roles for hydrons will not be evident.

III.H. Theoretical Studies on N₂ Binding at FeMoco

Since the definition of the structure of FeMoco, theoretical studies have appeared attempting to identify the manner by which N₂ binds to this cluster. The realization that the Mo site within the cluster is 6-coordinate, and therefore could be considered as coordinatively saturated, has led to what may be an overemphasis on looking at the Fe sites as the N₂ binding position. The salient features of the five studies reported so far will be discussed briefly, with an emphasis not on the basis of the calculations but the major conclusions of importance in giving an insight into the chemistry and biochemistry of nitrogen fixation.

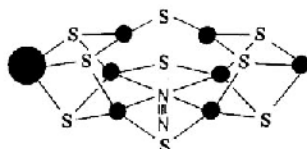
The results of extended Hückel calculations [140] indicate that binding of N₂ in an end-on fashion is favored, with one nitrogen atom bound to four Fe atoms of a face of the trigonal cavity as shown in Figure 25. These calculations also indicate three other important features of this cluster: (1) The HOMO is a singly occupied orbital in the approximate center of a block of Fe 3d-orbitals, but its exact identity could not be established since there are nine orbitals within 0.5 eV of one another. (2) Metal–metal bonding pervades the cluster and is particularly extensive in the trigonal cavity. (3) Three electron reduction of the cluster (which is necessary to drive the system to a state that will bind and activate N₂) does not dramatically affect the electron density of N₂. This is because the three electrons go into the 3d block, which has little effect on the N₂-binding properties of cofactor.

Ab initio density functional calculations [141] agree with the Hückel calculations that there is extensive metal–metal bonding within the cluster. However, the density functional calculations favor side-on binding of N₂ as shown in Figure 25, with N₂ binding to four Fe atoms on a face of the trigonal cavity. In addition, these calculations indicate that the bridging sulfur atoms are the most susceptible to changes in the redox level of the cluster.

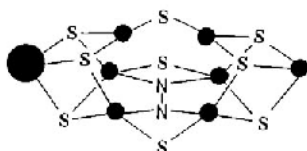
Intermediate neglect of differential overlap (INDO) (142) calculations favor yet another type of binding for N₂ in which it binds to cofactor inside the trigonal cavity with each nitrogen atom bound to three Fe atoms as shown in Figure 25. In addition, the INDO calculations indicate that the N atom at the Mo end is bound more strongly and is more negatively charged than the N at the Fe end of the cofactor. Although this mode of coordination has a degree of aesthetic simplicity and symmetry, it is difficult to understand how N₂ penetrates the exten-



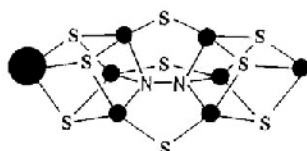
EXTENDED HÜCKEL



AB INITIO DENSITY FUNCTIONAL



INDO



CNDO

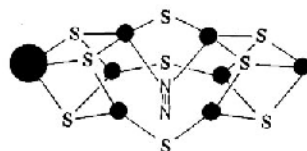
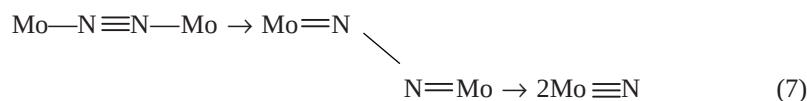


Figure 25 Coordination modes for N₂ bound to FeMoco, as predicted by various theoretical models.



sive metal–metal bonding inside the cavity. In addition, the cavity is ca. 0.05 nm too small to accommodate N_2 and so some expansion of the cavity is necessary before binding can occur, or, even more problematically, before hydronation and nitrogen–nitrogen cleavage can ensue. Recent EXAFS [143] results show that upon one electron reduction of cofactor the volume of the cluster actually contracts.

Before moving to the last type of calculation, it is worth noting that the cleavage of N_2 by what may be described as a wrenching motion inside the cavity of the cofactor is a reactivity that is mimicked in the unimolecular, stoichiometric cleavage of N_2 in the complex $[Mo(N(Ar)R)_3](\mu-N_2)$ ($R = Bu^t$, $Ar = Ph$ or $3,5-C_6H_3Me_2$) to give 2 mole-equivalents of $[MoN(N(Ar)R)_3]$ [144]. Theoretical studies on this type of cleavage indicate that it occurs through a so-called zig-zag transition state (shown in Eq. 7).



The simplicity of the reaction makes such a detailed mechanism difficult to substantiate; however, the experimentally determined activation parameters for the cleavage are $\Delta H^\ddagger = 5.6 \text{ kJ mol}^{-1}$ and $\Delta S^\ddagger = 0.69 \text{ J K}^{-1} \text{ mol}^{-1}$ and are in good agreement with those predicted from theory.

Finally, semiempirical complete neglect of differential overlap (CNDO) calculations [145] again emphasize N_2 binding to the trigonal cavity, but now with one nitrogen atom at the center of the cavity and the other on the outside of the cluster as shown in Figure 25.

Both CNDO and INDO calculations indicate that the bridging sulfur atoms in the cluster can act as hydron carriers. In particular, INDO calculations indicate that a N_2 bound in the so-called oblique arrow configuration (the energy minimized, optimal configuration [146] is that described by the *ab initio* calculations but with one N atom positioned between three sulfurs) contains a nitrogen atom (not coordinated to Fe) that is well positioned to bind hydrons from three bridging sulfur atoms of the cluster. After hydronation of the uncoordinated nitrogen, cleavage of the N–N bond ensues and NH_3 is released. The remaining nitrido species can now be hydronated in a similar fashion by hydrons on the same sulfur atoms.

This mechanism, involving sequential hydronation of N_2 initially at the nitrogen atom remote from the metal(s), followed by cleavage of the N–N bond, then hydronation of the resulting nitride, is, in essence, the same mechanism that has been developed on electron-rich, low-oxidation-state Mo phosphane complexes (Section III). The only real difference is that in this case the N_2 is bound to four Fe sites in a cluster.



IV. CATALYTIC SUBSTRATE REDUCTION BY SYNTHETIC IRON-SULFUR-BASED CLUSTERS

An important target in the development of understanding how nitrogenases work at the atomic level is establishing the fundamental substrate binding and transformation chemistry on extracted cofactor. However, cofactor is just one member of what is now a large family of structurally related Fe-S-based clusters. Consequently, in investigating the reactivity of cofactor it is important to be able to identify what reactivity is peculiar to cofactor and what is just a general characteristic of all Fe-S-based clusters.

When FeMoco is extracted from the MoFe protein it is no longer capable of converting N_2 into NH_3 , or indeed transforming any nitrogenase substrate. This together with the results of site-directed mutagenesis experiments (see Section III. C) on the MoFe protein clearly indicate a subtle interplay between the inorganic cluster and amino acid residues that are only hydrogen-bonded to it in the cluster cavity. Strategies for “switching on” the reactivity of extracted cofactor are beginning to emerge from two independent lines of research: first, identifying how synthetic Fe-S-based clusters accomplish stoichiometric transformations of simple substrates by electron- and hydron-transfer reactions, and, second, consideration of the crystal structures of the protein and in particular the hydrogen bonding interactions between polypeptide and cluster. Ultimately, by studying the reactivity of a synthetic, functioning nitrogen fixing system based on FeMoco we will be able to define, at the atomic level, how the enzyme transforms the substrates into products.

There are several reports in the literature concerning both stoichiometric and catalytic substrate transformations using synthetic Fe-S-based clusters. We will briefly outline the salient features of these studies before we discuss the approaches being adopted to establish how these reactions are accomplished at the atomic level.

IV.A. Dinitrogen and Hydrazine Reduction

Model systems composed of Mo complexes and Fe-S clusters can give facile N_2 reduction to NH_3 under mild conditions. The Fe-S cluster is considered to act only as an electron transfer catalyst from reducing agents, such as BH_4^- and $S_2O_4^{2-}$, to the Mo complexes at which the reduction occurs [147–149]. Similarly the reduction of N_2H_4 to NH_3 can be achieved by a catalyst of sodium molybdate, L-cysteine, and $NaBH_4$ with a turnover number of 4.2 NH_3 mol/(Mo-cysteine complex mol)hr (148).

Tanaka and colleagues have reported the catalytic reductions of both N_2 [150] and N_2H_4 [151] to NH_3 by the electrochemically reduced species of the clusters $[Fe_4S_4(SPh)_4]^{2-}$, $[(4-Fe)^{2-}]$, or $[MoFe_3S_4(SPh)_3]^{3-}$, $[(Mo-$



$\text{Fe}]^{3-}$) in protic solvents. The reduced species of the clusters are generated by controlled potential electrolysis (c.p.e.) at a Hg electrode using a potential that will form $[4\text{-Fe}]^{3-}$, $[4\text{-Fe}]^{4-}$, or $[\text{Mo-Fe}]^{5-}$. Although N_2 reduction was observed in such systems, the current efficiency was very low, with a maximum of 1.6% for the $[4\text{-Fe}]^{3-}$ cluster in methanol/tetrahydrofuran (MeOH/thf). The total yield of NH_3 based on the cluster was 195% for 4 days. It is surmised that most of the electrons transferred from the electrode are consumed in H_2 evolution from the solvent.

The reduction of N_2H_4 was conducted in H_2O or MeOH/thf solution containing $[\text{Fe}_4\text{S}_4\text{L}_4]^{2-}$ or $[\{\text{MoFe}_3\text{S}_4\text{L}_3\}_2(\mu\text{-L})_3]^{3-}$ ($\text{L} = \text{SPh}$ or $\text{SCH}_2\text{CH}_2\text{OH}$) under c.p.e. conditions. The $[\text{Mo-Fe}]^{3-}$ cluster suspended in aqueous solution reduces N_2H_4 to NH_3 at pH 12 (Eq. 8) with concomitant evolution of H_2 (Eq. 9).



Twenty-six moles of NH_3 and 19 moles of H_2 are produced per mole of cluster in 4 hr. The catalytic activity of $[\text{Mo-Fe}]^{m-}$ is superior to that of $[4\text{-Fe}]^{n-}$ with respect to NH_3 formation, and in both cases, higher pH favors the formation of NH_3 . The maximum current efficiency using $[\text{Mo-Fe}]^{m-}$ catalyst in MeOH/thf is 97%.

The catalytic reduction of N_2H_4 to NH_3 has been accomplished with single cubane clusters [66,69] of the type $[\text{MoFe}_3\text{S}_4\text{Cl}_3(\text{polycarboxylate})(\text{NCMe})]^{2-}$ and $[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{dmf})_3]^-$ using 2,6-dimethylpyridinium salts ($[\text{Hlut}]^+$) as acid and $[\text{Co}(\eta^5\text{-C}_5\text{H}_5)_2]$ as the reductant. The Mo cluster has a turnover of 100 molecules of N_2H_4 within 30 min, but the V cluster does not produce NH_3 so rapidly.

These systems have not been analyzed by a detailed kinetic study since the precipitation of ammonium salts occurs as the reaction proceeds. Nonetheless, there is some evidence that the N_2H_4 reduction site is the Mo or V. Thus the cluster, $[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{HBpz}_3)]^{2-}$, containing the nonlabile pyrazolylborate ligand does not convert N_2H_4 to NH_3 . In addition, the cluster $[\text{VFe}_3\text{S}_4\text{Cl}_3(\text{bpy})(\text{PhNHNH}_2)]^-$ has been isolated and the coordination of the PhNHNH_2 to the V site authenticated by x-ray crystallography.

Analogous studies with *cis*- $\text{MeN}=\text{NMe}$ and $[\text{MoFe}_3\text{S}_4\text{Cl}_3(\text{tccat})(\text{N-CMe})]^{2-}$ give exclusively MeNH_2 (152), in contrast to the enzyme, which gives a mixture of MeNH_2 , NH_3 , and CH_4 [153]. In addition, the cluster $[\text{MoFe}_3\text{S}_4\text{Cl}_3(\text{tccat})(\text{NH}_2\text{Me})]^{2-}$ has been isolated, strongly indicating that the Mo is the site of *cis*- $\text{MeN}=\text{NMe}$ binding and activation.

IV.B. Unsaturated Hydrocarbons

The noncatalytic homogeneous reduction of C_2H_2 to C_2H_4 (60% yield), using the system $[\text{Fe}_4\text{S}_4(\text{SPh})_4]^{3-}$ /ethanoic acid/ethanoic anhydride, has been reported



[154] with ethanoic acid as the source of hydrons. With C_2D_2 the principal product was identified as *cis*- $C_2H_2D_2$: the same stereoselectivity as observed with the enzyme. Furthermore, C_2H_2 can be catalytically reduced to C_2H_4 using a mixed-metal catalytic system, $[Fe_4S_4(SPh)_4]^{2-}/Mo^{IV}/(Gly-Gly-Cys)_n/NaBH_4$ in MeOH/thf [155,155a]. In addition diphenylethyne can be reduced catalytically and stereospecifically to *cis*-diphenylethene by $NaBH_4/[Fe_4S_4(SPh)_4]^{2-}$ [156].

C_2H_2 reduction to C_2H_4 , with concomitant H_2 evolution, is also catalyzed by the electrochemically reduced species of $[4-Fe]^{n-}$ and $[Mo-Fe]^{m-}$ [157]. In MeOH/thf the reduced species of $[4-Fe]^{n-}$ and $[Mo-Fe]^{m-}$ reduce hydrons from the MeOH to evolve H_2 , and the rate of H_2 evolution increases in the order $[Mo-Fe]^{5-} < [4-Fe]^{3-} < [4-Fe]^{4-}$. When C_2H_2 -saturated MeOH/thf is used as solvent, H_2 evolution is drastically depressed and C_2H_2 is reduced to C_2H_4 . Practically no H_2 evolution occurs in the reaction of C_2H_2 with $[4-Fe]^{3-}$ or $[Mo-Fe]^{5-}$. Similar behavior is demonstrated by nitrogenase [158]. Catalysis of C_2H_2 reduction with $[4-Fe]^{4-}$ is about six times faster than that with $[4-Fe]^{3-}$ or $[Mo-Fe]^{5-}$. A Michaelis constant of $K_M = 1.4$ mmol and $V_{max} = 2.7 \times 10^{-4}$ mmol sec^{-1} has been calculated for the reduction of C_2H_2 by electrochemically reduced $[4-Fe]^{n-}$. The K_M is larger than that of nitrogenase ($K_M = 0.1-0.3$ mmol) [159]. The strict stereospecificity observed in the nitrogenase reduction of C_2H_2 with D_2O [76] is not mirrored in the electrocatalytic systems.

Ferrocenethiolate substituted analogs of the Fe-S and Mo-Fe-S clusters, $[Fe_4S_4(SFc)_4]^{2-}$ and $[MoFe_3S_4(SFc)_3(\mu-X)_3]^{3-}$ ($Fc = C_5H_4FeC_5H_5$, $X = EtS$ or MeO), exhibit more negative reduction potentials [160] than the corresponding thiophenolate clusters [161]. On c.p.e. of dispersions of the clusters in C_2H_2 -saturated water (pH 7.0) containing a surfactant, not only is C_2H_4 evolved but so are a small amount of C_2H_6 and a considerable amount of H_2 . The mole ratio of C_2H_4/C_2H_6 evolved (about 11:1) is essentially independent of the cluster; however, the concomitant H_2 evolution is dependent on cluster type. The mole ratio C_2H_4/H_2 varies with the cluster in the order $[MoFe_3S_4(SFc)_3(\mu-OMe)_3]^{3-} < [MoFe_3S_4(SFc)_3(\mu-SEt)_3]^{3-} < [Fe_4S_4(SFc)_4]^{2-}$; the rate of C_2H_4 formation increases in the same order. Thus the Fe-S cluster exhibits not only a higher activity but also a better selectivity for C_2H_2 reduction than the Mo-Fe-S cluster.

The heteronuclear single-cubane cluster, $[MoFe_3S_4Cl_3(tccat)(NCMe)]^{2-}$, catalytically converts C_2D_2 stereospecifically to *cis*-CHDCHD (together with a small amount of ethane) (162), using $[Hlut]^+$ as the hydron source and $[Co(\eta^5-C_5H_5)_2]$ as the reductant. The catalyst accomplishes more than 15 turnovers in about 2 hr, and the rate of the catalysis show (1) a first-order dependence on the concentration of acid; (2) a zero-order dependence on the concentration of reductant, and (3) a nonlinear dependence on the concentration of C_2H_2 . The rate data have been analyzed by Michaelis-Menten kinetics and the values $K_M = 17.9$ mmol dm^{-3} and $V_{max} = 1.1 \times 10^{-4}$ mol min^{-1} determined. A temperature

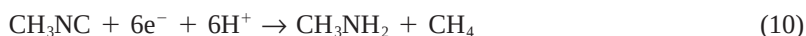


dependence study of the reaction rate gave $E_a = 2.15 \text{ kJ mol}^{-1}$ and $\Delta S^\ddagger = -7.7 \text{ J K}^{-1} \text{ mol}^{-1}$. The negative value of $\Delta S^\ddagger$ has been interpreted in terms of an ordered transition state.

The authors propose that Mo is the site where the C_2H_2 binds and is transformed. Thus when the analogous $[\text{MoFe}_3\text{S}_4\text{Cl}_3(\text{CO})_3]^{3-}$ cluster is employed, the rate of reduction of the C_2H_2 dramatically decreases but is not suppressed completely. It is argued that this is because the CO ligands are nonlabile and effectively block C_2H_2 from binding to the Mo site. The low reactivity to C_2H_2 reduction by these clusters is interpreted as being slower C_2H_2 reduction on the Fe sites. However, it is difficult to rule out completely the possibility that C_2H_2 is always converted to C_2H_4 at the Fe sites, and changing the ligands around Mo merely modulates the reactivity of the cluster by perturbing the electron distribution in the cluster. Certainly, the simple all Fe clusters, $[\text{Fe}_4\text{S}_4\text{Cl}_4]^{3-}$ and $[\text{Fe}_4\text{S}_4(\text{SPh})_4]^{3-}$, can convert C_2H_2 to C_2H_4 and have no choice but to do it at an Fe site. Clearly, a more detailed understanding of the way in which changing the ligands around one metal affects the rest of the cluster is needed.

IV.C. Alkylisocyanides and Alkylcyanides

Nitrogenase mediates the six-electron reduction of methylisocyanide to CH_3NH_2 and CH_4 (Eq. 10) [163] and of methylcyanide to C_2H_6 and NH_3 (Eq. 11) [164].



The catalytic reduction of CH_3NC by the electrochemically reduced species $[\text{4-Fe}]^{4-}$ in MeOH/thf under c.p.e. [165] generates CH_4 and small amounts of C_2H_6 and C_2H_4 , together with a trace of C_3H_8 ; this is similar to the reduction by nitrogenase, though the concomitant H_2 evolution is large ($\text{H}_2/\text{CH}_4 \approx 140$) compared with that in the nitrogenase reaction ($\text{H}_2/\text{CH}_4 = 3$) [166]. Similar results are obtained for the reduction of CH_3NC by $[\text{4-Fe}]^{3-}$ and $[\text{Mo-Fe}]^{5-}$ except that with the former catalyst C_2H_6 is a main product. The formation in solution of an isocyanide-cluster species has been demonstrated [167]. When MeOH/thf is the solvent the reduction product, CH_3NH_2 , reacts with HCHO , an oxidation product of the MeOH , giving a variety of amines and related compounds.

The electrolysis of CH_3NC in an aqueous suspension of $[\text{4-Fe}]^{n-}$ (pH 7.2) gives reaction products in the mole ratio $\text{CH}_4:\text{C}_2\text{H}_6:\text{C}_2\text{H}_4:\text{H}_2 = 1.0:0.29:0.16:320$, which are of the same order as those obtained in MeOH/thf ($1.0:0.05:0.03:140$). The amount of CH_3NH_2 formed is about 10 times greater than the amount of hydrocarbon formed.

CH_3CN , when similarly reduced in an aqueous suspension by $[\text{4-Fe}]^{4-}$ or $[\text{Mo-Fe}]^{5-}$ formed by c.p.e. (at pH 7.0), produced C_2H_6 , C_2H_4 and NH_3 . The



amount of NH_3 produced is 95% of the total moles of C_2H_6 and C_2H_4 formed and no $\text{C}_2\text{H}_5\text{NH}_2$ is detected. In addition, $\text{C}_2\text{H}_5\text{NH}_2$ is completely inactive under such reduction conditions, indicating that the reduction does not proceed via this species.

IV.D. Azides

The multielectron reduction of alkylazides catalyzed by various $[\text{4-Fe}]^{n-}$ and $[\text{Mo-Fe}]^{m-}$ clusters has been achieved by c.p.e. in homogeneous systems and at modified glassy-carbon electrodes [168–169]. Dithionite reduction of alkylazide is catalyzed by $[\text{Fe}_4\text{S}_4(\text{SC}_6\text{H}_4\text{-4-n-C}_8\text{H}_{17})_4]^{2-}$ [170] and $[\text{MoFe}_3\text{S}_4(\text{SR})_3(\text{Q})\text{X}]^{2-}$ (R = alkyl- or aryl-; Q = 3,6-disubstituted catecholate or tetrasubstituted catecholate; X = solvent) in aqueous micellar solutions [54,170].

The catalyzed reduction of N_3^- by $[\text{Mo-Fe}]^{3-}$ modified glassy-carbon electrode, $[\text{Mo-Fe}]/\text{GC}$, under c.p.e. conditions in water at $\text{pH} = 6$ produced N_2 , NH_3 , and N_2H_4 , whereas only N_2 and NH_3 were formed at $\text{pH} = 12$ (Eqs. 12 and 13) [171].



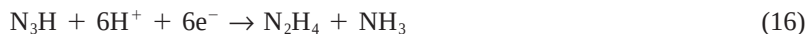
The $[\text{Mo-Fe}]/\text{GC}$ electrode cannot catalyze the eight-electron reduction to NH_3 (Eq. 14). A distinct dependence of pH on the rates of formation of N_2H_4 and NH_3 suggests that the reactions take place independently (Eqs. 12 and 13).



The rate of N_2H_4 formation was linearly proportional to the concentration of N_3H (which exists as an equilibrium mixture with N_3^- in aqueous solution (Eq. 15)),



whereas the rate of N_2 evolution was controlled by the N_3^- concentration. The formation of N_2H_4 is best represented by Equation 16.



Such observations suggest that N_2H_4 and N_2 are formed from precursors in which N_3H and N_3^- are coordinated to the Mo-Fe cluster [171].

IV.E. Nitrite

Nitrogenase reduces nitrite by a six-electron process to NH_3 [172]. The $[\text{Mo-Fe}]/\text{GC}$ electrode catalyzes, under c.p.e. conditions in water at $\text{pH} 10$, both the



assimilatory and dissimilatory reduction of nitrite to NH_3 and N_2O , respectively, depending on the electrolysis potential [173]. A $[\text{NBu}_4][\{\text{MoFe}_3\text{S}_4(\text{tc-cat})(\text{SPh})_2\}_2(\mu\text{-SPh})_2]$ -modified GC electrode, under similar electrolysis conditions, gives either NH_3 or N_2 [174].

None of the clusters used in the electrochemical reductions described previously is a particularly good synthetic model for FeMoco as the clusters' stoichiometry, geometry, and metal oxidation states do not correlate with those found for isolated FeMoco. The problems with interpretation of the mechanism of the substrate reductions have been delineated [54]. The active reduced cluster species (e.g., $[\{\text{MoFe}_3\text{S}_4(\text{SR})_3\}_2(\mu\text{-SR})_3]^{5-}$) have been characterized only by the potentials at which they are generated. Also, reduced cluster species are generally unstable in water and protic solvents and therefore would not be expected to exist over the period required for substrate reduction. Assuming there is no breakdown to separate Fe and Mo sulfides, which themselves are catalytically active, these results do suggest, however, that some Fe-S and Mo-Fe-S species are capable of nitrogenase substrate reduction.

IV.F. Hydronation of Fe-S Clusters

So far the discussion on synthetic clusters illustrates the accumulating information that a variety of nitrogenase substrates can be transformed by simple hydronation reactions at reduced synthetic Fe-S-based clusters. The next level of detail must address the mechanisms of these transformations. Already we have indicated several cases where kinetic studies have been performed. The major problem with the approaches taken so far, looking directly at substrate transformation, is that they can lead to erroneous conclusions. This is because in this approach the experimenter relies on the kinetics to define the number of species essential to accomplish the transformation. For example, the order with respect to hydrons has been established in several of the catalytic systems discussed and invariably found to be one. It is tempting to jump to the conclusion that only one hydron is necessary to activate the cluster. However, studies on the hydronation of Fe-S clusters show that the kinetics of simple hydronation reactions is much more complicated.

Since the binding of hydrons to Fe-S clusters is not associated with a discernible spectroscopic change (IR, NMR, or UV-visible spectrum), an approach that couples hydronation and substitution reactions has been developed: effectively using the large spectral changes associated with substitution of these clusters together with the way that the acid perturbs the rate of substitution to get information about the site and number of hydronations. The acid used in these studies is the relatively weak $[\text{NH}_4\text{Et}_3]^+$, and the nucleophile is most commonly a thiolate ion $[\text{RS}]^-$.

A variety of structurally different Fe-S clusters have been studied mecha-



nistically, including $[\text{Fe}_4\text{S}_4\text{X}_4]^{2-}$ [175]; $[\{\text{MoFe}_3\text{S}_4\text{X}_3\}_2(\mu\text{-SR})_3]^{3-}$ [176] ($\text{X} = \text{SR}, \text{SAr}, \text{or Cl}$); $[\text{Fe}_2\text{S}_2\text{Cl}_4]^{2-}$; $[\text{S}_2\text{Mo}(\mu\text{-S})_2\text{FeCl}_2]^{2-}$ [177]; and $[\text{Cl}_2\text{Fe}(\mu\text{-S})_2\text{V}(\mu\text{-S})_2\text{FeCl}_2]^{3-}$ [177]. Obviously, the details of the individual studies differ, but there are five features of the reactivity that appear to be entirely general for this class of molecule.

1. Both associative and dissociative substitution pathways can operate, in both the absence and the presence of acid.
2. The associative mechanism involves rapid binding of the nucleophile to the tetrahedral Fe site, followed by rate-limiting dissociation of the leaving group.
3. In the presence of $[\text{NH}_4\text{Et}_3]^+$ the rate of the reaction exhibits a nonlinear dependence on the ratio $[\text{NH}_4\text{Et}_3]^+ / [\text{NH}_4\text{Et}_3]$, consistent with rapid hydronation of the cluster followed by rate-limiting substitution.
4. The pK_a 's of all the hydronated clusters (in MeCN) fall in the relatively narrow range $17.9 < \text{pK}_a < 18.9$, even with clusters containing halide ligands, which cannot be hydronated by $[\text{NH}_4\text{Et}_3]^+$. This indicates that hydronation is at a common site on the cluster core: most probably a lone pair of electrons on a bridging sulfur [177,178].
5. Hydronation of a bridging sulfur atom adjacent to the site of substitution is the most labilizing, presumably because in this position it most effectively competes for the π -electron density on the substitutionally active Fe, thus weakening the Fe-leaving group bond [177].

One aim of mechanistic studies on the reactions of Fe-S-based clusters is to understand how each component of the cluster contributes to the reactivity of the whole entity. Systematic study of the reactions of the various clusters under the same conditions has revealed the effect that Mo has on the reactivity of the Fe sites.

The acid-catalyzed substitution reactions of $[\text{Fe}_4\text{S}_4(\text{SPh})_4]^{2-}$ occur by a dissociative mechanism exclusively. However, $[\{\text{MoFe}_3\text{S}_4(\text{SPh})_3\}_2(\mu\text{-SPh})_3]^{3-}$ contains a substitutionally inert Mo atom (formally replacing an Fe). Substitution at the Fe sites in the subcluster of this species occurs predominantly by an associative mechanism. Simplistically, the replacement of one Fe by Mo in the cube changes the electron distribution within the cluster and renders the Fe sites more electron-deficient.

A similar effect is observed in the analogous reactions of $[\text{Fe}_4\text{S}_4\text{Cl}_4]^{2-}$ and $[\{\text{MoFe}_3\text{S}_4\text{Cl}_3\}_2(\mu\text{-SEt})_3]^{3-}$, although in this case the Fe_4S_4 cluster undergoes substitution by a mixture of associative and dissociative mechanisms whereas the Mo-containing cluster undergoes substitution exclusively by an associative mechanism.

That the inclusion of Mo within the cluster makes the Fe sites behave as though they are electron-deficient is reflected in the ability of these clusters to



bind transiently a variety of small molecules (CO , N_3^- , CN^- , halide, and N_2O). The binding of these species to intact clusters is detected by the perturbation afforded to the rate of substitution, and analysis of the kinetics allows determination of the binding constant for each molecule. Invariably the binding of any particular molecule is always “tighter” to the Mo cluster.

These effects of Mo on the reactivity of the Fe sites in the cluster suggest that the role of the Mo in cofactor is only to modulate the reactivity of the Fe sites, giving them a greater affinity to bind substrates. Clearly this effect is not attributable to the Mo alone, but rather to the Mo and its ligands. In a recent study [179] systematic variation of the ligands on a Mo changes the reactivity of a neighboring Fe site from predominantly associative substitution to predominantly dissociative substitution. Electron-releasing ligands on Mo favor a dissociative mechanism on Fe whereas electron-withdrawing ligands on Mo favor an associative mechanism.

V. CONCLUDING REMARKS

The solution of the x-ray crystallographic structures of the nitrogenase proteins has provided a great deal of information but has not solved the fundamental problem of how the enzyme activates and reduces N_2 . It is now possible to formulate much more informed hypotheses on the enzyme's mode of action, but still major questions remain, e.g., Where on FeMoco does N_2 bind? How is it activated for reduction and how is this activation coupled to MgATP hydrolysis? Are electron and hydron transfers coordinated by the enzyme? What are the intermediate steps in N_2 reduction? Are these steps the same for all three nitrogenases?

It is clear that there is still much to learn about this fascinating and extremely important enzyme. The answers to the questions posed will come only from multidisciplinary studies involving structural and spectroscopic analysis, enzymology, genetic modification of the enzyme, and model chemistry. Together these approaches should allow the detailed understanding of the enzymic process at the atomic level that in turn could lead to more effective exploitation of the biological process and its underlying chemistry.

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8

Biological Iron–Sulfur Clusters with Catalytic Activity

Wilfred R. Hagen

*Wageningen Agricultural University, Wageningen, and
University of Nijmegen, Nijmegen, The Netherlands*

I. INTRODUCTION

I.A. Biological Iron–Sulfur Clusters

By definition biological iron–sulfur clusters are structures of sulfide and iron ions forming a core that is externally coordinated by a protein. As a result of many years of descriptive biochemical studies (cf. [1–5]) one can formulate a more specific operational definition in the form of a list of characteristics for which until now no counter-examples have been found. The irons are always high-spin Fe(III) or high-spin Fe(II). The sulfide ions form μ_2 or μ_3 bridges. The external ligands are always coordinated to the irons. Naturally occurring ligands always include, and predominantly so, S, thiolato side groups from cysteine; and occasionally N, imidazole side groups from histidine, or O, carboxylato side groups from aspartate. Selenium (selenide or selenocysteine) so far has not been found to be a part of naturally occurring iron–sulfur clusters. In some proteins one, and not more than one, of the iron ions can be coordinated by nonprotein ligands, e.g., water. Through strong exchange coupling the Fe/S core ions form a single paramagnetic entity whose ground state has a well-defined system spin S.

A more specific operational definition excludes the unique clusters of the nitrogenase enzymes (which are treated in Chapter 7) from our considerations: biological iron–sulfur clusters in this context only deal with Fe and do not contain any other metal. Under physiological conditions they can occur in two, and not more than two, oxidation states. All clusters are either dinuclear or one of the



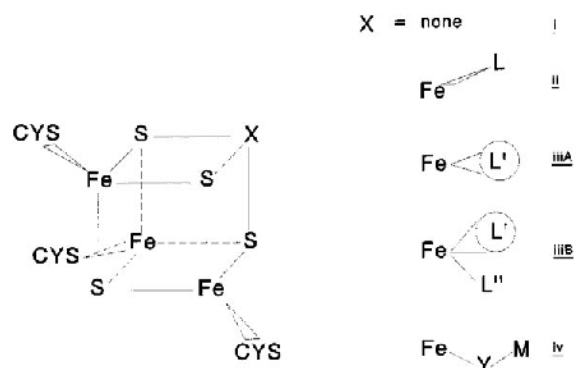


Figure 1 Variants to the cubane motif. L is a general ligand, i.e., a protein side group or a water or a hydroxo group. L' is a didentate substrate. L'' is a water molecule.

variants of the cuboidal trinuclear or tetranuclear structure. It will be shown that catalytic clusters are probably only of the latter type. These very few basic structures are illustrated in Figure 1.

I.B. Functions of Iron–Sulfur Clusters

The discovery of the different dinuclear or cuboidal-type biological iron–sulfur clusters is associated with their natural occurrence in two oxidation states. They can all function as one-electron transferring agents. This redox function has been well established in many studies over a period of almost five decades [1–5]. However, electron transfer is generally not considered to be a catalytic activity. It is typically a stoichiometric transfer between two complex redox proteins. Mechanistically, it is probably best described as “outer sphere” or not involving the breaking and making of “covalent” bonds other than those related to hydrons.

Several other possible biological functions of iron–sulfur clusters have been proposed as summarized in Table 1. Let us briefly discuss this list in detail to indicate why the majority of the entries are excluded from our definition of catalytic iron–sulfur clusters.

For a long time Fe/S clusters in the enzyme complexes of the respiratory chain of oxidative phosphorylation have been suggested to be directly involved in energy transduction, e.g., in the generation of a proton-motive force. A specific example is the putative cubane, center N2, in NADH:Q oxidoreductase [6]. One could formally write the process as a catalysis of the reaction $H^+_{in} \rightarrow H^+_{out}$.



Table 1 Established, Putative, and Proposed Biological Functions of Iron–Sulfur Proteins

Function	Example ^a
Electron transfer	Many ferredoxins
Energy transduction	NADH:Q oxidoreductase
Sensing	Ferrochelatase
Regulation	SoxR, FNR, IRP
Biosynthesis	NIFU
Structural role	Endonuclease III
Hydrolysis	Aconitase
Redox catalysis	Fe hydrogenase

^a NADH, reduced nicotinamide-adenine dinucleotide; Q, coenzyme Q; FNR, fumarate nitrate reduction; SoxR, superoxide activated regulatory protein; IRP, iron regulatory protein; NIFU, nitrogen fixation gene U product.

However, direct involvement, if any, of the Fe/S clusters in a spatial transfer of hydrons is presently still hypothetical [7].

Fe/S clusters in regulatory enzymes have been proposed to act as sensors in such a manner that, upon detection of a measurand, the cluster disintegrates and activity stops. Putative examples are NO sensing by the [2Fe-2S] cluster in the terminal enzyme of heme synthesis, ferrochelatase [8], and O₂ sensing by the [4Fe-4S] cluster in the regulatory enzyme of purine nucleotide biosynthesis, glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase [9]. This is of course not a catalytic activity, since the cluster is destroyed in the action.

Occasionally storage of iron has been proposed as a function of Fe/S proteins (cf. Chapter 8 in [4]). There are no recent reports on this possibility.

Relatively recently Fe/S proteins have been found to function in the regulation of biosynthesis. This can be by promoting deoxyribonucleic acid (DNA) transcription, e.g. the [2Fe-2S] containing *Escherichia coli* superoxide-activated (SoxR) transcription activator [10–12], or the presumably [4Fe-4S]-containing *E. coli* transcription factor fumarate nitrate reduction (FNR) [13,14]. Alternatively, the Fe/S protein can act by interference with messenger ribonucleic acid (mRNA) translation, i.e., the iron regulatory proteins (IRPs) [15,16]. These interactions are stoichiometric, therefore not catalytic. Presumably, they are also a form of sensing, namely, of oxidants and/or iron [17].

The biosynthesis of Fe/S clusters may well proceed via other, possibly more simple Fe/S clusters as intermediates. The products of the genes *nifS* and *nifU* in *Azotobacter vinelandii* are thought to collaborate in nitrogenase metallocluster biosynthesis; NIFS is a sulfide donor; NIFU is a [2Fe-2S] protein. The



latter may be a donor of iron ions or of complete iron–sulfur building blocks [18]. As with the sensing function this would imply that the cluster is not invariant, therefore this is also not a catalytic activity from the point of view of the cluster.

Superoxide may leach iron from iron–sulfur clusters. It has recently been proposed that this may be functional in DNA oxidation, namely, to increase hydroxy radical production [19]. Again, the cluster is destroyed in the act.

E. coli endonuclease III is the prototype of DNA repair enzymes that contain a [4Fe–4S] cluster [20]. The cluster has four Cys ligands and does not appear to be involved in the catalysis. It may have a structural, in particular, a charge-compensating, role [21,22].

This leaves only the two last entries of Table 1 for our further consideration.

I.C. Catalytic Iron–Sulfur Clusters

Through our definition of iron–sulfur clusters, mononuclear iron centers (e.g., rubredoxin) have been excluded from consideration. For convenience, we also do not consider the two exceptional clusters of nitrogenase in this chapter (see Chapter 7). Thus far, no dinuclear (with one possible exception [23], see later discussion) or trinuclear iron–sulfur clusters have been identified that functionally fall in one of the two categories: nonredox catalysts or redox catalysts. Therefore, at this time all established and putative catalytic iron–sulfur clusters are cubanes. Furthermore, they are all asymmetric cubanes: i.e., they have the schematic structure given in Figure 1, where X is not empty and also not $\text{Fe}-\eta^1-\text{S}-(\text{Cys})$. The iron at position X is always discernible from the other three irons. Following the nomenclature introduced by the groups of Beinert and Münck (cf. [15]) we label the iron at position X with a subscript a, Fe_a . The other irons are then Fe_b 's, or, in the favorable cases when they can be mutually distinguished (e.g., in Mössbauer spectroscopy) they are labeled with subscripts b1 through b3. In crystallographic studies a different nomenclature has been used, namely, Fe4 for Fe_a and Fe1 through Fe3 for the Fe_b 's [15].

All presently known nonredox enzymes with a cubane as catalytic center are hydrolases (see later discussion). In these enzymes Fe_a is formally equivalent to the tetrahedral Zn(II) site in hydrolytic zinc enzymes. Thus, when the enzyme is ready to act but not yet in turnover (the substrate-free enzyme), its cluster is probably best described as structure *ii* in Figure 1 with X a hydroxyl group. In shorthand notation this could be written as $[\text{Fe}_a\text{OH}-3\text{Fe}_b-4\text{S}]^{1+}(\text{Cys})_3$ or as $[\text{Fe}_a3\text{Fe}_b-4\text{S}]^{2+}\text{OH}(\text{Cys})_3$ where it has been assumed that, on average, each iron is formally $\text{Fe}^{2.5+}$.

The existence of hydrolytic iron–sulfur enzymes is well established. Contrarily, redox-catalytic iron–sulfur centers are presently still hypothetical at least



in the sense that no structural information is available yet on the substrate-catalytic center complex (cf. nitrogenase) or even on the catalytic center proper (cf. Fe-hydrogenase).

II. HYDROLYTIC (NONREDOX) IRON–SULFUR ENZYMES

II.A. Overview

Hydration and/or dehydration reactions are frequently catalyzed by metalloproteins. Examples are proteins containing nickel (urease), zinc (e.g., peptidases), molybdenum (the hydratase partial reaction of formate oxidoreductase), tungsten (acetylene hydratase). An obvious difference between Ni, Zn, on the one hand, and Fe, Mo, W, on the other, is that the first are directly coordinated to the protein whereas the latter are also part of a cofactor. With reference to the Fe/S cluster in aconitase it has been suggested that cofactor coordination may provide an added flexibility to the active site, in particular to the substrate binding domain [15].

Aconitase was the first protein to be identified as containing a catalytic iron–sulfur cluster [24–26]. It was also readily established that the redox properties of the $[4\text{Fe-4S}]^{(2+;1+)}$ cluster do not play a role of significance in biological functioning: the $1+$ oxidation state has some 30% of the activity of the $2+$ state [25]. Since then several other enzymes have been identified or proposed to be nonredox iron–sulfur catalysts. They are listed in Table 2. It appears that all are involved in stereospecific hydration reactions. However, these proteins are considerably less well characterized than aconitase. In particular, no crystal structural information is available yet. Therefore, later we summarize structural and mechanistic information on aconitase, noting that many of the basic principles are expected to be relevant to the other enzymes of Table 2.

Table 2 Hydrolytic (Nonredox) Iron–Sulfur Enzymes and Their Substrates

Enzyme (metabolic route)	Substrate/product
Aconitase (citric acid cycle)	Citrate/isocitrate
Isopropylmalate isomerase (Leu biosynthesis)	α -Isopropylmalate/ α -hydroxy- β -carboxy-isocaproate
Fumarase A (bioenergetics)	Fumarate/L-malate
L-Serine dehydratase (Ser degradation)	L-Serine/ pyruvate
Dihydroxyacid dehydratase (Val, Ile biosynthesis)	2,3-dihydroxycarboxylic acid/ 2-keto carboxylic acid



II.B. Aconitase: The Protein and the Active Site

Aconitase is the trivial name for citrate dehydratase *cis*-aconitate hydratase (EC 4.2.1.3). It catalyzes the reversible isomerization reaction of citrate into isocitrate via the intermediate *cis*-aconitate (Figure 2). It is a water-soluble, monomeric protein. In eukaryotic cells aconitase is located in the mitochondrial matrix. In prokaryotes the enzyme occurs in the cytoplasm. The pig heart enzyme consists of 754 amino-acid residues, providing a molecular mass of 83 kDa [27]. Aconitase from other sources has similar size. The porcine protein is synthesized with a mitochondrial targeting sequence. The mature, functional protein can be (over)expressed in *Escherichia coli* [28].

The three-dimensional structure [29–34] of the protein is made up of four domains. Each domain contains α helices and β strands. The first three, N-terminal domains bear mutual similarity, and their fold is similar to a domain found in dehydrogenases. The topology of the fourth, C-terminal domain appears to be unique. The first three domains can be thought of as forming a base on which the fourth domain hinges somewhat like a lid on a shallow box. The [4Fe-4S] cluster is at the interface of the four domains. The lid is ajar, forming a cleft from the outside to the active site; however, this cleft is filled with side chains and water molecules, and therefore transfer of substrate or product (or intermediate; see later discussion) requires spacial rearrangement, i.e., conformational changes.

The [4Fe-4S] cluster is coordinated by three Cys from domain 3. The fourth ligand, i.e., to Fe₄, is not from the protein. In the substrate-free, active enzyme it is a hydroxo group [35]. In the substrate-bound complex this becomes a water molecule [35]. All four domains are thought to contribute amino acids (some 21 in total, cf. [15]) to a broadly defined active site, i.e., the cluster, reactive side groups, and a network of hydrogen bonds including the substrate binding do-

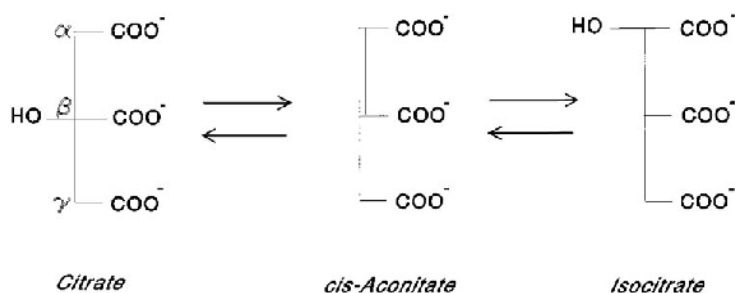


Figure 2 The aconitase reaction: a reversible isomerization by means of subsequent dehydration–hydration reactions.



main. For example, domain 1 contains His101 and domain 4 contains Ser642. Both residues are thought to be directly involved in catalysis as hydron-transfer groups.

II.C. Aconitase: Catalytic Mechanism

A detailed model for the enzyme mechanism has been developed by Beinert and Kennedy and their collaborators on the basis of extensive spectroscopic studies, isotope labeling, kinetic experiments, several crystal structures, and site-directed mutagenesis experiments. As usual for an enzymological work in progress, the hardness of individual aspects of the model ranges from well established to conjectural.

Essential features of the model, as it presently stands, are summarized in the ten-step catalytic cycle of Figure 3. In this scheme, the six-carbon substrate/product has been drawn as a four-carbon plus R compound for clarity. Thus, it is not necessary to draw out the right-hand side of the cycle fully as the structures are identical to those of the left-hand side except for an interchange of the groups $C_\alpha\text{—H}$ and $C_\beta\text{—R}$ (see also Figure 2). Going through the subsequent steps of the reaction cycle and discussing the experimental evidence for the different intermediates, we start at the top with the substrate-free enzyme and then step counter-clockwise through the cycle.

1. The iron depicted is of course Fe_a . The supporting cuboidal $[3\text{Fe}_b\text{—}4\text{S}]$ structure has been reduced to a circular symbol because it is thought not to change structure during catalysis. The external ligand to Fe_a is hydroxo: an O is found in the crystal structure [31] and a single deuterium is observed in ^2H -ENDOR at ligand exchange in $^2\text{H}_2\text{O}$ [35].
2. Isocitrate has been bound by the enzyme. Fe_a is 5- or 6-coordinate on the basis of Mössbauer spectroscopy [36,37]. The ENDOR studies with ^{17}O -labeled citrate and the inhibitor nitroisocitrate (nonexchangeable hydroxyl group) indicate significant binding to Fe_a only of β -carboxyl and of hydroxyl [38]. ^2H -ENDOR shows that the sixth ligand has been hydronated [35]. We draw this water ligand inside the circle to indicate a remarkable fact: there is no evidence that this ligand participates in the catalytic mechanism (other than its charge-compensating effect on Fe_a). The indicated interactions with the protein are deduced from the crystal structures with isocitrate and with nitroisocitrate [31]. Fe_a has now been drawn as popped out of a slot because it is moved away by approximately 0.2 Å from the $[3\text{Fe}_b\text{—}4\text{S}]$ structure. Ser642 and His101 form hydrogen bonds as indicated.
3. Ser642 has abstracted a hydron from C_α ; a carbanion has been formed. That Ser642 has a dehydronated hydroxyl group implies that its $\text{p}K_a$



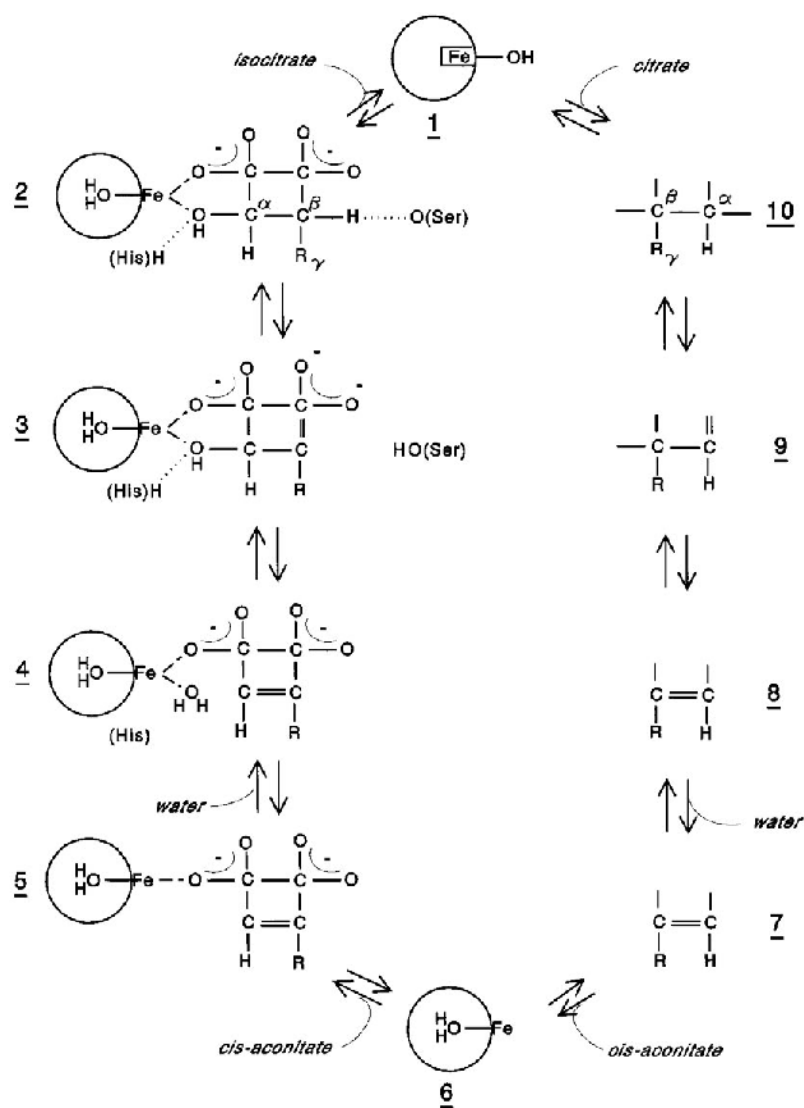


Figure 3 The catalytic cycle of aconitase. The circle symbolizes the $[3Fe_4S]$ structure that carries the reactive Fe_a . Note that the right-hand side of the scheme is the mirror image of the left-hand side with respect to the $C_\alpha C_\beta C_\gamma$ fragment.



is of the order of 7, i.e., much lower than in free Ser. The crystal structures indicate the Ser —O^- to be close to the C_β hydron and to be stabilized by hydrogen bonds [31]. Also, of all the point mutations analyzed thus far, Ser642Ala results in the most drastic reduction in activity by five orders of magnitude [28].

4. Attack of the carbanion onto the hydrogen-bonding N-H of His101 results in the removal of the hydroxo group from C_α , and with the hydron of His101 it temporarily forms a second water ligand on Fe_a . By now the substrate has been converted to *cis*-aconitate. The *cis*-aconitate complex has not been crystallized. *Trans*-aconitate is an inhibitor of the enzyme; in its crystal structure Fe_a is five-coordinate [32]; therefore, it is a useful model for the next intermediate.
5. The second water ligand leaves the Fe_a coordination sphere, leaving it five-coordinate. We have now arrived at a crucial step in the reaction mechanism. The *cis*-aconitate has to “flip” from its isocitrate binding mode to its citrate binding mode, i.e., a 180° rotation is required to interconvert $\text{C}_\alpha\text{—H}$ and $\text{C}_\beta\text{—R}$. It is hypothesized (cf. [15]) that this “flip” is brought about by displacement of the *cis*-aconitate by another molecule of *cis*-aconitate. This implies release of the *cis*-aconitate into solution and formation, if only transient, of **6**.
6. This state is formally the free enzyme; again, however, its conformation is probably not exactly that of **1**; it may be an activated form that allows for ready release and binding of *cis*-aconitate. In the scheme this is indicated by leaving Fe_a in its “popped-up” position.
7. A *cis*-aconitate molecule from solution binds in the citrate binding mode. All subsequent steps, i.e., the formation of **8**, **9**, **10**, and the final release of the citrate, are simply the reverse of the corresponding steps in the left-hand part of Figure 3, except for the inverted positions of the C_α and C_β groups. As a consequence, there are no irreversible steps in the mechanism.

II.D. Critical Evaluation

In short, the reaction mechanism consists of a dehydration, a flip, and a hydration. The first and the last steps appear to be well defined on the basis of spectroscopy, crystallography, and chemical common sense. The details of the flip, perhaps the key feature of the mechanism, are less clear. There are no crystals of the *cis*-aconitate complex (in fact, there should be two different complexes, **5** and **7**). The free-enzyme intermediate **6** has not been isolated. Displacement of one *cis*-aconitate by another one is expected to require significant conformational changes in the cleft from the catalytic center to the protein surface. It has not been established that the leaving and entering *cis*-aconitases are not, in fact, one and the



same molecule. In this option *cis*-aconitate need not necessarily diffuse into solution before becoming rebound. The activated **6** form of the enzyme might allow for a localized flip somewhere along the cleft's trajectory.

A second point, worthy of further consideration, is the apparently humble role of the sixth, hydroxo/water ligand to Fe_a as a charge compensator. One may wonder whether there is any additional chemistry yet to be identified. Also, it remains to be seen whether the two water ligands bound to Fe_a in structures **4** and **8** are really as inequivalent as they have been drawn in Figure 3.

II.E. Other Hydratases: Reaction Mechanism

To what extent is the aconitase reaction mechanism applicable in the description of the enzyme activities of Table 2? The substrates of aconitase and isopropylmalate isomerase [39,40] are similar in the representation used in Figure 3: only the R group changes from acetyl to isopropyl. Therefore, the reaction mechanisms can be expected to be similar. Specifically, isopropylmalate isomerase should have the equivalent of the two binding modes of the intermediate found in aconitase. The situation for fumarase [41–44] is different in part. The R group is now a hydron so the two mirror-images of Figure 3 are identical for this enzyme. Only one binding mode is required, and there is no need for displacement of intermediate. Consequently, demands on conformational flexibility of the enzyme can be expected to be less than for aconitase.

The substrates of serine dehydratase [45,46] and dihydroxyacid dehydratase [47] differ from citrate in more than just R. The different substrates are compared in Figure 4. They all have in common the central $\text{HO}^*-\text{C}-\text{CH}^{**}\text{COO}^-$ fragment, indicating that all enzymes should have the following features:

1. Coordination of hydroxy to Fe_a .
2. Some H bridge to this $-\text{OH}^*$ (His101 is not strictly conserved in aconitases).
3. Release of water from Fe_a , H bridge of the serine base to $-\text{H}^{**}$ (Ser642 is conserved in aconitase and in isopropylmalate isomerase).
4. Abstraction of this $-\text{H}^{**}$ by the base. It is not obvious from Figure 4 which, if any, substrate atoms should additionally coordinate to Fe_a . The coordination number has not been established.

As with fumarase, the reactions catalyzed by serine dehydratase and dihydroxyacid dehydratase do not require release and rebinding of intermediate. The reaction mechanisms should be describable as adapted versions of one-half only of Figure 3.

This section will be concluded with a note on an apparently exceptional system. The dihydroxyacid dehydratase from spinach has been reported to contain a $[\text{2Fe-2S}]$ cluster [23]. This would then be the very first example of a noncubane iron–sulfur enzyme. The authors list three observations that, together, “conclu-



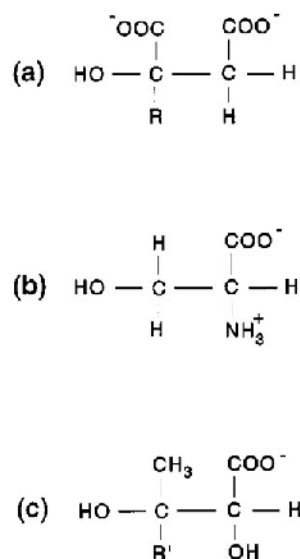


Figure 4 Analogous substrates of hydrolytic Fe/S enzymes. (a) Citrate (R = acetyl), isopropylmalate (R = propyl), malate (R = H); (b) serine; (c) dihydroxyvalerate (R' = methyl), dihydroxymethylvalerate (R' = ethyl).

sively demonstrate” the presence of a [2Fe-2S] cluster: (1) 2 Fe/S per protein monomer; (2) 1 EPR spin per 1.9 Fe; (3) characteristic *g* values and EPR temperature dependence [23]. However, the evidence might not be complete:

1. The reported *g* values are not typical either for [2Fe-2S] or for [4Fe-4S].
2. The EPR data have only been reported for one temperature, 30 K.
3. A “preliminary value” for the oxidation–reduction potential, $E_{m,8} \approx -470$ mV, indicates that complete reduction by dithionite and, therefore, spin quantification may have been difficult to achieve. It would seem that additional studies are required to establish firmly the nature of the Fe/S cluster in this enzyme before considering the equivalent of an Fe_a in a [2Fe-2S] cluster.

III. REDOX IRON–SULFUR ENZYMES

III.A. Overview

A catalytic redox iron–sulfur cluster is defined by having two properties: (1) the coordination of the cluster (or one of its constituting atoms) changes during catal-



ysis by direct interaction with substrate; (2) the oxidation state of the cluster changes functionally during catalysis; i.e., the cluster transfers electrons to/from the substrate. At present, these combined properties have not been firmly established for any enzyme. Several putative candidates are listed in Table 3, and we note that all the substrates are small inorganic molecules. The first entry, sulfite reductase, is perhaps the least convincing example. It is given here to illustrate a problem of definition. *E. coli* assimilatory sulfite reductase contains a siroheme whose iron is bridged through a cysteine sulfur to a [4Fe-4S] cubane [48]. In the crystal structure a coordinating phosphate is found at the distal side of the heme, and this is the likely binding site for the substrates, sulfite, or nitrite. It would thus appear that we can associate catalysis with the siroheme and electron transfer with the cubane. However, the crystal structure indicates that the two metal centers form a bridged, therefore integrated unit. Also, before catalysis can start, the enzyme has to be reduced, and the structure of this form has not been determined. Furthermore, the redox properties of the isolated enzyme are unusual: the E_m 's of both the heme and the cubane appear to be much lower than that of the substrate/product couples [49]. Properties of the more complex dissimilatory sulfite reductases are even less understood ([50], and refs. therein). The bottom line is that for these enzymes we do not precisely know whether and, if so, to what extent the iron-sulfur cluster is directly involved in the catalytic mechanism.

There are several other systems like sulfite reductase, i.e., complex redox enzymes containing an Fe/S cluster that is somehow related to activity but that is eluding the rigor of our definition of a catalytic redox iron-sulfur cluster. A recent example is the glycyl-radical enzyme ribonucleotide reductase of anaerobically grown *E. coli*. This $\alpha_2\beta_2$ complex presumably contains a [4Fe-4S] cluster bridged between the two β subunits [51]. Very recently, it has been proposed [52] that this cluster is a redox catalyst because its EPR spectrum in the reduced enzyme instantly changes upon binding of the activator S-adenosylmethionine.

Table 3 Possible Redox-Catalytic Iron-Sulfur Enzymes, Their Substrates, and the Putative Structures of Their Catalytic Center

Enzyme	Substrate	Catalytic center	Comments
Sulfite reductase	$\text{SO}_3^{2-}/\text{HSO}_3^-$	[4Fe-4S]-Cys-siroheme	See Chapter 7
Nitrogenase	N_2	Homocitrate-[MFe ₇ S ₈], M is Mo, V, Fe	
CO dehydrogenase	CO	[4Fe-4S]-X-Ni	See Chapter 9
“Prismane” protein	Unknown	[4Fe-2S-2O]	
Fe hydrogenase	H_2	Unknown	



It is thought that the activator becomes reduced by the excess electron of the Fe/S cluster and then cleaves to produce a 5'-deoxyadenosyl radical, which in turn produces the glycyl radical [52]. So the evidence of the redox-catalytic nature of the Fe/S cluster is not very strong, as there is no indication of direct coordination of the activator or its products to the cluster.

The next two entries to Table 3 are cited for completeness. Nitrogenase is treated in Chapter 7 and CO dehydrogenase in Chapter 9. Nitrogenase contains a very complex iron–sulfur cluster that includes another metal, molybdenum or vanadium. The crystal structure of the Mo variant has been determined. There is a third variant, “alternative” nitrogenase [92], whose cluster apparently does not contain any heterometal. That cluster would thus be a perfect candidate for our definition of a redox-catalytic iron–sulfur cluster. Unfortunately, this third nitrogenase has thus far been characterized to a much lesser extent than the other two forms. For *all* nitrogenases holds that the binding of N₂ to the cluster has not been established [53]; therefore, formally these enzymes have not yet been positively identified as redox iron–sulfur catalysts.

For CO dehydrogenase no crystal structure has been published. On the basis of a combination of spectroscopies an active-center structure has been proposed that features a [4Fe-4S] cubane bridged through an unknown ligand to a protein-bound nickel ion. This would seem to be a situation analogous to that discussed for sulfite reductase. On the basis of resonance Raman spectroscopic experiments it has been concluded that the substrate CO binds directly to one of the Fe ions of the cubane [54]; however, this claim has recently been retracted [55]. The reader is referred to Chapter 9 on nickel enzymes.

The last entry in Table 3 is Fe hydrogenase, or, iron-only hydrogenase, to set it apart from NiFe hydrogenases. The latter do contain iron–sulfur clusters, which are, however, involved in electron transfer only (cf. Chapter 9). The crystal structure of Fe hydrogenase has not been determined. Its catalytic center is thought to be an iron–sulfur cluster. The most recent models propose a cluster containing 6Fe [56–59], which is probably not correct (see later discussion). For a meaningful discussion of the present state of matters we first turn to the “prismane” protein. This is another protein that has been proposed to contain a 6Fe cluster [62]. A recent crystal-structure determination has shown this to be incorrect, and the analysis has important implications for Fe hydrogenase.

III.B. The “Prismane” Protein

The unusual “prismane” protein is probably a redox catalyst, although its biological function has not been established. It has been isolated from sulfate-reducing bacteria as a 60 kDa monomer, but homologous genes have been identified in a wide range of microorganisms [60]. Its trivial name refers to the core structure [6Fe-6S] in synthetic compounds that contain the anion (Fe₆S₆L₆)³⁻ in which L



is, e.g., a thiolato ligand [61]. On the basis of elemental analysis and comparative EPR spectroscopy the “prismane” protein was initially proposed to contain a single [6Fe-6S] cluster [62]. Solution of the crystal structure to 1.7 Å [60] has shown that it does not contain a single 6Fe cluster but rather two 4Fe clusters, one of which is a regular [4Fe-4S] cubane. The second 4Fe cluster has a novel-structure that is a mixture of structural elements from iron-sulfur clusters, iron-oxo clusters, and a persulfido group. It has therefore been given the trivial name “hybrid” cluster. A schematic representation of the hybrid cluster [60] is given in Figure 5. The position X marks electron density from an as yet unidentified ligand, possibly O. Its occupancy in the crystal is low, $\leq 10\%$. This means that in the majority of molecules the Fe5 is coordinatively unsaturated. The overall structure is suggestive of an active site ready to accommodate a substrate to be coordinated by Fe5 and Fe7. The cubane is close to the surface and some 12 Å away from the hybrid cluster, i.e., well positioned to act in electron transfer between an external redox partner and the active-site hybrid cluster [60].

Thus, the hybrid cluster is a putative iron-sulfur redox catalyst. It is, however, a very uncommon cluster (perhaps only comparable to the nitrogenase active site) in two aspects: (1) it is a hybrid cluster: i.e., it contains intrinsic building blocks that are distinctly “strange” to iron-sulfur clusters; and (2) it can exist in more than two (in fact, four [63]) oxidation states.

The finding that the “prismane” protein does not contain a prismane cluster

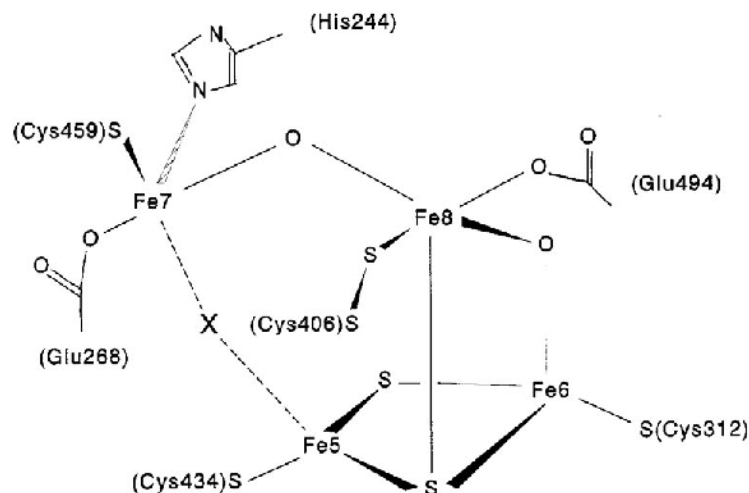


Figure 5 A sketch of the structure of the hybrid cluster in the “prismane” protein. The approximate core structure is [4Fe-2S-2O]. Cys406 carries a persulfido sulfur. X is an unidentified bridge of low occupancy in the crystal structure.



of course weakens the case for a 6Fe cluster in hydrogenase [56–59]. There is also a second aspect in which the reevaluation of the prismane protein is relevant to an analysis of the active site in hydrogenase. A mutant in which the Cys ligand to Fe5 has been replaced by Ser produces stable prismane protein, which, however, lacks the hybrid cluster. This has allowed for an unambiguous analysis of the quite unusual EPR spectrum of the [4Fe-4S] cluster [60]. The reduced cubane is $S = 3/2$; however, its EPR effective g values grossly deviate from theoretical values predicted by the standard $S = 3/2$ rhombogram (a graph relating effective g values to rhombicity). This implies that the system is “spin-admixed” i.e., subject to strong spin-orbit coupling through a low-lying excited state [64,65]. In practice, this means that one relatively pronounced absorption-shaped feature with a high g value (in this case $g \sim 4.5$) is readily observed, whereas the other two g values are difficult to detect. A similar spectrum has been observed for the active site of Fe-hydrogenase, suggesting that a regular cubane could be a part of its catalytic center.

III.C. Fe Hydrogenase: Structure

Fe hydrogenases are approximately 60 kDa enzymes that contain from 11 to 22 Fe and a similar amount of S^{2-} ; no other metals are present ([66] and refs. therein). The enzymes from *Desulfovibrio vulgaris*, strain Hildenborough, and from *Clostridium pasteurianum* have been characterized in detail spectroscopically (reviewed, e.g., in [59]). They have also been crystallized, but thus far no crystals suitable for x-ray analysis have been produced. The *D. vulgaris* enzyme contains two regular [4Fe-4S] cubanes for electron transfer (named F clusters) and an active site of undetermined structure (named an H cluster). The *C. pasteurianum* enzyme contains the same clusters and in addition a [4Fe-4S] and a [2Fe-2S] cluster (the F' clusters). Comparison of structural-gene sequences available from these and four other Fe hydrogenases indicates variability in the N-terminal electron transfer part, but a conserved C-terminal binding domain for the H cluster. This latter domain contains five Cys and one His [67–71].

Since the first purifications of Fe hydrogenases in the early 1970s a range of different models for the H-cluster active site have been proposed including mononuclear iron and clusters of 2, 3, 4, and 6Fe [56,57,72–77]. At least in part this changing stoichiometry reflects improvements in purification, elemental analysis, and spectroscopy. The more recent models propose the H cluster to contain approximately 6 Fe on the basis of elemental analysis [56,57] and a putative 3.3 Å Fe–Fe distance indicated by EXAFS spectroscopy [58]. The data are indicative at best, because counting Fe in proteins has an uncertainty typically of the order of 10% (i.e., 1–2Fe), and because no EXAFS on 6Fe models has been published.

D. vulgaris Fe hydrogenase is purified aerobically in an inactive form. Dur-



ing reductive activation it exhibits an EPR signal ($g = 2.07, 1.96, 1.89$ [66]) indicative of $[4\text{Fe-4S}]^{1+}$. Upon completion of activation this signal never reappears but another signal, consisting of a single sharp absorption-shaped peak at $g = 5.0$, appears [78,79]. Although it was originally assigned to an $S = 2$ system [78], subsequent parallel-mode EPR measurements indicate that the spin is half-integer. With reference to the unusual EPR of the regular cubane in the prismane protein we can now conclude that the $g = 5.0$ line is probably from a "spin-admixed" $S = 3/2$ system, and $[4\text{Fe-4S}]^{1+}$ is a likely candidate. A similar $g = 5$ line has recently been reported for a model containing the $[4\text{Fe-4S}]^{1+}$ core [80]. $S = 3/2$ -like resonances have also been observed in the EPR of *C. pasteurianum* Fe hydrogenase [81]. Apparently a $[4\text{Fe-4S}]$ cubane is part of the H-cluster structure.

The active site in NiFe hydrogenases is a dinuclear NiFe cluster in which the metals are bridged by two Cys sulfurs and a third, unidentified ligand (presumably oxygen) [82]; additionally, the Fe is coordinated by three nonprotein ligands, two CN and one CO [83] (cf. Chapter 9). The $-\text{Fe}(\text{CN})_2\text{CO}$ moiety (Fe_c for short) gives a unique fingerprint of sharp lines in Fourier transform infrared (FTIR) spectroscopy [83]. A comparative FTIR study on Fe/S and/or Ni proteins (including all entries in Table 3) revealed that no other protein exhibits this fingerprint *except* the Fe hydrogenases [84]. This strongly implies that the active center of Fe hydrogenases also contains a moiety equal/similar to $-\text{Fe}(\text{CN})_2\text{CO}$.

Oxidized, active Fe hydrogenase exhibits an EPR spectrum ($g = 2.10, 2.05, 2.01$ [74]) that could come from low-spin $\text{Fe}_c(\text{III})$. MCD spectroscopy excludes the possibility of a so-called HiPIP, $[4\text{Fe-4S}]^{3+}$ cluster [85,86]. It thus appears that the active H cluster contains *both* an $-\text{Fe}(\text{CN})_2\text{CO}$ -like moiety *and* a regular $[\text{Fe-4S}]$ cubane, i.e., a total of 5Fe.

III.D. Fe Hydrogenase: Reaction Mechanism

The overall reaction catalyzed is $\text{H}_2 \rightleftharpoons 2\text{H}^+ + 2\text{e}^-$. The cleavage of H_2 is heterolytic, i.e. a hydron and a hydride are produced [87]. Stabilizing H^- can conveniently be envisioned to occur at an Fe site, but to stabilize H^+ requires a base. This base could be part of the cluster (e.g., a sulfur) or be provided by the protein. Recent 2D-ESEEM studies [88] have provided evidence that the single conserved His residue (His371 in the *D. vulgaris* enzyme) is close to the species that gives rise to the $g = 2.10$ spectrum, i.e., presumably the $-\text{Fe}(\text{CN})_2\text{CO}$ -like moiety. Thus, His371 is a good candidate for the H^+ stabilizing base. This proposal, together with the structural information given earlier, is the basis for the hypothetical picture of the catalytic center in Fe hydrogenase in Figure 6. The picture integrates the following ingredients: a regular $[4\text{Fe-4S}]$ cubane, a separate $-\text{Fe}(\text{CN})_2\text{CO}$ -like moiety, five conserved Cys in the H-cluster-binding region,



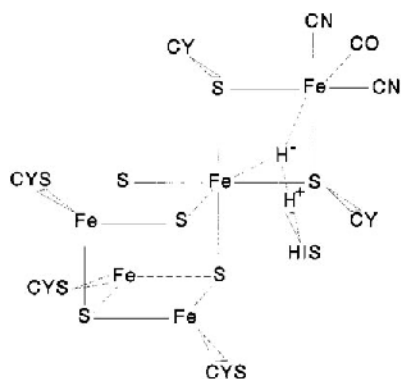


Figure 6 Hypothetical, speculative structure of the H cluster, the catalytic center in Fe hydrogenase that splits molecular hydrogen heterolytically. The structure has been conceived by integration of the following building blocks: a cubane, an FeC_3 fragment, five conserved Cys, one conserved His base.

one conserved His close to the Fe, and an H_2 molecule ready to be heterolytically cleaved.

As ascribed, the EPR spectrum with $g = 2.10$ can be low-spin $\text{Fe}_c(\text{III})$. When the isolated enzyme is reductively titrated this signal disappears at a potential $E_{m,7} \approx -0.3 \text{ V}$ [65]. This would seem to indicate that the putative $\text{Fe}_c(\text{III})$ form is not relevant, at least not to hydrogen-production activity. The cubane is a one-electron acceptor as it can shuttle between the $2+$ and $1+$ oxidation states. Therefore, if the active center were to take up a total of two electrons, then the oxidation state of the Fe_c would, as least formally, shuttle between II and I. Recently, a redox transition in Fe hydrogenase with an E_m below the H_2/H^+ potential has been observed in direct electrochemistry [89]. This “superreduced” state has not been studied by spectroscopy. It might well correspond to the formal $\text{Fe}_c(\text{I})$ state. For NiFe hydrogenases $\text{Fe}_c(\text{I})$ has recently been proposed as a key intermediate in the catalytic cycle [90] (cf. Chapter 9).

With reference to the hypothetical structure in Figure 6 we can now begin to formulate a reaction mechanism for Fe hydrogenases. One may take the hydrogen-oxidation route: (1) the H cluster is oxidized by the F clusters to the two-electron oxidized state. We assume that the cubane is $[\text{4Fe-4S}]^{2+}$ and Fe_c is (II). (2) The H cluster binds molecular hydrogen, inducing a charge asymmetry in the manner depicted in Figure 6. (3) A hydron is abstracted by the base His371, and the charge of the remaining hydride is distributed over the cubane and Fe_c . (4) The hydron on His371 is released in solution. (5) The second hydron is abstracted possibly by the same His371; this leaves the H cluster in the reduced state, i.e.,



$[4\text{Fe-4S}]^{1+}\text{-Fe}_c(\text{I})$. (6) The F clusters are reoxidized by the enzyme's natural redox partner (e.g., cytochrome c_3 [91]), and the cycle can start again.

It should be clear that this reaction mechanism and the structure in Figure 6 are both conjectural to a considerable extent. A crystal structure is the major piece of information lacking. The model is based on presently available spectroscopic, redox-chemical, and protein-sequence information. It predicts that His371 is essential for activity, but not for structural integrity, of the H cluster. It also predicts that in fully reduced Fe hydrogenase the H cluster contains two (possibly coupled) $S = 1/2$ systems.

IV. CONCLUDING REMARKS

Iron-sulfur-containing proteins are ubiquitously present in nature, where they exert a broad range of functions (cf. Table 1). Some of these functions can be defined as catalytic, and these have been addressed in this chapter.

In particular, the dehydration/hydration catalysis in intermediary metabolism, by proteins that contain an asymmetric cubane, is well established. The present picture is predominantly based on the extensive studies with aconitase for which a detailed reaction mechanism has been proposed. The dehydration and hydration steps in this mechanism are well understood; however, the details of the "flip" of the intermediate are still to be determined.

Redox catalysis by iron-sulfur clusters is not well understood. In fact, it is not at all certain that genuine iron-sulfur clusters with this activity do exist. Several hybrid systems are being studied with a view to possible direct involvement of their Fe/S part in catalysis. There is still a distinct lack of structural information in this area.

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9

Catalysis by Nickel in Biological Systems

Richard Cammack

King's College London, London, England

Pieter van Vliet

Leiden University, Leiden, The Netherlands

I. INTRODUCTION

The last few years have seen an increased recognition of the importance of nickel in the catalysis by enzymes of plants and microorganisms [1–6]. This review of a rapidly developing area concentrates on the catalytic functions of nickel, in those enzymes that have been demonstrated to contain it naturally. Other enzymes, which are activated by different ions, including nickel, are not covered here, but are listed in [7]. Since the previous edition [8], the status of the enzymes has changed considerably. Carbon monoxide dehydrogenase (CODH) is now recognized as being homologous to part of the acetyl coenzyme A synthase (ACS), but since it has a different function, it is classed as a separate enzyme. A new nickel enzyme, superoxide dismutase, has been added. This brings the list of distinct types of nickel-containing enzymes to six (Table 1). Advances have been made recently, notably in the areas of molecular biology, spectroscopy, and inorganic modeling. Most important, crystallographic studies have uncovered the structures of three types of enzymes.

Nickel enzymes are particularly prominent in the metabolism of anaerobic bacteria. For example, the methanogenic bacteria, which are classified as Archaea, an ancient division of living organisms, can grow on a mixture of H_2 and CO_2 to produce methane [9–11]. The metabolism of methanogens involves three



Table 1 Nickel Enzymes

Enzyme	Ni center	Reaction	Distribution
Urease EC 3.5.1.5	High-spin dinuclear Ni(II)	Urea $\rightarrow$ NH ₃ + CO ₂	Bacteria, plants
Hydrogenase EC 1.12.1.2; 1.2.2.1; 1.18.99.1	Dinuclear Ni(III)/(II)/(I?) + Fe(II)	2H ⁺ + 2e ⁻ $\leftrightarrow$ H ₂	Bacteria
Methyl CoM reductase EC 1.8.-.-	Ni tetrapyrrole (hydrocorphin)	CH ₃ -CoM $\rightarrow$ CH ₄	Methanogenic bacteria
CO dehydrogenase EC 1.2.99.2	Ni-Fe ₄ S ₄ cluster	CO + [acceptor] $\rightarrow$ CO ₂	Photosynthetic bacteria
Acetyl CoA synthase EC 1.2.99.2	Ni-Fe ₄ S ₄ cluster	CO ₂ + [CH ₃]-corrin + CoA + 2e ⁻ $\rightarrow$ CH ₃ COCOA	Methanogenic and acetogenic bacteria
Superoxide dismutase EC 1.15.1.1	Ni center	2 O ₂ ^{-•} + 2H ⁺ $\rightarrow$ O ₂ + H ₂ O ₂	<i>Streptomyces</i> sp.



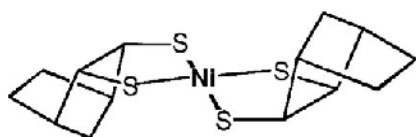
types of nickel enzymes. Methyl CoM reductase (MCR) is involved in the energy-yielding pathway for methane production; acetyl CoA synthase (ACS) is central to the pathway of CO₂ fixation; and hydrogenases are used to supply reducing equivalents for the reactions. The acetogenic bacteria, such as *Clostridium thermoaceticum*, which are not Archaea, are also able to use H₂ and CO₂, and they use ACS to produce acetate [12,13].

It is noteworthy that the substrates or products are dissolved gases: hydrogen, oxygen, carbon monoxide, carbon dioxide, methane, ammonia. However, the enzymes show no common pattern, either in the chemical state of nickel or in the type of reaction catalyzed. Their nickel-containing sites are remarkably diverse (Table 1), and in four enzymes the active center comprises groups in addition to the nickel ion.

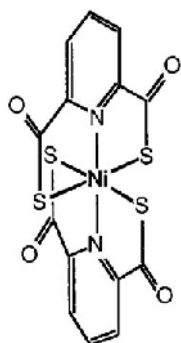
The genes for a considerable number of nickel enzymes have now been cloned and sequenced. Genetics has revealed the existence of ancillary proteins in the cell for nickel ion uptake, storage, and insertion into the relevant proteins. For example, the assembly of the NiFe hydrogenases requires about 20 ancillary genes [14–17]. The genes, which are organized differently in different organisms, include high-affinity nickel transporters that pump nickel ions into the cell with the consumption of adenosine triphosphate (ATP). The nickel-uptake system in *Escherichia coli* consists of five proteins, *NikABCDE*. There are also nickel permeases that specifically allow nickel through the cell membrane, such as that encoded by *hoxN* in *Alcaligenes eutrophus* [18]. The ancillary proteins are of interest for studies of catalysis because manipulation of them permits the functional investigation of different parts of the enzyme.

The relevant chemistry of nickel complexes has been reviewed [7,19]. Nickel can take up oxidation states from Ni(0) to Ni(IV), with Ni(II) the most stable, for example, in aqueous solutions of salts. Ni(0) is a well-known carbonylation and hydrogenation catalyst. Hydrides and carbonyls of the higher oxidation states are less common. For nickel sites in proteins, the formal oxidation state may also be difficult to determine as a result of charge delocalization. This is particularly the case for mixed-metal cluster complexes, and here model complexes are a valuable indication of their behavior. From inorganic chemistry it is known that the various oxidation states of nickel have preferred geometries; for example, Ni(II) may be octahedral (and high-spin, $S = 1$) or square planar (and low-spin, $S = 0$), although the coordination geometry is often highly distorted by the location of ligands. Model complexes are also useful to establish the effects, for example, of different ligand types. For example, it has been shown that thiolate ligands tend to promote more negative redox potentials in complexes such as those shown in Scheme 1 [20] and Scheme 2 [21].





Scheme 1



Scheme 2

II. UREASE

II.A. Function

Urease (urea amidohydrolase) is an enzyme first identified over a hundred years ago in bacterial extracts [22]. The presence of urease is a virulence factor for some pathogenic bacteria [23,24]. It is now known to occur also in plants, fungi, and invertebrates (see [24,25] for reviews). Urease from jack bean was the first enzyme to be crystallized, in 1926. Almost 50 years later its metal content was reexamined and it was found to contain two atoms of nickel per subunit [26]. Finally in 1995 the crystal structure of the enzyme from the enteric bacterium *Klebsiella aerogenes* was determined [27]. Amino-acid sequence comparisons predict that the structures of the plant and bacterial enzymes are similar, although with different subunit arrangements.

II.B. Composition and Structure

The composition, visible spectroscopy, and catalytic properties of urease have been reviewed by Blakeley and Zerner [25] and by Hausinger [2]. The urease from jack bean is typical of the enzymes from plants. It has a protein of relative



molecular mass 545,000, consisting of a hexamer of identical 97,791 Da subunits, organized in a trigonal bipyramidal arrangement [28]. The urease from *K. pneumoniae* consists of three types of subunit, α (60.3 kDa), β (11.7 kDa), and γ (11 kDa), in a stoichiometry $(\alpha\beta\gamma)_3$. Its structure is thus a trimer of trimers, with one dinuclear nickel center in each subunit.

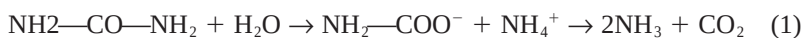
The apourease, lacking nickel, may be obtained from cells of plants or bacteria grown in nickel-free media [29,30]. Reconstitution of the enzyme with nickel was difficult until it was recognized that hydrogencarbonate was necessary [31]. Incubation of apourease with high concentrations of nickel and hydrogen carbonate led to incorporation of 2Ni per subunit, but restored only 15% of the activity. Mutation studies have implicated the products of four genes, *ureD*, *ureE*, *ureF*, and *ureG*, in the energy-dependent assembly of the nickel center in its active form. These are located in the same operon as the genes *ureA*, *ureB*, and *ureC* encoding the α , β , and γ subunits of the urease protein [32]. It is interesting that the sequence of *ureE* contains a histidine-rich region that might bind nickel. The sequence of *ureG* contains a potential nucleotide-binding motif [33], which suggests it is involved in the energy-dependent insertion of nickel.

Optical spectroscopy [25] and EXAFS spectroscopy [34] indicated that the ligands to nickel were nitrogens and/or oxygens. Data from magnetic circular dichroism (MCD) [35] and magnetic susceptibility were consistent with high-spin ($S = 1$) Ni(II) ions, although there was disagreement as to whether a coupling between the spins on the nickel ions could be detected [36,37]. Reactivity of the apoenzyme and holoenzyme with diethyl pyrocarbonate and site-directed mutagenesis studies [38] indicated that some of the ligands to nickel are histidines.

A clearer picture emerged when the crystal structure of *K. pneumoniae* urease was determined [27]. The nickel atoms in the center, Ni-1 and Ni-2, are 3.5 Å apart. They are bridged by a carbamyl group, formed from CO₂ and a lysine residue, explaining the requirement for hydrogen carbonate in reconstitution. The other ligands are two histidines for Ni-1 and an aspartate and two histidines for Ni-2.

II.C. Catalytic Mechanism

Urease catalyzes the hydrolysis of urea to carbamate, which then spontaneously decomposes to ammonia and hydrogen carbonate:



This reaction differs from the spontaneous hydrolysis of urea to ammonium and cyanate, which is 10²⁴ times slower. Urease is a highly specific and efficient enzyme. Its other substrates, of which the best are semicarbazide, formamide, acetamide, and *N*-methylurea, are hydrolyzed at very low rates [39].



From a study of the enzyme kinetics with a range of substrates and inhibitors, and the chemistry of related metal-ion complexes, Dixon et al. [39] proposed a model of the environment of the active site and reaction cycle. Although differing in details from the structure as now determined, this mechanism provided a basis for understanding the functions of the two metal ions. One nickel ion (Ni-1) binds the urea, and the other nickel ion (Ni-2) binds a hydroxide ion that makes a nucleophilic attack on the urea, leading to the formation of a tetrahedral intermediate.

From the crystal structure of urease, Jabri et al. [27] proposed that urea binds through its carbonyl oxygen, whereas the $-NH_2$ hydrogens are hydrogen-bonded to residues in the protein (Figure 1). The structure of the site is such that water molecules in the active site do not coordinate optimally to the nickel ions in the substrate-free form. As a result, the binding of urea is favored [40]. A loop of polypeptide forms a flap that covers the active site once urea is bound. This flap includes cysteine 319, which had been believed to be catalytically important [41] and is one of the residues proposed to hydrogen-bond to the urea nitrogens. Mutation of this cysteine to alanine leads to decrease, but not necessarily loss, of activity.

The structure of the complex of urease with urea in the active site is unknown, because the enzyme-substrate intermediate is very short-lived and has not been trapped. Nevertheless, a number of inhibitors of urease that bridge between the nickel atoms are known. Acetohydroxamate is the most studied and binds slowly but with high affinity ($K_i = 4$ mM [25]). Phosphoroamide is also a slowly binding inhibitor. 2-Thioethanol causes the appearance of sulfur-to-nickel

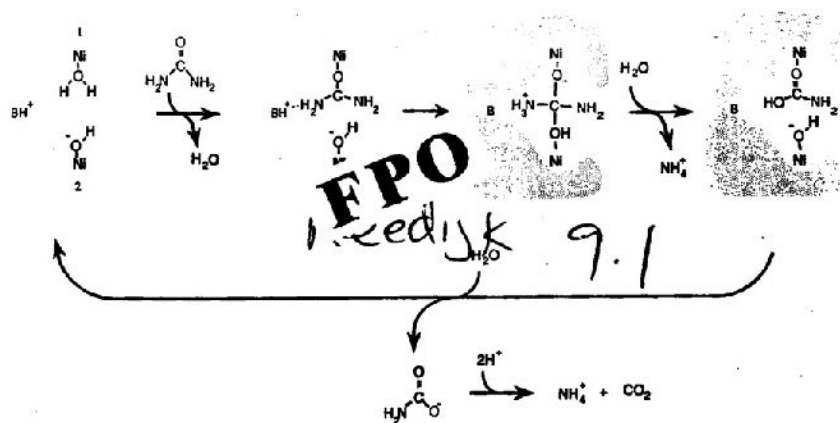
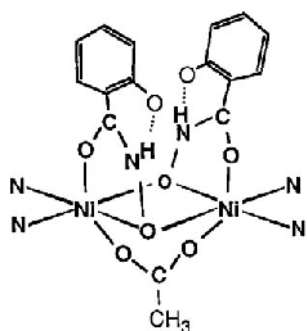


Figure 1 Model of the structure of the active site of urease, after Jabri et al. [27], and reaction cycle. (After Refs. 21,161.)





Scheme 3

charge-transfer bands, and a marked decrease in paramagnetism as observed by MCD and magnetic susceptibility [35,36]. Deviations from Curie law behavior were interpreted in terms of a strong antiferromagnetic coupling ($J = -40 \text{ cm}^{-1}$) between the nickel ions, consistent with the formation of a sulfur bridge between the nickel atoms.

The structure of the complex of acetohydroxamate with a Cys319-Ala mutant of *K. aerogenes* urease has been determined [42]. The hydroxyl group of acetohydroxamate bridges the nickel ions. A similar bridging arrangement is seen in a model for the active site by Stemmler et al. (Scheme 3) [43]. Karplus et al. [40] have suggested that histidine 320 could act as a catalytic acid to hydronate a urea nitrogen. This explains the inhibitory effects of bulky modifications of the Cys319, which could prevent the approach of the histidine to the substrate.

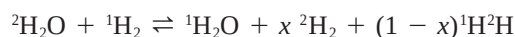
III. HYDROGENASE

III.A. Function

Hydrogenases catalyze the production or consumption of hydrogen gas, with suitable electron donors or acceptors:



The metal-containing hydrogenases also catalyze hydrogen isotope exchange [44], for example, between $^1\text{H}_2$ gas and deuterium oxide, yielding varying proportions of isotopically labeled hydrogen gas:



and interconversion of ortho- and parahydrogen (which differ in the relative orientations of the proton spins) [44]. These reactions indicate that the enzyme catalyzes heterolytic cleavage of H_2 . Thus, even before the type of hydrogen-activating site was known, mechanisms that involved the formation of hydride species (reviewed in [45]) were proposed.

Three distinct classes of hydrogenases have been established. The [Fe] hydrogenases contain only iron–sulfur clusters, and the active site for reaction with H_2 is a special iron–sulfur cluster known as the H cluster [46] (Chapter 8). The [NiFe] hydrogenases contain nickel in the catalytic site, of which a subclass, the [NiFeSe] hydrogenases, also contain selenium, which, in the form of selenocysteine, is a ligand to nickel. Nickel is believed to participate in the hydrogen activation. A third enzyme, N5,N10-methylenetetrahydromethanopterin dehydrogenase, isolated from methanogenic bacteria, is capable of producing and consuming hydrogen gas with coenzyme F-420 (deazaflavin) as acceptor, but no metal centers have been detected in it [47,48] and it does not catalyze the reduction of compounds such as methylviologen.

The physiological roles within each class of hydrogenases are diverse. Nevertheless, [Fe] hydrogenases are distinct from [NiFe] hydrogenases in that [Fe] hydrogenases show 10–100 times higher hydrogenase activity than the nickel hydrogenases, whereas the affinity for molecular hydrogen of [Fe] hydrogenases is about 100 times lower. In most cases, metabolic hydrogen production involves [Fe] hydrogenases, for example, in the fermentation of sugars by *Clostridium* sp., whereas [NiFe] hydrogenases are more often hydrogen-uptake enzymes. An exception is the [NiFe] hydrogenase of the extremely thermophilic archaeobacterium *Pyrococcus furiosus* (optimal temperature $>96^\circ\text{C}$ [49]), which catalyzes hydrogen production at a 4–12-fold higher rate than uptake [50]. It should be noted, however, that the relative rates of hydrogen uptake and production measured in vitro depend on the electron acceptors and donors employed.

Many nickel hydrogenases are membrane-bound and involved in respiratory chains. Such hydrogenases are found in bacteria that can grow by the oxidation of hydrogen, such as *E. coli* [51], the nitrogen fixer *Azotobacter* [52], the hydrogen-oxidizing bacterium *Alcaligenes eutrophus*, and the photosynthetic bacteria *Rhodobacter capsulatus* [53], *Chromatium vinosum* [54], and *Thiocapsa roseopersicina* [55]. Dihydrogen oxidation on the outer surface of the bacterial membrane leads in a straightforward way to the generation of a transmembrane H^+ potential (e.g., [55]). Further hydrons may be pumped across the membrane by an energy-yielding respiratory chain. The proton motive force is used by membrane-bound ATPase to form ATP. Periplasmic hydrogenases are anchored to the membrane by a hydrophobic peptide and feed electrons into the respiratory chain at the level of quinone. Other membrane-bound electron carriers may also be involved. For the hydrogenase of *Wolinella succinogenes*, a third protein sub-



unit coded by the *hydC* gene has been shown to be a membrane-bound cytochrome *b* [56], which presumably transfers electrons to menaquinone.

Other types of energy metabolism that involve uptake hydrogenases include sulfate respiration and methanogenesis. In sulfate-reducing bacteria the hydrogenases are involved in dihydrogen production and consumption [57]. In methanogenic bacteria, some of the hydrogenases use as an electron acceptor coenzyme F-420 (deazaflavin), which serves as a reductant for various steps in the reduction of one-carbon intermediates. Of others the initial acceptor is unknown, and these are referred to as methylviologen-reducing hydrogenases, since, like most hydrogenases, they can reduce this artificial acceptor. In fact, some methanogens contain both [NiFe] and [NiFeSe] hydrogenases, as well as the nonmetal hydrogenase methylenetetrahydromethanopterin dehydrogenase. So they can express five different types.

III.B. Structure and Composition

The protein compositions of a variety of [NiFe] hydrogenases are summarized in Table 2. All [NiFe] hydrogenases comprise at least two subunits (large and small) of approximately 60 and 30 kDa, containing a nickel center and at least two [4Fe-4S] clusters. The NAD-reducing hydrogenases of hydrogen bacteria such as *A. eutrophus* contain additional subunits with a [2Fe-2S] cluster and flavin mononucleotide (FMN) [58]. These additional subunits show homology with the flavoprotein subunits of complex I (reduced NAD- [NADH]-ubiquinone reductase) of the respiratory chain [59,60]. The methanogenic-F-420-reducing hydrogenases contain a third subunit, which in *Methanobacterium thermoautotrophicum* was shown to be homologous with the second subunit of the F420-reducing formate dehydrogenase and which is proposed to contain FAD.

The crystal structure of the oxidized [NiFe] hydrogenase of *Desulfovibrio gigas* has been resolved [61,62]. A schematic view of this enzyme is shown in Figure 2. The large subunit contains the catalytic site and is buried into the protein. The small subunit contains the iron-sulfur clusters, which together with the nickel show an approximately linear arrangement. This arrangement of redox centers in the enzyme supports the generally accepted view that the iron-sulfur clusters in [NiFe] hydrogenases function as electron transfer components between the catalytic site and secondary electron carrier, which in the case of the *D. gigas* hydrogenase is a multiheme cytochrome such as cytochrome *c*₃. Studies by spectrophotometry and cyclic voltametry demonstrated electron transfer between the soluble hydrogenases from sulfate-reducing bacteria, which was rapid ($k = 6.5, 108 \text{ M}^{-1} \text{ sec}^{-1}$) [63].

A surprising observation is that the [3Fe-4S] cluster is placed between the two [4Fe-4S] clusters. The [3Fe-4S] cluster has a midpoint potential of -70 mV



Table 2 Properties of Hydrogenases

Type	Source	Location	Metabolic function	Subunit M_r in kDa	Composition	Acceptor/donor
Fe	<i>Clostridium pasteurianum</i> Hydrogenase I	Cytoplasm, soluble	H ₂ production	64	[H] + 4[4Fe-4S]	Ferredoxin
Fe	<i>Clostridium pasteurianum</i> Hydrogenase II	Cytoplasm, soluble	H ₂ consumption		[H] + 2[4Fe-4S]	Ferredoxin
Fe	<i>Desulfovibrio vulgaris</i> , Hildenborough strain	Periplasm, soluble	H ₂ consumption/production	46 + 10	[H] + 2[4Fe-4S]	Multiheme cytochrome <i>c</i>
NiFe	<i>Desulfovibrio gigas</i>	Periplasm, soluble	H ₂ consumption/production	61 + 28	Ni/Fe + [3Fe-4S] + 2[4Fe-4S]	Cytochrome <i>c</i> ₃
NiFe	<i>Alcaligenes eutrophus</i>	Cytoplasm, soluble	H ₂ consumption	55 + 23 + 67 + 26	Ni/Fe + [3Fe-4S] + n [4Fe-4S] + [2Fe-2S] + FMN	NAD ⁺
NiFe	<i>Wolinella succinogenes</i>	Membrane-bound	H ₂ respiration	64 + 32 + 19	Ni/Fe + Fe-S + cyt <i>b</i>	Menaquinone
NiFe	<i>Pyrococcus furiosus</i>	Soluble	H ₂ production	2(46 + 27 + 24)	Ni/Fe + [2Fe-2S] + n [4Fe-4S]	Ferredoxin
NiFeSe	<i>Desulfomicrobium baculatum</i>	Soluble	H ₂ consumption	57 + 31	Ni/Fe + 2[4Fe-4S]	Cytochrome <i>c</i> ₃
NiFeSe	<i>Methanococcus voltae</i> , deazaflavin-reducing	Soluble	H ₂ consumption	48 + 33 + 80	Ni/Fe + n [4Fe-4S]	F-420



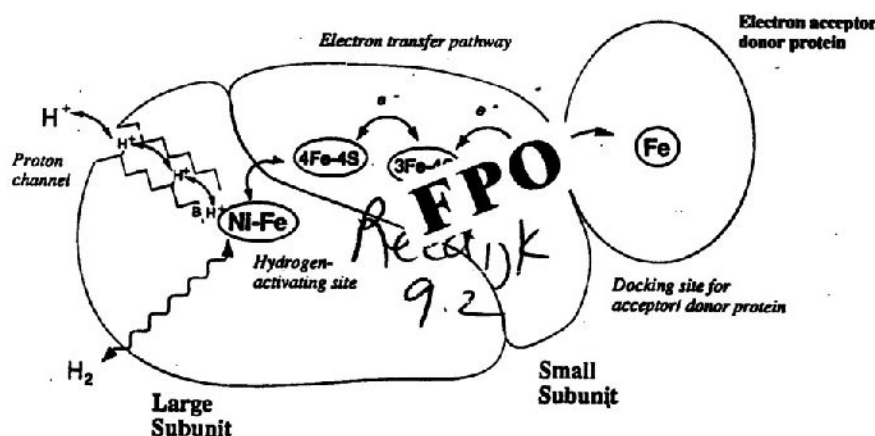


Figure 2 Schematic view of the structure of [NiFe] hydrogenase based on the crystal structure [61,62].

(see [64] and references therein), which is significantly higher than those (-291 mV and -340 mV) of the $[4\text{Fe-4S}]$ clusters [64], which are close to the midpoint potential of H_2 activation (-310 mV) of the enzyme [65]. This raises the question as to whether the $[3\text{Fe-4S}]$ cluster is involved in electron transfer. However, the crystal structure shows that the clusters are relatively close together with an edge-to-edge distance of $10 \text{ \AA}$ or less. At such relatively short distances between redox centers, the high midpoint potential of the $[3\text{Fe-4S}]$ cluster is not the rate-limiting factor of electron transfer between the catalytic site of hydrogenase and cytochrome c_3 [66].

For a range of [NiFe] hydrogenases it has been shown that hydrogenase maturation involves the removal of the C-terminal end of the large subunit (reviewed in Refs. 67,68). The size of the cleaved C-terminal peptide is variable, depending on the organism. It has been shown for hydrogenase 3 of *E. coli* that the C-terminal extension of the precursor form is removed by a specific protease [69]. During processing, nickel is incorporated into the subunit prior to C-terminal cleavage [70,71]. The three-dimensional structure of the *D. gigas* hydrogenase revealed that the C terminus that provides ligands to the nickel is deeply buried in the subunit. This finding supported the proposal by Rossmann et al. [71] that the C-terminal extension keeps the hydrogenase precursor in a specific conformation to allow functional incorporation of nickel. That this is indeed the case is indicated by recent observations that a mutant of hydrogenase 3 of *E. coli* lacking the C-terminal extension of the large subunit is unable to bind nickel [72].



The *HypX* gene, which has sequences analogous to those of folate enzymes and dehydratases, may be involved in formation of diatomic ligands to the NiFe center [16]. Other genes appear to have a regulatory function, such as the genes, called *HupUV* or *HoxBC* in different species, that resemble the large and small subunits of the NiFe hydrogenase itself and have been implicated in sensing either nickel or hydrogen [17,18].

Since the discovery of nickel in hydrogenases it was assumed that that was the sole metal center involved in activation of hydrogen. However, the crystal structure shows that the catalytic site is dimetallic and that adjacent to the nickel an iron atom is present [61,62]. The catalytic site exhibits an unusual ligand composition and coordination geometry. The nickel is pentacoordinate with a distorted square pyramidal geometry. The iron is hexacoordinate with a distorted octahedral geometry [61,62]. The nickel and iron are bridged by two cysteine sulfurs [61] and also by an unidentified ligand. In view of the enzymological properties of the enzyme this third bridging ligand is proposed to be an oxygen species [62].

Three of the ligands to iron appear to be diatomic nonprotein molecules [61,62]. The identity of these ligands is not clear, but recent Fourier transform infrared (FTIR) studies of [NiFe] hydrogenases revealed the presence of three unique bands in the 1910–2101 cm^{-1} frequency region of the FTIR spectrum [62,73]. These FTIR bands were assigned to the nonprotein diatomic ligands, and their frequencies point to the presence of CN, CO, or NO, although NO is less favored [62]. Further investigations after ^{15}N and ^{13}C labeling resulted in shifts of the three FTIR bands with a pattern that indicates the presence of one CO and two CN molecules bound to the iron [74].

III.C. States of the Catalytic Site of the Enzyme

Enzymological and redox potentiometric studies by EPR have indicated that the catalytic sites of the oxygen-stable [Ni-Fe] hydrogenases exhibit at least six enzymologically distinct states [75–78]. These are schematically represented in Figure 3, which depicts a speculative model for the hydrogenase redox cycle, as discussed later.

Most [NiFe] enzymes are reversibly inactivated by molecular oxygen. After isolation under air the enzyme exhibits two rhombic EPR signals originating from the nickel [79,80]. These are designated Ni-A and Ni-B (see, e.g., Ref. [75]). In *D. gigas* these EPR signals have g values $g_{x,y,z} = 2.32, 2.23, 2.01$ and $g_{x,y,z} = 2.34, 2.16, 2.01$, respectively. The Ni-A EPR signal is dominant under those conditions. Rapid reduction under anaerobic conditions, by e.g., dithionite results in an EPR silent state (Ni-SU) while the enzyme remains inactive. The enzyme is activated upon subsequent incubation in the reduced state for several hours. Therefore, the Ni-A and the Ni-SU states have been termed “unready” [75,81].



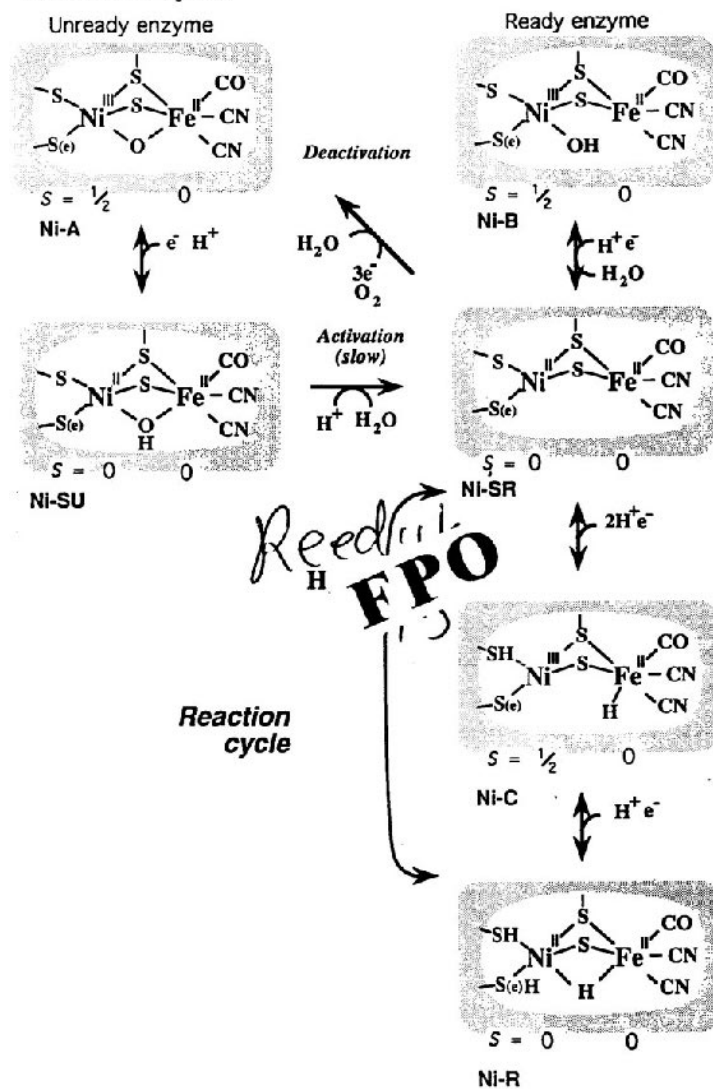
Activation cycle

Figure 3 Speculative model for the hydrogenase enzyme cycle such as that from *D. gigas*. The highest oxidation states of the enzyme are at the top, and each step down corresponds to a one-electron reduction. Some hydrons that are transferred to sites in the protein are not shown. Redox states of the iron-sulfur clusters are omitted.



The mechanism responsible for the slow activation is not understood, but it has been suggested that some structural changes or dissociation of an oxygen species from the catalytic site are involved [75,81].

After activation, at least three states can be distinguished by EPR: (1) an EPR-silent state (Ni-SR); (2) an EPR-detectable state (Ni-C), which is formed upon reduction of Ni-SR; and (3) another EPR-silent state (Ni-R), which is formed upon further reduction of the Ni-C state. The Ni-C EPR signal in *D. gigas* hydrogenase exhibits g values $g_{x,y,z} = 2.19, 2.15, 2.01$. Anaerobic oxidation of activated hydrogenase results in the formation of a large amount of the Ni-B EPR signal, whereas oxidation by air exposure results in significantly smaller amounts of Ni-B and Ni-A is also formed. Like the Ni-A state, the Ni-B state does not activate hydrogen [75,81]. However, unlike in the Ni-A state, reduction of the Ni-B state is accompanied by rapid activation of the enzyme. Therefore, the Ni-B state is also referred to as the "ready" state [75,81]. The Ni-SU and the Ni-SR states are probably electrochemically equivalent [81]. Nevertheless, Fernandez et al. [75,81] observed a relatively slow conversion of the Ni-SU to the Ni-SR state. They concluded that this transition was the rate-limiting step in activation and that it involved a slow intramolecular entropically determined process. The EPR signals described earlier have been observed in a number of different hydrogenases (see, e.g., Ref. [8]). The interconversions between the other states are thought to correspond to one-electron redox transitions [75–78]. However, under conditions where molecular hydrogen is used as a reductant in the absence of mediators, the Ni-C to Ni-R conversion was observed to involve an apparent two-electron redox process [82].

To investigate the mechanism underlying the effect of molecular oxygen in inactivating [NiFe] enzymes, van der Zwaan et al. [83] used labeled molecular oxygen ($^{17}\text{O}_2$) to oxidize the reduced, active hydrogenase of *C. vinosum*. After oxidation, a mixture of the Ni-A and Ni-B EPR signals was observed. Both signals were broadened in comparison to those observed after oxidation by $^{16}\text{O}_2$. Similar effects of $^{17}\text{O}_2$ have been observed in *D. gigas* hydrogenase (R. Cammack, M. J. Payne, E. C. Hatchikian, unpublished observations, 1988). Thus it seems that the formation of Ni-A and Ni-B is accompanied by binding of an oxygen species in the vicinity of the nickel [83]. This oxygen species may correspond to the putative ligand observed in the crystal structure that forms a bridge between the two metal centers of the catalytic site [62]. This is supported by observations indicating that the bridging ligand is absent in crystals of hydrogenase from *D. baculatum* that were reduced by hydrogen gas (J. C. Fontecilla-Camps, personal communication, Albertville, France, 1997).

Nevertheless, the Ni-B EPR signal also appears after oxidation of the active enzyme under anaerobic conditions. Assuming that the Ni-B formation is accompanied by binding of an oxygen species to the catalytic site, this suggests that besides molecular oxygen, water can also provide the putative bound oxygen



species. However, ^{17}O -labeled water had no effect on the Ni-A and Ni-B EPR signals [78]. This apparent discrepancy may be explained by the presence of molecular oxygen during those experiments as indicated by the appearance of the Ni-A EPR signal. We observed that under strict anaerobic conditions Ni-B exclusively is formed upon oxidation of the active enzyme (in the presence of mediators), but that the formation of Ni-B occurs on a much slower time scale (minutes) than that of Ni-A under air (seconds) (V. Fernandez and R. Cammack, unpublished observations, 1986). Thus it is conceivable that the accessibility and/or binding affinity of molecular oxygen to the catalytic site is significantly higher than those of water. It can thus be speculated that the enzymological differences between the Ni-B and Ni-A states are due to differences in the nature and/or binding to the catalytic site of the putative oxygen species (Figure 3).

The state giving rise to the Ni-C EPR signal has until now been the only state of the active enzyme that is paramagnetic. Therefore many magnetic resonance spectroscopic investigations have focused on the nature of this state. Replacement of $^1\text{H}_2\text{O}$ by $^2\text{H}_2\text{O}$ in samples of *C. vinosum* hydrogenase was shown to result in a narrowing of the Ni-C EPR signal of about 0.5 mT but not of the Ni-A or Ni-B signal [84]. This indicated the presence of exchangeable hydrons in the vicinity of the nickel center. Furthermore, after illumination at liquid nitrogen temperatures the Ni-C EPR signal was converted into another EPR signal [84], which in this review is designated Ni-L. The effect of light was reversed by annealing of the sample to 200 K [84]. The rate of photoconversion of Ni-C to Ni-L was significantly slowed (a factor of 6) after $^1\text{H}_2\text{O}$ to $^2\text{H}_2\text{O}$ exchange, but the Ni-L EPR signal was unmodified [84]. These results led to proposals that the Ni-C state could be an intermediate in the mechanism of heterolytic cleavage and that molecular hydrogen or hydride species could be bound to the nickel center [84]. The effects of illumination and $^2\text{H}_2\text{O}$ described have been observed in various hydrogenases, including that of *D. gigas*, but the extent to which they occur is variable, depending on the species [85]. On the basis of EPR studies in the presence of the competitive inhibitor CO, Van der Zwaan et al. [83] favored the possibility that in the Ni-C state molecular hydrogen is associated to the nickel.

Independent studies using different techniques have indicated the presence of exchangeable hydrons near the nickel center. Using electron spin echo envelope modulation (ESEEM) spectroscopy, Chapman et al. [86] detected the presence of hydrons near the nickel in the Ni-C state that were exchangeable in active but not in oxidized, inactive hydrogenase. These hydrons exhibited a relatively weak magnetic interaction with the nickel and were proposed to correspond to hydrons outside the first coordination sphere of nickel [86]. These hydrons also were observed in the Ni-L state [86]. Investigations by Q-band (35 GHz) electron nuclear double resonance (ENDOR) spectroscopy indicated two types of exchangeable hydrons in the Ni-C state exhibiting magnetic interactions with the



nickel of 4.4 and 16.6 MHz [87]. The 16.6 MHz coupling is consistent with a hyperfine interaction giving rise to the 0.5 mT broadening of the Ni-C in $^1\text{H}_2\text{O}$. Since the 16.6 MHz coupled hydrons were not observed after generation of Ni-L by illumination, these hydrons were proposed to be the putative photolabile species associated to nickel [88]. Upon reoxidation the 16.6 MHz coupling disappeared, in contrast to the 4.4 MHz coupling, which also was observed after reoxidation to the Ni-B state [88].

Knowledge of the identity of the observed exchangeable hydrons and of the mechanism responsible for the light-induced conversion of Ni-C to Ni-L may be important for understanding of the enzymological nature of the Ni-C state. That these hydrons originate from the association of a water molecule to the catalytic site is not very likely since exchange with ^{17}O -labeled water had no effect on the Ni-C EPR signal [78]. It has been proposed that in the Ni-C state molecular hydrogen [78,83] or hydride [89] is bound to the nickel center. However, the hyperfine couplings of exchangeable hydrons in the vicinity of the nickel detected so far are significantly weaker than those expected for hydrogen species directly associated to the nickel, which would give rise to couplings of hundreds of megahertz [90]; in the case of *D. baculatum* hydrogenase, the splitting of the EPR spectrum by ^1H is negligible [85]. Association of the more strongly coupled exchangeable hydrogen species outside the first coordination sphere of nickel also was suggested from analysis of the splittings of Ni-L EPR signals due to spin-spin interaction between the nickel and the proximal reduced iron-sulfur cluster [91]. In addition, removal of hydrogen by extensive argon flushing of hydrogen-activated hydrogenase had no effect on the Ni-C EPR signal [82,92], whereas isotope exchange experiments indicated rapid (milliseconds) exchange between unbound substrate (molecular hydrogen) and its cleavage products H^+ and H^- . Thus, it seems unlikely that the Ni-C EPR signal represents a Ni-H or Ni- H_2 species. Hence, it is possible that H_2 reacts with the Ni-SR state to form Ni-R in a two-electron process. The Ni-C may subsequently be generated after one-electron oxidation by the proximal [4Fe-4S] cluster (Figure 3) [92].

III.D. Roles of Iron and Nickel in the Enzyme Cycle

In view of the dimetallic character of the catalytic site, various models can be proposed for the redox behavior of the metals in the enzyme cycle (see, e.g., Ref. 89). Nevertheless, the following EPR spectroscopic data indicate that the catalytic site contains redox-active nickel, whereas iron is diamagnetic low-spin Fe(II) throughout the enzyme cycle: (1) The EPR signals observed in [NiFe] hydrogenases originate from paramagnetic nickel, as evidenced by the strong hyperfine interaction with ^{61}Ni [79,80,93]; (2) the nuclear interaction with ^{57}Fe with paramagnetic nickel has been detected (see, e.g., [64]). The valencies of



nickel in the various states of the enzyme are not established, although it is generally believed that the Ni-A and Ni-B EPR signals observed in the oxidized enzyme originate from Ni(III) (reviewed in [78]). However, EXAFS studies show that the shifts of the x-ray absorption edge (K-edge) upon conversion of Ni-A to Ni-C are significantly smaller than those expected for two-electron reduction of nickel [88,94]. The origin of this discrepancy is unknown, but in the context of the model for the enzyme cycle described (Figure 3), it may be speculated that Ni-C corresponds to Ni(III) and the two-electron reduction results in a hydride that is associated to iron.

Relevant to the possible role of the nickel–iron center in the mechanism of heterolytic cleavage of H_2 is a recent FTIR study by van der Spek et al. [95] of a range of [Fe] and [NiFe] hydrogenases. In this study three unique FTIR bands in the 1910–2100 cm^{-1} frequency region were observed in all [Fe] and [NiFe] hydrogenases investigated, indicating that the presence of these bands in the FTIR spectrum is characteristic of all metal-containing hydrogenases. It was concluded that there could be significant structural and functional similarities between the catalytic sites of [NiFe] and [Fe] hydrogenases [95]. Nevertheless, as already noted, a specific enzymological difference between [Fe] and [NiFe] hydrogenases is that [Fe] hydrogenases exhibit significantly higher hydrogenase activity ($V_v(H_2\text{oxidation}) = 9000\text{--}50,000 \mu\text{mol min}^{-1} \text{mg}^{-1}$) and a significantly lower substrate affinity ($K_m \sim 7 \mu\text{M}$) than [NiFe]-hydrogenases ($V_v \sim 700 \mu\text{mol min}^{-1} \text{mg}^{-1}$, $K_m \sim 0.07 \mu\text{M}$). From this comparison it may be considered that the high substrate affinity in [NiFe] hydrogenases is due to the presence of nickel in the catalytic site and that nickel is essential for substrate binding and activation. Indeed, the structure of the catalytic site allows for initial association of hydrogen to the nickel, which exhibits a vacant binding site in an axial position [61,62]. It is conceivable that nickel initially stabilizes the hydride formed during heterolytic cleavage and that subsequently the hydride is transferred to the iron via a bridging position between the two metal centers [89]. From studies with model compounds it may be proposed that the hydron formed by heterolytic cleavage is stabilized by one of the sulfur ligands in a base-assisted mechanism (reviewed in [89]).

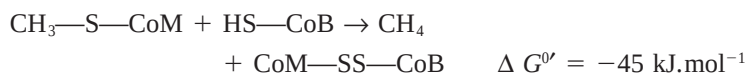
IV. METHYL CoM REDUCTASE

IV.A. Function

Methanogenesis is a highly specialized form of energy metabolism for which the methanogenic bacteria are uniquely adapted. The process of energy capture involves at least two steps in which transmembrane potentials are generated. In



addition, the process requires a number of unusual coenzymes [96] (Figure 4). Methyl CoM reductase (MCR) catalyzes the final step of methane production, the reduction of methyl coenzyme M by coenzyme B:



In the bacterial cells the final stage of energy capture takes place during the rereduction of CoM-SS-CoB by an electron transfer process in the membrane, which involves hydrogenase. Thus methane release and the redox process are separated [9]. This discovery explained the apparently conflicting observations that the energy of methane production was conserved by a chemiosmotic process (i.e., involving a transmembrane hydron gradient), but that methyl coenzyme M reductase in some methanogens was not associated with the membrane [97].

IV.B. Properties of F-430

The chemical and spectroscopic properties of the cofactor F-430 have been reviewed [98,99]. The structure of the macrocycle (Figure 4) was elucidated by x-ray crystallography and NMR spectroscopy [100]. The free cofactor, which is present in substantial amounts in the cells, has an absorption maximum at 430 nm, hence its name. In the enzyme, the absorption maximum is blue shifted to 420 nm. The pentamethyl ester of F-430 is soluble in organic solvents and can be reduced to the Ni(I) state under aprotic conditions, resulting in an absorption peak shift to 382 nm [101], or can be oxidized to the Ni(III) state, giving an absorption peak at 368 nm [102].

The macrocycle that binds nickel in cofactor F-430 is a tetrapyrrole structure containing an unusually large number of saturated bonds, and is described as a tetrahydrocorphin (being a hybrid of a porphyrin and a corrin). In contrast to the planar porphyrins, the ring structure is puckered and flexible. Changes in average Ni-N bond lengths have been observed by EXAFS, from 1.9 Å in the low-spin state to 2.1 Å in the high-spin state, which is more than enough to accommodate any changes in ionic radius occurring on reduction [103]. Computer analysis of the conformation indicated that the tetrahydrocorphin structure can accommodate both square-planar and trigonal-bipyramidal coordination geometries, which would facilitate the reduction of Ni(II) to Ni(I) [104,105].

The coordination and catalytic properties of the F-430 nickel in MCR have been examined by optical, EXAFS, and resonance Raman spectroscopy. These indicated that the nickel is probably 6-coordinate, and that no sulfur ligands are involved [106,107]. The magnetic properties of the Ni(II) state of F-430 have been investigated by MCD spectroscopy [108,109]. In both the free coenzyme and the protein-bound form, the 6-coordinate nickel is high-spin Ni(II) ($S = 1$)



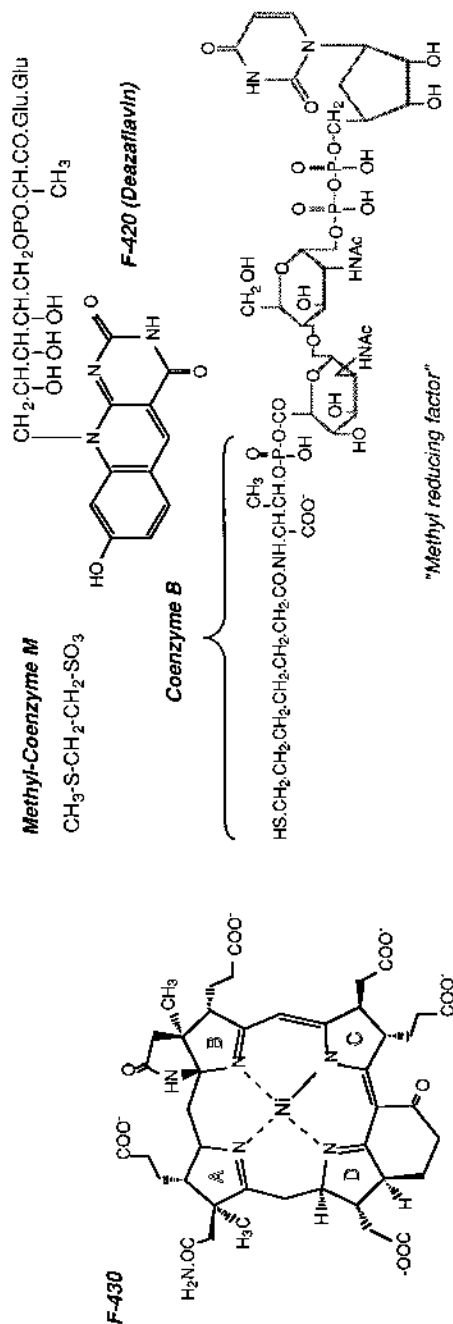


Figure 4 Coenzymes of methanogenesis. F-430, coenzyme M (2-thioethanesulfonate), and coenzyme B (7-thioheptanoyl-threonine-O-phosphate [sometimes abbreviated HS-HTP]). Methyl-reducing factor is a structure proposed on the basis of NMR and mass spectrometry, in which the phosphate group of CoB is linked by a carboxylic-phosphoric anhydride to a UDP disaccharide, uridine 5'-(2-acetamido-2-deoxymannopyranosyl)-2-acetamido-2-deoxy-glucopyranosyl-diphosphate [171]. The CoB moiety without it appears to be functionally active in the enzyme reaction.



with an axial zero-field splitting $D = 9 \text{ cm}^{-1}$. This value is consistent with oxygen ligands to the nickel in the protein.

IV.C. Properties of the Enzyme

As isolated from various methanogenic species, MCR consists of three different protein subunits arranged as $\alpha_2\beta_2\gamma_2$, containing two molecules of the nickel-containing yellow cofactor F-430. Two different types of MCR, which can be distinguished by a difference of about 5 kDa in their γ subunits, have been observed. It has recently been shown that there are distinct genes for the two types of enzyme in *M. thermoautotrophicum* [110], which differ somewhat in catalytic properties and are expressed preferentially at different phases of the growth of the bacterial culture.

Methyl coenzyme M reductase has been extracted from strains of the thermophilic bacterium *M. thermoautotrophicum* and intensively studied. When isolated, it contains both CoM and CoB [111,112]. The enzyme is irreversibly inactivated by oxygen. Even when extracted anaerobically it loses activity within a few hours [113], producing the inactive, EPR-silent state $\text{MCR}_{\text{silent}}$ in which the nickel is presumably Ni(II).

Like hydrogenase, MCR undergoes changes between various states that differ in activity and spectroscopic properties [114,115]. If, prior to extraction, the bacterial cells are gassed with CO_2/N_2 before isolating the enzyme, the enzyme is obtained in the MCR_{ox} state, which shows a characteristic axial EPR signal, MCR-ox1 ($g_{x,y,z} = 2.226, 2.147$) [116]. In this state the enzyme has relatively low activity. It can be reactivated in vitro by the strong reductant Ti(III) citrate. The activated protein shows the EPR signal MCR-red1 and an optical absorption band at 750 nm, indicative of Ni(I) [115]. The MCR_{ox} state gradually loses activity, forming the state $\text{MCR}_{\text{ox/silent}}$.

In enzyme preparations having high specific activity, obtained by anaerobic isolation, the EPR signals MCR-red1 ($g_{x,y,z} = 2.24, 2.054$) and MCR-red2 ($g_{x,y,z} = 2.28, 2.23, 2.18$) have been observed. These signals were also observed in whole cells of *M. thermoautotrophicum*, where they responded to treatments with H_2 and CO_2 . These observations indicate that the states giving these EPR signals are relevant to the active enzyme. Because they were observed in whole bacterial cells after reduction with hydrogen they are likely to represent the Ni(I) rather than the Ni(III) oxidation state [116]. Furthermore, the axial MCR-red2 signal is very similar to that of the pentamethyl ester of F-430, reduced to its Ni(I) state under aprotic conditions [101], and quite unlike the spectrum of the oxidized cofactor [102]. The difference between the two states is not established but appears to be related to the presence of substrates in the active site. When methyl CoM was added to the purified enzyme the MCR-red2 signal disappeared and



the MCR-red1 signal increased. When CoB was added, the MCR-red2 signal was enhanced, whereas the MCR-red1 signal was unchanged [114].

The specificity of MCR for the methyl donor gives an indication of the mode of substrate binding [117]. The ethyl and difluoromethyl derivatives are poor substrates, giving rise to low activities. The sulfide sulfur could be replaced with selenium but not with oxygen. Replacement of the sulfonate by carboxylate also retained some activity, indicating that the sulfonate does not participate directly in the reaction. A number of sulfonate derivatives were shown to be inhibitors, including 2-bromoethanesulfonate ($K_i = 4 \mu\text{M}$) [117], 3-bromopropanesulfonate, and 2-azidoethanesulfonate ($K_i = 1 \mu\text{M}$) [118].

IV.D. Structure of the Active Site

The crystal structure of MCR has recently been determined at high resolution, which has made it possible to describe the structure of the active site in detail [119]. The structure of the protein contains a number of modified amino acids. One of these appears to be glycine with sulfur replacing the carbonyl oxygen and is close to the CoB-binding site. Each α subunit contains the F-430 cofactor in which the nickel is coordinated to the amide oxygen of a glutamine from the other α subunit. The two F-430 groups are separated by 50 Å and thus are catalytically independent. They have a rather flat conformation. The free coordination site of nickel is situated in a hydrophobic pocket, facing a hydrophobic channel, 6 Å wide and 30 Å long, leading to the surface.

Two forms of the enzyme have been crystallized. Both contain CoM in the pocket, and the channel is occupied by CoB. The reactants are thus isolated from the solvent. In the $\text{MCR}_{\text{ox/silent}}$ state the CoM is in the reduced state and the thiol group is close to the nickel. In the $\text{MCR}_{\text{silent}}$ state the CoB and CoM form a disulfide and the sulfonate group of CoM is directed at the nickel (Figure 5). The implication is that CoM moves around the active site pocket in the reaction cycle, which explains why a small molecule is required.

IV.E. Catalytic Mechanism

Ahn et al. [120] have shown that the stereochemistry of the reaction with isotopically labeled ethyl-CoM is consistent with a net inversion of symmetry at the methyl carbon. This was interpreted as due to an inversion during transfer to the nickel, followed by retention of symmetry during protolysis.

Possible chemical mechanisms of methane release have been investigated by studies of the properties of isolated coenzyme F-430 and related nickel complexes. Jaun and Pfaltz [121] demonstrated the formation of methane from the Ni(I) state of F-430 with methyl iodide or methyl sulfonium as methyl donor, though methyl CoM was not effective. With zinc as reductant the reaction became



Methyl-CoM reductase

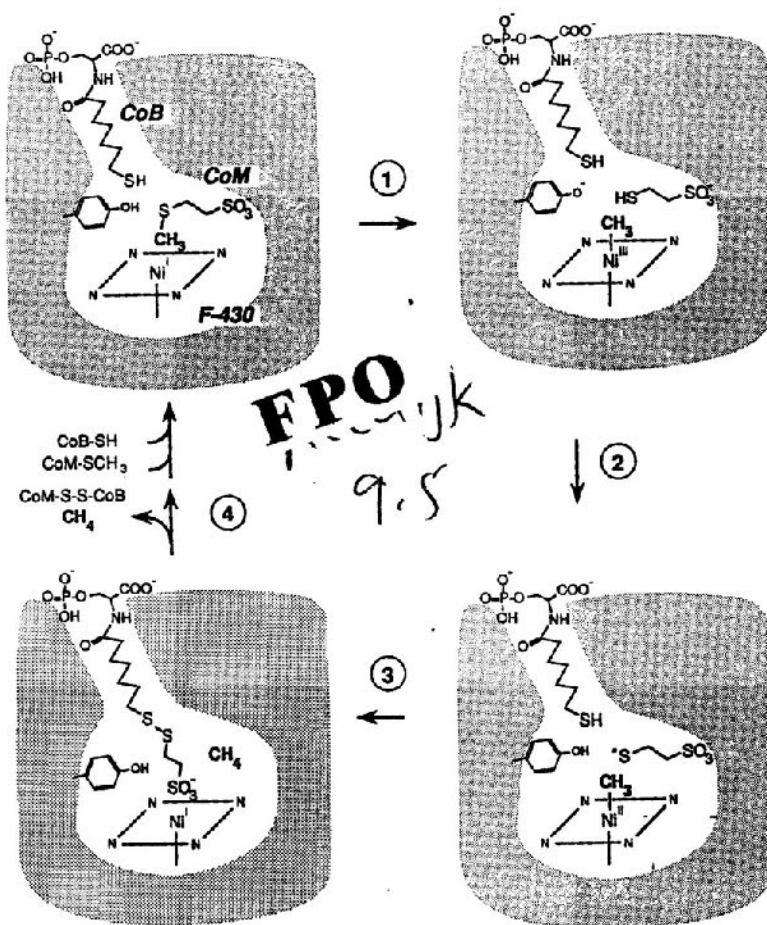


Figure 5 Proposed catalytic cycle of MCR. Methyl CoM binds first to the active site pocket, followed by CoB, which plugs the channel. The methyl group is transferred from CoM to nickel (step 1). The donor for the hydron that is taken up is suggested here to be a tyrosyl residue in the active site. The Ni(III)-methyl group is a strong oxidizing species and oxidizes the CoM-SH to a thiyl radical CoM-S[•] (step 2). Release of the methyl group takes place by hydronation of the methyl-Ni(II), while the CoM forms a disulfide with CoB, with electron transfer to the Ni(I); these two processes are combined in step 3. Repulsion of the sulfonate group by the Ni(I) would favor the release of the product disulfide from the active site (step 4). (Adapted from Ref. 119.)



catalytic. These reactions were proposed to involve the methyl derivative of the Ni(II) state, with the final step a protolysis. These experiments were in dimethylformamide as solvent. Krone et al. [122] showed that in aqueous solution the free coenzyme F-430 could catalyze the reduction of methyl chloride to methane with Ti(III) citrate as reductant.

A mechanism based on the crystal structure and other data [119] is shown in Figure 5. It may be noted that the forms of the enzyme that are crystallized represent stable states that probably do not take part in the enzyme cycle. In particular the methane or methyl group is absent, and the nickel is oxidized from Ni(I) to Ni(II). Nevertheless the observed structure of the active site provides the basis for understanding the catalytic cycle. In Figure 5 the numbering of the steps follows approximately the sequence proposed by Ermler et al. [119]. A critical step is the reduction of nickel, with the formation of the mixed disulfide CoB-SS-CoM. It was previously hypothesized that the sulfur of CoB was a ligand to the nickel during the reaction, but in the protein they are separated by 8.7 Å. The most likely redox mechanism is long-distance electron transfer, which implies that a radical is involved. Radical processes are consistent with the hydrophobic nature of the active site pocket. It may be significant that the thio-glycine residue protrudes into the active-site cavity and might play a role in electron transfer.

V. CARBON MONOXIDE DEHYDROGENASE

V.A. Function

Nickel-containing enzymes have been isolated from anaerobic bacteria, which catalyze the reversible oxidation of carbon monoxide to carbon dioxide:



These enzymes are classified as oxidoreductases and, following biochemical convention, are called carbon monoxide dehydrogenase (CODH), despite the fact that CO obviously contains no hydrogen. Unlike the molybdenum-containing CO dehydrogenases of aerobic carbon monoxide-oxidizing bacteria [123], the nickel-containing CO dehydrogenases are sensitive to dioxygen and are isolated under anaerobic conditions. Some carbon monoxide dehydrogenases, known as acetyl CoA synthases, are also able to synthesize acetyl coenzyme A, from a methyl-corrinoid protein, and CoA. These will be described in the next section. The properties of CODH and acetyl coenzyme A synthase (ACS) have recently been reviewed by Ragsdale and Kumar [124].

Bonam, Ludden, and coworkers isolated CODH from the purple photosynthetic bacterium *Rhodospirillum rubrum* [125]. The enzyme was a single 61.8



kDa polypeptide. It contained 1.4 Ni, 9 Fe, 8 S, and 0.8 Zn per molecule of protein. The enzyme is induced in the cells by carbon monoxide.

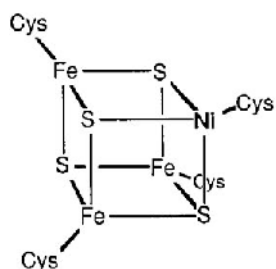
The operon encoding CODH in *R. rubrum* has been cloned and sequenced. It comprises, in addition to the *cooS* gene encoding CODH, a membrane-bound 22 kDa iron-sulfur protein, encoded by the *cooF* gene, containing a [4Fe-4S] cluster [126], a membrane-bound hydrogenase (*cooH*) [127], and several genes involved in nickel insertion [128]. It was suggested that the *cooF* protein serves as the electron carrier in vivo, linking CODH to a nickel-containing hydrogenase, forming a CO hydrogen oxidoreductase system that is analogous to the formate: hydrogen lyase of bacteria such as *E. coli* (see [129]).

Carbon monoxide dehydrogenase of *R. rubrum* exhibits EPR signals that correspond with various oxidation states [130]. EPR signal C_{red1} ($g_{x,y,z} = 2.01, 1.81, 1.65$) was observed on reduction with dithionite or CO. The midpoint potential was estimated to be -111 mV for the *R. rubrum* enzyme [131]. The signal was only observed when Ni was present in the enzyme, and its spectral line width was broadened in samples enriched with either ^{61}Ni or ^{57}Fe , demonstrating that it arises from a paramagnetic cluster containing both nickel and iron [129]. Further reduction below -530 mV induced the second signal, C_{red2} ($g_{x,y,z} = 1.98, 1.86, 1.75$). These signals are associated with nickel-containing iron-sulfur cluster C. In addition the protein contains a [4Fe-4S] cluster B ($E_m = -418$ mV, EPR in the reduced state with $g_{x,y,z} = 2.04, 1.95, 1.89$), which is presumably involved in electron transfer.

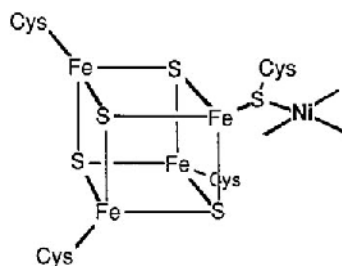
Cluster C in *R. rubrum* CODH is a useful model for the CO-binding sites in both CODH and ACS and has the advantage that its spectroscopic properties are not complicated by signals from the other Ni-F-S cluster, A, found in ACS. The enzyme containing the cluster in a nickel-free form can be isolated from cells grown on nickel-free medium. The nickel-deficient enzyme contains the usual complement of iron and sulfide and is even more sensitive to dioxygen than the native enzyme. The active enzyme was restored to a considerable extent by addition of 10 mM Ni^{2+} [132]. This is the only nickel enzyme in which facile insertion of Ni^{2+} has so far been reported. On the basis of this observation an analogy was drawn [130] with the introduction of a nickel ion into [3Fe-4S] clusters, such as that of *Pyrococcus furiosus* ferredoxin, to give a [3FeNi-4S] cluster (Scheme 4) [133]. An analog cluster of this type has been synthesized [134]. However, the oxidized nickel-free CODH does not give the $g = 2.01$ EPR signal that is expected for an oxidized [3Fe-4S] cluster. Moreover, EXAFS studies of the holoenzyme indicated that the nickel is 5-coordinate with 2S and 3 N/O ligands, but iron was not detected in the immediate vicinity [135]. The spectroscopic properties of cluster C were interpreted in terms of a [4Fe-4S] cluster weakly coupled via an intervening ligand to a high-spin Ni(II) site (possibly a cysteine sulfur) (Scheme 5) [136].

There is evidence that nickel-iron-sulfur cluster C is the site of reaction





Scheme 4



Scheme 5

with CO in the *R. rubrum* enzyme. Electron transfer from carbon monoxide to the iron–sulfur clusters did not occur in the nickel-free form of CO oxidoreductase [137]. Moreover, cyanide, a slow-binding, reversible inhibitor, induced the disappearance of signal C_{red1} [129]. Its inhibitory action was prevented by the presence of CO or by the absence of nickel [138]. This implies that it binds at or near the CO-binding site.

The mechanism of the redox reaction between CO and CO_2 at cluster C is expected to be similar to that in ACS [139], which will be described in the next section (Figure 6a), with the difference that the electron acceptor is the *cooF* protein instead of ferredoxin.

VI. ACETYL-CoA SYNTHASE

VI.A. Function

The CODH of *C. thermoaceticum* [140] and *Methanothrix soehngenii*, when isolated under strict anaerobic conditions, can catalyze reversibly the oxidation of



CO to CO₂ in a similar way to the CODH of photosynthetic bacteria, and also the synthesis of acetyl coenzyme A [140].



where the corrin is part of a corrinoid–iron–sulfur protein. They are therefore described as acetyl CoA synthases. The synthesis of acetyl CoA appears to require an additional protein subunit and the A cluster, which comprises iron, sulfide, and nickel. The physiological electron donor or acceptor is ferredoxin [141], but methyl viologen is commonly used experimentally.

In methanogens such as *M. thermoautotrophicum*, acetate is synthesized from CO₂ and H₂ and used as a carbon source for growth, while the production of methane is coupled to the building up of a proton motive force to produce ATP. Methanogens such as *Methanosarcina barkeri* and *Methanotherix soehngenii* can grow on acetate and produce methane, by a reversal of the acetyl CoA synthase reaction [11,142,143]. In acetogenic bacteria, e.g., *C. thermoaceticum*, the production of acetate is the carbon source and also is used for the generation of ATP [12,144]. The pathway differs from that of the methanogens in that it involves formate dehydrogenase and uses tetrahydrofolate instead of tetrahydromethanopterin.

Acetyl coenzyme A synthase of *C. thermoaceticum* has a (αβ)₂ dimer-of-dimers structure with subunit molecular masses 78 kDa (α) and 71 kDa (β). Analysis indicated 12 Fe, 14 S, 2 Ni, 1 Zn per αβ dimer. CO dehydrogenase activity resides in the β subunit (AcsA). This subunit has 46% identity (75% homology) with the *R. rubrum* CooS protein and appears to contain cluster C. Cluster A is located at least partially in the α subunit (AcsA); ACS of *Methanosarcina barkeri* comprises five subunits α, 84–93 kDa; β, 63 kDa; γ, 53 kDa; δ, 51 kDa; ε, 20 kDa [141]. The CODH activity is associated with the α subunit.

Like *R. rubrum* CODH, *C. thermoaceticum* ACS contains a [4Fe-4S] cluster B and a CO-oxidizing cluster C. The latter can be distinguished by its specific inhibition by cyanide ($K_i < 10 \mu\text{M}$) [145].

VI.B. Carbon Monoxide Dehydrogenase Reaction

Evidence that the CODH activity is a function of cluster C was obtained by rapid-freeze EPR studies [124]. The reaction of cluster C with CO occurred within 10 ms, consistent with a role in CO oxidation; reaction of the other clusters was much slower.

A mechanism for the oxidation of carbon monoxide at cluster C has been proposed [139]; in it CO and water bind to the cluster (Figure 6a). By analogy with cyanide binding, water is proposed to bind to the high-spin Ni(II) and CO to an iron atom of the cluster. After dehydronation of the water molecule the resulting hydroxide attacks the CO to form –COOH. Dehydronation of this spe-



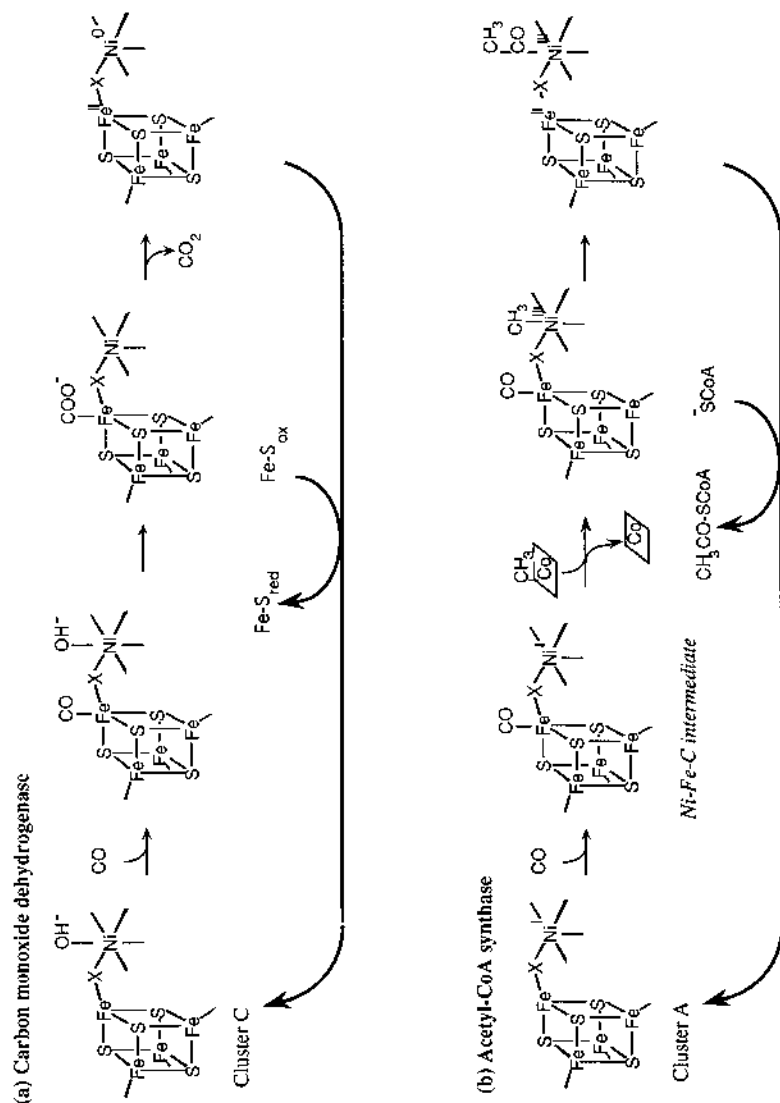


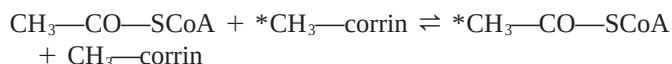
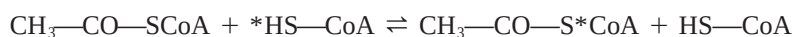
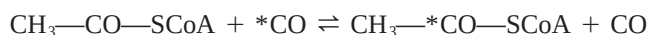
Figure 6 Proposed mechanism of CODH and acetyl ACS. (a) Oxidation of CO at cluster C; (b) synthesis of acetyl CoA at cluster A. No structure for these enzymes is available and the model allows other possibilities. The form of the Fe-S cluster may not be a cubane type; in (a), the CO₂ could bind to Fe instead of Ni; the valences of Fe and Ni have not been established.



cies yields CO_2 and a reduced cluster, which is reoxidized in a reaction with ferredoxin. The reaction has been modeled by square-planar dinuclear clusters of nickel(II) with N, O, S ligand sets, which were able to oxidize CO to CO_2 in aqueous solution with methyl viologen as electron acceptor [146].

VI.C. Acetyl CoA Synthase Reaction

Acetyl coenzyme A synthase of *C. thermoaceticum* is able to catalyze the whole reaction for reductive synthesis of acetyl coenzyme A from carbon dioxide, a methylated corrinoid/iron-sulfur protein, and coenzyme A. The enzyme catalyzes several exchange reactions [147–149]:



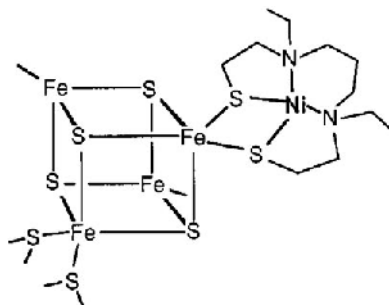
These indicate that the enzyme has specific binding sites for the methyl, carbonyl, and CoA groups. The kinetics of the exchange reactions show that they are relevant to the reaction cycle [147]. Measurements of the rate of the reaction under controlled redox potential conditions showed that binding of the methyl group requires a reduced site on the ACS [149]. The exchange takes place with retention of stereochemistry at the methyl group, consistent with an organometallic intermediate [150,151]. Further experiments on the redox potential dependence of the acetyl CoA–HS–CoA exchange reaction showed that although binding of CoA to the enzyme is independent of reduction, the heterolytic cleavage of the carbonyl–sulfur bond depends on the reduction of a center with a midpoint potential below -486 mV [152]. This was proposed to be a nucleophilic redox-active metal center. Its potential is consistent with the center's giving the Ni-Fe-C EPR signal.

A number of different paramagnetic clusters have been observed by EPR spectroscopy in reduced *C. thermoaceticum* ACS [153–155]. Most interesting, association of CO with the enzyme results in a paramagnetic species, known as the Ni-Fe-C intermediate, with g values $g_{x,y,z} = 2.074, 2.028$ [153]. Hyperfine broadening of the EPR signal in enzyme enriched in ^{61}Ni , ^{57}Fe , or ^{13}C demonstrated that this species contains nickel and iron and is strongly coupled to ^{13}C from carbon monoxide [155]. A combination of spectroscopic studies concluded that this is a nickel–iron–sulfur cluster A that is implicated in the catalytic cycle of acetyl CoA synthesis (Figure 6b) [153]. More detailed measurements of the hyperfine interactions of the paramagnetic cluster of *C. thermoaceticum* CODH enriched with ^{61}Ni , ^{57}Fe , and ^{13}C were made by ENDOR at 35 GHz [156].

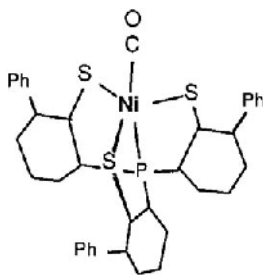


The ENDOR results were consistent with a stoichiometry NiFe_3S_4 . As with cluster C, this might suggest an analogy with the $[\text{3FeNi-4S}]$ cluster formed by insertion of Ni into the $[\text{3Fe-4S}]$ cluster (Scheme 4) [133]. However, this proposal is not consistent with the spectroscopic properties of the cluster of CODH. In both of the $[\text{3FeNi-4S}]$ clusters, the EPR spectra indicated an $S = 3/2$ ground state, whereas the Ni-Fe-C signal behaves as an $S = 1/2$ state. Furthermore, EXAFS spectroscopy showed that the nickel had sulfur ligands, possibly in a square-planar environment. No Fe atoms, at a distance expected for a $[\text{3FeNi-4S}]$ cluster, were detected [157,158]. An alternative possibility, supported by Mössbauer spectroscopy [154], is that the nickel-iron cluster consists of a $[\text{4Fe-4S}]$ cluster coupled through an intervening ligand to a nickel ion (Scheme 5). A model comprising a $[\text{4Fe-4S}]$ cluster linked by two bridging thiolates has been synthesized (Scheme 6) [159].

The chemistry of carbonyls of higher-valent nickel and iron has not been extensively studied, but a model thiolate complex of Ni(II) with carbon monoxide (Scheme 7) and its Fe(II) analog has been synthesized [160]. The methyl deriva-

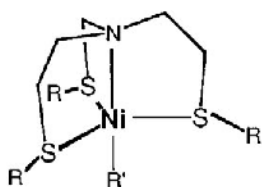


Scheme 6



Scheme 7





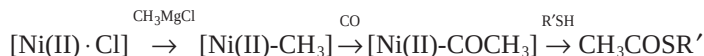
R = *i*-Pr, *t*-Bu; R' = CH₃-, -CO, CH₃-CO-

Scheme 8

tive was also investigated by transferring the methyl group from the corrinoid-iron-sulfur protein [161]. In this case, isotope substitution indicated that the methyl group was located on the nickel, as seen in methyl-CoM reductase.

Combining these observations, a mechanism can be proposed (Figure 6b) in which the reduced NiFe cluster A is involved in three steps: the transfer of CH₃ from the corrinoid/Fe-S protein, the binding of CO and formation of the CH₃-CO bond, and the thioester bond of acetyl CoA. In the first stage of the assembly of acetyl CoA, CO and the methyl group can bind to the center in a random order. This is followed by the formation of an acetyl derivative. The CoA then binds to the enzyme and acetyl CoA is released.

Stavropoulos et al. [162] synthesized the methyl, carbonyl, hydride, and acetyl coordinated complexes of the compound shown in Scheme 8 as models of a possible pathway to the formation of a thioester. The tripodal ligands N(CH₂CH₂SR)₃ are biologically relevant in using N and S coordination. Further, the sequence of reactions



was demonstrated, where R'SH = PhSH or EtSH, representing HS—CoA.

Tucci and Holm [163] have demonstrated an alternative reaction scheme, starting from the complex [Ni(bpy)(CH₃)₂(SR'')₂], where bpy is 2,2'-bipyridyl and R'' is aromatic. In this case, the thiol displaced one methyl group from the nickel as methane, in a reaction reminiscent of methyl-CoM reductase, and coordinated to the nickel. Further addition of CO liberated CH₃COSR'' in high yield. This reaction demonstrates the feasibility of a reaction in which both the thiol and acetyl groups coordinate the nickel.

VII. SUPEROXIDE DISMUTASE

A newly discovered nickel enzyme is superoxide dismutase (SOD) from the actinomycete *Streptomyces* sp [164,165]. In its protein properties, this enzyme is



distinct from the three other known superoxide dismutases, which contain, respectively, copper/zinc, iron, and manganese centers. The Ni-SOD showed no immunological cross-reaction with the latter enzymes [166]. The nickel-containing enzyme from *Streptomyces coelicolor* showed a different pattern of expression from another SOD, which contained iron and zinc [167]. Its molecular mass was estimated to be 13.4 kDa, which is lower than for the other superoxide dismutases. It contained approximately 0.74 g-atom nickel per molecule; Fe, Zn, Mn, Cu were present only at very low levels. It was sensitive to inhibition by cyanide but less sensitive to azide and H_2O_2 . After extraction of the nickel, activity was partially restored by metal ions, 12.5% by Ni^{2+} , 2.1% by Fe^{2+} , 4.6% by Mn^{2+} .

The EPR spectrum of SOD from *Streptomyces* showed rhombic symmetry ($g_{x,y,z} = 2.304, 2.248, 2.012$), with a strong three-line splitting on the $g_z = 2.012$ [164,165]. This spectrum is similar to those observed in tetragonal complexes of Ni(III) with peptides, where the splitting is due to an axial ^{14}N ligand [168,169]. A possible histidine ligand was suggested. This appears to be yet another unique type of metalloenzyme active site. The only possible analogy of the reactivity of this site with dioxygen is the formation of the Ni-A state in NiFe hydrogenase, although the latter has thiolate ligands and not nitrogen. The mechanism of superoxide dismutases generally involves the alternate reduction and oxidation of the metal site [166]. In this case the states involved would be Ni(II) and Ni(III).

VIII. CONCLUDING REMARKS

The nickel-containing enzymes have very different coordination sites, and there are few similarities between them. In different ways they exploit the unique redox and coordination chemistry of nickel. Methyl-Ni(III) complexes occur in MCR and ACS, two of the few examples of organometallic intermediates in nature. Unlike the methyl-Co(III) derivatives in enzymes containing vitamin B_{12} , the methyl-Ni(III) species in MCR is readily reduced, with a facile release of methane [1,121]. Presumably it is the arrangement of functional groups around the nickel site that prevents the release of methane from the methyl-nickel intermediate in ACS. Dioxygen is a product of superoxide dismutase and also reacts reversibly in some hydrogenases to form a ligand to nickel in the oxidized state. On the other hand, MCR, CODH, and ACS are extremely sensitive to oxygen. Carbon monoxide is a substrate for ACS and CODH and an inhibitor of hydrogenase.

In urease, the active site with a pair of nickel ions is unique. A large number of esterases and phosphatases contain dinuclear clusters of zinc and/or iron, and in these a metal-bound hydroxide or water molecule is a common feature [170]. In urease the active site is rather rigid and designed to favor binding of urea over that of solvent water, and for this purpose nickel may be preferable to zinc, which has more flexible coordination geometry.



There are parallels between the biological and industrial chemistries of nickel. Three of the nickel enzymes listed in Table 1 catalyze reactions that correspond to industrial processes, namely, hydrogenation, desulfurization of methyl coenzyme M to give methane, and carbonylation of a methyl group to give acetyl coenzyme A, which correspond to industrial processes, for which the use of nickel catalysts is uniquely suited. A consideration of the mechanism of the industrial catalysts may throw light on its biochemical role. On the other hand, improved understanding of the geometry, ligands, and oxidation state changes of the active sites in the nickel enzymes might allow the development of more efficient industrial catalysts.

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10

Oxygen Activation at Nonheme Iron Centers

Lawrence Que, Jr.

University of Minnesota, Minneapolis, Minnesota

The activation of dioxygen in biological systems has long attracted the interest of chemists and biochemists because of the metabolic importance of the substrates and products of such reactions. The efficiency and specificity of the enzymes that activate dioxygen have raised important mechanistic questions as to the nature of the chemical species that carry out such reactions. The nonheme iron enzymes represent the most diverse subset of enzymes associated with oxygen activation, in terms of both the nature of the active site and the types of oxidations that are carried out. Since the publication of the first edition of this book, there have been many significant advances in the nonheme iron field; in particular, the number of crystal structures of nonheme iron enzymes has tripled. This chapter will summarize highlights of the many enzymes that fall in this category. For greater depth, the reader is directed to a number of reviews that have recently appeared [1–5].

I. THE HEME PARADIGM FOR OXYGEN ACTIVATION

Because of its many functions in mammalian metabolism, cytochrome P-450 is the most studied and best understood oxygen activating metalloenzyme [6]. Substantial mechanistic insight has been obtained for the role of the heme cofactor in catalyzing a variety of oxidative transformations, including the hydroxylation of unactivated alkanes [7], a subject that is covered in detail in Chapter 11. Oxygen activation at a heme center can thus be regarded as the paradigm to which other systems are compared. The principal features of the cytochrome



P-450 mechanism are summarized in Figure 1. Dioxygen binds to an iron(II) porphyrin center and is reduced by two electrons to a peroxo species. Subsequent O—O heterolysis transforms it into a formally iron (V)-oxo species, which is proposed to be responsible for the oxygen transfer chemistry.

Although a high-valent intermediate has not yet been directly observed in the catalytic cycle of cytochrome P-450, its proposed involvement is based on two observations: First, the reaction of heme peroxidases with H_2O_2 affords an intermediate called Compound I, which is spectroscopically characterized as an oxoiron(IV) porphyrin radical complex [8]. Second, Fe(III)-cytochrome P-450 and synthetic Fe(III) porphyrin complexes are known to react with oxygen atom donors such as peroxides, PhIO, and ClO^- to generate a species capable of performing the same reactions as the native enzyme (Figure 2). This reaction bypasses the Fe(II) oxidation state in the P-450 cycle and is known as the peroxide shunt [9]. In alkane hydroxylations, the putative terminal oxo ligand inserts into the C—H bond in a two-step process: (1) a hydrogen atom is abstracted from substrate by the high-valent iron-oxo species generating an alkyl radical in a solvent cage, and (2) the alkyl radical is then trapped by the iron(IV)-hydroxo species to form the C—O bond via a process termed *oxygen rebound* (Figure 2).

Thus, in this heme paradigm, the porphyrin plays an active role in accessing the high-valent oxidation state required for the substrate oxidations. Many of the proposed mechanisms for the nonheme iron oxygen activating enzymes follow this mechanistic model. An important question is how analogous chemical reactions can be carried out in the absence of a porphyrin ligands; alternatively, what

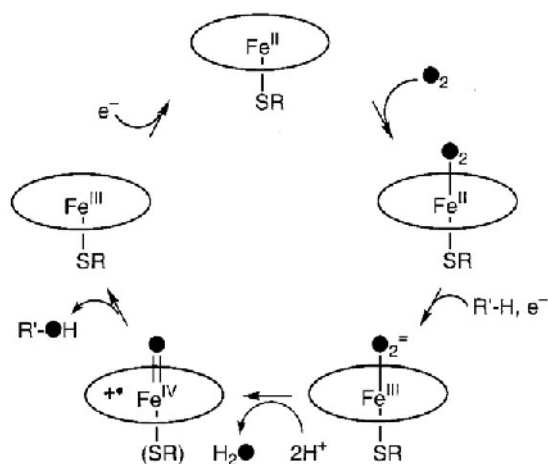


Figure 1 Catalytic cycle of cytochrome P-450.



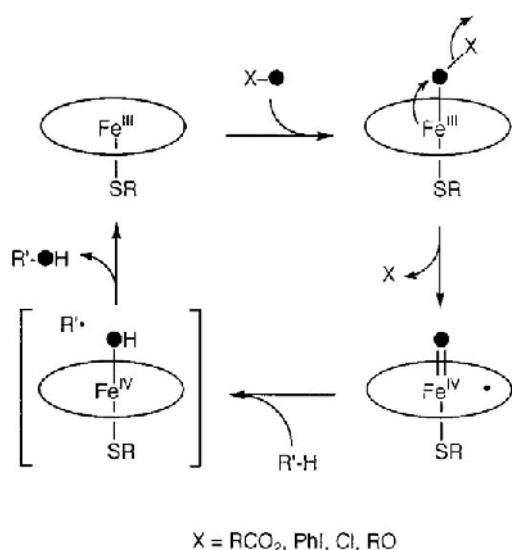


Figure 2 Peroxide shunt pathway for cytochrome P-450.

modifications of this paradigm are required to effect the interesting transformations catalyzed by the nonheme iron enzymes?

II. NONHEME IRON SYSTEMS

There are a number of nonheme iron systems that utilize O_2 to effect organic transformations of clear metabolic significance. Though there is less information regarding these nonheme iron enzymes relative to cytochrome P-450 and other heme enzymes, the situation continues to improve as more investigators turn their attention to these less understood systems. Indeed crystal structures of a number of enzymes discussed in this chapter have been solved within the past few years. The reactions catalyzed by these nonheme iron enzymes require a greater mechanistic diversity with respect to oxygen activation; in many cases, substrate or cofactor coordination to the metal center is necessary prior to oxygen binding. Features of the cytochrome P-450 mechanism can undoubtedly be recognized in the proposed catalytic cycles of these nonheme iron systems.

II.A. Bleomycin

Bleomycins (BLMs) (Figure 3a) are a family of glycopeptide-derived antibiotics that have the ability to bind and degrade deoxyribonucleic acid (DNA), which



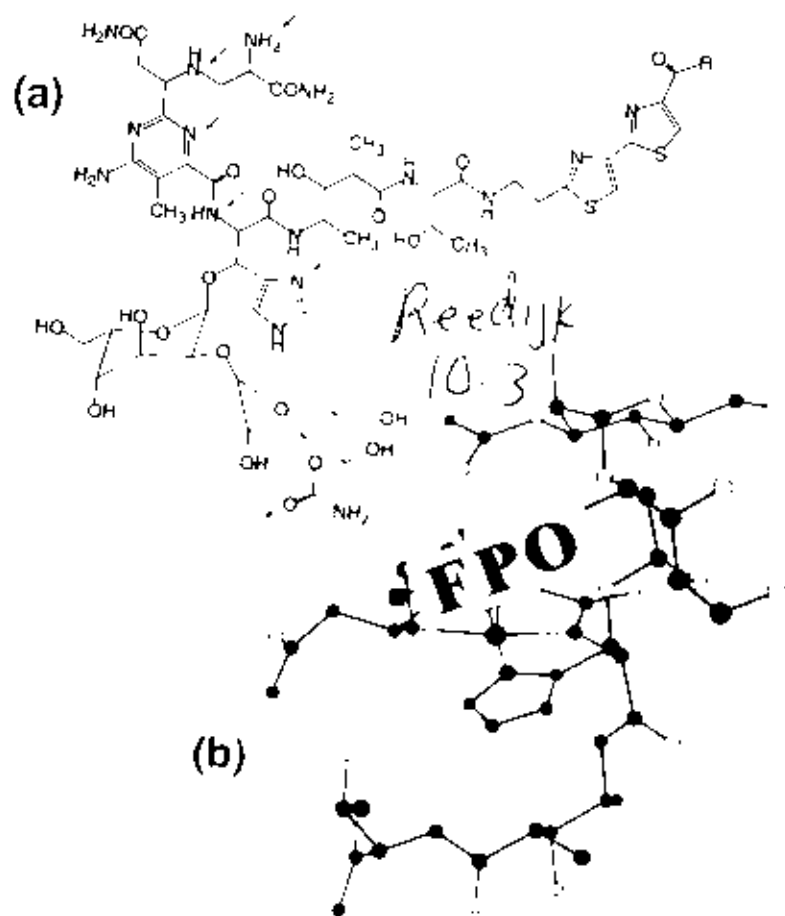


Figure 3 Structure of (a) bleomycin and (b) its metal complex.

is believed to be responsible for their antitumor activity [10]. Because the DNA cleavage reaction requires Fe(II) and O_2 , bleomycin is included in this discussion of nonheme iron enzymes despite the fact that it is not strictly an enzyme. The coordination chemistry of the metallo BLMs has been investigated using a variety of spectroscopic techniques [10]. Recent two-dimensional nuclear magnetic resonance (2D NMR) studies on Co(III)BLM—OOH, both free in solution and bound to a self-complementary dodecanucleotide [11], have provided significant insight into the solution structures of the complexes. On the basis of these studies, there



are six possible binding sites as indicated by the arrows in Figure 3a: namely, the pyrimidine, the imidazole, and the amide nitrogen of the β -hydroxyhistidine moiety; the primary and secondary amines; and the mannose carbamoyl group. Magnetic circular dichroism (MCD) studies suggest that Fe(II)BLM is 6-coordinate [12], so it is possible that all six proposed ligands bind to the metal center in Fe(II)BLM, but one of these must then dissociate upon O_2 binding. The NMR solution structure of Co(III)BLM—OOH (Figure 3b) favors the participation of the five nitrogen ligands with the hydroperoxide occupying the sixth site instead of the carbamoyl group [13]. This structural arrangement is supported by a subsequent NMR investigation on the paramagnetic Fe(II) complex itself [14].

The oxygen chemistry of Fe(II)BLM has been elucidated by Peisach and Horwitz using a combination of spectroscopic techniques [15,16]; it is summarized in the reaction cycle shown in Figure 4. Like deoxyhemoglobin, deoxymyoglobin, and Fe(II)-cytochrome P-450, Fe(II)BLM is a high-spin iron(II) complex, with one position available for the binding of O_2 and analogs such as CO, NO, and isonitriles. Upon O_2 binding, the iron center is converted to a diamagnetic (and therefore EPR silent) low-spin center [16], like the O_2 adducts of heme proteins. The β -hydroxyhistidine amide, the only anionic ligand in the putative coordination sphere, is proposed to play an important role in lowering the $Fe^{III/II}$ redox potential into a range suitable for O_2 binding [17], and strong backbonding from the pyrimidine ligand is suggested to reduce the tendency of the oxy complex to dissociate into Fe(III)BLM and superoxide [12].

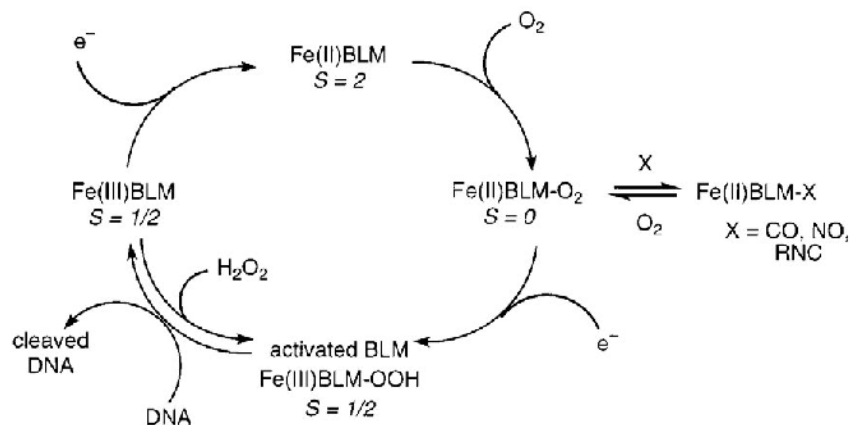


Figure 4 Reaction cycle of Fe-bleomycin.



As for cytochrome P-450, the introduction of an electron converts Fe(II)BLM—O₂ to the active species called *activated BLM* the last intermediate detectable prior to DNA cleavage [15]. Activated BLM can also be formed by the addition of H₂O₂ to Fe(III)BLM, by analogy to the peroxide shunt in cytochrome P-450. This strong correspondence between the chemistry of Fe(II)BLM and that of cytochrome P-450 has led investigators to consider whether activated BLM is formally an Fe^V=O species. For example, it hydroxylates naphthalene and 4-deuterioanisole (to 4-methoxy-2-deuteriophenol with concomitant NIH shift), epoxidizes olefins, and demethylates N,N-dimethylaniline [10]. Furthermore, Fe(III)BLM can act as an oxygen atom transfer agent using donors such as PhIO and periodate [18]. Thus, by analogy to the high-valent chemistry of cytochrome P-450 and its models, the involvement of what is a formally Fe^V=O species in bleomycin chemistry is strongly implicated.

However, spectroscopic studies of activated BLM indicate that it is not an Fe^V=O species. It exhibits an $S = 1/2$ EPR spectrum with g values at 2.26, 2.17, and 1.94 [15], which is typical of a low-spin Fe^{III} center. This low-spin Fe^{III} designation is corroborated by Mössbauer and x-ray absorption spectroscopy [16,19]. Furthermore, EXAFS studies on activated BLM show no evidence for a short Fe—O distance, which would be expected for an iron-oxo moiety [19]. These spectroscopic results suggest that activated BLM is a low-spin iron(III) peroxide complex, so the two oxidizing equivalents needed for the oxidation chemistry would be localized on the dioxygen moiety, instead of on the metal center. This Fe(III)BLM—OOH formulation has been recently confirmed by electrospray ionization mass spectrometry [20] and is supported by the characterization of related synthetic low-spin iron(III) peroxide species, e.g., [Fe(pma)O₂]⁺ [21] and [Fe(N4py)OOH]²⁺ [22]. The question then arises whether the peroxide intermediate is itself the oxidant in these reactions or the precursor to a short-lived iron-oxo species that effects the cytochrome P-450-like transformations. This remains an open question and the subject of continuing interest.

The DNA degradation by FeBLM results in the formation of two types of monomeric products, free nucleic acid bases and base propenals, suggestive of at least two distinct mechanisms for cleavage [10]. The elegant O₂ pulse-chase experiments of Stubbe and Kozarich [23] have substantiated the details of a unified mechanistic scheme that explains all the observed products (Figure 5). The first step of this scheme involves the abstraction of the C-4'—H of a ribose by activated BLM to generate a C-4' carbon radical. This was established unequivocally using poly(dA-[4'-³H]dU), where isotope effects of 7–12 for the formation of both monomeric products were observed. The C-4' radical then partitions down two pathways. Under aerobic conditions, it picks up a molecule of O₂ to form an alkylperoxy radical, which is then reduced to a hydroperoxide. Cleavage of the hydroperoxide via a Criegee-type mechanism yields the observed base



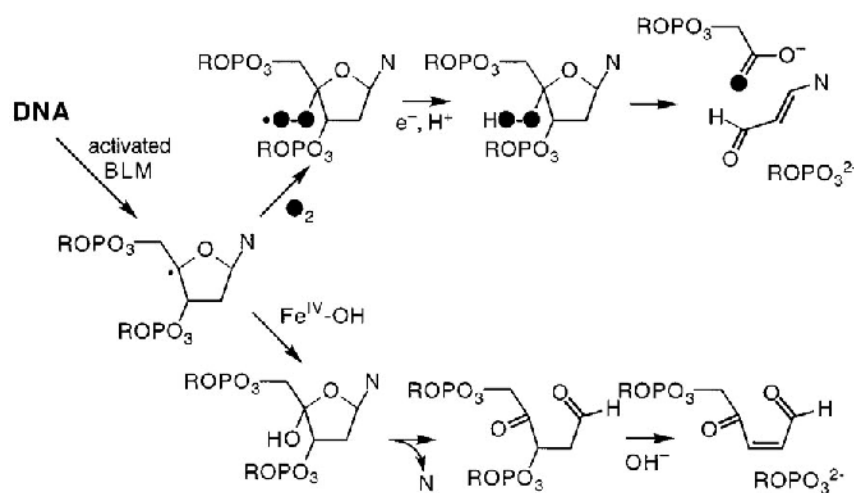


Figure 5 Unified mechanism for DNA degradation by Fe-bleomycin.

propenal and phosphoglycolate products. Under anaerobic conditions, the C-4' radical is converted to an alcohol, which releases the nucleic acid base and yields an oxidized sugar moiety in the intact DNA strand. Labeling studies show that solvent, not O_2 , is the source of the 4-keto oxygen in the product [24]. The observation that the intermediate ribose C-4' radical can be trapped by O_2 indicates that rapid oxygen rebound does not occur in this system, thereby distinguishing the BLM mechanism from that of cytochrome P-450.

II.B. Diiron Enzymes: Methane Monooxygenase, Ribonucleotide Reductase, and Fatty Acid Desaturase

Nonheme proteins with diiron active sites have emerged in the past decade as a new subclass of iron proteins [1,25,26]. These diiron sites are bridged by an oxo group and/or didentate carboxylates and often found to be involved in dioxygen binding and/or activation. Four of these proteins have been characterized crystallographically: hemerythrin (Hr) [27], the R2 protein of ribonucleotide reductase (RNR R2) (EC 1.17.4 group) [28,29], the hydroxylase component of methane monooxygenase (MMOH) (EC 1.14.13.25) [30–32], and fatty acid desaturases [33]. Hemerythrin is a multimeric protein responsible for oxygen transport in marine invertebrates [34]. Ribonucleotide reductase, a key enzyme in DNA biosynthesis [35], consists of two proteins, R1 and R2. R1 contains the substrate



and effector binding sites and the conserved thiols that provide the electrons for the reaction, while R2 harbors the diiron center and the tyrosyl radical (at residue 122) that is catalytically essential for initiating the deoxygenation of the ribonucleotide. In this enzyme, the diiron center is not directly involved in catalysis but functions to generate the tyrosyl radical in the assembly of the active enzyme during the course of the cell's life cycle [36]. Methane mono-oxygenase is isolated from methanotrophs that utilize methane as their energy and carbon source [37]. This enzyme consists of three components: a hydroxylase component that contains the diiron site at which methane hydroxylation occurs, a reductase component that mediates electron transfer between reduced nicotinamide-adenine dinucleotide (NADH) and the hydroxylase, and component B, which serves to ensure the efficient coupling of O_2 reduction and substrate oxidation. Fatty acid desaturases, exemplified here by stearyl acyl carrier protein Δ^9 -desaturase ($\Delta 9D$) (EC 1.14.99.5), are responsible for the conversion of saturated fatty acids into their unsaturated derivatives in plant metabolism [38]. For $\Delta 9D$, the reaction requires the fatty acid substrate to be conjugated to a carrier protein as well as a reductase and ferredoxin to mediate the reduction of O_2 by NADH. This subclass continues to grow. On the basis of sequence comparisons, toluene monooxygenases and phenol hydroxylases seem likely to have nonheme diiron active sites as well [39]. This group of enzymes thus catalyzes a range of dioxygen-dependent reactions that rival in versatility those that occur at heme active sites.

II.B.1. Hemerythrin, the "Prototypical" Diiron Nonheme Protein

Hemerythrin (Hr) was the first of this group to be characterized in detail and is thus considered the prototype. (As will be seen later, however, this notion is incorrect, as Hr turns out to be distinct from the other members of this class.) The structures of deoxyhemerythrin and oxyhemerythrin (Figure 6) show a novel (μ -hydroxo or oxo)bis(μ -carboxylato)diiron site with five terminal His ligands [27]. The binding of O_2 at the vacant sixth terminal site of deoxyHr involves the transfer of two electrons from the diiron(II) center and the hydron of the hydroxo

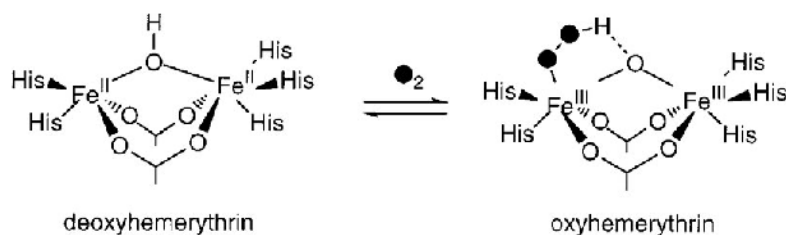


Figure 6 Active site structures of deoxyhemerythrin and oxyhemerythrin.



bridge to form a (μ -oxo)diiron(III) center with a terminal hydroperoxide that is hydrogen bonded to the oxo bridge [40]. Little structural change is involved in this process, thereby allowing reversible O_2 binding.

DeoxyHr has a (μ -hydroxo)bis(μ -carboxylato)diiron(II) core with an Fe-Fe distance of 3.3 Å [27]. The iron(II) centers are in the high-spin state [41] and coupled antiferromagnetically (J in the range of -13 to -38 cm^{-1}) [42,43]. The diiron(II) core and its properties have been duplicated in synthetic complexes [44,45].

In contrast, oxyHr has a (μ -oxo)bis(μ -carboxylato)diiron(III) core, whose structural and physical properties are quite well established and have been modeled by a number of synthetic complexes [25,46]. The oxo bridge in the diiron(III) form defines many of its distinctive properties: short Fe- μ -oxo bonds (ca. 1.8 Å), strong antiferromagnetic coupling ($J = -120(10)\text{ cm}^{-1}$ for $H = -2J S_1 \cdot S_2$), and large Mössbauer quadrupole splittings ($\Delta E_Q \geq 1.3\text{ mm/sec}$). The carboxylate bridges bend the Fe—O—Fe unit to an angle of 120° – 130° , resulting in an Fe-Fe separation of 3.2(1) Å and giving rise to a series of characteristic visible features ($\epsilon = 100$ – $1000\text{ M}^{-1}\text{cm}^{-1}$) [47,48] and a Raman vibration near 500 cm^{-1} [49] associated with a bent Fe—O—Fe unit. In addition, oxyHr has an intense visible band near 500 nm that is associated with a peroxide-to-Fe(III) charge transfer transition [40]. Resonance Raman studies confirm this assignment with the observation of $\nu\text{Fe—OO}$ and $\nu\text{O—O}$ features at 503 and 844 cm^{-1} , respectively, both of which downshift appropriately upon ^{18}O substitution [40]. That the bound dioxygen is present as a hydroperoxide is indicated by the downshift observed for $\nu\text{Fe—OO}$ in D_2O . More interestingly, the symmetric $\nu\text{Fe—O—Fe}$ feature at 492 cm^{-1} upshifts by 4 cm^{-1} in D_2O [40], an observation best rationalized by a hydrogen bond from the hydroperoxide to the oxo bridge. The presence of the hydrogen bond to the oxo bridge is corroborated by the weaker antiferromagnetic coupling observed for oxyHr (-77 cm^{-1}) relative to that for metHr, the diiron(III) form without the hydroperoxide ligand (-134 cm^{-1}) [50].

II.B.2. Active Site Structures

The core structures of the diiron(II) and diiron(III) forms of RNR R2, methane mono-oxygenase hydroxylase component (MMOH) and $\Delta 9D$, shown in Figure 7, are strikingly similar but significantly different from that of Hr, which belies an earlier notion that Hr was prototypical of this class. Table 1 compares some of the key properties of the active sites of the nonheme diiron enzymes. Unlike in deoxyHr, the iron(II) ions in these enzymes are all coordinatively unsaturated, raising the possibility that O_2 can bridge the diiron center. The diiron(II) centers all have two carboxylate bridges but no third bridge derived from solvent as found in deoxyHr [27]. For RNR R2_{red} and $\Delta 9D$ _{red} the two carboxylates bridge in a didentate fashion, resulting in Fe-Fe distances of 3.9 and 4.2 Å, respectively



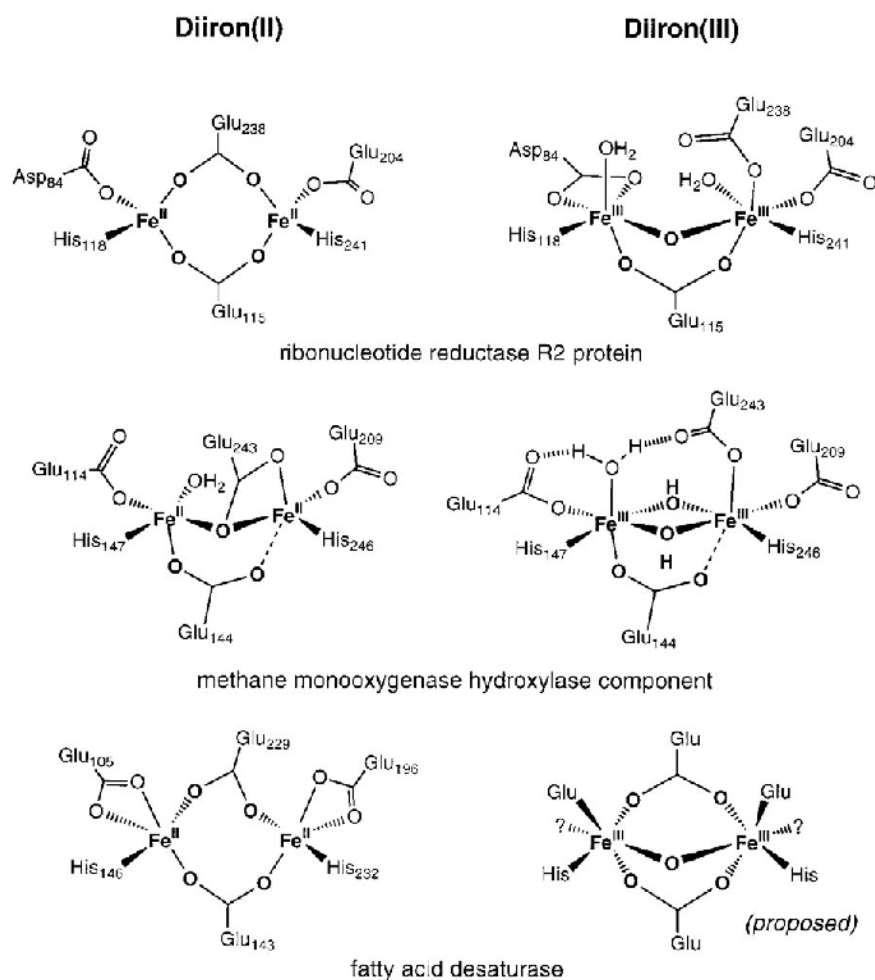


Figure 7 Active sites of RNR R2, MMOH, and $\Delta 9D$.



Table 1 Properties of Diiron Sites in Proteins

Protein	$r(\text{Fe-Fe})^a$ (Å)	$\angle\text{Fe-O-Fe}^a$	$\delta [\Delta E_Q]$ (mm/sec)
DeoxyHr (Refs. 27,41)	3.3	128°	1.14 [2.76]
OxyHr (Refs. 27,41)	3.2	128°	0.51 [1.96]
			0.52 [0.95]
MetHr (Refs. 27,41)	3.2	127°	0.46 [1.57]
RNR R2 _{red} (Refs. 29,58)	3.9	N/A	1.26 [3.13]
RNR R2 _{ox} (Refs. 28,63–65)	3.3	[138°]	0.53 [1.65]
	(3.2)	(130°)	0.46 [1.57]
RNR R2-X (Refs. 92,93)	(2.5)	(ca. 90°)	0.55 [0.9]
			0.26 [0.6]
MMOH _{red} (Refs. 31,59,60)	3.3	96°	1.30 [3.14]
MMOH _{ox}			
<i>M. capsulatus</i> Bath	3.1	110°, 119°	0.50 [1.05]
(Refs. 30,31,60)	3.4	132°	
<i>M. trichosporium</i> OB3b	3.0	105°	0.50 [0.87]
(Refs. 32,59)			0.51 [0.91]
MMOH-P (Ref. 77)			0.66 [1.51]
MMOH-Q			
<i>M. trichosporium</i> OB3b	(2.5)	(ca. 90°)	0.17 [0.53]
(Refs. 74,78)			
<i>M. capsulatus</i> Bath			0.14 [0.55]
(Ref. 77)			0.21 [0.68]
$\Delta 9\text{D}_{red}$ (Refs. 33,38)	4.2	N/A	1.30 [3.04]
			1.30 [3.36]
$\Delta 9\text{D}_{ox}$ (Refs. 38,39,68a)	(3.15)	[123°]	0.53 [1.54]

^a Values derive from x-ray crystallography, except those in parentheses, which are from EXAFS, or those in brackets, which are from Raman studies.

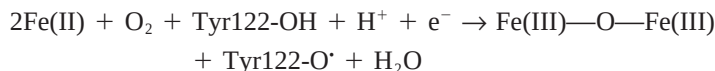
[29,33]. A similar distance of 4.3 Å is found in the synthetic complex $[\text{Fe}_2(\mu\text{-O}_2\text{CCH}_3)_2(\text{tmpa})_2]^{2+}$ [51]. The diiron(II) center of MMOH differs somewhat from the other two in having an Fe-Fe distance of 3.3 Å, which is due to the presence of a monodentate carboxylate bridge [31]. Such a bridging arrangement is modeled by $[\text{Fe}_2(\text{biphme})_2(\text{O}_2\text{CH})_4]$ [52]. The monodentate carboxylate bridge may explain why MMOH_{red} exhibits an integer spin EPR signal at $g = 16$ [53] whereas RNR R2_{red} and $\Delta 9\text{D}_{red}$ are EPR-silent. Such signals have been observed in some synthetic diiron(II) complexes that have acute Fe—μ—O—Fe angles [52,54,55]; it is proposed that the acute angles give rise to ferromagnetic interactions between the iron(II) ions and these integer spin signals. This $g = 16$ signal has proved to be a convenient probe for the diiron(II) state of MMOH, which is



spectroscopically rather inaccessible [56,57], but it is not clear at present what mechanistic implications, if any, the different bridging mode engenders.

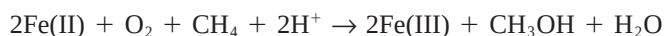
Another major distinction between Hr and the other diiron proteins is the nature of the terminal ligands. Hemerythrin (Hr) has five terminal His ligands, while the other three all have two terminal His and two terminal carboxylate ligands. This difference is also manifested in the Mössbauer isomer shifts of the high-spin diiron(II) forms of these proteins, 1.14 mm/sec for Hr [41] and 1.26–1.30 mm/sec for the others [38,58–60] (Table 1). These active site ligands derive from a pair of Asp/Glu-X-X-His sequence motifs in each active site. The presence of such a pair is the first hint that a new enzyme may contain a nonheme diiron active site, as in the case of $\Delta 9D$ and perhaps for toluene monooxygenases and phenol hydroxylase as well [39]. This difference in terminal ligands and the coordinative unsaturation of both iron ions in the active sites of RNR R2, MMOH, and $\Delta 9D$ may serve as the structural basis for eliciting O_2 activation instead of reversible O_2 binding.

The introduction of O_2 to RNR R2_{red} converts it to a diiron(III) form, R2_{ox}, concomitant with the input of an electron from an external source and the oxidation of Tyr122, i.e.,



One of the oxygen atoms from O_2 is incorporated as the oxo bridge between the iron(III) ions [61], and one of the bridging carboxylates is converted to a terminal ligand [28,29] in what has been termed a carboxylate shift [62]. Thus the bis(μ -carboxylato)diiron(II) core becomes a (μ -oxo)(μ -carboxylato)diiron(III) core with magnetic [63], Mössbauer [64], and resonance Raman [65] properties similar to those of metHr. The Fe-Fe separation in R2_{ox} is 3.3 Å from crystallography [28] and 3.22 Å from EXAFS [66]. Tyr122 is 5 Å away from the diiron cluster and appears to be hydrogen bonded to Asp84 in the reduced form [29]. Support for this interaction is found in the 70 mV decrease in the redox potential of the diiron(III) center between the wild-type protein and its Y122F mutant form [67]. This hydrogen bonding interaction presumably facilitates the abstraction of the hydrogen atom during the oxidation process.

Exposure of MMOH_{red} to O_2 converts the diiron(II) center to its diiron(III) form concomitant with the hydroxylation of methane [56], i.e.,

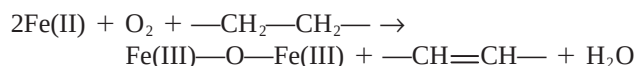


As in the case of RNR R2, one of the bridging carboxylates, Glu243, undergoes a carboxylate shift to become a terminal ligand [31]. However, unlike in RNR R2, an oxo bridge is not present in MMOH_{ox}, accounting for weak antiferromagnetic coupling between the iron(III) ions as well as the absence of any visible absorp-



tion feature [59]. There are three crystal structures of MMOH_{ox} , and the iron ions are bridged by at least one hydroxide and a didentate carboxylate in each of these structures [30–32]. The methane mono-oxygenase hydroxylase component (MMOH) from *Methylococcus capsulatus* Bath has an additional aqua bridge or an additional acetate bridge derived from the buffer, yielding Fe-Fe separations of 3.1 and 3.4 Å, respectively [30,31], whereas the MMOH from *Methylosinus trichosporium* OB3b has a second hydroxo bridge, resulting in an Fe-Fe separation of 3.0 Å [32]. The latter can also have different core structures in frozen solution, as demonstrated by EXAFS determined Fe-Fe distances of 3.0 and 3.4 Å [68]. Thus, the diiron(III) site of MMOH_{ox} appears to be rather flexible. The three core structures observed for MMOH_{ox} can be related to the RNR R2_{ox} core by the equilibria shown in Figure 8. At present, it is not clear what factors favor the different structures.

Presumably $\Delta 9\text{D}_{\text{red}}$ reacts with O_2 in a manner analogous to that of RNR R2_{red} and MMOH_{red} to produce a diiron(III) center concomitant with the desaturation of substrate, i.e.,



Unfortunately, a crystal structure of $\Delta 9\text{D}_{\text{ox}}$ is not currently available because of

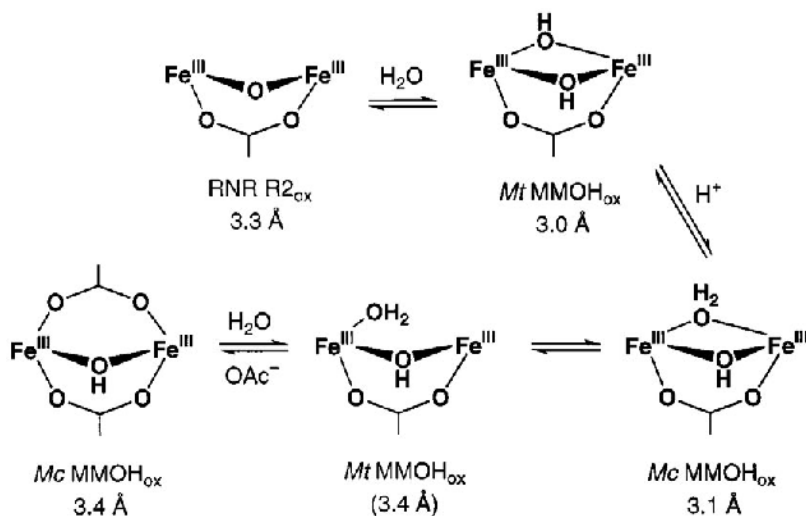


Figure 8 Proposed equilibria relating the different diiron core structures of MMOH_{ox} and R2_{ox} . Distances shown are derived from crystallography, except the value in parentheses, which is from EXAFS.



its susceptibility to photoreduction by x-rays [33]. However, the presence of an oxo-bridged diiron(III) center in $\Delta 9D_{ox}$ is indicated by a large Mössbauer quadrupole splitting [38] and a $\nu Fe-O-Fe$ feature at 530 cm^{-1} in its resonance Raman spectrum [39]. The $\nu Fe-O-Fe$ frequency of 530 cm^{-1} corresponds to an $Fe-O-Fe$ angle of 120° , which is 10° smaller than that found for RNR $R2_{ox}$. In agreement, preliminary EXAFS data on $\Delta 9D_{ox}$ also show an Fe-Fe distance of $3.15\text{ \AA}$, which is shorter than that found in RNR $R2_{ox}$. These two observations lead to the proposal that $\Delta 9D_{ox}$ has a $(\mu\text{-oxo})\text{bis}(\mu\text{-carboxylato})\text{diiron(III)}$ core, suggesting that, unlike RNR R2 and MMOH, $\Delta 9D$ may not undergo a carboxylate shift during its oxidation from the diiron(II) form to the diiron(III) form.

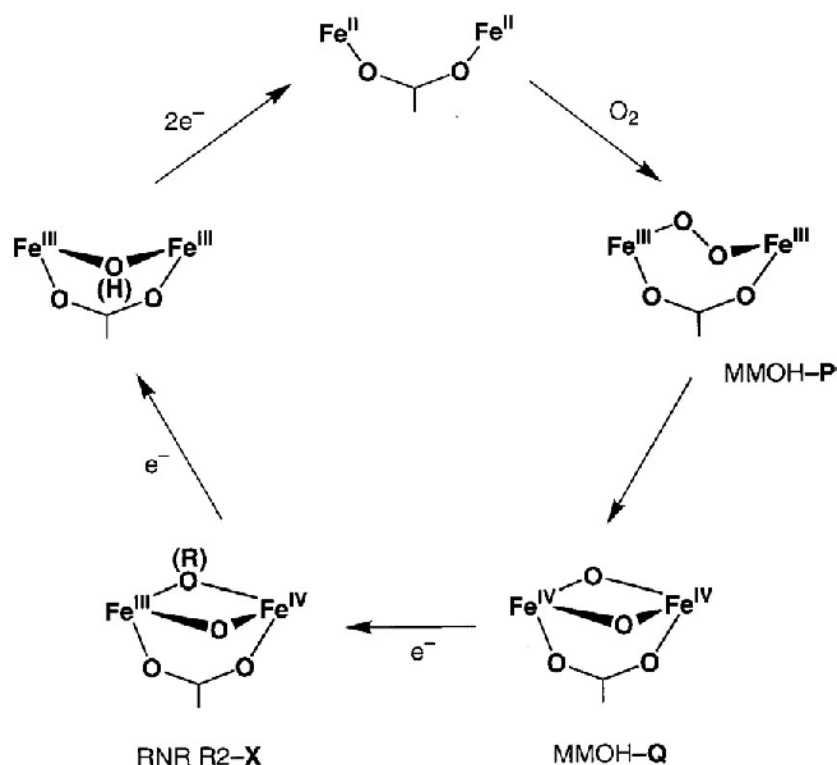


Figure 9 Common oxygen activation mechanism proposed for nonheme diiron enzymes.



II.B.3. Oxygen Activation Mechanism

Given the similarities among the active sites of RNR R2, MMOH, and $\Delta 9D$, it is attractive to consider a common mechanism for oxygen activation by nonheme diiron centers [69,70] (Figure 9). The first step is the binding of O_2 to the diiron(II) center. As in deoxyHr, the diiron(II) unit in these enzymes provides the two electrons necessary to reduce dioxygen, yielding a diiron(III) peroxide species. This intermediate then converts to a diiron(IV) species or, upon one-electron reduction, into an iron(III)iron(IV) species that can carry out the critical substrate oxidation steps in the enzyme mechanisms. The diiron(IV) intermediate effects the two-electron substrate oxidations of MMOH and $\Delta 9D$; the iron(III)iron(IV) species performs the one-electron oxidation of Tyr122 in RNR R2. These high-valent species are diiron analogs of iron-oxo intermediates associated with heme enzymes, i.e., heme peroxidase compounds I and II [8]. It may be argued that the second iron in the diiron(IV) species serves the function of the porphyrin in storing an oxidizing equivalent. The structures proposed for intermediates in this common mechanism are based on synthetic precedents (see next section); identical or related structures have been proposed by others, particularly with respect to the mechanism of MMOH [1,4,71–73]. A caveat to keep in mind: such a general scheme is a working model and will need to be tailored to individual enzymes, so particular intermediates may not be observable in particular cases and variations of the core structures and electronic descriptions are possible.

Rapid kinetic experiments provide direct spectroscopic evidence for some of these proposed intermediates in the reaction of O_2 with $MMOH_{red}$ and RNR $R2_{red}$. Two intermediates, **P** and **Q**, have been characterized in the reaction cycle of MMOH [57,74–77]. $MMOH-P$ was initially postulated by Lipscomb and coworkers from the kinetics of the disappearance of the characteristic $g = 16$ integer EPR signal of $MMOH_{red}$ [57] and subsequently identified spectroscopically by Lippard and coworkers [75–77]. $MMOH-P$ exhibits a Mössbauer spectrum with one quadrupole doublet ($\delta = 0.66$ mm/sec; $\Delta E_Q = 1.51$ mm/sec). Its isomer shift suggests that the metal ions are in the high-spin iron(III) state, though the observed δ value is somewhat higher than is typical for high-spin Fe(III) (0.45–0.55 mm/sec). The fact that only one doublet is observed indicates that the two iron sites must be in similar environments and antiferromagnetically coupled to each other. In addition, $MMOH-P$ exhibits a visible absorption band in the 600–700 nm region ($\epsilon = 1500 M^{-1}cm^{-1}$) associated with a peroxo-to-iron(III) charge transfer transition. Excitation into this band resulted in the observation of a Raman $\nu O-O$ band near 905 cm^{-1} , which is similar to those found for synthetic complexes with a μ -1,2-peroxo bridge (see next section), but the Raman result has been retracted [76]. Nevertheless, it is likely that $MMOH-P$ has a (μ -1,2-peroxo)diiron(III) core.

$MMOH-P$ converts in a first-order process to **Q**, the species directly respon-



sible for the hydroxylation of methane [57,77]. MMOH-Q is associated with comparably intense electronic absorption features at 330 and 430 nm ($\epsilon \sim 5000 \text{ M}^{-1}\text{cm}^{-1}$). Its Mössbauer properties ($\delta \sim 0.17 \text{ mm/sec}$) indicate that the two metal ions are in a higher-valent state and in quite similar environments. High-field studies show a diamagnetic ground state, indicative of an antiferromagnetically coupled diiron center. Taken together, these results assign MMOH-Q as a diiron(IV) species.

Structural insights into MMOH-Q have recently been obtained from tandem rapid-freeze-quench Mössbauer and EXAFS experiments [78]. New features corresponding to one scatterer per iron each at 1.8 and 2.5 Å are observed in samples containing a significant fraction of the intermediate; these features are absent in the spectra of MMOH_{red} and MMOH_{ox}. The 1.8 Å distance is too long to be associated with a terminal oxo atom as found for Compounds I and II of the heme peroxidases (1.64 Å) [79] and most likely arises from the Fe—O bonds of a (μ -oxo)diiron core. The 2.5 Å distance is associated with an Fe scatterer and is significantly shorter than the Fe-Fe distances found in the diiron(II) and diiron(III) forms of MMOH [30–32]. Such a short distance requires a rather compact diiron core structure with at least two single atom bridges, as observed in $[\text{Fe}_2(\mu\text{-OH})_3(\text{Me}_3\text{tacn})_2]^+$ (2.5 Å) [80] and $[\text{Mn}_2(\mu\text{-O})_2(\mu\text{-O}_2\text{CCH}_3)(\text{Me}_3\text{tacn})_2]^{2+}$ (2.6 Å) [81]. Thus a number of possible structures for MMOH-Q can be drawn, but only the $\text{Fe}_2(\mu\text{-O})_2$ diamond core shown in Figure 9 is precededented in high-valent synthetic diiron complexes (see next section).

The reaction of MMOH-Q with alkane substrates has been investigated. A mechanism of alkane hydroxylation following the heme paradigm would entail hydrogen atom abstraction from substrate by MMOH-Q to form an alkyl radical followed by C—O bond formation by oxygen rebound. Consistent with this mechanism, substantial kinetic isotope effects (KIEs) have been observed for the single turnover reaction of MMOH with methane, indicating that the C—H bond is cleaved in the transition state [82]. The intramolecular KIE value based on product analysis is large (9 for CH_2D_2); the intermolecular KIE value, obtained by measuring the decay of MMOH-Q in the presence of CH_4 or CD_4 , is 50–100. The latter is the largest KIE value observed in biology and is not currently understood. The lifetime of the putative alkyl radical has been assessed with the use of chiral substrates and radical clock probes. The oxidation of chiral ethane afforded alcohol products with some retention of stereochemistry [83,84]. The lack of complete scrambling of configuration suggests that if an alkyl radical were formed, its lifetime must be very short, on the order of 10^{-13} sec, the time scale of a single bond vibration. Similarly, studies with ultrafast radical clock probes did not reveal evidence for probe rearrangement [85], also implying a very short lifetime for the intermediate alkyl radical. These observations call into question whether an alkyl radical is actually involved in the MMOH mechanism as in the heme paradigm and have led to an alternative mechanism in which both



atoms of the scissile C—H bond interact with the high-valent center of MMOH-**Q** leading to O—H bond formation, C—H bond cleavage, and C—O bond formation in a concerted but not totally synchronous manner [85]. Clearly these mechanistic issues require further investigation.

The corresponding high-valent intermediate in the assembly of the diiron(III) center-tyrosyl radical cofactor of RNR R2 has also been identified by Stubbe and coworkers and designated as **X** [86,89]. This intermediate decays to the (μ -oxo)diiron(III) form at a rate commensurate with the appearance of the tyrosyl radical. Intermediate **X**, formally Fe(III)Fe(IV), exhibits an isotropic $S = 1/2$ spin EPR signal near $g = 2$, which is split by the introduction of ^{57}Fe and broadened by $^{17}\text{O}_2$ in the assembly reaction. These observations as well as Mössbauer results show that the unpaired spin must be associated with the diiron center [88,89].

The appropriate electronic description for the diiron center in RNR R2-**X**, either Fe(III)—O—Fe(III) or Fe(III)Fe(IV), has been a matter of discussion. The former description was favored initially, because the isotropic nature of the EPR signal appeared to preclude the involvement of an anisotropic Fe(IV) ion [89]. Subsequently the latter Fe(III)Fe(IV) description gained credence because of two developments: electron nuclear double resonance (ENDOR) evidence showing that one of the iron sites in RNR R2-**X** was indeed anisotropic [90] and the synthesis of a bona fide Fe(III)Fe(IV) complex that exhibited a similarly isotropic signal [91]. The “Fe(IV)” ion is associated with a Mössbauer isomer shift of 0.26 mm/sec [90]; this value is too low for a typical high-spin Fe(III) but higher than expected for high-spin Fe(IV). Therefore, RNR R2-**X** is best described to date as an antiferromagnetically coupled high-spin Fe(III)—high-spin Fe(IV) center with significant metal-ligand covalency, thereby conferring some radical character on the bridging oxo ligand.

The core structure of RNR R2-**X** has been probed by a number of methods. Rapid-freeze-quench Mössbauer and EXAFS experiments show that RNR R2-**X**, like MMOH-**Q**, has an Fe—O bond of 1.8 Å and an Fe-Fe distance of 2.5 Å [92], so the two intermediates may share the same core structure despite differences in the oxidation states. More information can be derived from **X**, however, since it is EPR active. Indeed, ^{17}O -ENDOR has been useful in demonstrating that the two atoms of O_2 are incorporated into **X** but have different A values [93], suggesting that a simple $\text{Fe}_2(\mu\text{-O})_2$ core derived from a (μ -peroxo)diiron(III) species may be unlikely. Analysis of the $^1\text{H}/^2\text{H}$ -ENDOR of the exchangeable hydrons of **X** suggests that the oxygen atom with the smaller A value is associated with a terminal water ligand on the Fe(III) site [94]. The oxygen atom with the larger A value is proposed to be the oxo bridge implicated in the EXAFS experiment [92] and the precursor to the oxo bridge in the diiron(III) form of RNR R2 [61].

To date, **X** is the only intermediate in the RNR R2 cycle that is well characterized. Another species called **U** was observed in initial experiments and associ-



ated with a visible absorption near 570 nm when the *apo*R2 protein was reacted with substoichiometric amounts of Fe(II) in the presence of O₂ [86,95]. Intermediate **U** was suggested to be a diiron(III)-peroxo intermediate in the initial report [86] and a tryptophan radical in a subsequent paper [96]; however, it is not clear at present whether **U** is a catalytically competent species. More recently, preliminary evidence for a **P**-like species has been found in the reaction of preformed RNR R2_{red} with O₂, based on the observation of a Mössbauer doublet with parameters identical to those of MMOH-**P** [96]. If substantiated, this observation would support the notion that the carboxylate-rich diiron active sites utilize a common mechanism for activating O₂.

The oxidative mechanism of RNR R2 differs from that of MMOH in requiring an additional electron, since Tyr122 provides only a single electron. This electron is needed to convert “**P**” to **X**. It has been shown that external reductants such as excess Fe(II) or ascorbate can provide this electron in *in vitro* reconstitution reactions [87,97]. Since the diiron site is buried 10 Å below the protein surface, a long-range electron transfer pathway is required to deliver this extra electron to the diiron center. Such a pathway involving a number of amino acid residues has been proposed from examining the crystal structure of R2 [98].

Despite the differences in their electron requirements, there is one further observation that appears to tie the oxidative mechanisms of RNR R2 and MMOH together—the hydroxylation of Y208 to 3,4 dihydroxyphenylalanine (DOPA) in the F208Y variant of RNR R2 [99]. F208 is a residue in the putative O₂ binding pocket of RNR R2. In the absence of O₂, the Y208 residue in the mutant protein is unchanged, but the protein acquires an intense blue color (λ_{max} 700 nm) upon exposure to O₂ that is associated with a catecholate-to-Fe(III) charge transfer band by resonance Raman studies [100]. X-ray crystallography shows that Y208 has been hydroxylated to form a bound DOPA residue (Figure 10) [99]. The

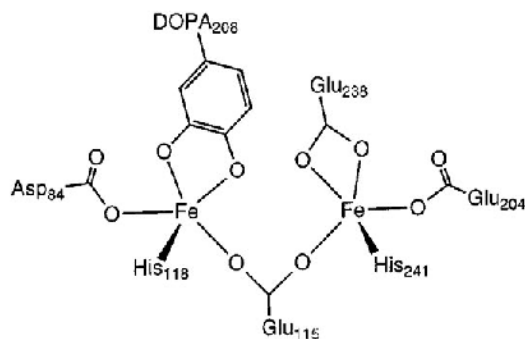


Figure 10 Active structure of DOPA208 RNR R2.



replacement of F208 by the more electron-rich tyrosine residue may provide a suitable target for two-electron oxidation by the as yet unobserved high-valent intermediate analogous to MMOH-Q in the RNR R2 cycle. Interestingly, the oxygen atom incorporated into DOPA208 derives from solvent water, not from O₂ [100]. Given the similarities of the diiron sites of RNR R2 and MMOH and this parallel in their chemistries, it may be expected that the mechanistic scheme proposed in Figure 9 would extend to fatty acid desaturases and arene hydroxylases when their catalytic mechanisms become better characterized.

II.B.4. Reactivity Models

Our understanding of the oxygen activation chemistry of the nonheme diiron enzymes has been greatly enhanced by synthetic complexes that mimic the reactivity of the diiron centers with dioxygen. For example, a number of synthetic nonheme iron(II) complexes that can bind O₂ have been characterized in the past few years [101–106]. The O₂ adducts derive from iron(II) precursors of two general types. [Fe(Tp^{3,5-i-Pr2})(O₂CPh)(CH₃CN)] [101,105] is a mononuclear 6-coordinate complex with a labile solvent ligand; it binds O₂ reversibly in toluene at –20°C to form a 2:1 adduct. On the other hand, [Fe₂(N-Et-hptb)(O₂CPh)]²⁺ is a diiron(II) complex of a dinucleating ligand based on a 2-hydroxy-1,3-diaminopropane backbone with pendant benzimidazoles; it has two 5-coordinate iron centers and binds O₂ irreversibly in CH₂Cl₂ at –60°C to form a 1:1 adduct [102,104]. O₂ binding becomes reversible when the pendant benzimidazoles are replaced with 6-methylpyridines [103,106].

Both of these types of O₂ adducts have been crystallized, and three structures were reported in rapid succession in 1996 (Figure 11). [Fe₂(μ-O₂)(Tp^{3,5-i-Pr2})₂(O₂CCH₂Ph)₂] is a (μ-1,2-peroxo)diiron(III) species supported by two bridging carboxylates [107], whereas [Fe₂(μ-O₂)(N-Et-hptb)(OPPh₃)₂]³⁺ has a (μ-1,2-peroxo)(μ-alkoxo)diiron(III) core [108]. A variation of the latter is [Fe₂(μ-O₂)(Ph-bimp)(O₂CPh)]²⁺, where Ph-bimp is a dinucleating ligand that provides a phenolate instead of an alkoxide to act as a bridge between iron centers and pendant imidazoles with bulky phenyl substituents as terminal ligands [109]. Its structure reveals an adduct with a (μ-1,2-peroxo)(μ-phenoxo)(μ-benzoato)diiron(III) core and represents the first of the three crystal structures reported.

The three structures reveal a number of interesting features for these O₂ adducts. In all three cases, the O₂ complexes derive from coordinately unsaturated iron(II) precursors, assuring rapid formation of the adducts. The O—O bond lengths are 1.42 ± 0.01 Å, in agreement with the peroxide formulation for the 1,2-dioxygen bridge. The Fe^{III}—O_{peroxo} bond lengths average 1.88 Å, comparable to Fe^{III}—OH bond lengths [110] and consistent with the basicity of the peroxide ligand. The peroxide coordinates trans to equivalent ligands in the structures of [Fe₂(μ-O₂)(μ-O₂CCH₂Ph)₂(Tp^{3,5-i-Pr2})₂] and [Fe₂(μ-O₂)(N-Et-hptb)(OPPh₃)₂]³⁺



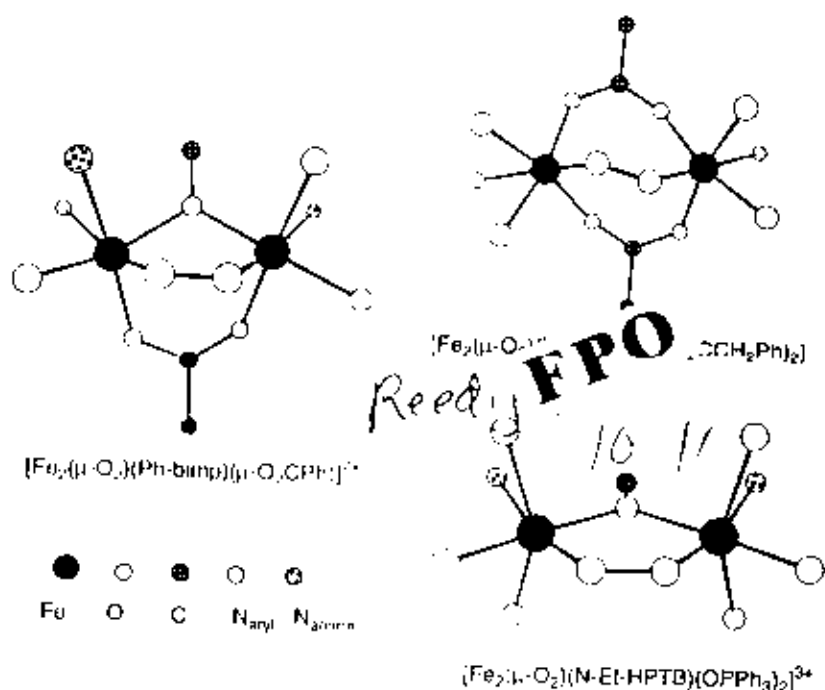


Figure 11 Core structures of synthetic diiron-O₂ adducts.

and thus acts as a symmetric bridge between two essentially equivalent iron centers in both complexes. In contrast, the peroxide in $[\text{Fe}_2(\mu\text{-O}_2)(\text{Ph-bimp})(\text{O}_2\text{CPh})]^{2+}$ coordinates *trans* to an imidazole on one iron and *trans* to an amine on the other iron, resulting in distinct iron sites with Fe—O_{peroxo} bonds that differ by almost 0.1 Å in length.

The spectroscopic properties of these synthetic O₂ adducts serve as useful benchmarks for methane monooxygenase (MMO) intermediate **P**. These adducts exhibit a resonance-enhanced Raman feature near 900 cm⁻¹, which downshifts the expected 40–50 cm⁻¹ with ¹⁸O₂ [104–106]. Two of the adducts exhibit the unusual Mössbauer isomer shift of 0.66 mm/sec first observed for MMOH-**P** [77], which is about 0.1 mm/sec higher than typical of a high-spin iron(III) site, suggesting that such an isomer shift may be associated with iron sites that can bind O₂. However, this suggestion must be tempered by the fact that oxyHr and $[\text{Fe}_2(\mu\text{-O}_2)(\text{N-Et-hptb})(\text{O}_2\text{CPh})]^{2+}$ exhibit isomer shifts typical of high-spin iron(III) centers [41,102], so the chemical significance of the unusual isomer shift is not yet understood. Nevertheless, the excellent match in the properties



of MMOH-**P** and $[\text{Fe}_2(\mu\text{-O}_2\text{CCH}_2\text{Ph})_2(\text{Tp}^{3,5\text{-iPr}_2})_2]$ makes a (μ -1,2-peroxo)bis(μ -carboxylato)diiron(III) core structure a good working model for MMOH-**P**.

The extended lifetimes of these synthetic O_2 adducts, albeit requiring low temperature in some cases, provide some insight into the factors that modulate the reactivity properties of (μ -peroxo)diiron(III) species [111]. It is clear that the introduction of sterically bulky groups contributes significantly to the stabilization of the adducts, thereby allowing their isolation and characterization. Electronic factors also play an important role in determining the stability of the O_2 adducts, as illustrated by studies involving substitutions of the carboxylate bridge. For example, replacement of the benzoate bridge in $[\text{Fe}_2(\mu\text{-O}_2)(6\text{-Me}_4\text{-hptp})(\text{O}_2\text{CPh})]^{2+}$ with trifluoroacetate afforded a thermally more stable O_2 adduct [106]. Similarly, the rates of decomposition at -10°C of a series of $[\text{Fe}_2(\mu\text{-O}_2)(\text{N-Et-hptb})(\text{O}_2\text{CC}_6\text{H}_4\text{-X})]^{2+}$ adducts correlate with the σ values of the phenyl ring substituent X, affording a ρ value of -1.1 [108]. These observations indicate that the stability of the (μ -1,2-peroxo)diiron(III) unit can be modulated by the amount of electron donation by the ligands, with increasing electron density required to activate the O-O bond. This correlation rationalizes the effect of Ph_3PO or dimethyl sulfoxide (dmsO) on the O_2 adducts of $[\text{Fe}_2(\text{L})(\text{O}_2\text{CPh})]^{2+}$ ($\text{L} = \text{hptb}$ and htpb) [109]. In the crystal structure of $[\text{Fe}_2(\mu\text{-O}_2)(\text{N-Et-hptb})(\text{OPPh}_3)_2]^{3+}$, the anionic carboxylate bridge has been replaced by neutral phosphane oxide ligands, thereby decreasing the electron density at the diiron center and stabilizing the O_2 adduct [108]. When extended to the nonheme diiron proteins, this correlation can be used to rationalize the use of a histidine-rich diiron center for a reversible O_2 binding protein such as hemerythrin and a carboxylate-rich diiron center for O_2 activating enzymes such as RNR R2, MMOH, and $\Delta 9\text{D}$.

The next step in the proposed mechanism for nonheme diiron enzymes is the conversion of the peroxo intermediate into a high-valent species. To date, none of the synthetic diiron O_2 adducts described gives rise to a high-valent intermediate that can be characterized. Only one, $[\text{Fe}_2(\mu\text{-O}_2)(\text{hptp})(\text{OPPh}_3)_2]^{3+}$, has been found to oxidize a substrate related to those of the enzymes of interest, i.e., 2,4-di-*tert*-butylphenol, which is analogous to Tyr122 of RNR R2 [87], but none of these can effect hydrocarbon oxidation. This lack of reactivity may derive from the restrictions imposed by the ligand design in order to stabilize the O_2 adducts.

The only synthetic high-valent complexes of relevance to the nonheme diiron enzymes derive from the reaction of $[\text{Fe}^{\text{III}}_2(\mu\text{-O})(\text{L})_2(\mu\text{-H}_3\text{O}_2)]^{3+}$ ($\text{L} = \text{tris}(2\text{-pyridylmethyl})\text{amine}$ [tmpa] and its ring-alkylated derivatives) and H_2O_2 [70,91,110,112]. It is suggested that the presence of the oxo group and the readily displaceable H_3O_2^- bridge is the key to achieving this higher-valent chemistry. Thus treatment of these diiron(III) complexes with H_2O_2 affords $[\text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2(\text{L})_2]^{3+}$ (Figure 12). Though their instability has thus far prevented crystallographic characterization, the use of low-temperature spectroscopy has allowed



the formulation of a structure for these complexes. First, electrospray ionization mass spectrometry determined the molecular composition of the high-valent species to be $[\text{Fe}_2(\text{O})_2(\text{L})_2]^{3+}$ [110]. For the charge to balance, this formulation required one iron center to be Fe(III) and the other to be Fe(IV), a notion confirmed by Mössbauer for the 6-Me-tmpa complex [91]. The EXAFS spectra of these complexes revealed a strong feature in the second coordination sphere that could only be associated with an Fe-Fe distance of 2.9–3.0 Å [110]. Such intense features at similar distance are typical for complexes with $\text{M}_2(\mu\text{-O})_2$ diamond cores [113–116]. Taken together, these data strongly implicate a structure with an $\text{Fe}_2(\mu\text{-O})_2$ diamond core for the $[\text{Fe}_2(\text{O})_2(\text{L})_2](\text{ClO}_4)_3$ complexes (Figure 12).

There are two types of $[\text{Fe}^{\text{III}}\text{Fe}^{\text{IV}}(\mu\text{-O})_2(\text{L})_2](\text{ClO}_4)_3$ complexes that have been characterized thus far (Table 2). An $S = 3/2$ species is obtained for $L = \text{tmpa}$ or a 5-alkylated derivative [110], and an $S = 1/2$ species is formed when $L = 6\text{-Me-tmpa}$ [91]. The different electronic properties can be determined by considering the spin states of the iron ions in the two types of complexes. The $S = 1/2$ species derives from the antiferromagnetic coupling of a high-spin Fe(III) ($S_1 = 5/2$) and a high-spin Fe(IV) ($S_2 = 2$) ion. This affords a valence localized pair, as indicated by the distinct isomer shifts of the metal ions corresponding to two different oxidation states ($\delta = 0.48$ mm/sec for the Fe^{III} site and $\delta = 0.08$ mm/sec for the Fe^{IV} site). On the other hand, the $S = 3/2$ species exhibits only one set of Mössbauer parameters ($\delta = 0.14$ mm/sec), consistent with a valence-delocalized low-spin Fe^{III} ($S_1 = 1/2$) and low-spin Fe^{IV} ($S_2 = 1$) pair. The change in spin state from low spin to high spin upon the introduction of a 6-methyl group has been explained with steric arguments [117]. These synthetic complexes

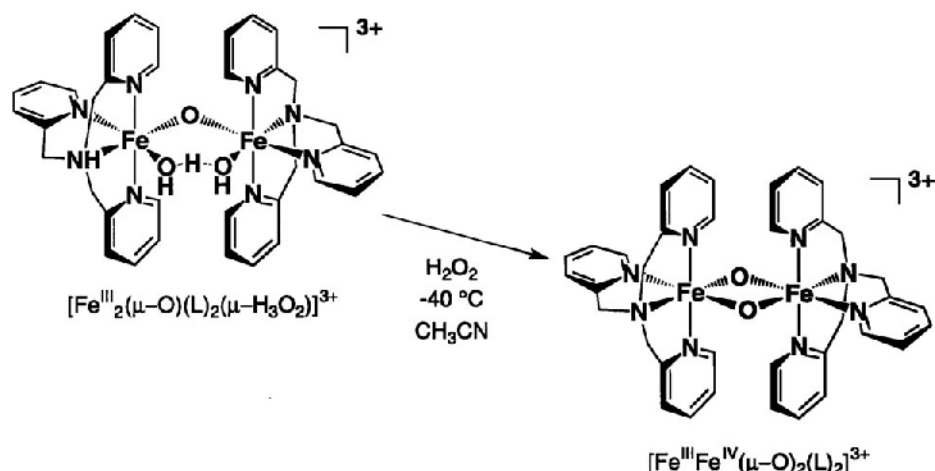


Figure 12 Formation of the $[\text{Fe}_2(\mu\text{-O})_2]^{3+}$ diamond core.



Table 2 Properties of Synthetic $[\text{Fe}_2(\mu\text{-O})_2(\text{L})_2]^{3+}$ Complexes^a

	5-R ₃ -tmpa (R = H, CH ₃)	6-Me-tmpa
EPR	$S = 3/2$	$S = 1/2$
Mössbauer δ	One iron site, 0.17 mm/sec	Two iron sites, 0.48 and 0.08 mm/sec
λ_{max}	616 nm (green)	360 nm (Brown)
Spin state	Low-spin Fe(III)/Fe(IV)	High-spin Fe(III)/Fe(IV)
Exchange coupling	Valence delocalized via ferromagnetic and/or double exchange	Valence localized via anti-ferromagnetic coupling
Fe-Fe distance (EXAFS)	2.9 Å	

^a Data from Refs. 70, 91, 110, and 111.

demonstrate that the Fe(IV) oxidation state can be attained in a nonheme ligand environment and stabilized by the bis(μ -oxo) core structure.

Significantly, these synthetic high-valent diiron species model properties of the high-valent intermediates of the nonheme diiron enzymes. For example, $[\text{Fe}_2(\mu\text{-O})_2(6\text{-Me-tmpa})_2]^{3+}$ has EPR properties that resemble those of RNR R2-X [91], which have been helpful in clarifying the nature of the diiron center of RNR R2-X [90]. Furthermore $[\text{Fe}_2(\mu\text{-O})_2(\text{tmpa})_2]^{3+}$ has been shown to effect oxidations related to those carried out by the nonheme diiron enzymes [118]. This complex can oxidize 2,4-di-*tert*-butylphenol to its phenoxy radical, mimicking the oxidation of Tyr122 by RNR-R2 X in the assembly of the diiron(III)-tyrosyl radical cofactor found in active RNR R2. It also oxidizes cumene to afford cumyl alcohol and α -methylstyrene, analogous to hydroxylation and desaturation reactions catalyzed by MMO and fatty acid desaturases, respectively. Because of its somewhat limited oxidizing power, $[\text{Fe}_2(\mu\text{-O})_2(\text{tmpa})_2]^{3+}$ does not attack hydrocarbons like cyclohexane. Such a reaction presumably requires a diiron(IV) complex, as utilized by MMOH, but synthetic analogs of this intermediate are not yet available. Nevertheless, it is clear that the high-valent $[\text{Fe}_2(\mu\text{-O})_2]$ diamond core displays a versatile oxidative reactivity that encompasses the range of reactions catalyzed by the nonheme diiron enzymes. This versatility supports the notion that a high-valent species with an $\text{Fe}_2(\mu\text{-O})_2$ diamond core (or some variation thereof) may be the common feature of the oxidative mechanisms of this family of enzymes.

II.C. Mononuclear Iron Enzymes

Because of the increasing availability of protein structures, our understanding of nonheme iron enzymes with mononuclear active sites has also developed significantly since the first edition of this book. In addition to the previously reported



crystal structure of an intradiol cleaving catechol dioxygenase, those for an extradiol cleaving catechol dioxygenase, a pterin-dependent hydroxylase, and isopenicillin N synthase have also been solved. In some cases, corresponding structures for substrate and/or inhibitor complexes are also available. These structures corroborate and amplify the insights derived from earlier spectroscopic studies and allow the focus to shift to questions of mechanism.

II.C.1. Intradiol Cleaving Catechol Dioxygenases

The catechol dioxygenases serve as part of nature's strategy for degrading aromatic molecules in the environment [119] that derive from a variety of natural products (lignin, for example) as well as man-made sources. The enzymes are found in soil bacteria such as pseudomonads and bacilli and act in the last step of transforming aromatic precursors into aliphatic products [120,121]. These enzymes are called dioxygenases because the elements of dioxygen become incorporated into the product [122,123]. The catechol substrates can be oxidatively cleaved by nonheme iron dioxygenases in two distinct modes (Figure 13). The intradiol cleaving enzymes require Fe(III); the extradiol cleaving enzymes utilize Fe(II).

The crystal structure of the intradiol cleaving protocatechuate 3,4-dioxygenase (PCD) (EC 1.13.11.3) from *Pseudomonas putida* [124] reveals a multimeric enzyme consisting of 12 $\alpha\beta$ protomers with the active site found at the interface between α and β subunits. The iron center has a trigonal bipyramidal coordination environment with Tyr447 and His462 as the axial ligands and Tyr408 and His460 as the equatorial ligands, all derived solely from the β subunit (Figure 14). The third equatorial ligand is assigned to a solvent molecule. These ligands appear to be the only bases present in the active site pocket.

The iron coordination environment defined by the crystal structure corresponds remarkably well to that proposed earlier on the basis of spectroscopic studies [125]. The presence of two distinct tyrosine ligands was indicated by its resonance Raman spectrum and a visible spectrum with two tyrosinate-to-

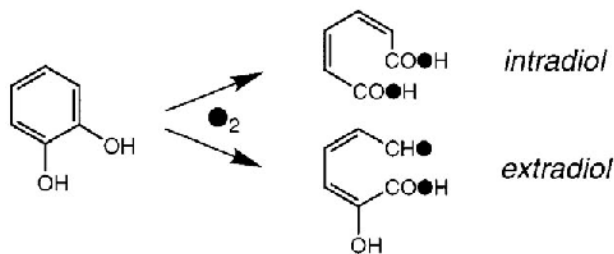


Figure 13 Modes of catechol cleavage.



iron(III) charge transfer bands of different energy [126,127]. The participation of histidines was inferred from the observation of a low-energy $\nu\text{Fe}-\text{N}(\text{Im})$ feature at 276.5 cm^{-1} [128], which downshifted to 274 cm^{-1} in D_2O [129], and the presence of second and third shell EXAFS features ascribable to imidazole ring atoms at ca. 3.0 and 4.3 Å from the metal center [128]. That solvent water was bound to the metal center was demonstrated by the line broadening found in the EPR spectrum of the native enzyme from *Brevibacterium fuscum* when dissolved in H_2^{17}O [130]. Subsequent analysis of the first shell EXAFS data showed that the solvent water must be bound as hydroxide [131].

Spectroscopic changes upon anaerobic addition of substrate indicate that it must bind to the active site iron. EPR studies of the protocatechuate 3,4-dioxygenase–3,4-dihydroxyphenylacetate (PCD-dhpa) complex showed that the sharp $g = 4.3$ EPR signal was broadened when either catecholate oxygen was labeled with ^{17}O [132], suggesting the coordination of both catecholate oxygens. Furthermore, a new ligand-to-metal charge transfer band appeared at low energy in the visible absorption spectra of the enzyme–substrate (ES) complexes. Excitation into this band elicited resonance Raman vibrations characteristic of a chelated dianion [133,134], so substrate binding must entail the loss of both its hydrogens. Since the only bases in the PCD active site are the iron ligands, two ligands must be displaced upon substrate binding. Given the solvent accessible surface of the PCD active site, plausible candidates for displacement are the equatorial hydroxide and one of the axial ligands. These ideas have been confirmed by the recently solved crystal structures of PCD complexed with protocatechuate (3,4-dihydroxybenzoate) and 3,4-dihydroxyphenylacetate (dhpa) [134,135], which reveal a 5-coordinate iron site with a didentate catecholate displacing the hydroxide and Tyr447 (Figure 14).

The primary question of bioinorganic interest is the role of the iron center.

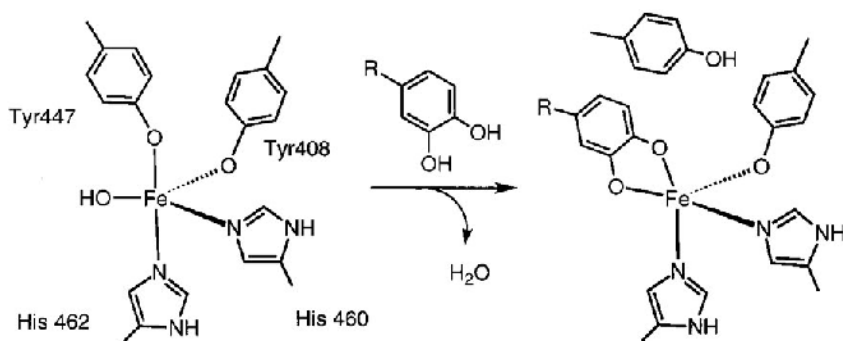


Figure 14 Active sites of PCD as isolated and its substrate complex.



It was clear at the start that the native enzyme had a high-spin iron(III) center [136] and that the enzyme mechanism involved initial substrate binding followed by O_2 attack [137]. An attractive mechanism postulated early in these studies suggested the reduction of the Fe(III) by substrate, followed by dioxygen binding to the Fe(II) center. However, EPR and Mössbauer studies showed that the iron retained its high-spin iron(III) oxidation state even after the substrate bound [138,139]. Stopped-flow kinetic studies revealed the involvement of several intermediates subsequent to O_2 attack on the ES complex. Since these intermediates retained their visible chromophores, it was deduced that the metal center remained Fe(III) in these species [140,141]. (Reduction of native enzyme by dithionite resulted in a colorless solution, which regained its burgundy color upon exposure to O_2 .) These results have led to a proposed substrate activation mechanism in which the coordination of catechol to the iron(III) center activates the catechol for direct attack by O_2 [142] (Figure 15).

Besides removing the substrate hydrons, the enzyme active site promotes the activation of the substrate in a number of ways. Several lines of evidence indicate that the substrate is unsymmetrically chelated: i.e., one of the

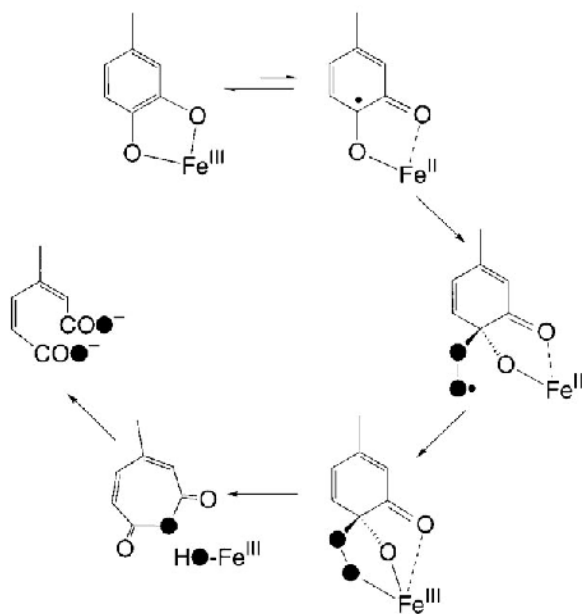


Figure 15 Proposed substrate activation mechanism for the intradiol cleaving catechol dioxygenases.



Fe—O_{catecholate} bonds is weaker than the other. This is consistent with early steady-state inhibition kinetics results showing that *p*-hydroxybenzoates are significantly better inhibitors of PCD than the corresponding *meta* isomers [142]. Resonance Raman data on the PCD-dhpa complex using specifically [¹⁸O]-labeled dhpa show that the Fe—O4 stretch is 70 cm⁻¹ higher in energy than the Fe—O3 vibration [134]. More importantly, the crystal structures of both PCD ES complexes reveal that the Fe—O4 bond is ca. 0.2 Å shorter than the Fe—O3 bond [134,135]. This bond length asymmetry may result from different ligands trans to the catecholate oxygens (anionic Tyr408 vs. neutral His462) as well as the presence of hydrogen bonding interactions to O3 from Arg457. The longer Fe—O3 bond may allow the substrate to undergo keto-enol tautomerization at C-3, generating a carbanion at C-4 (Figure 16, X = C) that then reacts with O₂. Consistent with this notion is the observation that 2-hydroxypyridinones (Figure 16, X = N) are slow but tight binding irreversible inhibitors of PCD [143,144]. Their inhibitory properties are postulated to result from the preference of the inhibitor for its ketonized form, mimicking the structure of the activated substrate. Crystal structures of these inhibitor complexes show that the inhibitors bind in the same manner as the substrate [135]. In support of the keto-enol tautomerization, closer scrutiny of the structures of the ES complexes shows that there is a strictly conserved Arg457 residue in the active site, situated close to C-4 of the substrate, that appears poised to stabilize increased electron density at this carbon and facilitate the attack of O₂ [135]. However, the expected distortion of C-4 toward a more tetrahedral geometry upon ketonization at C-3 is not large enough to be apparent in the crystal structures.

A problem with the formation of the ternary ES—O₂ adduct is that it entails an attack of triplet O₂ on the singlet substrate, which is a forbidden reaction. However, the presence of iron(III) may provide a mechanism for relaxing this barrier, as suggested by biomimetic studies showing that oxidative cleavage of catechols can be catalyzed by iron(III) centers [145,146]. Building on this important early work, Que and coworkers synthesized a series of [Fe(L)(dbc)] complexes where L is a tetradentate tripodal ligand based on trimethylamine with

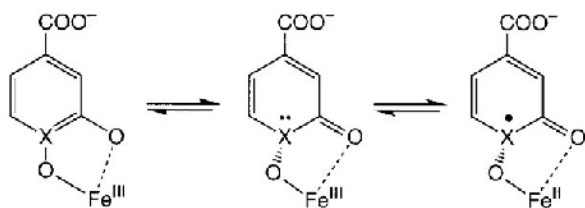


Figure 16 Proposed activated forms of the bound substrate in PCD.



pendant pyridine, carboxylate, or phenolate ligands ($H_2dbc = 3,5\text{-di-}tert\text{-butylcatechol}$) [147–149]. Three of these complexes were structurally characterized and found to have a 6-coordinate metal center with a chelated dianionic dbc ligand. All the $[Fe(L)(dbc)]$ complexes reacted with O_2 to yield the intradiol cleavage product. Notably the yield of intradiol cleavage product increased and approached quantitative conversion with increased Lewis acidity of the iron center, as indicated by the decreasing energies of the catecholate-to-iron(III) charge transfer bands. More significantly, the rate of reaction was accelerated by three orders of magnitude for the ligand with three pendant pyridines relative to that with three pendant carboxylates. Thus the most Lewis acidic metal center as indicated by the energy of the catecholate-to-iron(III) charge transfer transition was the most reactive. It was therefore proposed that increased Lewis acidity of the iron center enhanced the covalency of the iron-catecholate bond and increased the semiquinone character of the bound catecholate (Figure 16, $X = C$), an idea that was corroborated by the increase in the upfield NMR shifts of the catecholate hydrons in the various complexes [148]. (This Fe(III)-catecholate/Fe(II)-semiquinone tautomerization would also be favored by the asymmetric chelation of the catecholate and ketonization at C-3 suggested from the enzyme studies [Figure 16].) Thus the induction of radical character into the substrate would relax the singlet–triplet barrier for the reaction and promote the attack of O_2 on the substrate.

The peroxy intermediate derived from O_2 attack on the activated substrate is proposed to act as a tridentate ligand to the iron(III) center as shown in Figure 15. Molecular modeling of this adduct using the structure of the PCD ES complex as a starting point shows that such a structure is reasonable [133]. This coordination mode is preceded in the structure of the O_2 adduct of $[Ir(III)(triphos)(dbc)]^+$ [150,151].

The decomposition of the peroxy intermediate leads to the oxidative cleavage of the substrate. Two modes of decomposition have been considered. An early favorite was the formation of a dioxetane, which was favored because it easily explains the dioxygenase nature of the reaction. However, it has been argued by Hamilton [152] that an intermediate with such a four-membered ring requires too much energy to justify its formation in an enzyme reaction. The alternative, shown in Figure 15, involves a Criegee-type rearrangement of the peroxy intermediate to form a muconic anhydride and an Fe(III)—OH moiety. Such a rearrangement could perhaps be promoted by coordination of the peroxide to the iron center. To maintain the dioxygenase nature of the reaction, the Fe(III)—OH moiety that attacks the anhydride would have to be protected from exchange with bulk solvent. It is clear from an analysis of the products that an anhydride is in fact formed in the biomimetic reactions [147–149], and evidence for an anhydride intermediate in the enzymes has been obtained from $^{18}O_2$ labeling studies using slower substrates. The reaction of catechol 1,2-dioxygenase with 1,2,3-trihydroxybenzene affords α -hydroxy-*cis,cis*-muconic acid, which is



only partially doubly labeled [153]. The fact that some of the product is only singly labeled is a result that cannot be explained by the dioxetane mechanism and favors the anhydride mechanism. The loss of label is presumably a result of some exchange of the Fe(III)—OH species with solvent that is due to the slowness of the reaction.

II.C.2. Extradiol Cleaving Catechol Dioxygenases

In contrast to their intradiol cleaving counterparts, the extradiol cleaving catechol dioxygenases utilize mainly Fe(II) and represent the more common oxidative cleavage pathway in nature. Because of the difficulty of probing Fe(II) centers, our understanding of these enzymes is not as well developed. This situation has improved in recent years, and the first crystal structures of an extradiol cleaving dioxygenase, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) (EC 1.13.11.39) are now available [154–156]. Crystal structures of both the active Fe(II) enzyme and the inactive Fe(III) enzyme reveal a square pyramidal iron site with His146 as the apical ligand and His210, Glu260, and two solvent molecules in the equatorial plane (Figure 17). This picture concurs with previously obtained spectroscopic (near infrared circular dichroism [IR CD], MCD, and x-ray absorption spectroscopy [XAS]) data on catechol 2,3-dioxygenase (2,3-CTD) (EC 1.13.11.2) [157,158], and NMR studies of 2,2',3-trihydroxybiphenyl dioxygenase (EC 1.13.11 class) [159]. Sequence comparisons with a number of extradiol dioxygenases [160], including a Mn(II)-dependent enzyme [161], show that these three residues are strictly conserved in this superfamily and thus likely to be the ligands to the metal centers of these enzymes as well.

The anaerobic addition of catechol to catechol 2,3-dioxygenase (2,3-CTD) results in the formation of an ES complex that retains the Fe(II) oxidation state of the as-isolated enzyme as indicated by a number of spectroscopic (Mössbauer [162,163], XAS [158], near IR CD and MCD [157]) methods. These methods also indicate that the iron center remains 5-coordinate. Crystallographic studies

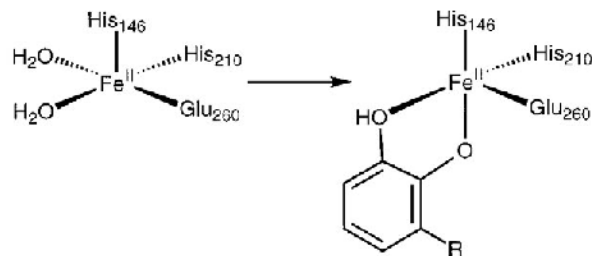


Figure 17 Active sites of BphC as isolated and its substrate complex.



of the BphC ES complex are consistent with this conclusion and show that the substrate coordinates in a didentate fashion (Figure 17) (J.F. Bolin, personal communication, 1996) [155]. One catecholate oxygen occupies the vacant sixth site in the as-isolated enzyme, and the other catecholate oxygen displaces the water ligand *trans* to His210. The other water ligand is presumably lost, if the 5-coordinate iron(II) site indicated by spectroscopy is to be maintained.

Like the PCD ES complex, the BphC ES complex also has an unsymmetrically chelated catecholate [155]. A similar bond length asymmetry was also deduced from EXAFS studies on the 2,3-CTD-catechol complex, which showed four O/N scatterers at 2.10 Å and one scatterer at 1.93 Å [158]. This short bond is comparable in length to one Fe—O_{catecholate} bond in the crystal structure of a synthetic Fe(II)-catecholate complex, [Fe(6-Me₃-tmpa) (H-dbc)]⁺ [164]. The significant asymmetry in catechol binding in the synthetic complex stems from the presence of a didentate but monoanionic catecholate. The much weaker affinity of Fe(II) for catecholate explains why the monoanionic ionization state is favored in the Fe(II) complex, a situation that is also likely to apply in the ES complexes.

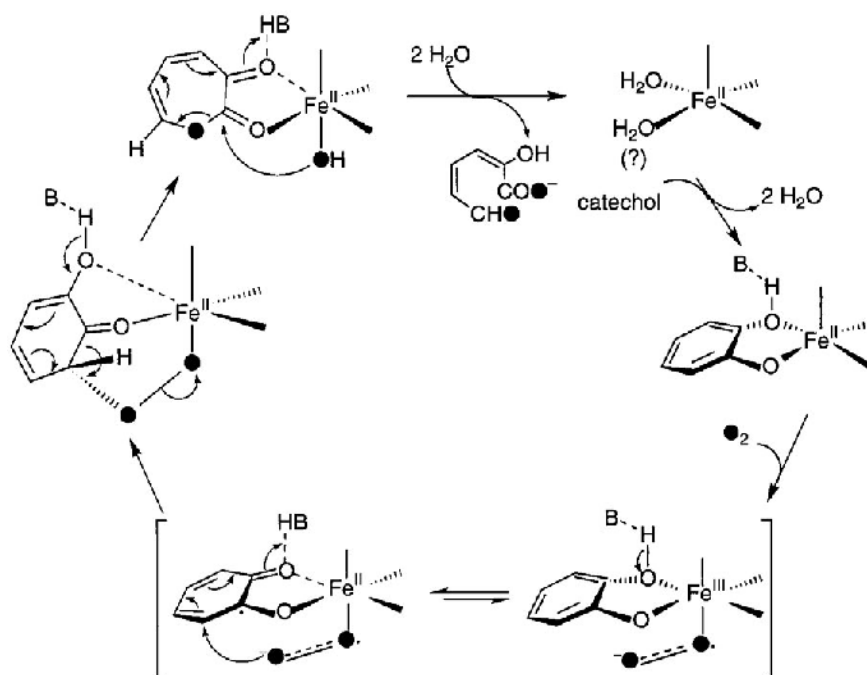
The binding of substrate primes the iron center to interact with O₂. The as isolated Fe(II) enzyme is not air sensitive, but the coordination of substrate introduces another anion into the metal coordination sphere and presumably shifts its potential to more negative values, thereby making the metal center able to bind O₂. No ternary ES—O₂ complex of an extradiol dioxygenase has been observed, presumably because of its rapid decomposition to product. However, NO has proved useful as an odd-electron O₂ surrogate and forms a ternary ES—NO complex that provides useful spectroscopic information [165,166]. Upon binding of NO, the normally EPR-silent high-spin Fe(II) center (*S* = 2) is converted into an EPR-active *S* = 3/2 center with EPR signals near *g* = 4. Many of these spectra are sharp and allow hyperfine line broadening from ¹⁷O-labeled substrates to be observed. The introduction of ¹⁷O at either catecholate oxygen of the substrate protocatechuate broadens the EPR features associated with the ES—NO complex of protocatechuate 4,5-dioxygenase (4,5-PCD) (EC 1.13.11.8) [165,166], indicating that the substrate persists as a didentate ligand in the ternary complex. These results show that there are three available coordination sites on the iron center for exogenous ligand binding, two for substrate and one for NO (and presumably O₂).

Table 3 compares some key properties of the enzyme–substrate complexes of the intradiol and extradiol cleaving dioxygenases. A mechanism for extradiol cleavage must account for the differing metal requirements of the two classes of enzymes and their distinct regiospecificities. A key difference is the reactivity of the ES complexes toward NO. Whereas the ES complexes of extradiol enzymes readily react with NO to form ES—NO adducts, those of intradiol enzymes do not react with NO unless the Fe(III) center is reduced prior to exposure to NO [167]. Thus the Fe(II) center in the extradiol cleaving enzymes appears



Table 3 Comparison of the Key Properties of the Dioxygenase–Substrate Complexes

	Intradiol cleaving	Extradiol cleaving
Metal center	5-Coordinate Fe(III)	5-Coordinate Fe(II)
Endogenous ligands	1 Tyr, 2 His	1 Glu, 2 His
Substrate binding mode	Didentate dianionic	Didentate monoanionic
Reaction with NO	Prior reduction required	Immediate
Proposed mechanism	Electrophilic attack of bound substrate by O_2	Nucleophilic attack of bound substrate by O_2^-

**Figure 18** Proposed mechanism for the extradiol cleavage of catechol.

electronically poised to react with NO (and O₂ by inference); indeed the coordination of the substrate significantly increases the affinity of the iron center for NO [165,166]. It would thus appear that the extradiol enzymes carry out oxidative cleavage by O₂ activation, rather than substrate activation.

A mechanism for extradiol cleavage is proposed in Figure 18 [5,158]. Substrate binds first to the iron(II) center, followed by O₂, to form a ternary complex akin to the ES—NO complex described earlier. Electron transfer from metal to O₂ in the Fe(II)—O₂ adduct results in a superoxide-like moiety and imparts nucleophilic character to the bound O₂. This in turn generates semiquinone character on the bound substrate, which is attacked by the nascent superoxide to form a peroxy intermediate that decomposes by a Criegee-type rearrangement to the observed product.

There are two carbons at which the attack can occur, a carbon of the enediol unit or a carbon adjacent to the enediol unit. Examination of the crystal structure of the BphC ES complex suggests that attack at an enediol carbon would not require a significant change in the conformation of the active site. However, such an attack would give rise to a peroxy intermediate very similar to that proposed for the intradiol cleavage mechanism (see Figure 15), so it is not clear how extradiol cleavage can be ensured. To circumvent this problem, we have proposed that nucleophilic superoxide attacks on the carbon adjacent to the enediol via a Michael-type addition [158]. The decomposition of this peroxy intermediate would only afford extradiol cleavage. This distinction between the attack of substrate by a nucleophilic superoxide in the extradiol cleavage mechanism and that by an electrophilic dioxygen in the intradiol cleavage mechanism serves as an attractive model to explain the regiospecificity of cleavage in these enzymes.

Although the proposed mechanism appears plausible, much work remains to be done to substantiate the individual steps. Thus far, reaction conditions have not been identified that would allow some of the steps to be kinetically resolved. Recently, however, Bugg et al. found evidence for the development of semiquinone character on the substrate as it goes through the extradiol cleavage cycle of 3-(2',3'-dihydroxyphenyl)propionate 1',2'-dioxygenase (MphB) (EC 1.13.11 class) [168] by using a substrate analog with a cyclopropyl side chain. Substituents on the cyclopropyl ring underwent *cis-trans* isomerization in the course of ring cleavage, implying the involvement of a cyclopropylcarbinyl radical species in the mechanism. In Figure 18, a semiquinone species is proposed to form after O₂ binds to the enzyme–substrate complex. Bugg has also reported evidence for the participation of the lactone intermediate subsequent to O—O cleavage in the mechanism for MphB [169]. When H₂¹⁸O is introduced into the solvent, the carboxylate group of the extradiol cleavage product becomes labeled; conversely, extradiol cleavage product derived from ¹⁸O₂ shows some loss of label. These experiments demonstrate that solvent can access the active site during the reaction and exchange with the Fe(II)—OH moiety needed to open the lactone ring.



As with the intradiol enzymes, important insights into the role of the metal center may also derive from appropriate functional models. Dei et al. reported that $[\text{Fe}(\text{tacn})(\text{dbc})\text{Cl}]$, upon exposure to O_2 , afforded nearly exclusive extradiol cleavage of the bound catecholate in 35% yield [170]. Building on these observations, Ito and Que have recently found that prior treatment of the complex with AgBF_4 to remove the bound chloride can elicit quantitative extradiol cleavage, suggesting that the availability of a coordination site is important for this reaction [171]. Surprisingly, this model is an iron(III) complex, not an iron(II) species as found in the enzymes, demonstrating that such reactions can also be carried out by iron(III). The availability of a complex capable of eliciting quantitative extradiol cleavage provides an opportunity to carry out systematic mechanistic studies of this reaction and, in particular, to determine whether the key C—O bond forming step involves nucleophilic attack of substrate by superoxide as proposed in Figure 18.

II.C.3. Pterin-Dependent Hydroxylases

The hydroxylations of phenylalanine, tyrosine, and tryptophan to tyrosine, DOPA, and 5-hydroxytryptophan, respectively, are catalyzed by hydroxylases that depend on Fe(II) and a tetrahydropterin cofactor [172]. These are neurologically important enzymes in the biosyntheses of dopamine, epinephrine, and serotonin. These enzymes have been the subject of a recent comprehensive review [3], and readers are referred to this paper for a discussion in more depth. Since the publication of this review, the crystal structure of tyrosine hydroxylase (TyrH) (EC 1.14.16.2) has been reported [173]. The iron active site (Figure 19) is square pyramidal like that of BphC with His331, His336, and Glu376 forming a triangular face and two solvent molecules completing the coordination sphere. Significantly, the three endogenous ligands in TyrH are completely conserved in all known sequences of the three pterin-dependent aromatic amino acid hydroxylases [3]. Such a geometry is consistent with spectroscopic studies of the iron site in phenylalanine hydroxylase (PheH) (EC 1.14.16.1) [174].

It is clear that the iron site is accessible to exogenous ligands besides solvent. Tyrosine hydroxylase is isolated under certain circumstances as the inactive iron(III) form complexed with noradrenaline or epinephrine [175,176], which are metabolites of DOPA and act as feedback inhibitors of the enzyme. The complexation of these catecholamines engenders a characteristic blue–green color characterized by resonance Raman spectroscopy as arising from a catecholate-to-iron(III) charge transfer transition. The energy of the charge transfer bands can be used to infer an iron site of relatively high Lewis acidity with a maximum of one anionic ligand [175,177], in agreement with the crystal structure of TyrH [173]. Similar spectral changes are observed when catechol is bound to Fe(III)-PheH [177].



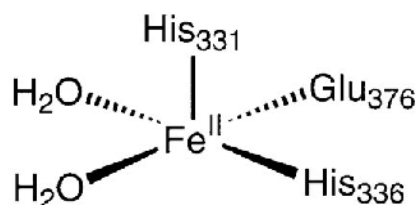


Figure 19 Active site of tyrosine hydroxylase.

Like BphC, Fe(II) is the catalytically important oxidation state [178], but unlike for BphC, there is no evidence that substrate coordinates to the iron center. Although tyrosine can potentially coordinate to the metal center, neither phenylalanine nor tryptophan is able to do so. Oxygen activation must therefore be initiated by a mechanism other than that proposed for the extradiol cleaving enzymes, if a common mechanism is to be shared by these three pterin-dependent enzymes. Steady-state kinetic studies suggest an ordered mechanism in which the pterin cofactor binds to the enzyme pocket first, followed by substrate, and then O_2 ; the rate limiting step appears to be the formation of the hydroxylating intermediate [179]. Since tetrahydropterin is structurally related to dihydroflavin, it can serve as the electron-rich species that initiates oxygen activation and form a hydroperoxypterin intermediate (Figure 20), in a role analogous to the role flavins play in the flavoenzymes [180]. In support of this role is the observation of a 4a-hydroxy adduct, which forms as a by-product of substrate hydroxylation [181]. However, unlike the dihydroflavins, the tetrahydropterins react with O_2 more slowly [182], so one role of the Fe(II) center may be to facilitate the formation of a peroxy intermediate (Figure 20).

Another proposed role for the Fe(II) center is to facilitate the heterolytic

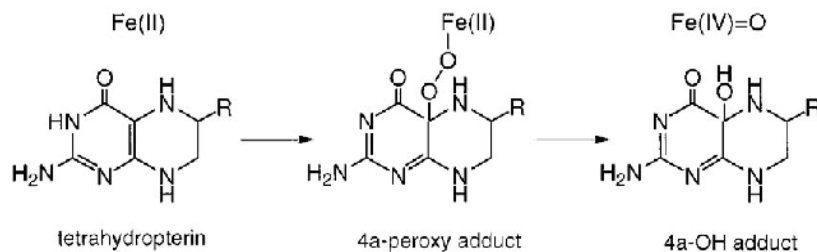


Figure 20 Proposed roles of the iron center and the pterin cofactor in the oxygen activation mechanism for pterin-dependent hydroxylases.



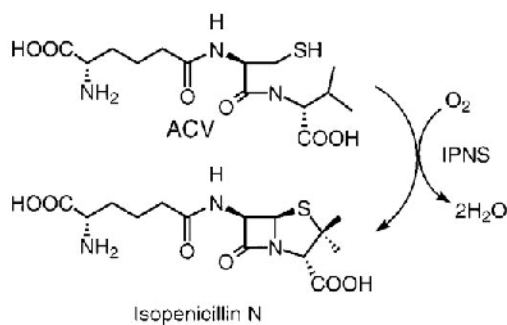


Figure 21 Reaction catalyzed by isopenicillin *N* synthase.

cleavage of the O—O bond of the peroxypterin species to generate an Fe(IV)=O species capable of hydroxylating the aromatic ring (Figure 20). Although a hydroxyl radical derived from the homolysis of the peroxy intermediate has been considered as the oxidizing agent [183], such a species would not be expected to engender the NIH shift (hydrogen shift from C-4 to C-3 in the conversion of phenylalanine to tyrosine) that is characteristic of this enzyme [184]. Furthermore, the ability of PheH to epoxidize 2,5-dihydrophenylalanine [192] would seem inconsistent with a hydroxyl radical mechanism and favor a high-valent metal-oxo pathway. Studies on TyrH also support an iron-oxo intermediate [185]. The effect of the *para*-substituent on the action of TyrH on a series of 4-X-substituted phenylalanines correlates well with the substituent σ values giving rise to ρ values of ca. -5 . This very negative ρ value is consistent with a highly electrophilic oxygen species that attacks the aromatic ring to generate a cationic intermediate.

II.C.4. Isopenicillin *N* Synthase

Isopenicillin *N* synthase (IPNS) (EC 6.3.2 group) is an enzyme found in β -lactam antibiotic-producing microorganisms, such as *Cephalosporium*, *Penicillium*, and *Streptomyces*. This nonheme Fe(II)-dependent enzyme catalyzes the formation of isopenicillin *N* from δ -(L- α -aminoadipoyl)-L-cysteinyl-D-valine (ACV), a four-electron oxidation that requires concomitant reduction of dioxygen to water (Figure 21) [186,187]. Thus the enzyme is technically an oxidase, but oxygen activation must nevertheless take place to drive the two ring forming steps of the reaction.

Recent crystallographic and spectroscopic studies have elucidated the structure of the metal center [188]. The crystal structure of the Mn(II)-substituted IPNS from *Aspergillus nidulans* shows a six-coordinate metal center bound to



the enzyme via four endogenous protein ligands (His214, Asp216, His270, and Gln330) with the remaining two *cis* coordination sites occupied by two H₂O molecules (Figure 22).

The crystallographic information solidifies structural insights derived from biochemical and spectroscopic studies. The His residues were identified in NMR studies of Fe(II)IPNS [189] and electron spin echo envelope modulation (ESEEM) studies of Cu(II)IPNS [190], whereas the Asp residue was recognized in the NMR spectrum of Co(II)IPNS on the basis of the chemical shifts of its three C—H protons and their NOE interconnectivities [189]. However, the Gln330 ligand was a feature not anticipated by spectroscopic studies, since an amide is not a commonly observed ligand for iron centers and no diagnostic spectroscopic probes were available. All four ligands are strictly conserved in the sequences of IPNS enzymes from a number of sources, and all except Gln330 have been found to be essential for activity by site-directed mutagenesis experiments [191–194].

Spectroscopic studies of the enzyme–substrate complex show that the δ -(L- α -amino adipoyl)-L-cysteinyl-D-valine (ACV) thiolate coordinates to the metal center. First, there is a decrease in the Mössbauer isomer shift of the Fe(II) center from 1.2 to 1.0 mm/sec, indicating a more covalent Fe(II)–ligand environment [195]. Second, an intense band appears at 390 nm in the visible spectrum of Cu(II)IPNS upon addition of ACV, which is associated with a thiolate-to-Cu(II) charge transfer transition found for tetragonal copper(II) centers [196]. Last, EXAFS analysis of the Fe(II)IPNS-ACV complex indicates the presence of a sulfur scatterer at ca. 2.3 Å, which is a distance typical of Fe(II)-thiolate coordination [197,198]. The very recently elucidated crystal structure of the Fe(II)IPNS-ACV complex confirms the thiolate coordination [199].

Fe(II)IPNS becomes O₂-sensitive only after addition of ACV. This observation suggests that the coordination of the ACV thiolate primes the Fe(II) center

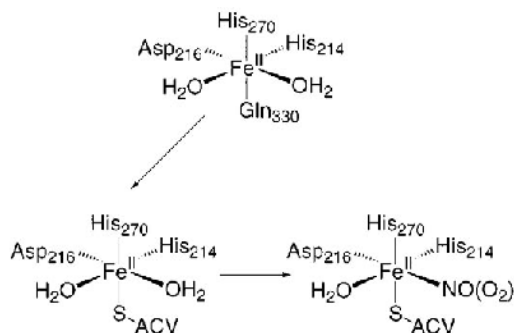


Figure 22 Active sites of Fe(II)IPNS and its binary and ternary complexes.



for its reaction with O_2 , presumably by shifting the Fe(III/II) redox potential to a more negative value, as suggested for the case of the extradiol cleaving dioxygenases as well. Unfortunately, there is as yet no spectroscopic evidence for an Fe(II)IPNS-ACV- O_2 adduct, as such an intermediate appears to be too reactive to be trapped. However, the corresponding Fe(II)IPNS-ACV-NO adduct has been studied [195]. As discussed in the catechol dioxygenase section, NO is useful as a surrogate for O_2 and affords a complex with rich spectroscopic properties. The fact that an NO complex can form demonstrates that there is one coordination site for NO (and presumably O_2); such an Fe-NO bond gives rise to the 1.75 Å scatterer required in the EXAFS fits of Fe(II)IPNS-ACV-NO [197]. Furthermore, this complex exhibits visible bands at 508 nm and 720 nm, which are red-shifted relative to those observed for Fe(II)IPNS-NO and Fe(II)IPNS-ASerV-NO, and elicits an $S = 3/2$ EPR spectrum that shows a larger rhombicity ($g = 4.22, 3.81$, and 1.99 with $E/D = 0.035$) than that of Fe(II)IPNS-NO ($g = 4.09, 3.95$, and 2.0 with $E/D = 0.015$) [195]. These observations suggest that the thiolate remains coordinated to the iron center in this complex. That these features do not arise from endogenous cysteine residues has been established by site-directed mutagenesis [200]. Direct evidence for thiolate coordination is provided by EXAFS analysis, which shows the persistence of an Fe-S scatterer at 2.3 Å found in the ES complex [197]. Last, the EPR spectrum of the ES-NO complex is broadened by the introduction of $H_2^{17}O$, indicating that there is a solvent-derived ligand on the iron center [195]. Taken together, these results show that there must be three exogenous ligands to the iron center in this complex, requiring that one of the four endogenous ligands be displaced during the catalytic cycle. Indeed, recent crystallographic results on the ES and ES-NO complexes identify this residue to be the catalytically nonessential Gln330, which is displaced upon binding of ACV; furthermore, NO binds trans to Asp216 (Figure 22) [199].

Important insights into the reaction mechanism have been obtained by Baldwin and coworkers using isotopically labeled substrates and a host of substrate analogs [186,187]. These observations serve as the basis for the mechanism proposed in Figure 23. The reduction of O_2 occurs in two stages concomitant with the sequential formation of the β -lactam and the thiazolidine rings. In the first stage, O_2 is reduced to peroxide and the Cys thiol is oxidized to thioaldehyde (or some equivalent moiety); this stage is followed by nucleophilic attack of the valinyl peptide nitrogen on the thiocarbonyl carbon to form the β -lactam and regenerate the thiolate. When the ACV Cys residue is replaced by homocysteine or difluorohomocysteine, the valine residue is unaffected; only the homocysteine residue is oxidized and some incorporation of oxygen from O_2 is observed in the products (Figure 24) [201,202]. These results support the notion that the β -lactam ring forms first.

The second stage involves the abstraction of the valinyl C3-H and the subsequent transfer of the coordinated Cys sulfur to the carbon radical to form the



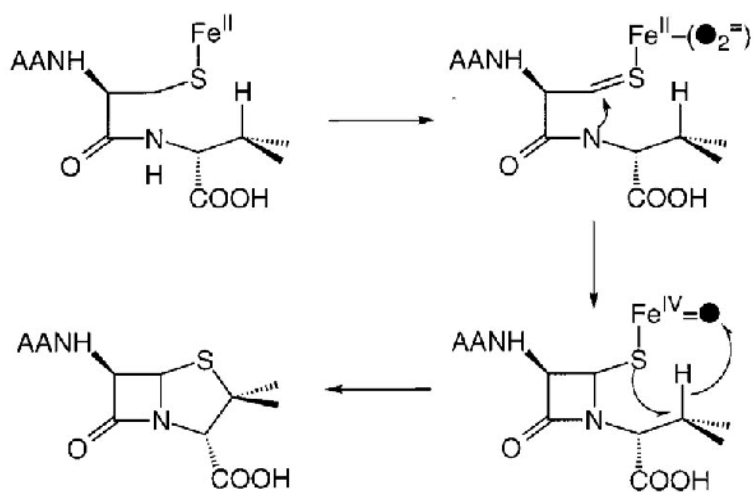


Figure 23 Proposed mechanism for Fe(II)IPNS.

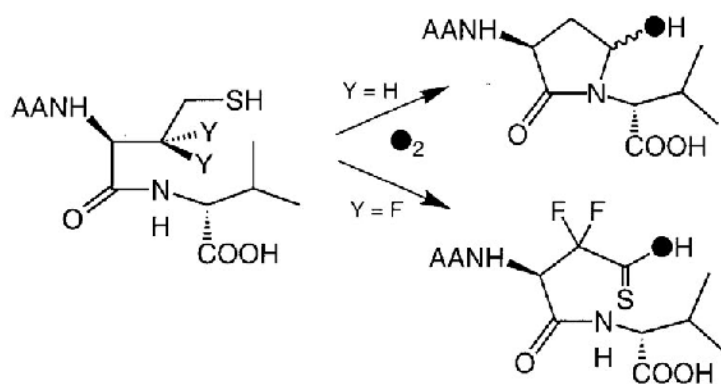


Figure 24 Reactions of ACV variants at cysteine.

thiazolidine ring. The participation of a carbon radical species is clearly indicated by the products observed in the β -cyclopropylalanyl analog (Figure 25), wherein the ring opening of a cyclopropylcarbonyl radical accounts for the formation of an eight-membered ring [203]. The involvement of an iron-oxo species is sug-



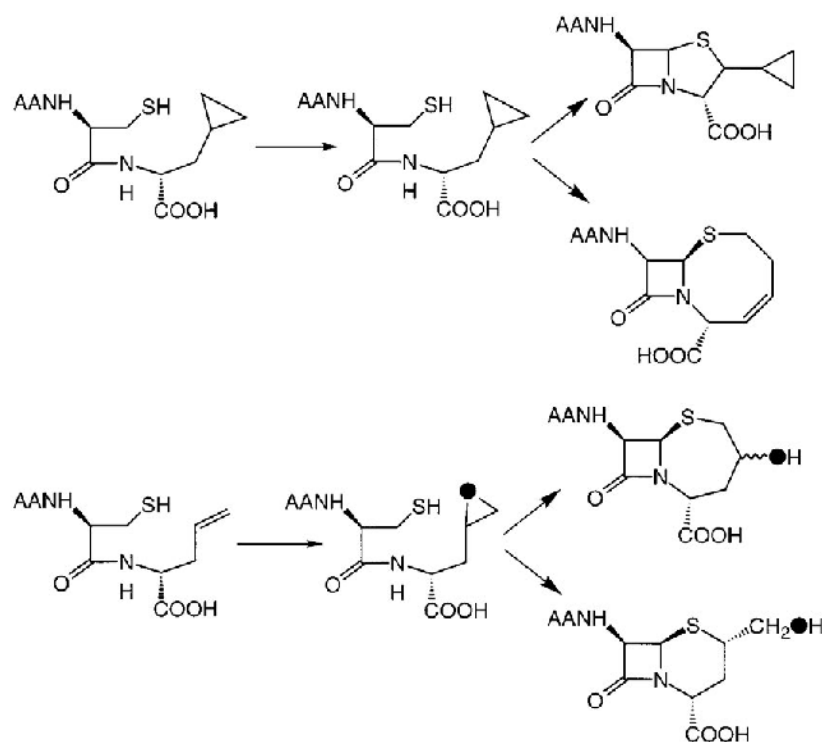


Figure 25 Reactions of ACV variants at valine.

gested by the studies of the allylglycyl analog (Figure 25), whose products must derive from an intermediate epoxide resulting from oxo transfer onto the double bond [204]. A key intermediate in the thiazolidine ring formation is the thiolate coordinated to an iron(IV)-oxo species (Figure 23). Reaction of the Val β -H with this species generates a radical that reacts with the coordinated thiolate to form the thiazolidine ring. Such a ligand transfer reaction has been observed in the oxidation of cyclohexane by $[\text{Fe}(\text{tmpa})\text{X}_2]^+/\text{BuOOH}$ ($\text{X} = \text{Cl}, \text{Br}, \text{or } \text{N}_3$) where the X group is transferred to the alkane substrate instead of the peroxidic oxygen when a stoichiometric amount of peroxide is used [205].

II.C.5. α -Keto Acid-Dependent Enzymes

The α -keto acid-dependent enzymes [206–208] catalyze a diverse array of reactions (Figure 26) involving functionalization of an unactivated C—H bond concomitant with the oxidative decarboxylation of a keto acid. For the hydroxylation



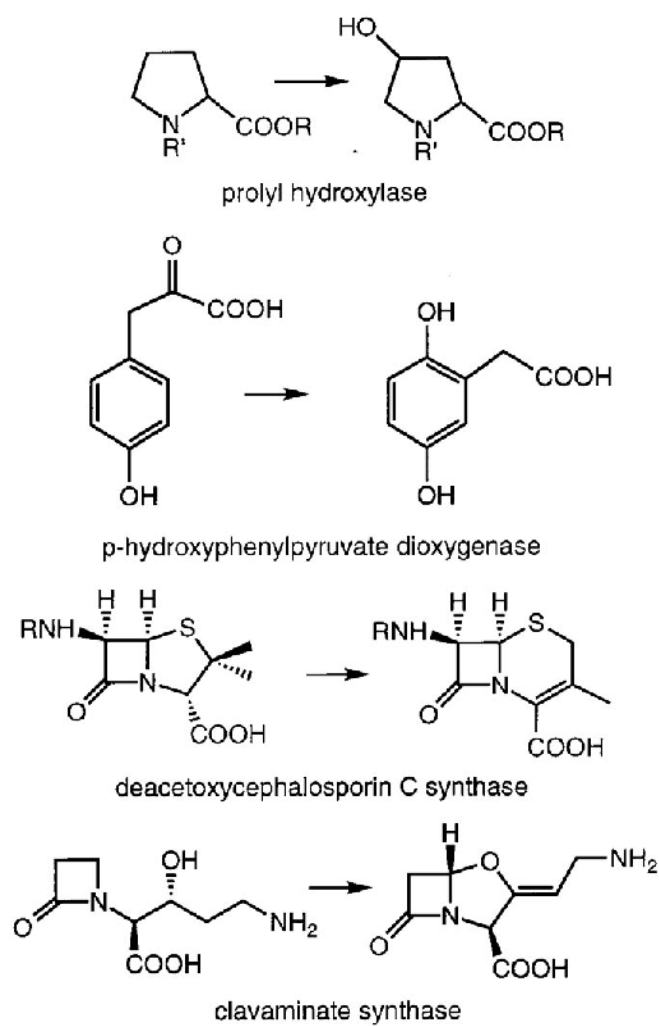
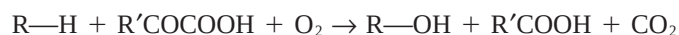


Figure 26 Some reactions catalyzed by α -keto acid-dependent enzymes.



reactions, one atom of dioxygen is incorporated into the product, and the other ends up on the carboxylate derived from the keto acid, i.e.,



Examples of such enzymes include those that hydroxylate prolyl and lysyl residues in procollagen and aspartyl residues in vitamin K-dependent proteins; such enzymes require α -ketoglutarate as cofactor. However, for *p*-hydroxyphenylpyruvate (HPP) dioxygenase (EC 1.13.11.27), the substrate also possesses the keto acid function in an intramolecular variant of the reaction (Reaction 2 of Figure 26); in addition, the transformation also entails a 1,2-alkyl shift. α -Keto acid-dependent enzymes also catalyze reactions wherein a C-H bond becomes functionalized by a heteroatom in the same molecule (Reactions 3 and 4 in Figure 26). Since dioxygen is not incorporated into product in these cases, deacetoxycephalosporin C (DAOC) synthase and clavamate synthase are technically oxidases, not oxygenases. In general, these enzymes require one equivalent of Fe(II) [209–211], an α -keto acid (usually α -ketoglutarate), and ascorbate for full activity. Other divalent metal ions do not elicit enzymatic activity but bind competitively to the active site [209–213]. The coordination environment around the iron center is only just beginning to be investigated. Unlike for the other enzymes in this review, there is as yet no crystal structure of an α -keto acid-dependent enzyme. However, sequence comparisons demonstrate that these enzymes have several homologous regions with an Asp and two His residues that are conserved in all the enzymes. Significantly, these conserved His and Asp residues correspond to the His and Asp ligands found in IPNS [191,192,214], suggesting that the iron center in these enzymes may be coordinated in a similar fashion.

Evidence for the involvement of histidine residues in the active site has been obtained from a number of experiments. Treatment with the histidine-selective reagent diethyl pyrocarbonate causes complete loss of activity in prolyl 4-hydroxylase (EC 1.14.11.2) [215] and 2,4-dichlorophenoxyacetate dioxygenase (TfdA) (EC 1.13.11 group) [211]. Site-directed mutagenesis of some of the conserved His residues in prolyl 4-hydroxylase [216] and aspartyl β -hydroxylase (EC 1.14.11.16) [217] also results in the loss of enzymatic activity. More recently, direct spectroscopic evidence for His coordination has been obtained in ESEEM studies of Cu(II)-substituted TfdA [218].

The metal coordination environment for HPP dioxygenase appears to be an exception to the 2-His-1-Asp active site discussed. This enzyme is isolated in the high-spin Fe(III) state as indicated by an intense EPR signal at $g = 4.3$. More significantly, it is deep blue in color (λ_{max} 595 nm) and exhibits a resonance Raman spectrum characteristic of a tyrosinate ligand [219]. Indeed, sequence comparisons of seven HPP dioxygenases from various mammalian and pseudomonad sources indicate the presence of a conserved Tyr residue [220,221]. It



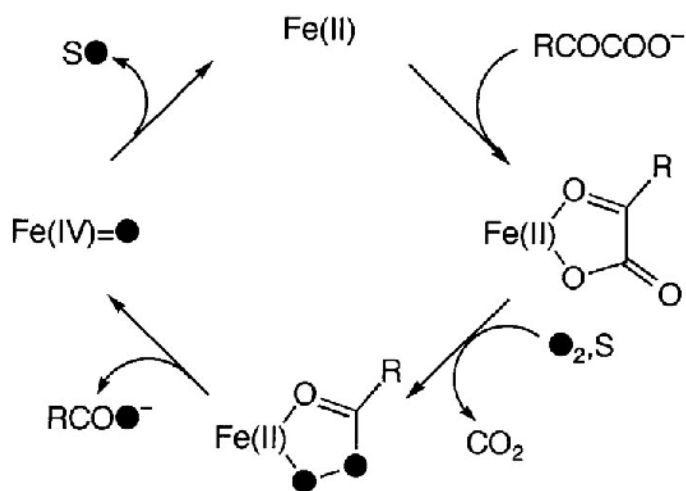


Figure 27 Proposed mechanism for the α -keto acid-dependent hydroxylases.

is important to note that this blue enzyme species is inactive and requires the addition of a reductant, which bleaches the blue color and activates the enzyme.

Despite the limited information about the coordination environment of the metal center, a mechanism for the α -keto acid-dependent enzymes has been proposed (Figure 27) [222] in which the α -keto acid binds to the iron center and primes it for O_2 binding. Attack of the bound O_2 on the coordinated α -keto acid at the C-2 position results in decarboxylation of the α -keto acid to give an Fe(II) -peroxy derivative that can react with substrate either directly or via a high-valent iron-oxo intermediate to give the oxygenated substrate, a carboxylic acid, and the starting Fe(II) enzyme.

This mechanism is consistent with a number of observations. Kinetic studies on prolyl 4-hydroxylase [223] and thymine hydroxylase (EC 1.14.11.6) [224] suggest that cofactor binds first, followed by O_2 . The bound O_2 appears to have superoxide character, as superoxide scavengers are competitive inhibitors of O_2 consumption [225,226]. It is also clear that the oxidative decarboxylation of the keto acid is a distinct phase of the mechanism from the alkane functionalization step, as these two phases can be uncoupled, particularly when poor substrate analogs are employed [227–229]. Evidence for an $\text{Fe(IV)} = \text{O}$ intermediate derives from studies with substrate analogs. Besides the hydroxylation of the 5-methyl group of thymine, thymine hydroxylase can also catalyze allylic hydroxylations, epoxidation of olefins, oxidation of sulfides to sulfoxides, and N-de-



methylation of amines [230], while HPP dioxygenase can catalyze sulfoxidations [231]. This reactivity is similar to that of cytochrome P-450 and suggests a similar active intermediate. Furthermore, it has been shown in deacetoxycephalosporin C (DAOC) synthase [232] and HPP dioxygenase [233] that ^{18}O from H_2^{18}O can be incorporated into the oxygenated product, as is consistent with an iron-oxo or iron-hydroxyl species in the mechanisms of DAOC synthase and HPP dioxygenase. However, there is evidence against the participation of a peracid intermediate. The use of peracid derivatives of the α -keto acids to substitute for the α -keto acid/ O_2 combination has not been successful in eliciting the desired oxidation in prolyl hydroxylase [234] and HPP dioxygenase [235], suggesting that either a peracid is not involved or such a species must be generated in the active site and cannot be introduced into the active site.

Recent model studies strongly support the proposed mechanism. The first crystal structures of Fe(II) complexed to benzoylformate show that an α -keto acid can coordinate to the iron as either a monodentate or didentate ligand [236]. Exposure of these $[\text{Fe}(\text{II})(\text{L})(\text{bf})]^+$ complexes ($\text{L} = \text{tmpa}$ or $6\text{-Me}_3\text{-tmpa}$) to O_2 results in the quantitative conversion of benzoylformate to benzoic acid and CO_2 , modeling the oxidative decarboxylation reaction characteristic of this class of enzymes. As with the enzymes, the use of $^{18}\text{O}_2$ in the model studies results in the incorporation of the label into the benzoate product. For $[\text{Fe}(6\text{-Me}_3\text{-tmpa})(\text{bf})]^+$, the rate of the oxidative decarboxylation increases as the substituent of the benzoylformate becomes more electron-withdrawing, affording a Hammett ρ of $+1.07$. This suggests that the oxidative decarboxylation involves a nucleophilic attack, most plausibly by the iron-bound O_2^- , on the keto carbon of benzoylformate to initiate decarboxylation as proposed in Figure 27.

Further support for an iron-bound active oxidant comes from the study of the $[\text{Fe}(\text{II})(\text{Tp}^{3,5\text{-Me}_2})(\text{bf})]$ complex [237]. This complex reacts with O_2 to form a species capable of stereospecifically epoxidizing olefins. For example, epoxidation of *cis*-stilbene gives only *cis*-stilbene oxide as the product, but *trans*-stilbene cannot be epoxidized, suggesting that epoxidation occurs at a sterically congested transition state, i.e., near the iron center. More studies are needed to provide insight into the nature of the active oxidant.

III. CONCLUSION

There has been much progress in recent years in our understanding of nonheme iron enzymes that activate dioxygen, particularly with respect to structure. From this vantage point, I have attempted to assemble the mechanistic ideas for these enzymes, using experimental evidence whenever available to support the proposed mechanisms. These mechanisms will evolve and need refinement as new data are reported. The various mechanisms discussed here appear to have a com-



mon thread; only details differ from enzyme to enzyme. As our efforts to understand these enzymes develop and mature, it will be interesting to discover how the various active sites control the metal environment and tailor the iron chemistry to effect the myriad transformations these enzymes catalyze.

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11

Dioxygen Activation at Heme Centers in Enzymes and Synthetic Analogs

Daniel Mansuy and Pierrette Battioni

University of Paris V, Paris, France

I. INTRODUCTION

All aerobic living organisms utilize dioxygen as an essential component for three main roles that are necessary to maintain life: the production of energy, the oxidative metabolism of endogenous compounds, and the defense of the organism against various kinds of infections [1,2] (Figure 1). In many species, the production of energy necessary for the cells utilizes the reduction of O_2 into H_2O in mitochondria, this highly exothermic reaction being coupled with adenosine triphosphate (ATP) production. The last step of this four-electron reduction of O_2 is performed by cytochrome oxidase, a system containing two hemes and three copper ions.

The second role of dioxygen is to act as the main oxygen atom donor in a variety of oxidation reactions involved in the biosynthesis or biodegradation of endogenous compounds such as amino acids, nucleotides, sugars, steroids, and fatty acids. It also acts as the main oxygen atom donor in the oxidative metabolism of exogenous compounds such as drugs and, in a more general manner, compounds from our environment. This oxidative metabolism of exogenous compounds, also called xenobiotics, is the first step of the elimination of these compounds from the body.

The third role of dioxygen concerns the defense of living organisms against infection [3]. Cells that are mainly involved in these defense mechanisms, such as leukocytes or macrophages, are able to reduce dioxygen into the superoxide anion $O_2^{\cdot-}$ through a reduced nicotinamide-adenine dinucleotide phosphate



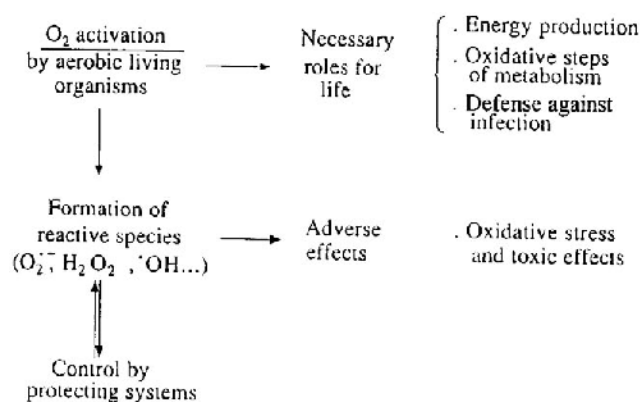


Figure 1 Useful and adverse effects of the activation of dioxygen by aerobic living organisms.

(NADPH) oxidase that becomes active after cell activation by an immune signal that appears as a result of the infection. Thanks to other enzymes such as myeloperoxidase, H₂O₂ formed in a first step by dismutation of O₂^{-•} is transformed into ClO⁻, which may react with some endogenous amines to give highly electrophilic *N*-chloroamines. All these potent oxidizing species formed locally by leukocytes and macrophages participate synergistically in the destruction of microbes.

In order to play these roles, dioxygen must be activated by proper enzymatic systems. Actually, although the direct reactions of O₂ with organic substrates are strongly exothermic and highly favored from a thermodynamic point of view, they are very slow and need to be catalyzed [4,5]. These two characteristics are at the basis of life in the presence of O₂. The kinetic barrier of reactions of O₂ with organic substrates prevents all organisms from being rapidly “burned” by O₂. This kinetic barrier is due to the electronic structure of O₂, which exists as a diradical (triplet state, *S* = 1), whereas most organic substrates are diamagnetic and exist in a singlet (*S* = 0) state. Therefore, it is easily seen that reactions between O₂ and organic substrates are slow, but also that reactions between O₂ and intermediate free radicals are very fast.

For the aforementioned reasons, every aerobic living organism contains a great variety of enzymatic systems that activate O₂ to utilize its specific properties. Most of these activations imply a reduction of O₂, which explains why several species derived from successive one-electron reduction of O₂ are produced in most tissues and organs. O₂^{-•} is the first species of this cascade of O₂ reduction (Figure 2). Its one-electron reduction leads to H₂O₂, which may be further reduced



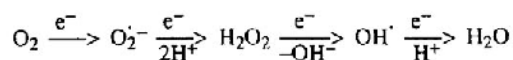


Figure 2 Species formed by successive one-electron reductions of dioxygen.

to give the very reactive free radical $\text{OH}^{\cdot}$. Two reactions of formation of $\text{OH}^{\cdot}$ from H_2O_2 are frequently encountered in living organisms: the Fenton reaction, which involves the reduction of H_2O_2 by Fe(II) complexes, and the Haber–Weiss reaction, which involves the reduction of H_2O_2 by $\text{O}_2^{\cdot-}$. Under normal conditions, such reduced species are formed in most tissues because of the aerobic life and the extensive use and necessary activation of O_2 . However, they are efficiently controlled by enzymatic systems specifically designed to limit their concentrations [3]. This is the case of superoxide dismutases, which catalyze the dismutation of $\text{O}_2^{\cdot-}$; of glutathione peroxidase, which catalyzes the reduction of H_2O_2 into H_2O ; and of catalase, which catalyzes the dismutation of H_2O_2 . However, this delicate equilibrium between the activation of O_2 and the formation of its reduction products, on one side, and the control of these products by proper enzymatic systems, on the other side, may be displaced toward an excessive formation of radicals derived from O_2 reduction. This may be the result of a lowered activity of the protecting enzymes for genetic or nutritional reasons or of an increased formation of radicals derived from O_2 in pathological situations or after the administration of some toxic molecules. The excessive accumulation of these O_2 -derived radicals then results in an oxidative degradation of various cell macromolecules such as proteins, nucleic acids, and membrane lipids that are particularly sensitive to oxidants. Such an oxidative stress appears to be implicated in several pathological conditions [3].

A wide variety of enzymatic systems are involved in the activation and use of O_2 by aerobic organisms [1]. Among them, the systems most frequently encountered particularly in mammals are hemoproteins; this indicates that iron porphyrins are particularly well-suited cofactors for the binding and activation of O_2 (Figure 3). Some hemoproteins, like hemoglobin and myoglobin, are responsible for the transport and storage of O_2 , because of its reversible binding to Fe(II) . The present chapter is focused on hemoproteins involved in the activation of O_2 . Actually, one may distinguish three main classes of hemoproteins that activate and use O_2 by different mechanisms. The first class catalyzes the insertion of the two oxygen atoms of the dioxygen molecule into substrates and are called dioxygenases. They do not require the consumption of a reducing agent. The second class catalyzes the insertion of only one oxygen atom of O_2 into substrates and reduces the second oxygen atom into water. Therefore, they consume two electrons, which generally come from NADPH or NADH and are called monooxygenases. The third class catalyzes the reduction of O_2 into H_2O by four electrons



Table 1 Main Hemoproteins Involved in the Binding and Activation of O₂

Hemoprotein	Iron axial ligand	Function
Hemoglobin	Histidine	O ₂ transport
Prostaglandin synthase	Histidine	Dioxygenation of arachidonic acid
Cytochrome P-450	Cysteinate	Mono-oxygenations $\text{RH} + \text{O}_2 + 2\text{e}^- + 2\text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O}$
Cytochrome <i>a3</i>	Histidine	O ₂ reduction $\text{O}_2 + 4\text{e}^- + 4\text{H}^+ \rightarrow 2\text{H}_2\text{O}$

from various electron donors and belonging to the category of oxidases. Interestingly enough, all these hemoproteins (Table 1) involve the same kind of cofactor in their active site, an iron porphyrin (Figure 3), and a common iron axial ligand from the protein (except cytochrome P-450), the imidazole from a histidine residue. However, because of a very different structure of the protein and distal environment of the heme, they have clearly different roles and/or catalyze different reactions (Table 1).

This chapter will describe the detailed mechanisms of O₂ activation by a main representative of each class of hemoproteins. Then, efforts to mimic oxygenases by using model systems based on metalloporphyrins will be reviewed. In fact, metalloporphyrin model systems have proved to be useful for three main objectives. The first is to determine the detailed structure of the iron complexes

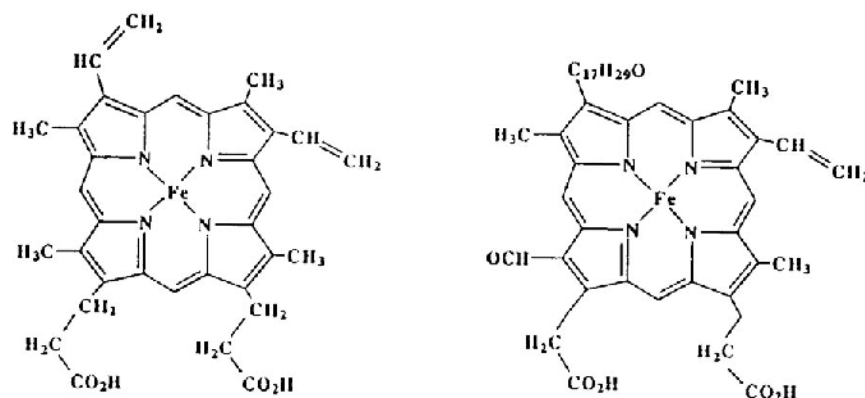


Figure 3 Formula of the hemes present in cytochromes *a* and *b*. Left: iron-protoporphyrin IX of cytochromes *b*. Right: heme of cytochromes *a* (C₁₇H₂₉O = CHOCH—CH₂—CH₂—CH=C(CH₃)—CH₂CH₂CH=C(CH₃)CH₂CH₂CH=C(CH₃)₂).

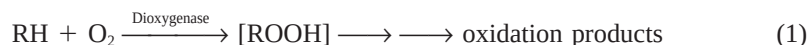


involved as intermediates in the catalytic cycle of dioxygen activation by the hemoproteins. Obviously, such a determination on the hemoproteins themselves is difficult because of their high molecular mass. The second interest is to improve understanding of the coordination chemistry and the detailed nature of the interactions of hemoproteins with substrates, as the compounds mimicking hemoprotein–iron–metabolite complexes can be easily studied by all the spectroscopic techniques and particularly by x-ray crystallography. The third interest of this approach using models is to build up catalytically active chemical systems able to reproduce the main reactions catalyzed by these hemoproteins. This could lead to interesting new catalysts for the selective oxidation of substrates by O_2 , which remains a difficult challenge in homogeneous catalysis and oxidation chemistry [6].

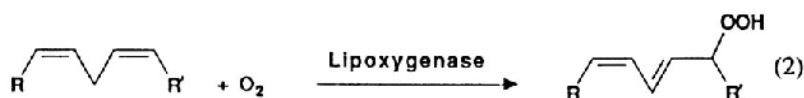
II. CATALYSIS OF O_2 TRANSFER INTO SUBSTRATES BY HEME-CONTAINING DIOXYGENASES

II.A. General Properties of Dioxygenases

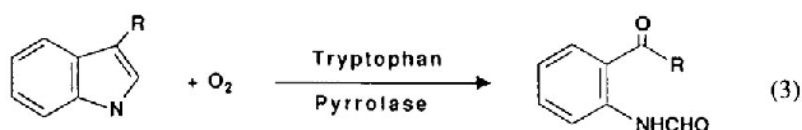
Dioxygenases catalyze the insertion of the two oxygen atoms of O_2 into substrates (Eq. 1) [1]. The main reactions catalyzed by dioxygenases occur on two main classes of substrates that are relatively reactive from a chemical point of view; these are unsaturated fatty acids containing reactive allylic CH_2 groups from 1,4-diene units and aromatic compounds such as tryptophan.



After the dioxygenase-catalyzed incorporation of the two oxygen atoms of O_2 , the first class of substrate, the unsaturated fatty acids, are very often transformed into the corresponding alkylhydroperoxides, whereas the second class, the aromatic compounds, generally undergo an opening of their aromatic ring after the oxidative cleavage of one of their carbon–carbon bonds (e.g., Eqs. 2 and 3). It is noteworthy that these substrates are relatively prone to undergo autoxidation reactions by O_2 , particularly in the presence of transition metal catalysts. In fact, dioxygenases appear to play two main roles: (1) to increase the rates of reaction of these substrates with O_2 , and (2) to control the chemo-, regio- and stereoselectivities of these reactions. Substrate oxidations by dioxygenases do not require the consumption of a reducing agent (Eq. 1), and, in most cases, the



role of the metal cofactor of their active site is to activate the substrate into a free radical species that rapidly reacts with O_2 . Most dioxygenases are iron enzymes; the iron is either only bound to amino acid residues, as in lipoxygenases,



or included in a porphyrin tetradentate ligand, as in prostaglandin synthetase or tryptophan pyrrolase.

II.B. Dioxygenation Catalyzed by Prostaglandin H Synthetase

II.B.1. Characteristics and Roles of Prostaglandin H Synthetase [7]

Prostaglandin H Synthetase (PGHS) catalyzes the first step of the biosynthesis of prostanoids (prostaglandins, prostacyclins, and thromboxanes), the bis-dioxygenation of arachidonic acid leading to the 9,11-endoperoxide 15-hydroperoxide, PGG_2 (Figure 4). This oxidation of arachidonic acid into PGG_2 , which involves the insertion of two O_2 molecules into arachidonic acid, is often called the *cyclooxygenase reaction*. Prostaglandin H Synthetase-Fe(III) also catalyzes the reduction of PGG_2 to the corresponding alcohol PGH_2 (peroxidase reaction) (Eq. 4). In a more general manner, PGHS-Fe(III) acts as a peroxidase and can catalyze the reduction of alkylhydroperoxides to alcohols by a great variety of electron-rich compounds such as phenols and anilines [7] (Eq. 5).

Nonsteroidal anti-inflammatory drugs (NSAIDs) exert their effects by inhibition of prostaglandin production, and the pharmacological target of most NSAIDs is PGHS (EC 1.14.99.1). In fact, two isoforms of PGHS are known [8a]. PGHS-1 (or COX-1), which is constitutively expressed in many tissues, is responsible for the physiological production of prostaglandins; PGHS-2 (or COX-2), which is induced by cytokines and endotoxins in inflammatory cells, is responsible for the high production of prostaglandins during inflammation. The x-ray structure of ovine COX-1 was published in 1994 [8b], that of murine COX-2 in 1996 [8c]. The two isoforms exhibit very similar structures that involve a (protoporphyrin IX) Fe(III) binding site responsible for the peroxidase activity and a substrate (or inhibitor) binding site in which the bisdioxygenation of arachidonic acid takes place. In its resting state, PGHS exists as a high-spin Fe(III) complex with a histidine axial ligand from the protein (His388 in PGHS-1). In both isoforms, a tyrosine residue is located between the heme and substrate binding sites and seems to play a key catalytic role.



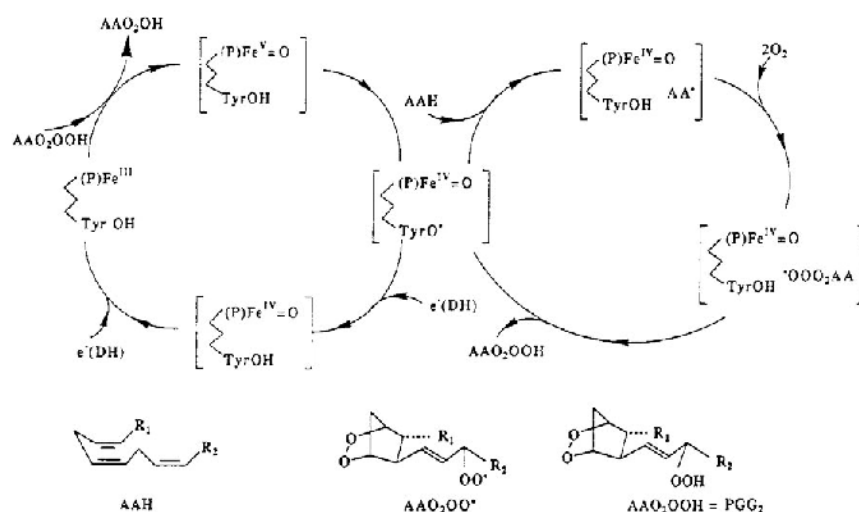
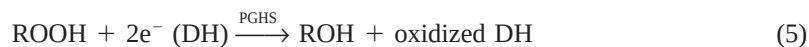
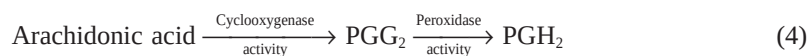
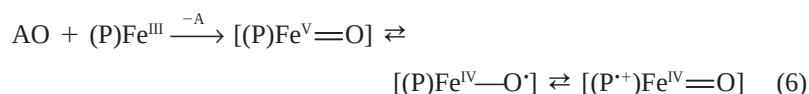


Figure 4 Catalytic cycles for the cyclo-oxygenase (dioxygenase) and peroxidase activities of prostaglandin synthetase. P = protoporphyrin IX and Tyr OH is the tyrosine residue of the protein oxidized by PGHS compound I; AAH = arachidonic acid; AAO₂OOH = PGG₂ = prostaglandin G₂; AAO₂OH = PGH₂ = prostaglandin H₂; DH = electron-donor substrate for the peroxidase activity of PGHS.



II.B.2. The Peroxidase Reaction of PGHS

The peroxidase activity of PGHS is comparable to that of better known peroxidases such as horseradish peroxidase (HRP). The catalytic cycle of HRP is shown in Figure 5 [9]. Its first step is the formation of an intermediate very often found in hemoproteins by transfer of an oxygen atom from various oxygen atom donors to the Fe(III) heme (Eq. 6). It is a high-valent iron-oxo species, at least formally a Fe(V)=O complex. In fact, the detailed electronic structure of this intermediate depends on the environment of the heme provided by the protein. In HRP, this intermediate (called *compound I*) is a (porphyrin radical-cation)-Fe(IV)=O complex, as shown by many spectroscopic techniques [9].



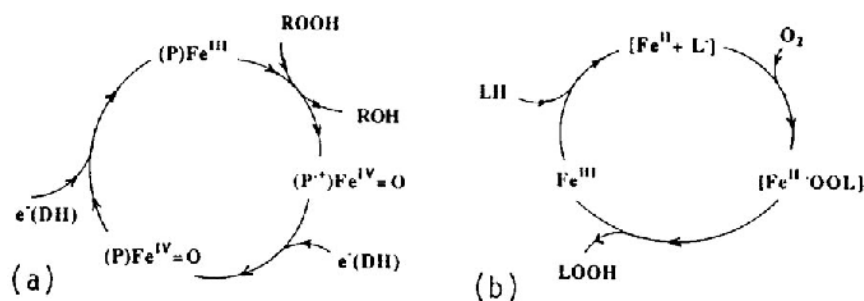


Figure 5 Catalytic cycles of horseradish peroxidase (a) and of soybean lipoxygenase (b). $ROOH = H_2O_2$ or an alkylhydroperoxide; DH = electron-donor substrate of peroxidase; LH = linoleic acid; $LOOH$ = 13-hydroperoxide of linoleic acid; $(P) Fe(III)$ = $Fe(III)$ (protoporphyrin IX).

On the contrary, in cytochrome *c* peroxidase, the intermediate is a (porphyrin) $Fe(IV)=O$ complex with a free radical derived from the one-electron oxidation of an amino acid residue in the vicinity of the heme [10]. The second step of the HRP catalytic cycle is the one-electron oxidation by compound I of HRP substrates that are generally electron-rich aromatic compounds. This leads to the second intermediate, called *compound II*, of the catalytic cycle, which is a (porphyrin) $Fe(IV)=O$ complex. The one-electron reduction of compound II by HRP substrates regenerates HRP in its resting iron(III) state (Figure 5a).

Spectroscopic studies of the interaction of $PGHS-Fe(III)$ with PGG_2 showed the very fast formation of an equivalent of compound I [11,12]. This intermediate then undergoes a fast conversion into a second species whose spectroscopic properties are consistent with a $Fe(IV)=O$ structure. This species exhibits an electron paramagnetic resonance (EPR) signal at $g = 2.005$ very similar to that reported for the tyrosyl radical present in the active site of the ribonucleotide reductase of *Escherichia coli* [13]. Thus, it seems that the compound I species of $PGHS$ rapidly oxidizes a tyrosine residue of the protein [12]. The following steps of the $PGHS$ peroxidase cycle should be very similar to those of the HRP cycle and should involve two successive one-electron oxidations of electron-rich aromatic substrates by the compound I equivalent of $PGHS$ (Figure 4).

II.B.3. Mechanism of O_2 Transfer by Prostaglandin H Synthetase

Several mechanisms have been proposed for the dioxygenation of arachidonic acid by $PGHS$ (the cyclooxygenase reaction). The involvement of free radical intermediates derived from the abstraction of a hydrogen atom at position 13 of arachidonic acid was proposed [14]. A mechanism deduced from that generally



accepted for lipoxygenases [15] appeared likely. In this lipoxygenase cycle [15] (Figure 5b), the key step is the abstraction of a bis-allylic hydrogen atom of arachidonic acid coupled with a one-electron reduction of the Fe(III) state of lipoxygenase to its Fe(II) state. The allylic free radical formed in this step rapidly reacts with O_2 and the derived peroxy radical oxidizes lipoxygenase-Fe(II) to its starting Fe(III) state with the release of the fatty acid hydroperoxide product. An alternative mechanism of lipoxygenase would involve the formation of an iron-carbon bond in a σ -alkyl Fe(III)-fatty acid complex and insertion of O_2 into this iron-carbon bond [16,17]. In agreement with the mechanism of Figure 5b, incubation of lipoxygenase-Fe(III) with arachidonic acid under anaerobic conditions is accompanied by the disappearance of the EPR signal of high-spin lipoxygenase-Fe(III) and the formation of EPR-silent lipoxygenase-Fe(II). Such a conversion of the EPR-active Fe(III) signal to an EPR-silent Fe(II) could not be observed upon incubation of PGHS-Fe(III) with arachidonic acid under anaerobic conditions [12], suggesting that the cyclooxygenase catalytic cycle should be different from that of lipoxygenase.

On the basis of the results obtained for the interaction of PGHS-Fe(III) with PGG_2 , another mechanism was proposed for cyclooxygenase [12]. As shown in Figure 4, this mechanism establishes a link between the cyclooxygenase and peroxidase cycles of PGHS and is consistent with reports showing that alkylhydroperoxides are necessary activators of the PGHS cyclooxygenase activity [18]. Activation of an allylic C—H bond of arachidonic acid would be performed by the tyrosyl radical of the compound I equivalent of PGHS. This free radical hydrogen abstraction would lead to a substrate allylic free radical, which would then react with two O_2 molecules to give the peroxy radical of PGG_2 . The peroxy radical would reoxidize the tyrosine moiety, leading to PGG_2 and regenerating the active [Fe(IV)=O tyrosyl radical] state of PGHS. However, it is noteworthy that this hypothesis remains controversial [19].

II.C. About the Mechanisms of the Activation of Substrates by Dioxygenases and the Role of O_2

If one considers the mechanisms described for the dioxygenation of polyunsaturated fatty acids by a heme-containing dioxygenase, PGHS, and by nonheme dioxygenases, the lipoxygenases, two common characteristics appear. The first is a very similar mode of activation of fatty acid allylic C—H bonds by a one-electron oxidation coupled to a hydrogen abstraction performed by an oxidized state of the enzyme [Fe(III) in lipoxygenases, Fe(IV)=O tyrosyl radical in PGHS]. The second common characteristic is the lack of participation of O_2 in this substrate activation. With both kinds of enzymes, O_2 only reacts with the free radical derived from the substrate. Therefore, there is no activation of O_2 by the iron catalyst in these dioxygenases. In fact, there is no evidence for O_2 binding



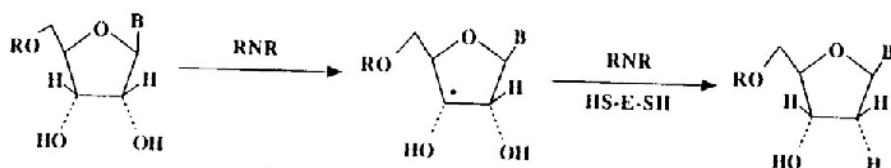


Figure 6 Reactions catalyzed by ribonucleotide reductase (RNR). HS-E-SH is the site of the enzyme responsible for the reduction step; SH is a cysteine thiol group.

to the iron in these enzymes. The main difference between the mechanism of the hemoprotein PGHS and the nonheme proteins, lipoxygenases, is the nature of the species able to oxidize the fatty acid C—H bond. The redox potential of lipoxygenase-Fe(III) is high enough to perform this reaction, whereas that of PGHS—Fe(III) is not. Therefore, PGHS—Fe(III) must be oxidized to the equivalent of a peroxidase compound I by its hydroperoxide product PGG₂ (or by other fatty acid hydroperoxides) in order to become active. The abstraction of a fatty acid allylic hydrogen is thus performed by a protein radical located close to the heme and produced by one-electron oxidation of an amino acid residue by PGHS compound I. Such a role of a free radical derived from a protein amino acid in the activation of a substrate has already been observed in other enzymatic reactions [20]. For instance, the ribonucleotide reductase of *E. coli* involves a unique active site that contains a dinuclear iron center, and a tyrosyl radical that is stable in the resting state of the enzyme [13]. This radical is essential for its catalytic activity and, more particularly, for the first step of the reduction of ribonucleotides. This step is the abstraction of a hydrogen atom from the 3'-position of the ribonucleotide by a protein radical to form a 3'-ribonucleotide radical (Figure 6) [20].

Actually, it is noteworthy that there are examples of heme-containing dioxygenases that bind O₂ with formation of a heme—Fe(II)—O₂ complex. This is the case of indolamine-2,3-dioxygenase (EC 1.13.11.11) and tryptophan-2,3-dioxygenase, which catalyze the insertion of O₂ into L-tryptophan to yield *N*-formylkynurenine [21]. The catalytic cycle involves the ternary complex L-tryptophan-iron(II) enzyme-O₂ as an active intermediate. In this ternary complex, which yields *N*-formylkynurenine and the Fe(II) enzyme, O₂ and/or the substrate is activated [21], a situation clearly different from that found for PGHS.

III. CATALYSIS OF O₂ ACTIVATION BY HEME-CONTAINING MONOOXYGENASES

There is a great variety of iron-containing monooxygenases; however, the heme-containing monooxygenases known so far are almost based on two classes of hemoproteins, the cytochromes P-450 and the closely related NO synthases [22].



III.A. Structure and Function of Cytochrome P-450-Dependent Monooxygenases [22]

Cytochrome P-450-dependent monooxygenases are widely distributed in living organisms (e.g., mammals, insects, fish, yeasts, plants). In humans, they exist in several organs and tissues with a maximum level in the liver. These monooxygenases are multienzymatic systems that are able to transfer electrons from NADPH or NADH to their terminal component, cytochrome P-450, which contains the site of activation of dioxygen and substrates. They catalyze the insertion of only one oxygen atom of O₂ into the substrate (Eq. 7).



Electrons from NADPH or NADH are transferred to the heme of cytochrome P-450 by an electron transfer chain involving in many cases a flavoprotein, cytochrome P-450 reductase, and, in some cases, other electron-transfer proteins such as iron sulfur proteins. Cytochrome P-450-dependent monooxygenases play two main roles in living organisms. Their first role is to catalyze specific oxidation steps involved in the biosynthesis or biodegradation of endogenous compounds like steroids or fatty acids. This role is played by cytochromes P-450, which are generally specific for their substrate and catalyze highly regio- and stereoselective reactions. The second role of cytochromes P-450 is in the oxidative metabolism and elimination from the body of exogenous compounds from our environment such as drugs, pesticides, or solvents. The cytochromes P-450 that seem to be in charge of the degradation of foreign compounds (xenobiotics) are generally less selective as they accept a broad variety of substrates. As a consequence, the cytochromes of this category are generally less regio- and stereoselective than those of the former category.

Because of their wide distribution in living organisms and their important roles in biochemistry, pharmacology, and toxicology, cytochromes P-450 have been extensively studied in the last 30 years. More than 500 cytochromes P-450 from various species have already been cloned and sequenced [23].

Actually, four crystal structures of prokaryotic cytochromes P-450, from *Pseudomonas putida*, *Bacillus megaterium*, a *Pseudomonad* catalyzing the hydroxylation of α -terpineol, and *Saccharopolyspora erythaea*, have been determined so far [24,25]. The resting state of these cytochromes P-450 involves a hexacoordinate Fe(III) geometry for the iron included in protoporphyrin IX and bound to two axial ligands, a cysteinate ligand from the protein and a water molecule. In the presence of its substrate (camphor), P-450cam from *Pseudomonas putida* shows a penta-coordinate state of the iron with cysteinate 357 as the only axial ligand. The camphor molecule is maintained close to the heme, mainly by a hydrogen bond between the camphor keto group and a tyrosine residue of the protein [24]. In a more general manner, various spectroscopic data indicate that all cytochromes P-450 reported so far have in common the following charac-



teristics: (1) the presence of iron(III) protoporphyrin IX as the only cofactor within a single polypeptide chain with a molecular weight between 40 and 60 kDa and (2) the binding of this heme to the apoprotein through an axial iron–cysteinate bond. Moreover, the various reactions catalyzed by all these cytochromes appear to involve a common catalytic cycle of dioxygen activation.

III.B. Catalytic Cycle of Cytochrome P-450 [22,25–30]

As with cytochrome P-450cam, all the cytochromes P-450 studied so far exist in their resting iron(III) state in the form of two Fe(III) complexes in equilibrium: a hexacoordinate low-spin iron complex with two axial ligands, the cysteinate and an OH-containing residue, presumably H₂O, and a pentacoordinate high-spin iron complex with the cysteinate as the only axial ligand (Figure 7). Binding of the substrate occurs on a protein site close to the heme and generally leads to a shift of this equilibrium toward the pentacoordinate complex. This high-spin Fe(III) P-450–substrate complex very often exhibits a higher redox potential and is more easily reduced by one electron provided by the electron-transfer chain. This leads to P-450-iron(II), a high-spin pentacoordinate complex with an accessible sixth coordination position. Therefore, many ligands, such as CO, isocyanides, imidazoles, pyridines, and phosphanes can bind to the iron(II), leading to P-450 complexes characterized by Soret bands between 440 and 460 nm. Dioxygen itself binds to P-450-iron(II), thus leading to a low-spin hexacoordinate complex that is not the hydroxylating species of the catalytic cycle.

The structure, coordination, and spin state of these first four intermediate complexes of the catalytic cycle have become better understood as a result of a comparison of their spectral properties with those of model iron–porphyrin complexes that have been prepared and completely characterized by x-ray analysis [29,30–33]. The intermediate of the catalytic cycle that may deliver its oxygen atom into substrates is derived from a one-electron reduction of the P-450-iron(II)-O₂ complex. This active oxygen complex is so short-lived that it has not been detected by any spectroscopic technique. Therefore, our understanding of its structure and reactivity is based on data from studies of its reactions with substrates, and on a comparison with analogous active oxygen complexes obtained with other hemoproteins or iron-porphyrins [34]. These data suggest a high-valent iron-oxo structure for this intermediate that would be formed by the heterolytic cleavage of the O–O bond of a possible Fe(III)–O–OH species derived from a one-electron reduction of the Fe(II)–O₂ complex. Accordingly, single oxygen atom donors such as C₆H₅IO, H₂O₂, or ROOH can replace O₂ and NADPH for the P-450-catalyzed oxidations of various substrates. The detailed electronic structure of the cytochrome P-450 high-valent active species is not known at present. Formally, it is an equivalent of the peroxidase compound I (see Section II.B.2). Actually, different formal structures may be written for the



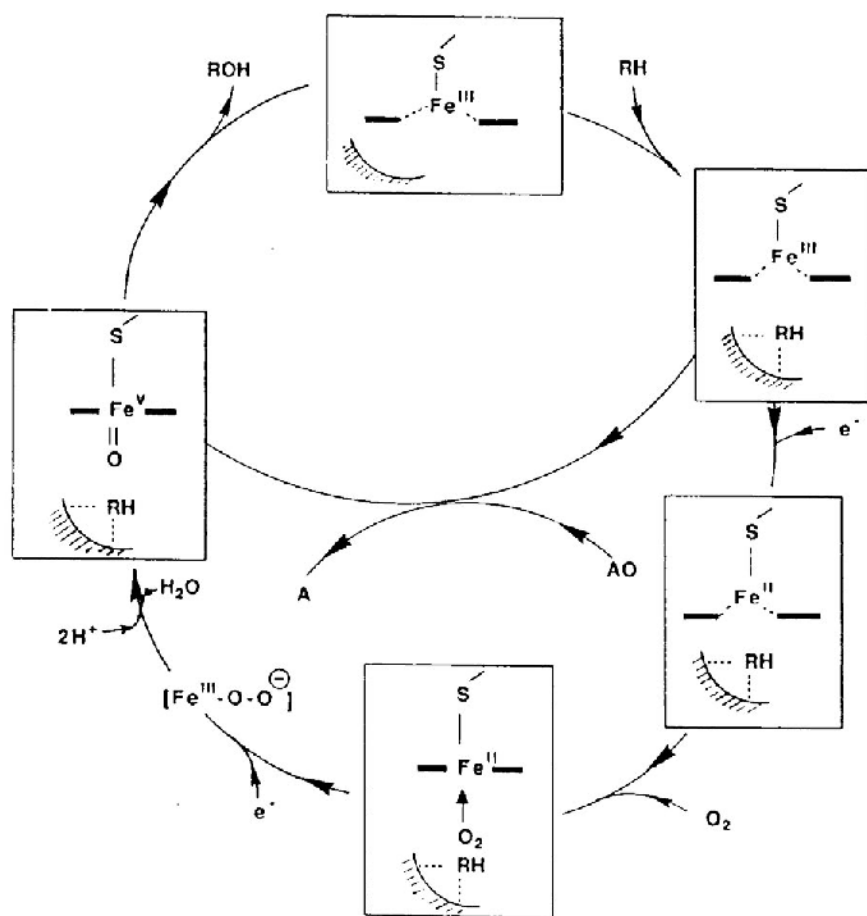


Figure 7 Catalytic cycles of cytochrome P-450, involving either O₂ and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) or a single oxygen atom donor AO.

state obtained upon interaction of a Fe(III) heme with an oxygen atom (oxenoid) from various oxygen atom donors, such as PhIO, H₂O₂, or a peracid. Some of them, Fe(V)=O; Fe(IV)—O[•], and (porphyrin)⁺ Fe(IV)=O, are given in Equation 6. A first model iron-porphyrin complex for such high-valent species has been prepared by reacting *meta*-chloroperbenzoic acid with iron(III)-*meso*-tetramesityl porphyrin [35]. Similar iron-oxo complexes have been obtained from reactions of various iron-porphyrins with oxygen atom donors [36]. Their [¹H] nuclear magnetic resonance (NMR), EPR, Mössbauer, and extended x-ray ab-



sorption fine structure (EXAFS) spectra indicate a (porphyrin-radical cation) Fe(IV)=O structure similar to that of horseradish peroxidase compound I. These complexes perform oxidations similar to those catalyzed by cytochromes P-450, suggesting that high-valent iron-oxo species equivalent to Fe(V)=O (Eq. 6) are most often responsible for cytochrome P-450-dependent monooxygenations.

III.C. Mechanisms of the Main Oxidations Catalyzed by Cytochromes P-450

The active oxygen species of cytochromes P-450 is reactive enough to transfer its oxygen atom to most organic compounds. However, the most frequently encountered reactions are (1) the hydroxylation of C—H bonds, (2) the epoxidation of double bonds, (3) the hydroxylation of aromatic rings, and (4) the transfer of an oxygen atom to compounds containing an N, S, or P heteroatom.

P-450-catalyzed hydroxylations of aliphatic C—H bonds most often involve a nonconcerted mechanism (Figure 8), which occurs in two steps: (1) an abstraction of the hydrogen atom by the P-450 active oxygen complex, which exhibits a free radical-like reactivity, and (2) an oxidation of the substrate-derived free radical formed in this step by the Fe(IV)—OH intermediate [34,37,38].

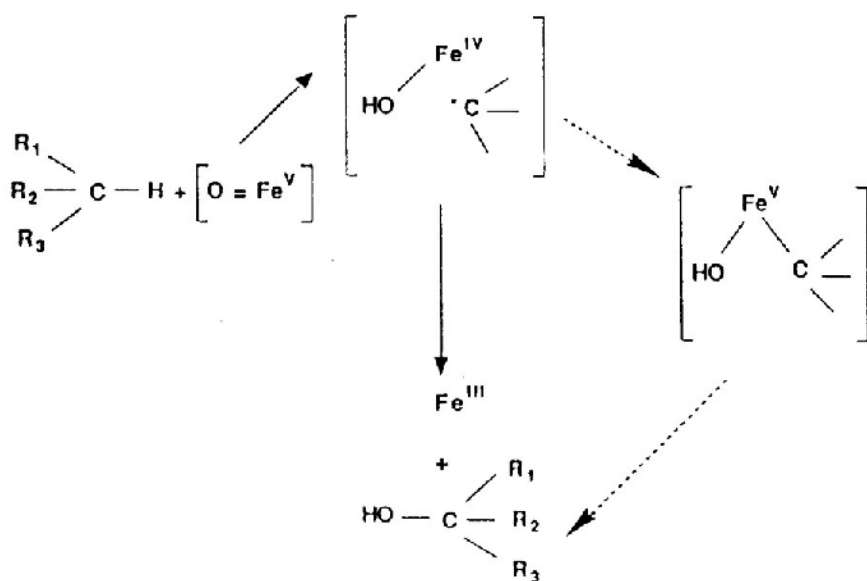


Figure 8 Generally accepted mechanisms for C—H bond hydroxylations catalyzed by cytochromes P-450.



Another mechanism has been proposed for the second step of the hydroxylation [30,39]. It postulates that the substrate-derived free radical binds to P-450-iron, leading to an intermediate high-valent σ -alkyl iron complex. A reductive elimination of the σ -alkyl and OH ligands that are in cis position on the iron should lead to the hydroxylated product and regenerate P-450-Fe(III). This mechanism would explain the formation of P-450-iron-metabolite complexes, which seem to involve an iron-carbon bond, during the hydroxylation of some substrates [30,40,41]. Model iron-porphyrin complexes involving such iron σ -alkyl (-aryl) bonds or iron-carbene bonds have been prepared and completely characterized [30]. In that regard, the x-ray structure of a P-450cam complex with a Fe(III)—Ph bond has been reported [42].

Several mechanisms have been proposed for the epoxidation of double bonds of alkenes by cytochromes P-450 [30,34,43,44]. The first step of this reaction could be an electron transfer from the alkene to P-450-Fe(V)=O, which would lead to a substrate radical-cation and P-450-Fe(IV)=O. Recombination of these two species within the active site would lead to a substrate-derived free radical (or cation) linked to the iron through a Fe—O—C—C moiety. Another mechanism for this first step of the epoxidation could be the free radical addition of the P-450 active oxygen complex to the alkene double bond with formation of the same free radical linked to the iron. The last step of the reaction should be the intramolecular transfer of the oxygen atom bound to the iron to the intermediate substrate-derived radical or cation (Figure 9). Most often, the substrate-derived carbon-centered species (radical or cation) reacts with the oxygen atom, leading to the formation of an epoxide. However, in the particular case of monosubstituted unhindered alkanes, this species may also react with a pyrrole nitrogen atom of the heme. This explains the formation of N-alkylated hemes (green pigments) during the in vivo oxidative metabolism of some monosubstituted alkenes [45] (Figure 9).

Cytochromes P-450 also catalyze the hydroxylation of aromatic rings. In most cases, these reactions involve the intermediate formation of arene oxides derived from the epoxidation of a double bond of the aromatic compound and an isomerization of these very reactive epoxides into the corresponding phenols.

Finally, cytochromes P-450 catalyze the oxidation of heteroatom-containing molecules, leading to products that derive either from the transfer of an oxygen atom to the heteroatom (N- or S-oxygenation) or from the oxidative cleavage of a carbon-heteroatom bond (N- or S-dealkylation) [46].

IV. REDUCTION OF O₂ BY CYTOCHROME *c* OXIDASE

IV.A. Nature and Role of Cytochrome *c* Oxidase (EC 1.9.3.1)

Cytochrome *c* oxidase (also called cytochrome oxidase) is the terminal component of the complex system that is present in many aerobic living organisms; it



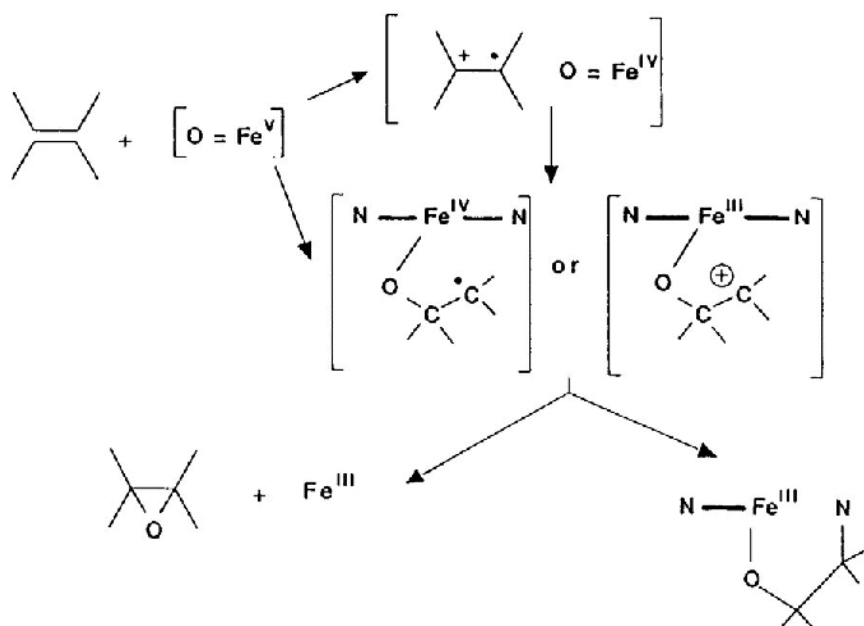


Figure 9 Generally accepted mechanisms for the oxidation of alkenes catalyzed by cytochromes P-450.

catalyzes the four-electron reduction of O_2 to H_2O , thus providing the energy necessary for the cells [47,48]. In the mitochondria of mammalian cells, electrons from various endogenous donors are transferred to cytochrome *c* oxidase as a result of a complex electron transfer chain. In fact, cytochrome *c* oxidase mediates the transfer of electrons from cytochrome *c*, the last component of the chain, to dioxygen. It catalyzes the reduction of O_2 into H_2O with a spectacular efficiency, its turnover rate being about 400 sec^{-1} [47]. Cytochrome *c* oxidase is a complex protein embedded in the inner membrane of the mitochondrion. For instance, bovine heart cytochrome *c* oxidase is a multicomponent membrane protein complex of 200 kDa involving 13 polypeptide subunits. Its x-ray structure (at $2.8 \text{ \AA}$) has recently been solved [49a]; it contains seven metal atoms, the two irons of hemes A, three coppers, one magnesium, and one zinc atom. Five of them are involved in electron transfer from cytochrome *c* to O_2 . The Cu_A center, which is close to cytochrome *c*, is a dimetallic $\text{Cu}-\text{Cu}$ site with two bridging cysteine ligands (Figure 10). Electrons are then transferred to cytochrome *a*, which involves a hexacoordinate $\text{Fe}(\text{III})$ ion bearing two axial histidine ligands. The site responsible for dioxygen binding and reduction involves heme a_3 and Cu_B . In



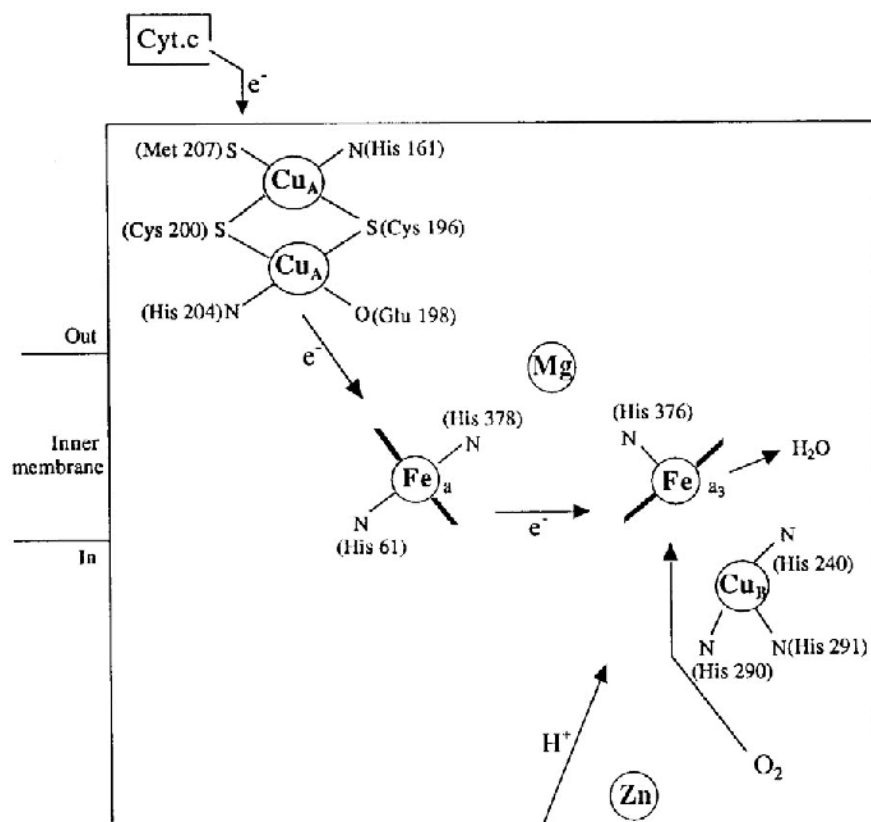


Figure 10 Schematic view of the polymetallic active site of bovine heart cytochrome *c* oxidase, deduced from its x-ray structure [49a].

the resting state of the enzyme heme a_3 iron(III) is pentacoordinate with only one axial imidazole ligand from His376; Cu_B(II) is coordinated by three imidazoles from His240, His290, and His291. The Mg ion is located between the Cu_A and heme a_3 —Cu_B sites. Zn(II) is far from the redox centers; it is bound to four cysteines in a tetrahedral configuration. The roles of these Mg(II) and Zn(II) centers remain to be determined.

The arrangement of the metal centers is in remarkable agreement with the structure, also reported in 1995 [49b], for another member of the superfamily of heme-copper oxidases, the cytochrome oxidase from *Paracoccus denitrificans*. The two structures show a strikingly similar coordination and arrangement of the five redox-active metals (the two irons and three coppers).



The Fe(III) and Cu(II) states of heme a_3 and Cu_B are EPR-silent. This result may be explained by magnetic coupling between the two paramagnetic centers. As the distance between heme a_3 iron and Cu_B is 4.5 Å in the bovine enzyme, and 5.2 Å in the bacterial enzyme, the EPR result would require the presence of a bridging ligand [49c,d]. The presence of an amino acid side chain randomly oriented so that the electron density could not be detected by x-ray crystallography is unlikely because all the amino acid side chains near the heme a_3 —Cu_B site have been assigned [49a]. The presence of bridging water molecules cannot currently be excluded.

IV.B. Mechanism of O₂ Reduction by Cytochrome *c* Oxidase

Reduction of O₂ to water occurs through a series of short-lived intermediates, yet to be fully characterized by time-resolved spectroscopies. Time-resolved absorption and Raman spectroscopies have already been most valuable in giving a preliminary idea of the nature of several intermediates involved in O₂ reduction by mammalian cytochrome *c* oxidase [50]. Binding of O₂ to the heme a_3 —Cu_B center only occurs after two-electron reduction of this center. The transfer of one electron from reduced Cu_B to the heme a_3 —Fe(II)—O₂ complex formally leads to a Fe(III)—O—O[−] peroxy intermediate, which is comparable to the Fe(III)—OO[−] intermediate of the cytochrome P-450 catalytic cycle (Figure 7). Then, transfer of an electron (the third electron received by resting cytochrome oxidase) to the peroxy intermediate results in O—O bond cleavage and formation of OH[−] (or a water molecule). The remaining oxygen atom is bound to heme a_3 iron in the form of an iron(IV)=O oxo complex, which is equivalent to peroxidase compound II (Figure 5). The transfer of a fourth electron (and a third hydron) to the heme a_3 —Cu_B center generates a heme a_3 —Fe(III)—OH intermediate. Finally, hydration of this ferric hydroxide species regenerates the resting state of the heme a_3 —Cu_B center after the release of a second water molecule (Figure 11).

V. CHEMICAL MODEL SYSTEMS FOR DIOXYGEN ACTIVATION BY OXYGENASES

V.A. Main Challenges

From an economic point of view, biomimetic systems able to perform selective oxidations by O₂ alone (dioxygenase models) would be much superior to monooxygenase models that perform similar oxidations but with the consumption of a reducing agent (according to the monooxygenase equation). However, as mentioned previously, dioxygenases only catalyze the oxidation of a limited number of substrates that are already relatively reactive by themselves and susceptible



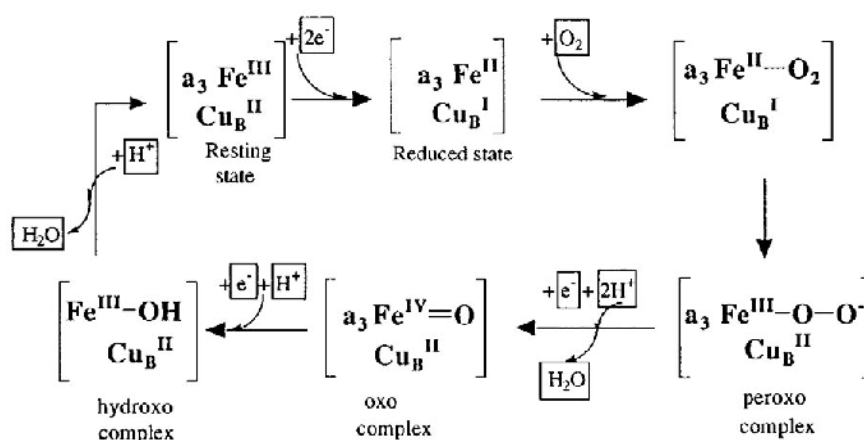


Figure 11 Reduction of O_2 into H_2O catalyzed by mammalian cytochrome c oxidase: possible intermediates formed in the heme a_3 - Cu_B center.

to autooxidation (unsaturated fatty acids, indoles, phenols, catechols, etc.). This could be the reason for the great effort to find chemical models for cytochrome P-450-dependent monooxygenases. Such models should activate O_2 with consumption of a reducing agent and catalyze the oxidation of a wide range of organic substrates including alkanes. Therefore, this section will be mainly devoted to monooxygenase models.

If one considers the catalytic cycle of substrate oxidation by cytochrome P-450 (Figure 7), a simplistic idea would be that an ideal model system should associate a synthetic iron-porphyrin, a thiolate ligand, a reducing agent, and O_2 . However, the catalytic cycle of cytochrome P-450 using O_2 itself is very difficult to mimic with such simple systems for the following main reasons: (1) the use of thiolate ligands appears difficult because of their fast oxidation by the strong oxidizing species formed in such systems; (2) in the absence of a steric protection of the porphyrin ring, the porphyrin is rapidly destroyed by oxidation by the strong oxidizing species produced during the reactions; and (3) the highly oxidizing $Fe(V)=O$ species should be rapidly reduced in the presence of the reducing agent used in stoichiometric amounts in monooxygenase reactions, and this reaction should severely compete with the oxidation of the substrate, thereby consuming the reducing agent.

In cytochrome P-450, this reaction is avoided as NADPH has no access to the P-450 active site. Therefore, it is clear that the alternate, shortened catalytic cycle of cytochrome P-450 using an oxygen atom donor AO, instead of O_2 and NADPH (Figure 7), is easier to mimic.



V.B. Model Systems for Cytochrome P-450 Using Single Oxygen Atom Donors

These considerations explain why the most efficient systems described so far to perform P-450-like oxidations are based on robust (oxidation-resistant) metalloporphyrin catalysts associated with different oxygen atom donors such as PhIO, ClO^- , H_2O_2 , alkylhydroperoxides, peracids, KHSO_5 , ClO_2^- , or tertiary amine *N*-oxides. The development of these systems and the results obtained have been the subject of several review articles [30,36,38,51–56]. Thus, the purpose of this section is not to describe the various model systems but only to mention briefly some of their major characteristics that are necessary to understand the development of physiologically relevant model systems of this cytochrome (using O_2 and NADPH). From the various metalloporphyrins tested as catalysts in such systems, Fe(III) and Mn(III) porphyrins gave the best results and reproduced quite well the different reactions catalyzed by cytochromes P-450 (i.e., C—H bond hydroxylation, alkene epoxidation, aromatic ring hydroxylation, N-oxidation of amines, S-oxidation of thioethers). The successful use of Mn(III) porphyrins as synthetic analogs of cytochrome P-450 might appear surprising. It is based on the very similar chemoselectivity of the active oxygen species formed in Fe(III) and Mn(III) porphyrin-catalyzed oxidations, which are in both cases high-valent metal-oxo complexes, formally Fe(V)=O and Mn(V)=O species.

The porphyrins of the first generation used at the beginning of these studies were meso-tetraarylporphyrins, such as Fe(TPP)Cl (Figure 12). Unfortunately, although many of the Mn(III) porphyrins were found more resistant to oxidative degradation than their Fe(III) analogs, simple metallotetra-arylporphyrins are generally rapidly degraded during the oxidations of poorly reactive substrates such as alkanes. Much better results were obtained with tetra-arylporphyrins involving meso-aryl groups bearing electron-withdrawing substituents, such as Mn(TDCPP)Cl (Figure 12). With these catalysts, efficient epoxidation of alkenes and hydroxylation of alkanes were observed even with oxidants known to degrade metalloporphyrins rapidly such as H_2O_2 . However, it is noteworthy that H_2O_2 or alkyl-hydroperoxides only gave satisfactory results in the presence of cocatalysts, such as imidazole, benzoic acid [54–56] and ammonium acetate [57], which facilitate the necessary heterolytic cleavage of the O—O bond of these hydroperoxides (Figure 13).

A third generation of Fe(III) and Mn(III) porphyrins bearing electron-withdrawing substituents such as halogens [58], SO_3H [59], and NO_2 [60] not only on their meso-aryl groups but also on the β -pyrrole positions, has been described. The structure of one of these catalysts, Fe(TDCP Cl_8P)Cl, is given in Figure 12. They appear much more active and are less rapidly degraded in the oxidation of hydrocarbons by PhIO [58a,d,g]. For instance, the hydroxylation of heptane by



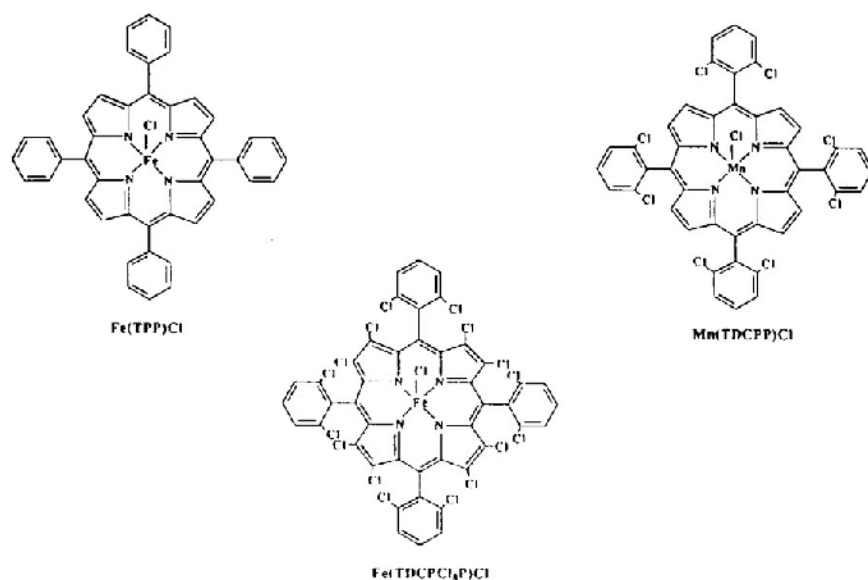
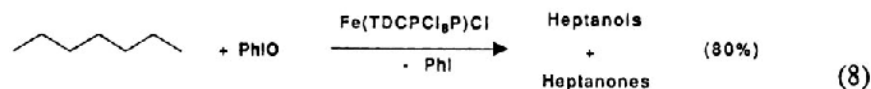


Figure 12 Examples of metalloporphyrins used in model systems of cytochrome P-450. TPP, *meso*-tetraphenylporphyrin; TDCPP, *meso*-tetrakis-(2,6-dichlorophenyl) porphyrin; TDCPCL₈P, *meso*-tetrakis-(2,6-dichlorophenyl)-β-octachloroporphyrin.

PhIO is catalyzed by [Fe(TDCPCL₈P)Cl] and occurs within a few minutes at room temperature with a 80% yield [58g] (Eq. 8). However, the first data indicate that,



in the particular case of oxidations by H₂O₂, these perhalogenated metalloporphyrin catalysts are oxidatively destroyed [58d,h]. Some of them have given very good results in drug oxidations [61,62].

Finally, it is very important to note that more sophisticated catalysts have been prepared either by introducing bulky and/or chiral substituent on the meso-aryl or pyrrole rings [for recent reviews see, for instance, 36,56e,56f,63] or by inserting the metalloporphyrin into various polymeric materials [64–75, for recent reviews see also 76]. These results have shown that this was a good strategy not only toward asymmetric oxidation catalysts, but also toward more regioselective and/or efficient and stable catalysts.



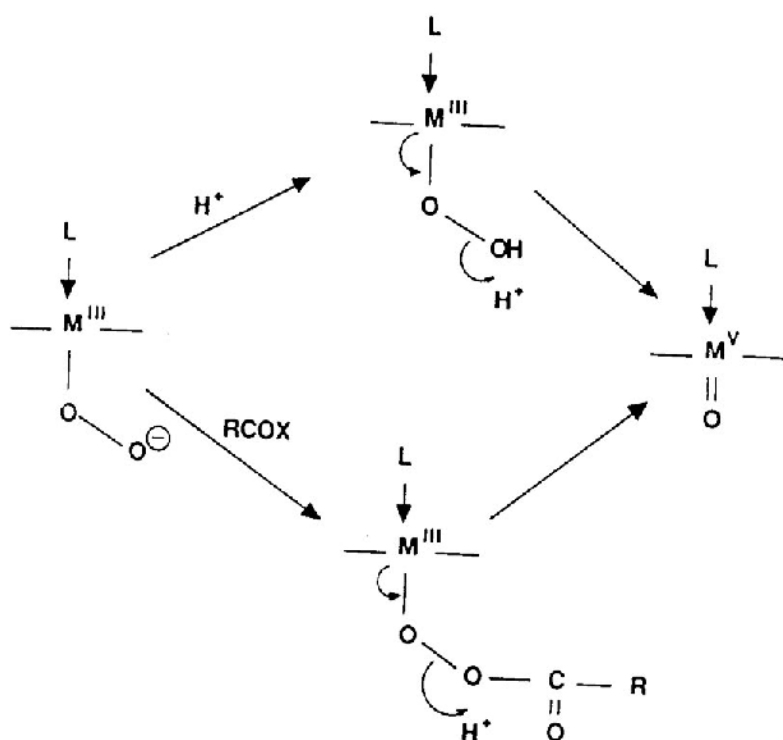


Figure 13 Formation of high-valent metal-oxo species by heterolytic cleavage of the $O-O$ bond of reduced O_2 (or of H_2O_2); advantageous role of H^+ and acylating agents. ($M = Fe$ or Mn .)

V.C. Model Systems for Cytochrome P-450 Using O_2 and a Reducing Agent

As mentioned earlier, there are additional problems in the search for efficient model systems that both mimic the long catalytic cycle of cytochrome P-450 and use O_2 and a reducing agent. First, the catalyst should bind O_2 with a sufficient affinity; this should eliminate some metalloporphyrins that are relatively electron-deficient and favor the use of imidazole cocatalysts as axial ligands that increase the binding affinity of $Fe(II)$ for O_2 . Second, the heterolytic cleavage of the $O-O$ bond of the $M(III)-O-O^-$ intermediate must be fast enough to avoid a competing homolytic cleavage. From model systems using H_2O_2 , peracids, or hydroperoxides, the presence of acid and base catalysts is essential to assist the departure



of the second oxygen atom, either as H_2O or as a better leaving group after reaction with an acylating agent (Figure 13). The presence of an electron-donating axial ligand at the metal to facilitate the heterolytic cleavage of the O—O bond is also very important. Accordingly, the heterolytic cleavage of the O—O bond of porphyrin— Fe(III)—O—O—COR complexes was found to involve a very low activation enthalpy (17 kJ/mol) and to be acid catalyzed [77]. Third, the high-valent metal-oxo species should not be reduced too rapidly by the reducing agent. All these parameters have been discussed in a very good review on the reductive dioxygen activation by use of artificial P-450 systems [53].

Many systems that use a Fe(III) or Mn(III) porphyrin catalyst and a reducing agent (some of them use imidazoles or pyridines as cocatalysts and an acylating agent) have been found to transfer one oxygen atom from O_2 to hydrocarbons. Borohydrides in the presence of Mn(TPP)Cl as catalyst were the first reducing agents used in such systems and gave encouraging results in the oxidation of alkenes by O_2 [64,66,78–84]. Sodium ascorbate was also used in the presence of catalytic amounts of Mn(TPP)Cl in a biphasic system (benzene-buffer pH 8.5) and in the presence of a phase-transfer agent [85,86]. This system was found able to epoxidize alkenes in a chemoselective manner and to hydroxylate alkanes with low yields. Hydrogen was also used as a reducing agent in the presence of catalytic amounts of colloidal platinum and gave interesting results particularly for alkene epoxidation in the presence of Mn(TPP)Cl and *N*-methylimidazole as catalysts [87–90]. Electrochemistry is another way to provide the electrons necessary for O_2 activation in the presence of Fe(III) or Mn(III) porphyrins [72,73,91–94]. The addition of stoichiometric amounts of acid chlorides or acid anhydrides to these systems led to better rates and yields based on the consumed electrons [91,92,94]. The best yield (about 60%, based on the reducing agent) was obtained for cyclo-octene epoxidation by O_2 in an electrochemical system using the Mn(TPP)Cl catalyst and benzoic anhydride as an acylating agent. However, the rate of this epoxidation remained low because of a slow electron transfer [92]. In such model systems it is very difficult to obtain both good rates, which require a fast electron transfer from the reducing agent to the metalloporphyrin, and good yields (based on the reducing agent), which requires a reduction of the active metal-oxo species by the reducing agent slow enough to allow the transfer of its oxygen atom to substrates. These appear to be contradictory requirements. In cytochrome P-450-dependent monooxygenases, the problem of electron transfer is solved by a complete separation between the Fe(V)=O species and NADPH and a proper positioning of the substrate relative to the active species. In fact, in the case of poorly reactive and/or poorly positioned substrates, cytochrome P-450-dependent monooxygenases reduce O_2 in H_2O_2 or H_2O either partly or totally [22,95].

A model system based on a closer analogy with monooxygenases was



studied; it involved a dihydropyridine as a reducing agent, and a water-soluble anionic Mn(III) porphyrin, *N*-methylimidazole, and a flavin mononucleotide as catalysts [53,96]. The presence of catalytic amounts of the flavin mononucleotide led to a 1600-fold increase of the rate of electron transfer from the dihydropyridine to the Mn-porphyrin. Further increases of this rate were obtained with Mn-porphyrin catalysts, where the flavin was covalently linked to the porphyrin or where the Mn-porphyrin and the flavin were both covalently bound to cyclodextrin [53]. The Mn(III)-porphyrin/*N*-methylimidazole/flavin mononucleotide/dihydropyridine system was found to epoxidize nerol with a rate of nine turnovers per minute and yields based on the reducing agent of about 30% [96]. A similar system based on a Fe(III) porphyrin and a dihydropyridine was found able to perform the selective cleavage of carbon–carbon bonds of 1,2-diols by O₂ [97].

Efficient model systems using Zn as a reducing agent have also been reported. A first system used Zn amalgam as a reductant in the presence of methylviologen as an electron transfer catalyst, a Fe(III) porphyrin catalyst, and acetic anhydride as a stoichiometric acylating agent [98]. Hydroxylation of cyclohexane by this system occurs with a high kinetic isotope effect ($k_H/k_D = 7$) and with yields up to 30% (based on O₂ consumed) and rates up to 1.1 turnover/min. Another system used Zn powder as a simple reducing agent and CH₃COOH as a hydrogen ion source, in the presence of catalytic amounts of Mn(TPP)Cl and *N*-methylimidazole [99]. At least from a qualitative point of view, this system performs the main reactions catalyzed by cytochrome P-450, such as the epoxidation of alkenes and phenanthrene, the hydroxylation of alkanes and aromatic rings like naphthalene, the S-oxidation of thioethers, and the N-dealkylation of amines [99]. Rates and yields of the oxidations performed by this system were relatively good (i.e., rates of 2, 3, and 0.5 turnovers/min and yields based on Zn of 70%, 50%, and 15%, for the sulfoxidation of di-*n*-butylthioether, the epoxidation of cyclo-octene, and the hydroxylation of cyclo-octane, respectively).

An artificial system mimicking electron transfer in monooxygenases and based on a multicomponent vesicular assembly has been reported [100]. In this system, electrons derived from the enzymic decarboxylation of pyruvic acid reduce a synthetic membrane-spanning Mn(III) cholesteryl porphyrin and mediate subsequent dioxygen activation and transfer. The membrane-spanning porphyrin, tetrakis-[ortho-(3-hydroxy-5-cholenoylamino)phenyl] porphyrin, was used for regioselective epoxidation of steroids by PhIO [101–103]. The electron transfer chain organized in dipalmitoylphosphocholine vesicles consists of pyruvate as the electron donor, pyruvate oxidase (which catalyzes the decarboxylation of pyruvate and the reduction of an enzyme-bound flavin adenine dinucleotide [FAD]), and an amphiphilic flavin that directly transfers electrons to the Mn(III) porphyrin. This system was found able to catalyze the oxidation of ethylbenzene to acetophenone by dioxygen and pyruvate [100].



VI. CONCLUSION

All the proteins described in this chapter and that catalyze very different reactions involving O_2 exhibit the following common characteristics: (1) the presence of an iron porphyrin cofactor, (2) an axial iron ligand coming from the protein that is in most cases a histidine imidazole (except for cytochrome P-450), and (3) the intermediate formation of high-valent iron-oxo species, formally equivalent to $Fe(IV)=O$ or $Fe(V)=O$, which are key intermediates in the enzymatic reactions. The different reactions performed by these iron-oxo species and the different uses of O_2 by the hemoproteins are due to the very different environments of the heme in these hemoproteins and to a more or less rapid electron transfer. As a function of its environment, the iron-oxo species may either be reduced into H_2O if electrons are easily transferred to the heme or oxidize a substrate or a protein amino acid that could be present in close proximity in the active site (Figure 14).

In cytochrome *c* oxidase, the presence of five metallic centers, which may all exist in a reduced state, transfer one electron, and are in close proximity in the active site (Figure 10), allows a very fast reduction of the cytochrome $a_3-Fe(IV)=O$ species transiently formed during O_2 reduction and a complete reduction of O_2 into H_2O . In this case, because of a very restricted access of substrates to the active site or cytochrome a_3 and this very fast electron transfer to cytochrome a_3 , reduction of O_2 into H_2O occurs without any substrate oxidation and without accumulation of species derived from O_2 , such as $O_2^{\cdot-}$, H_2O_2 , or $OH^{\cdot}$. The active site of cytochrome P-450 is very different as it provides easy access to organic substrates that are maintained by the protein in close proximity to the

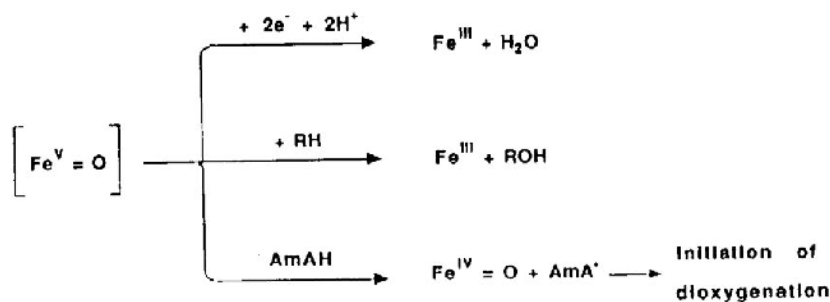


Figure 14 Main possible fates of the high-valent iron-oxo species formed as intermediates in O_2 -activating hemoproteins (RH is a substrate; AmAH is an amino acid residue close to the heme in the active site).



heme. Moreover, cytochrome P-450-dependent monooxygenases are organized to transfer rapidly two electrons from NADPH (or NADH) to O_2 . This leads to a $Fe(V)=O$ species that is not rapidly reduced by electrons from the reductase and has enough time to transfer its oxygen atom into well-located substrates. Another very different situation occurs in the case of prostaglandin synthase, which is not coupled to an electron transfer chain and whose $Fe(V)=O$ species does not seem able to react directly with arachidonic acid. This species oxidizes a tyrosine residue of the protein, thus creating a free radical center that will be able to abstract an allylic hydrogen of arachidonic acid and to start the dioxygenation reaction (Figure 4).

As far as the formation of these high-valent iron-oxo complexes is concerned, either by direct reaction of H_2O_2 with $Fe(III)$ —hemoproteins or after the binding of O_2 to $Fe(II)$ —hemoproteins and its subsequent two-electron reduction, heme model studies have underlined the great importance of the presence of acid and base catalysts and of the nature of the iron axial ligand for the necessary heterolytic cleavage of the $O—O$ bond of $Fe(III)—O—OH$ intermediates. Efficient chemical model systems using $Fe(III)$ or $Mn(III)$ porphyrin catalysts have been found to perform cytochrome P-450-like monooxygenations of various substrates including alkanes. Their rates and yields (based on the consumed reducing agent) should be improved by taking into account the following properties, which could provide the basis for the high efficiency of cytochromes P-450: (1) the organization of NADPH, the electron transfer chain, and cytochrome P-450, which allows the necessary specific transfer of two electrons to O_2 to give the $Fe(V)=O$ species; (2) the presence of the axial thiolate ligand, which could facilitate the heterolytic cleavage of the $O—O$ bond of $Fe(III)—O—OH$ and modulate the intrinsic reactivity of the $Fe(V)=O$ species; and (3) the role of the protein to maintain the substrate to be oxidized in close proximity of the heme in order to accelerate its oxidation by the iron-oxo species. It is also noteworthy that the binding of the substrate to a protein site close to the heme very often shifts the equilibrium between the low-spin and high-spin states of resting cytochrome P-450- $Fe(III)$ toward the more reducible high-spin state and controls the electron transfer to the heme.

Dioxygenases like prostaglandin synthase use dioxygen in a very different manner as O_2 is not activated in their catalytic cycle. Their main role is not to activate O_2 but to activate the substrate into a free radical that rapidly reacts with O_2 (Figures 4 and 5). Besides this role as an initiator of the auto-oxidation of substrates, dioxygenases also control the conformation of substrates in their active sites and the fate of the intermediate free radicals. This is the basis of the regio- and stereoselectivity of the reactions catalyzed by dioxygenases. Obviously, the species that perform the activation of substrates in dioxygenases (an amino acid free radical in the case of prostaglandin synthetase, or $Fe(III)$ in the case of lipoxigenases [Figure 5]) are much less potent than the $Fe(V)=O$ active species of



cytochrome P-450-dependent monooxygenases. This is why, contrary to monooxygenases, dioxygenases only catalyze the oxidation of substrates that are already reactive by themselves. From a chemical point of view, catalysts that would catalyze the selective oxidation of the wide range of substrates of monooxygenases (including alkanes) by a dioxygenase-like reaction—by using O_2 itself without consumption of a reducing agent—would be particularly interesting. It has been reported that Fe(III) polyhalogenated porphyrins catalyzed the oxidation of alkanes such as propane or butane by O_2 into the corresponding alcohols and ketones [58e,58i]. These oxidations are related to alkane autooxidations and involve the intermediate formation of free radicals $R^\cdot$ and $ROO^\cdot$ derived from the alkane RH [58e,58i,104]. Oxidation of alkanes to ketones and alcohols by O_2 was also observed in the presence of photoactivated iron porphyrins [105]. Here, again, free radicals $R^\cdot$ and $ROO^\cdot$ derived from alkanes appear as intermediates. However, those oxidations also seem to involve transient high-valent iron-oxo complexes. Therefore, such iron porphyrin-catalyzed oxidation of alkanes could partly exhibit some dioxygenase-like characteristics, even though they are presently much less selective than dioxygenases.

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12

Biological and Biomimetic Catalysis of Manganese Redox Enzymes and Their Inorganic Models

Jan Wikaira and Sergiu M. Gorun

Brown University, Providence, Rhode Island

I. INTRODUCTION

In the last three decades there has been increasing interest in bioinorganic chemistry in general, and the structural and functional modeling of metalloproteins in particular. This chapter is concerned with manganese redox enzymes and their synthetic models. Of the ever-increasing number of manganese-containing biomolecules now recognized [1–15] a few are known to be redox in nature. These include manganese-containing superoxide dismutase (EC 1.15.1.1), catalase (EC 1.11.1.6), Mn-ribonucleoside reductase (EC 1.17.4.1), Mn-peroxidase, ligninase, the oxygen-evolving center (OEC) of photosystem II (PSII), and Mn-thiosulfate oxidase.

Much of the understanding of such structural aspects as oxidation states, coordination, and the types of bridges between the manganese centers comes from modeling work. More recently, biomimetic *functionality* has become the target of synthetic chemists, consistent with a high level of maturity and sophistication reached by the *structural* modeling. Consequently, in addition to the Mn containing enzymes, this review will focus on their *functional* models, especially those developed in the last five years.

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II. SUPEROXIDE DISMUTASE

II.A. Introduction

The superoxide dismutases (SODs) catalyze the disproportionation of superoxide ion (Eq. 1) [5] into peroxide and molecular oxygen:



Three distinct forms of superoxide dismutases, classified according to their active site metal ion(s), are known: Cu,ZnSOD found in eukaryotes and some bacterial pathogens, MnSOD found in prokaryotes and eukaryotic mitochondria, and FeSOD found in prokaryotes. The Mn- and Fe-containing SODs are closely related, but the Mn enzyme exhibits unique physiological characteristics. For example, it has been found in vivo that, in contrast to either Cu,Zn or FeSODs, production of MnSOD increases during periods of oxidative stress [16]. In addition, MnSOD is not inhibited by H_2O_2 and human MnSOD has a significantly longer serum half-life, 5–6 hr, compared with 6–10 min for Cu,ZnSOD. Currently MnSOD is regarded as a potential treatment in a number of medical conditions, e.g. oxygen-damaged retinal [17], pulmonary [18,19], neural [20], and renal [21] tissues; influenza [22]; postischemic reperfusion of organs [23]; acute and chronic inflammation including arthritis [24]; and cardiac damage [25]. The SODs are also believed to have an anticancer role [26]. Importantly, besides MnSOD, even their model inorganic complexes have been proposed as drugs [27].

The Mn and Fe enzymes [28], which are structural homologs [29] and contain a single metal at their active site, are unrelated to the dinuclear Cu,Zn proteins. The Mn and Fe ions cycle between the M^{II} and M^{III} states during catalysis. Several x-ray structures of MnSODs are known to date (Table 1) [5].

A preliminary structure of oxidized MnSOD from *Thermus thermophilus* determined at 2.4 Å resolution [30] has recently been refined to 1.8 Å

Table 1 Structurally Characterized MnSOD Enzymes

Active site chromophore	Resolution (Å)	Reference
<i>Thermus thermophilus</i>	2.4	30
Oxidized $[\text{Mn}^{\text{III}}(\text{N} \cdot \text{His})_3(\text{O}_2\text{C}-\text{R})(\text{OH}^-)]$	1.8	31
Reduced $[\text{Mn}^{\text{II}}(\text{N} \cdot \text{His})_3(\text{O}_2\text{C}-\text{R})(\text{H}_2\text{O})]$	2.3	31
Azide-bound form $[\text{Mn}^{\text{III}}(\text{N} \cdot \text{His})_3(\text{O}_2\text{C}-\text{R})(\text{H}_2\text{O})(\text{N}_3)]$	1.8	33
<i>Bacillus stearothermophilus</i> $[\text{Mn}^{\text{III}}(\text{N} \cdot \text{His})_3(\text{O}_2\text{C}-\text{R})]$	2.4	32
Human mitochondrial $[\text{Mn}^{\text{III}}(\text{N} \cdot \text{His})_3(\text{O}_2\text{C}-\text{R})(\text{H}_2\text{O})]$	2.2	16



resolution. A dithionite-reduced form was subsequently refined to 2.3 Å resolution [31]. Usually prokaryotic MnSODs are dimeric and eukaryotic MnSODs are tetrameric; however, the MnSODs from *T. thermophilus* and *T. aquaticus* are both tetrameric.

II.B. Metal Active Site Structure

The crystal structure of *T. thermophilus* in both oxidized and reduced forms (Figure 1) reveals a trigonal bipyramidally coordinated Mn(III) ion: two N (His83, His170) and one O (Asp166) donors lie in the equatorial plane, and another N (His28) and one solvent oxygen define the axial positions [31].

The nonprotein oxygen in the oxidized structure is a hydroxide ion, which compensates for the positive charge increase that occurs on oxidation of Mn^{II} to Mn^{III}. No coordinated water was located at the active site of MnSOD from *Bacillus stearothermophilus* [32], presumably because of the high degree of water disorder in the crystal lattice [33,34]. Little structural change is noted on Mn reduction in *T. thermophilus*, but, as expected, the Mn—O bond lengthens as a result of OH hydration and Mn ionic radius increase. The metal ions are embedded deep in the protein structure, thus being shielded from direct accessibility. This shielding is provided by a “gateway” [31], formed by the conserved residues His32 and Tyr36. The gateway may open by a Tyr36 flip [35] to allow substrate binding to Mn via a channel that connects the surface of the protein with the gateway [31].

The structure of human mitochondrial MnSOD (determined at 3 Å [36] and 2.2 Å [16] resolution), although similar to that of the MnSOD from *T. thermophilus* as far as the Mn coordination sphere and its tetrameric form are concerned, is more compact, as evidenced by the differences in the Mn...Mn dis-

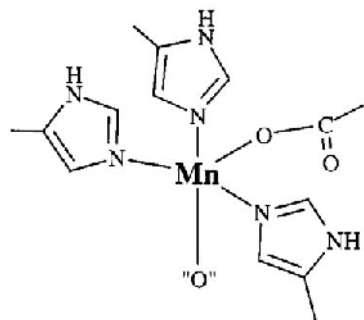


Figure 1 The active site of MnSOD (human kidney). O, H₂O (reduced form), or HO[−] (oxidized form).



tances: 40.7 Å and 42.0 Å in the former and 45.4 Å and 48.9 Å in the latter. The closer packing in the human MnSOD occurs because the N-terminal helices of the interconnecting hairpin region are longer than those in *T. thermophilus*.

The four subunits of the human mitochondrial MnSOD form two dimers and four extended helical hairpins, which assemble into two four-helix bundles at opposite ends of the dimer. The novel tetrameric interface of 820 Å² that forms (the dimer interface is 720 Å²) becomes the dominant factor in the tetramer assembly. The four-helix bundle also induces favorable Mn coordination geometry and forms a central tunnel, whose boundaries provide substrate (superoxide) recognition as a result of its positively charged Lys and Arg side chain lining. Importantly, the four-helix bundle interfaces seem to be unique to mitochondrial MnSOD and thus may be directly responsible for its previously mentioned extended serum half-life [16].

Early magnetic circular dichroism (MCD) spectroscopic studies over a wide temperature range, aimed at elucidating the catalytic mechanism (discussed later) [37], indicated that fluoride or azide binding to Mn^{III} in MnSOD (from *Escherichia coli*) resulted in displacement of an undetermined ligand in the active site, thus maintaining the 5-coordinate metal environment. The crystal structure of the azide-bound complex of *T. thermophilus* reveals that, in contrast, all four protein ligands and the axial water are retained, yielding an overall distorted octahedral geometry [33] (Figure 2).

This apparent contradiction has been resolved by recent variable temperature absorption spectroscopy studies on the native, azide-bound, and fluoride-bound forms of MnSOD from *E. coli* [38]. It was demonstrated that the Mn^{III} ion coordination number, in the anion-bound form, changes from 5 to 6 at low

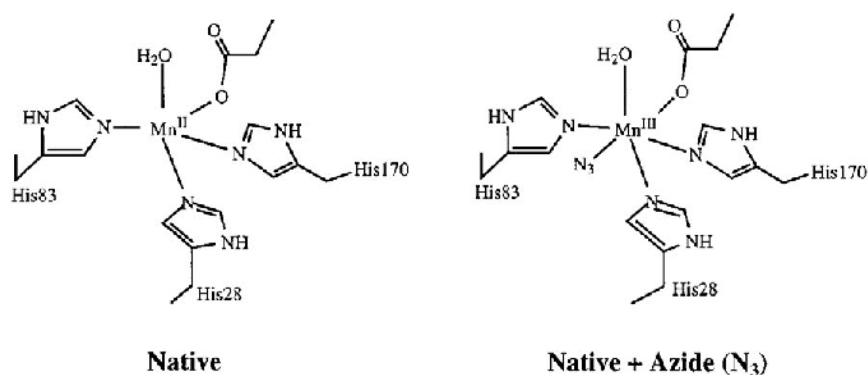


Figure 2 The active site of MnSOD from *Thermus thermophilus* and the azide complex (25°C) based on the x-ray coordinates [33]. (After Ref. 38.)



temperature; i.e., the active site structure is temperature dependent: the octahedral low-temperature azide or fluoride complex converts to a 5-coordinate complex at 295K, where both the native and the azide forms are 5-coordinate; that is, addition of the anion results in loss of an unidentified ligand [38]. This ligand is most likely to be either the bound water molecule or Asp166; the latter possibility is supported by an increase of 0.4 Å in the carboxylate-Mn bond length on azide binding in *T. thermophilus* [33]. The spectra of the low-temperature Mn-anion complexes of *E. coli* MnSOD are very similar to the 2°–6°C (‘‘low-temperature’’) spectrum of the superoxide bound form of *T. thermophilus* [39]. In this catalytically inactive form the loss of function was attributed to the 6-coordinate geometry of the Mn^{III} ion. It is conceivable that, as an adaptation to very different biological environments, this coordination transition takes place at distinctly different temperatures for MnSOD from highly thermophilic *T. thermophilus*, compared to that from mesophilic *E. coli* [38].

II.C. Catalytic Mechanism

The superoxide dismutation mechanism (Figure 3) [5] is consistent with the preceding structural data.

The superoxide anion binds to a vacant site on Mn^{III}, reduction to Mn^{II} takes place, and a dioxygen molecule is evolved upon hydronation of the OH

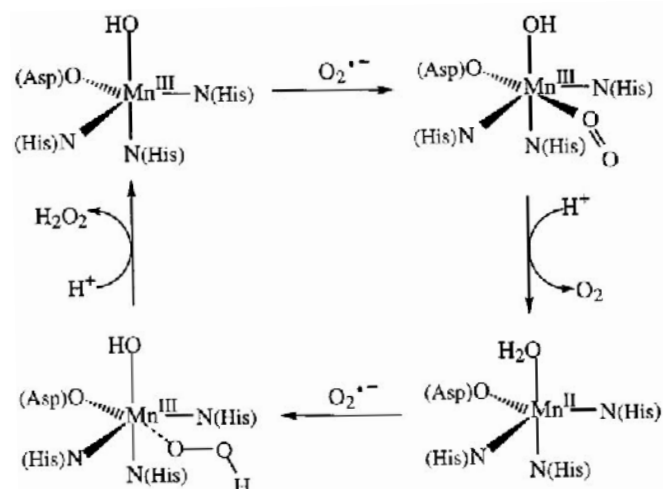


Figure 3 Superoxide dismutation mechanism under physiological conditions. (After Ref. 5.)



group. A second superoxide anion binds to Mn^{II} , which is oxidized, and a hydron transfers from the Mn^{II} bound water to the peroxide anion to form a hydroperoxo species. Dihydrogen peroxide release occurs upon hydronation of the Mn^{III} hydroperoxo intermediate.

The structural changes observed during the catalytic cycle are thus consistent with the proposed oxidation state changes and variation in the Mn coordination number. The latter changes have been termed the “5–6–5” mechanism [33].

The rate of electron transfer that occurs to/from the metal center is high. Structure based modeling of the active site of human MnSOD [40], which includes calculating the energies of both the oxidized and reduced states with either water or hydroxide bound to the metal, suggests the rate of this internal electron transfer is enhanced by electron-relaxation effects. In addition, a 0.17 V redox potential is calculated, a value that is low compared with the experimental values of 0.31 V for *E. coli* and 0.26 V for *B. stearothermophilus*, respectively. A potential of ~ 0.30 V seems to be optimal as it lies midway between the redox potentials of the two half reactions of the dismutation process [41].

The catalytic dismutation of superoxide is actually more complicated in *E. coli* [42] and *B. thermophilus* [43] Mn-SODs than that of either Cu or Fe proteins since it may involve an inactive form of the enzyme. The inactive form is believed [44] to contain a Mn^{III} -side-on peroxo unit (of the type shown in Figure 29) formed within the hydrophobic environment of MnSOD, in the absence of H^+ , by the oxidative addition of the superoxide ion to the Mn^{II} center. When H^+ ions are present, an active, end-on peroxo complex forms, yielding successively a bound hydroperoxide ion and free dihydrogen peroxide (cf. Figure 3). Thus, the key parameter that turns the reaction off or on may be the absence or presence of a H^+ ion [44].

II.D. Model Complexes

Several manganese complexes have recently been proposed as structural and functional models of SOD.

Manganese(II) complexes of hindered tris(pyrazolyl)borate ($\text{L1} = \text{HB}(3,5\text{-}i\text{-Pr}_2\text{pz})_3$) were shown to be both structural and functional mimics of MnSOD. The coordination geometry of one such complex [45], $[\text{Mn}(\text{OBz})(\text{H-3,5-}i\text{-Pr}_2\text{pz})(\text{L1})]$ **1** (Figure 4), is distorted trigonal bipyramidal with apical nitrogen ligands, similar to that of MnSOD.

The SOD activity measurements using the xanthine–xanthine oxidase nitro blue tetrazolium (NBT) method [45] indicated that a concentration of $0.75 \mu\text{M}$ of $[\text{Mn}(\text{OBz})(\text{H-3,5-}i\text{-Pr}_2\text{pz})(\text{L1})]$ **1** and $0.8 \mu\text{M}$ of the related $[\text{Mn}(\text{OBz})(\text{L1})]$ **2** complex exert the same SOD activity as one unit of native SOD. The high SOD activity of the tris(pyrazolyl)borate complexes was attributed to their structural similarities to the MnSOD active site [45].



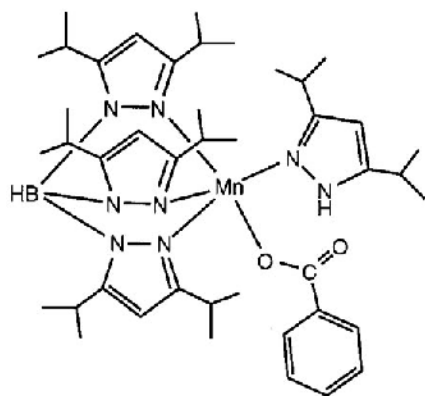


Figure 4 The Mn(OBz)(H-3,5-*i*-Pr₂pz)(L1) complex **1**.

A series of 4- and 5-coordinated Mn^{II} complexes of salen Schiff bases (Figure 5) [20] have either N₂O₂ or N₂O₂Cl chromophores and dismutation rates similar to those of the previously reported desferrioxamine [20,32a,45,46] complexes.

The salen complexes, however, retain SOD activity over weeks in solution, compared to only hours to days for the Mn-desferrioxamine complexes. Surprisingly, a positive charge at the manganese, expected to favor the binding of the superoxide anion, did not increase the SOD activity. For example, complex **7** (Figure 5) is more active than the cationic complex **3**, perhaps because the Mn coordination is more restricted in the latter. The activity of complexes of similar coordination geometry can be changed almost two orders of magnitude by variation of aromatic ring substituents, as indicated by a comparison of complexes **13** and **14** (Figure 5). This unexpected variation may be due to different three-dimensional steric interactions. This hypothesis is supported by the fact that, for each SOD mimic except **14**, rapid and complete inhibition by ethylenediaminetetra-acetic acid (edta) due to active site binding (as opposed to Mn removal) was noted. The exception, complex **14**, may sterically prevent edta, but not the smaller superoxo ion, from accessing the metal center. These complexes are also being considered for medical use.

A series of biomimetic Mn^{II} complexes based upon azacycloalkanes, with aneN_x (*x* = 4–7) (L2) (Figure 6) coordination environment, are being considered for the treatment of inflammatory disorders [27a,47].

Maximization of their SOD activity (measured via stopped flow ultraviolet visible [UV-Vis] spectroscopy) and stability was sought by systematically varying the ligands. Increased stability was an invariable result of increasing the num-



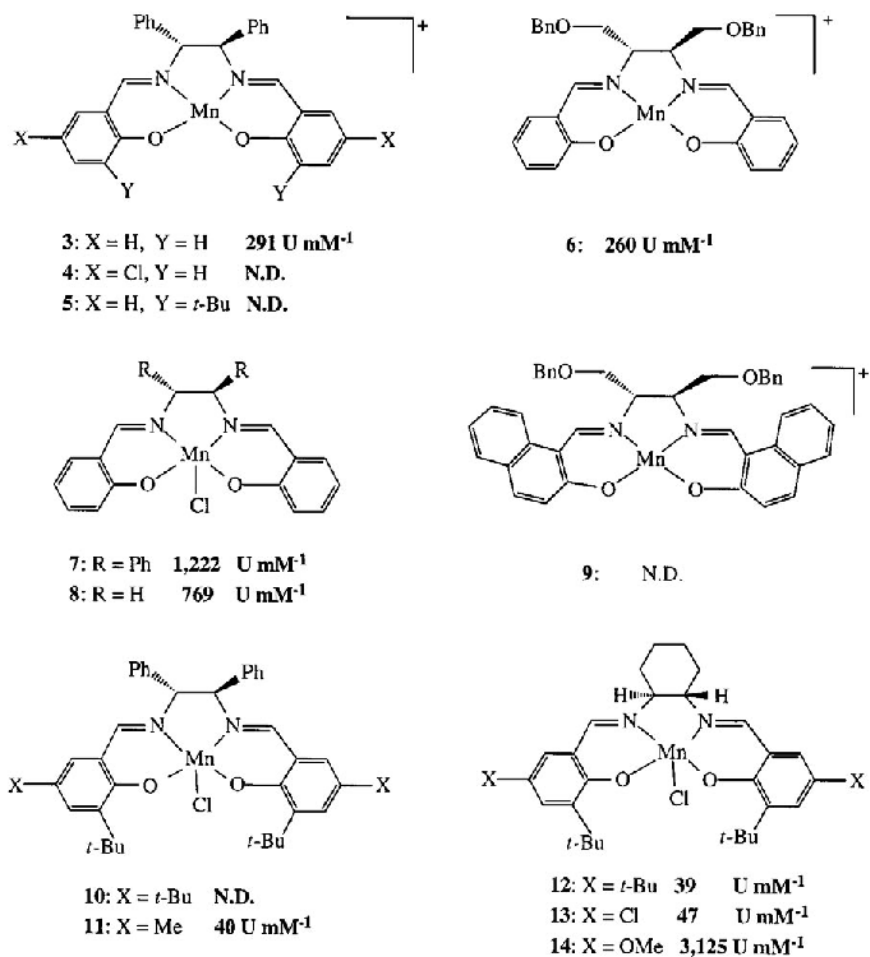


Figure 5 Structure and SOD activity of salen-based complexes. The SOD activity was calculated in Units per millimole (U mM⁻¹). N.D., not detectable. (From Ref. 20.)

ber of substituents of L2 (15, Figure 6) [47c], and the activity was also influenced by their position and stereochemistry. For example, C-substitution, as opposed to N-substitution [47c], increased both the kinetic and thermodynamic stabilities of the complexes, the latter to a much lesser degree. However, increasing the number of substituents did not ensure that the resultant Mn^{II} complex would possess any SOD activity. Inclusion of a single transfused cyclohexane group



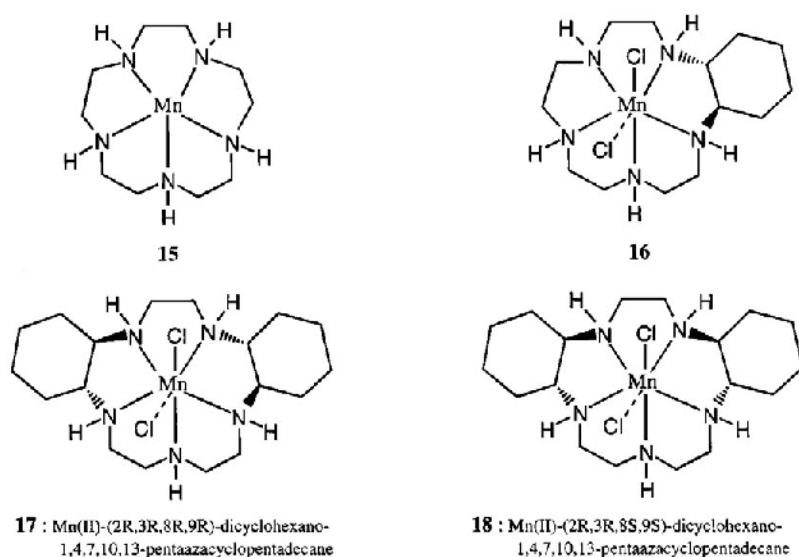


Figure 6 Mn^{II}-1,4,7,10,13-pentaazacyclodecane (L2) complexes. (From Ref. 47f.)

(**16**, Figure 6) into the macrocyclic ring doubled the catalytic activity of **16** over that of **15** and conferred greater stability both kinetically twofold enhancement) and thermodynamically ($\log K = 11.7$ cf. 10.9 for **15**) [47c]. Interestingly, two isomeric complexes, (2R,3R,8R,9R [**17**, Figure 6] and 2R,3R,8S,9S [**18**, Figure 6]), showed wildly different activity. Complex **17** was an excellent catalyst with a second-order rate constant comparable to that of native mitochondrial SOD (at pH 7.4 $k_{\text{cat}} = 1.2 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$), whereas **18** had no detectable SOD activity [47f] despite virtually identical $E_{1/2}$ of 0.74 V and similar dissociation constants.

What is the structural basis for such a difference in catalytic activity? The x-ray structure of the active **17** is not reported, but the structure of the inactive **18** reveals a pentagonal bipyramidal geometry of the Mn center, with two *trans* chlorides and equatorial nitrogens. The ligand is fully extended in order to accommodate this geometry. The solution geometry of the active **17** complex is believed to be octahedral, on the basis of (1) the structure of an active octahedral bis (nitrate) analog and (2) mechanistic studies that suggest that a pH-independent $S_N 1$ (dissociative) pathway is followed in parallel with a pH-dependent one. The latter pathway requires the existence of an octahedral aqua complex as a starting point. The dissociative pathway results in a rapid $\text{Mn}^{\text{II}} \leftrightarrow \text{Mn}^{\text{III}}$ equilibrium (and hence fast catalysis), suggesting similar, octahedral-type coordination environ-



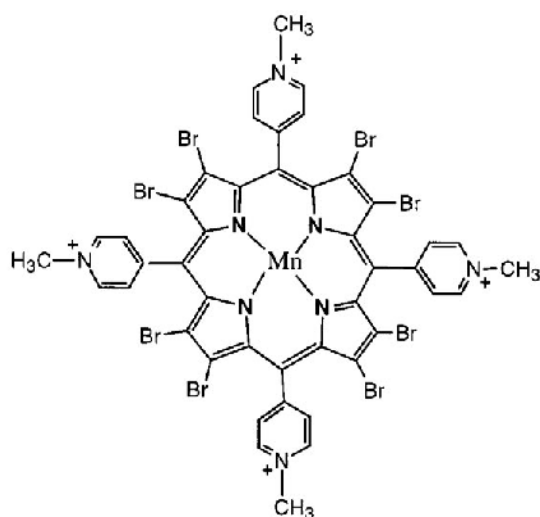


Figure 7 The manganese complex of 2,3,7,8,12,13,17,18-octabromo-5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl) porphyrin **20**.

ments for the two ions. The formation of an octahedral complex requires folding of the ligand, a conformational change easily achieved by **17** but not by **18**. Thus the substituents, which control the folding, also control the rates and the relative contributions of the two pathways to the overall rate of SOD activity [27a,47f].

Other SOD mimics that show promise for medical applications include porphyrin complexes such as Mn 5,10,15,20-tetrakis(*N*-methylpyridinium-4-yl) porphyrin ($\text{Mn}^{\text{III}}\text{TMPyP}^{5+}$) **19** [48] and its octabromo derivative **20** (Figure 7) [49].

The β -substitution with electron-withdrawing halogens results in a 60-fold increase in activity, $k_{\text{cat}} = 2.2 \times 10^8 \text{ M}^{-1} \text{ sec}^{-1}$ vs. $3.8 \times 10^6 \text{ M}^{-1} \text{ sec}^{-1}$, for the parent compound [49]. The enhanced activity has been ascribed to stabilization of the Mn^{II} state.

III. PEROXIDASES AND LIGNIN DEGRADATION

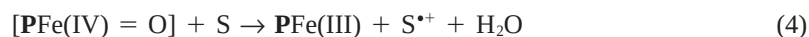
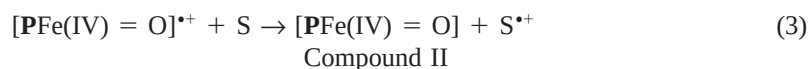
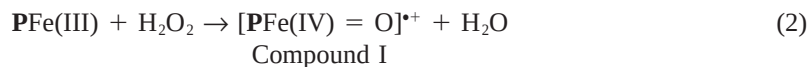
III.A. Introduction

In nature degradation of wood is accomplished almost exclusively by wood-rotting fungi such as *Phanerochaete chrysosporium* and *Ceriporiopsis subvermispora*. Lignin, which accounts for up to a third of the weight of wood, is



degraded in vivo by several extracellular enzymes secreted by these fungi (Figure 8).

Among the most studied fungi, *Phanerochaete chrysosporium* secretes at least two lignolytic peroxidases, namely, lignin peroxidase (LiP) and Mn peroxidase (MnP) [50]. Both LiP and MnP contain a heme iron center at their active site and both function according to the following simplified scheme (where **P** represents porphyrin) [50,51].



Substrate S, however, differs for the two peroxidases:

1. Veratryl alcohol (VA) (3,5 dimethoxy benzyl alcohol) for LiP;
2. Mn(II) for MnP.

Both the radical cation of VA and $\text{Mn}^{\text{III}}(\text{S}^{\bullet+})$ attack and degrade the lignin polymer; LiP also has the ability to oxidize nonphenolic aromatic molecules, most likely by attacking a benzylic C—H bond.

Understanding the MnP and LiP mechanism is important not only from a fundamental point of view but also because delignification of wood chips leaves behind “pulp,” the fibrous polysaccharides (hemicellulose and cellulose) that

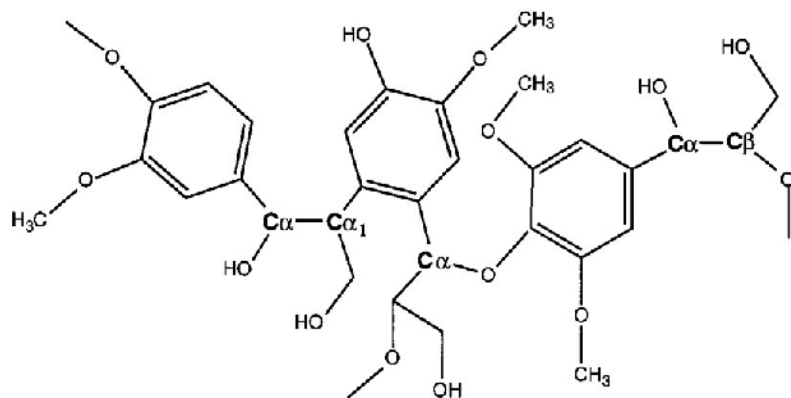


Figure 8 A fragment of lignin, a cross-linked phenyl propane polymer bearing free and methylated hydroxy groups.



will eventually form paper. In this chapter only the structure and catalytic mechanism of MnP will be discussed in some detail.

III.B. Structure of Mn Peroxidases and the Oxidation of Mn

The structure of MnP from *Phanerochaete chrysosporium* has been determined at 2.06 Å resolution [52]. Like most other peroxidases, it contains a heme Fe at its active site (the locus of H₂O₂ binding) and a Phe residue in the immediate environment of the proximal histidine. Notably, at this location a Trp is found in cytochrome *c* peroxidase and a Leu in *Arthromyces ramosus* peroxidase. Although the functional role of the Phe in MnP (and Leu in *A. ramosus*) is not entirely clear, in cytochrome *c* peroxidase the Trp191, present at the same location, both facilitates electron transfer (from ferrocytochrome *c*) and supports a free radical center (Compound I) [53,54]. Replacement of Trp191 by Phe in cytochrome *c* peroxidase obliterates these functions, thus suggesting the formation of a more common, porphyrin centered radical as Compound I of MnP[54].

Importantly, a Mn²⁺ binding site, distinguishable from two other Ca²⁺ sites, is observed in the structure of MnP [52]. In comparison with Ca²⁺, Mn²⁺ seems to be loosely bound (at full site occupancy, as determined by x-ray diffraction), suggesting that this Mn may be released in the environment subsequent to its oxidation. The Mn center is octahedrally coordinated exclusively by oxygen, from two glutamates, one aspartate, two waters, and one heme propionate. Thus, a net negative charge results at this site, a result that may favor the catalytically important oxidation of Mn^{II} to Mn^{III}. This oxidation may [55] or may not [52] require Mn binding by additional chelating anions, such as oxalate. The latter anion may bind by displacing a water molecule, thus increasing further the net negative charge at the Mn site, favoring its oxidation and facilitating the lignin degradation chemistry. The Mn^{II} to Mn^{III} oxidation, although favored by the specified chelation process, is ultimately driven by the reduction of H₂O₂ at the heme iron center (Eq. 2).

Elucidation of the Mn coordination sphere allows for further mechanistic insights into the Mn oxidation process; specifically the electron transfer pathway from Mn^{II} to the acceptor (delocalized porphyrin π radical or the Fe center) may take place via the heme propionate ligand [52]. Upon further chelation a (neutral) Mn^{III} complex may diffuse outside the enzyme and into the lignin polymer.

III.C. Lignin Degradation Mechanisms

Mn^{III} is a well-recognized one-electron oxidant and H⁺ abstractor, an ability exploited *inter alia* for free radical cyclizations [56]. The role of Mn^{III} in lignin degradation is to abstract electrons, producing phenoxy radicals, ultimately re-



sulting in C—C bond cleavage [57]. Also formed are carbon-centered radicals, which further react with dioxygen or reduce Mn^{III} to yield peroxides and reactive carbocations, respectively. This Mn-dependent degradation process was believed to be limited to phenolic lignins, as degradation of non-phenolic lignins is accomplished only by LiP, since Mn^{III} is thermodynamically unable to oxidize them. The Mn-dependent lignin degradation pathway, however, may not be unique. Recently the degradation of nonphenolic lignin models has been demonstrated with the MnP of *Ceriporiopsis subvermispora*, in the absence of LiP but in the presence of lipids [58], especially unsaturated lipids, which are more active than the saturated ones. The implication of the preceding observation and other mechanistic studies is that lipid peroxidation does occur first, in the presence of Mn and H_2O_2 . Thus, the peroxy, alkoxy, and perhaps hydroxyl radicals that form subsequently, rather than Mn^{III} , are the species responsible for lignin degradation [58,59]. These radicals, therefore, play the same role as the radical cation of VA generated by LiP and, indeed, oxidize similar substrates. The exact role of Mn in the preceding systems still remains to be clarified.

III.D. Biomimetic Functional Model Complexes for Lignin Degradation

Manganese(III) complexes of α -amino acids have been presented as structural models for the diffusible Mn ion of MnP [60].

The heterodimetallic complex $[(\text{P})\text{Fe}^{\text{III}}\text{—Mn}^{\text{II}}]$ **21** [61], in which a manganese bpy ligand is linked covalently to a fluorinated porphyrin, mimics the active site topology of MnP. The X-band electron paramagnetic resonance (EPR) result is consistent with magnetically isolated high-spin metal centers. Despite the lack of magnetic coupling (which suggests no Mn—X—Fe moieties), however, this complex catalyzes the $\text{C}_6\text{F}_5\text{I}(\text{OMe})_2$ -dependent reversible oxidation of Mn^{II} to Mn^{III} via a proposed rapid Mn^{II} to Fe^{IV} intramolecular electron transfer. The iron species, an $\text{Fe}^{\text{IV}}(\text{OMe})_2$ -porphyrin, is somewhat functionally analogous to the $[\text{PFe}(\text{IV})=\text{O}]$ ferryl species identified as compound II (see Eq. 4). The reduction of Mn^{III} back to Mn^{II} , which completes the catalytic cycle, is achieved stoichiometrically by the oxidation of 2,6-dimethylphenol to 2,2',6,6'-tetramethyldiphenoquinone.

Functional model complexes for LiP based on heme metal enzymes have also been obtained. Among them, halogenated [62] and sulfonated [63] metal porphyrins show ligninase activity and, usefully, degrade recalcitrant pollutants such as diphenyls and chlorinated aromatics in the presence of inorganic or organic single-oxygen donors. Importantly, porphyrin based lignolytic oxidations are shown to be truly biomimetic. For example, the $\text{C}_\alpha\text{—C}_\beta$ bond of lignin models, the analog of the key arylglycerol- β -aryl ether bond of lignin, is also cleaved



[62,63]. Furthermore, oxidation of veratryl alcohol by sulfonated metalloporphyrins yields the same two major products observed when LiP is used [63a].

Several functional ligninase models that do not contain key structural elements of LiP or MnP are worth mentioning since they do not use dihydrogen peroxide in the delignification process. Among them, robust polyoxometallate catalysts have been shown [64] to work well with dioxygen, thus making this chemistry commercially attractive.

IV. EXTRADIOL AROMATIC DIOXYGENASES

The incorporation of both oxygen atoms of dioxygen into ortho diphenols is accomplished in aerobic bacteria by several mononuclear iron enzymes via both substrate and dioxygen activation [65]. Depending upon the point of ring cleavage, these enzymes can be classified as either intradiol (ring cleavage *between* the two ortho OH groups) or extradiol (ring cleavage *next* to the two OH groups) dioxygenases. Importantly, even substrates derived from man-made aromatics, such as dichlorinated biphenyls, are degraded by these enzymes [66].

In addition to the more common iron dioxygenases, manganese-dependent dioxygenases, isolated from *Arthrobacter globiformis* and *Bacillus brevis* bacteria, have been recently shown to exhibit extradiol dioxygenase activity [67] by cleaving 3,4-dihydroxy phenyl acetate between the second and third carbons. Although the active site of these Mn enzymes has not been characterized by x-ray crystallography, two structures of Fe-dependent extradiol dioxygenases, from *Pseudomonas cepacia* [68] and *Pseudomonas* sp. strain KK S102 [68], are known. Both structures reveal an active-site Fe^{II} ion coordinated in a square pyramidal fashion by two histidines (one axial), one glutamate, and two cis water molecules. Additional imidazoles are present near the active site, consistent with the proposed reaction mechanism (see later discussion). The Mn and Fe enzymes may share similar metal coordination environments, although Mn coordination by three histidines, one glutamate, and one tyrosine has been hypothesized [67]. Although the existence of Mn dioxygenases is not in question, whether they are Mn redox enzymes or not is. On the basis of their similar chemistry and metal coordination environments it is tempting to draw a mechanistic parallel between the Fe and Mn based enzymes. This parallel, however, may not be entirely justified considering the reactivity difference between the two, most notably the dihydrogen peroxide inactivation of the Fe enzymes, but not of the Mn ones [67]. Furthermore, it is not clear whether the Fe extradiol oxygenases are metal-redox enzymes themselves. Although it is recognized that the enzymes “activate O₂” after substrate binding, the precise nature of “O₂ activation” is not known. A mechanistic sequence (Figure 9) based on ¹⁸O labeling [69] favors the formation of a semiquinone-superoxide Fe^{II} complex (**b**) followed by lactonization of a



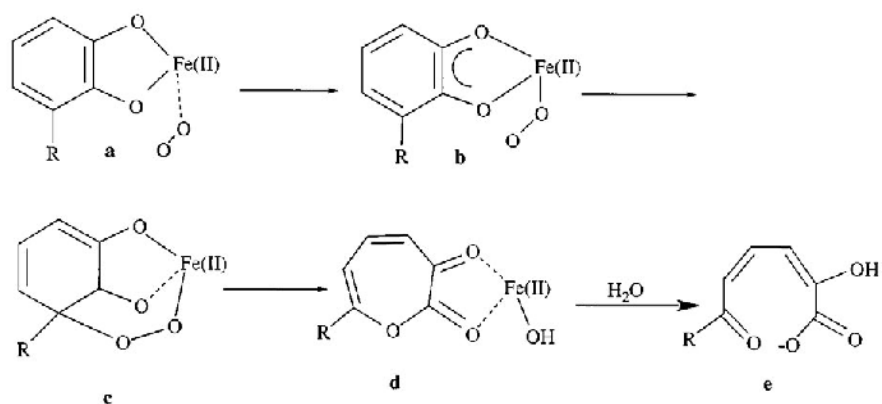


Figure 9 Proposed mechanism for extradiol cleavage.

peroxide intermediate (c and d) and hydrolysis of the lactone (e). The oxidation state of the Fe does not formally vary.

Alternatively, upon sequential binding of catechol and O_2 to the metal center, an Fe^{III} -superoxide-catechol intermediate is postulated to form [70]. Oxygen insertion to form a lactone follows in steps similar to those shown in Figure 9.

Formation of a superoxide ion is consistent with superoxide evolution from tightly bound iron nonmetabolizable substrate complexes [65]. Furthermore, the iron in such substrate complexes can be easily oxidized to Fe^{III} upon exposure to O_2 [65]. The latter observation suggests that the Fe might change its oxidation state during the catalytic process. If the Mn enzymes also function via a similar mechanism, then they should be considered bona fide Mn redox enzymes. A redox role for Mn in Mn dioxygenases will not be surprising considering the role Fe plays in Fe dioxygenases.

V. MANGANESE CATALASE AND CATALASE-TYPE ACTIVITY OF ARGINASE

V.A. Introduction

Catalases are enzymes that protect cells from oxidative damage by scavenging the dihydrogen peroxide produced during dioxygen reduction, an important function, considering that as much as 10% of the dioxygen used in cellular respiration may be reduced to dihydrogen peroxide [7]. Further undesired reactions of dihydrogen peroxide to yield hydroxyl radicals, potent oxidants capable of causing cellular damage, are thus prevented.



The catalases catalyze the disproportionation reaction of dihydrogen peroxide according to the following equation (Eq. 5) [5]:



On the basis of the structure of their active site, catalases may be classified as heme or nonheme enzymes. Those that contain heme iron are efficient catalysts, operating close to the diffusion limit, $\sim 10^8 \text{M}^{-1}\text{sec}^{-1}$. In iron catalases the metal is coordinated by four heme nitrogens and a proximal Tyr residue, which occupies the fifth coordination site. A catalytically required His is found on the distal side of the heme. In addition, a water molecule has also been observed close to the iron sixth coordination site.

The neutral, high-spin ferric (Fe^{III}) resting state reacts with peroxide to form a Fe^{IV} porphyrin-cation radical (compound I), which is subsequently reduced to the native resting state by the reaction with a second peroxide. Additional information on iron catalases can be obtained by consulting recent (post-1991) literature [71].

The nonheme catalases present in *Lactobacillus plantarum*, *Thermus thermophilus*, *Thermus albus*, and other bacteria contain dinuclear manganese centers at their active sites and cycle between $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ and $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ oxidation levels [3b]. However, the $[\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}]$ and $[\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}]$ levels are also known. The $[\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}]$, $[\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}]$, and $[\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}]$ oxidation levels are designated as the reduced, oxidized, and superoxidized forms, respectively. Kinetic studies [3b] indicate, as expected, that the disproportionation of H_2O_2 takes place via the following mechanism:



The favored peroxide disproportionation pathway requires two two-electron steps, with an optimal two-electron reduction potential of +0.86 V [5].

In manganese catalases the superoxidized $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ form has been shown to be inactive, and to date no function for the mixed-valence $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ form has been found.

V.B. Structure of the Enzyme Active Site

Early proposals suggested [72] that catalase contains a μ -oxo-bis(μ -carboxylato)-dimanganese core. The UV-Vis spectra of this structural motif present in model complexes exhibit 480–520 nm *d-d* absorptions [73] similar to the UV-Vis absorption bands of manganese catalases. The EPR studies of oxidized *T. thermophilus* catalase [74] also suggested a $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ μ -oxo-bis(μ -carboxylato) core as a possible structural motif for the active site.



Electron paramagnetic resonance has been used to investigate the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ and mixed valence ($\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ and $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$) states but not the $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ state, which is EPR-silent as a result of the fast relaxing, $S = 2$, ground state.

In general, the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$, $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$, and $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ species exhibit different multiline EPR signals, because the manganese ions are weakly exchange-coupled in the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ and the $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ forms [75]. Specifically, structural information on the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ state of *T. thermophilus* was obtained by least-squares fitting and simulations of EPR spectra. The metal ions exhibit a 3.8 Å Mn...Mn separation as determined by calculating the zero-field splitting of the single Mn ions in related $\text{Mn}^{\text{II}}\text{Zn}^{\text{II}}$ complexes. Multifrequency EPR and microwave polarization studies of the homovalent Mn^{2+} center of *L. plantarum* [76] indicated the presence of two high-spin ions weakly coupled ($J = -20 \text{ cm}^{-1}$) with relatively small dipole-dipole interactions and single-site zero-field splittings, typical of oxo/carboxylato bridged Mn complexes [77]. Fluoride binding and hydron uptake studies were used to demonstrate the coupling between fluoride binding and hydronation of the complex. The change in the spectra upon binding of two fluoride ions is indicative of a substantially reduced magnetic coupling ($J = -5.3 \text{ cm}^{-1}$) and is attributed to perturbation of the bridging ligands. It is possible that upon anion binding, hydrons are added to the bridging ligands, making them more labile and thus facilitating insertion of other ions into bridging positions of the dimanganese core [76]. An EPR comparison of two $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ and $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ complexes and the catalase from *T. thermophilus* used a new theoretical methodology, which allowed the accurate determination of the electron configuration, magnetic hyperfine constants, and D/J ratio of the Mn^{III} [78]. The EPR spectral simulations positively identify the $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ oxidation state in catalase, the first example of this oxidation state in any Mn protein.

The same EPR methodology was used to compare dimanganese(II, II) catalase from *T. thermophilus* with several dimanganese(II) complexes and the Mn center of arginase (rat liver) [79]. Multicomponent analysis of the individual EPR spectra for the three lowest electronic states of all the specified species allowed the Mn...Mn separation to be predicted correctly. For the phosphate form of $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ catalase, which exhibits a weak ($J = -5.6 \text{ cm}^{-1}$) exchange coupling [79], the 3.59 Å calculated intermetallic separation was in good agreement with the 3.6 ± 0.3 Å distance found in the 3.0 Å resolution crystal structure (discussed later) [80]. For $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ arginase this distance was predicted to be 3.36–3.57 Å, slightly higher than the 3.3 Å found in the x-ray structure (see later discussion) [81].

The extended x-ray absorption fine structure (EXAFS) spectrum of the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ catalase from *L. plantarum* [82] revealed a nearest neighbor at 2.19 Å and no evidence of a short Mn...O shell [82]. There is no evidence of a Mn—Mn vector. These EXAFS data support the coordination of one to two imidazoles per Mn in the superoxidized form and two to four in the reduced form, suggesting



that imidazole ligands are lost on conversion to the former. This would happen if the imidazoles were replaced by bridging oxo groups. The absence of a Mn—Mn vector eliminates $(\mu\text{-O})_2$ or $(\mu\text{-OH})_2$ bridged structures; dinuclear Mn structures with $(\mu\text{-carboxylato})_n$ or $(\mu\text{-OH})(\mu\text{-carboxylato})_n$ ($n = 1\text{--}3$) bridges, however, are consistent with the EXAFS data. On the basis of a comparison [83] of the magnetism of the free and phosphate-bound $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ sites of *T. thermophilus* catalase with model complexes, the $\mu\text{-oxo}$ species was designated as H_2O . This assignment is based on the similar exchange coupling interactions for the free enzyme and its phosphate form, $J = -2.4(2) \text{ cm}^{-1}$ and $J = -1.7(2) \text{ cm}^{-1}$, respectively. The slightly lower value for the phosphate form combined with its resistance to oxidation by either air or H_2O_2 suggests a bridging phosphate. This form of phosphate binding implies that a single carboxylate residue (not two) bridges the Mn in *T. thermophilus*, implying the remaining Mn coordination sites could be occupied by easily exchangeable ligands such as water. The latter was confirmed by the crystal structure [84].

The nature of the coordination sphere of the Mn ions in all states, including the $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$, has been elucidated by x-ray absorption spectroscopy (EXAFS and x-ray absorption near edge structure [XANES]). Investigation of the L-edge XANES of the catalase from *L. plantarum* showed that both the shape and the position of the Mn L-edges of model complexes and manganese salts varied dramatically with oxidation state [85]. Both reduced ($\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$) and superoxidized ($\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$) catalases were examined by fluorescence-detected soft x-ray absorption spectroscopy (XAS), and their Mn L-edge spectra found to be distinctly different. Ligand field atomic multiplet calculations and comparisons to model compounds were used to interpret the spectrum of reduced $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ catalase. In addition to providing the base for future use of these methods, the results support the coordination of predominately nitrogen and oxygen moieties to Mn.

V.C. Higher Oxidation States

Early EPR studies of *L. plantarum* $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ catalase [86] and $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ model complexes suggest that in this oxidation state the enzyme may have a bis($\mu\text{-oxo}$) bridged core (vs. single $\mu\text{-oxo}$ in $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$), in agreement with the EXAFS study [82]. Comparison of the magnitude of the hyperfine anisotropy of Mn^{III} ions in the two mixed valence ($\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ and $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$) oxidation states of Mn catalase identified a large spin density asymmetry for the $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ catalase, suggestive of a change in ligand coordination upon reduction. This change may be due to hydration and opening of the $(\mu\text{-O})_2$ bridges of $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ catalase to form a single $\mu\text{-OH}$ bridge, thus allowing for the formulation of the $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ catalase bridging core as $[\text{Mn}(\mu\text{-OH})(\mu\text{-carboxylato})_{(1-2)}\text{Mn}]$. A significant finding [78] was that the EPR spectrum of the $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ oxidation state can depart from the expected 16-line hyperfine pattern if the following criteria are not met: (1) the



intrinsic hyperfine constants for Mn^{III} and Mn^{IV} are about the same and (2) the spin projection of Mn^{III} is twice that of Mn^{IV} . The latter condition is not met for weakly coupled ions as exemplified by the $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ (L11) model complex (Figure 20), for which a 12-line X-band EPR spectrum had indeed been observed [87]. In this complex the magnetic exchange interaction, J , equals -10 cm^{-1} , a value that is an order of magnitude smaller than that usually seen in bis(μ -oxo) $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ complexes. The weak coupling is probably due to the long Mn...Mn separation (3.65 Å) and the presence of a single alkoxide bridge, a poor superexchange pathway mediator. The agreement of the EPR simulation with the observed data is excellent, thus extending the range of the theoretical simulation tools available to study weakly coupled ions.

The $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ (superoxidized) state of catalases was further subjected to several x-ray and magnetic resonance studies. The $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ core of *L. plantarum* was probed at three different EPR microwave frequencies, resulting in self-consistent g and A values. In turn, these parameters were used successfully to simulate both the low- and conventional frequency spectra. The EPR parameters agreed well with one another as well as with those of previous studies [88].

The nature of the protein ligands in the superoxidized state, particularly the number and type of N donors coordinated to the dinuclear metal core, was probed via pulsed EPR (ESEEM) studies of Mn catalase from *T. thermophilus* and a $[\text{Mn}_2(\text{bpy})_4(\mu\text{-O})_2](\text{ClO}_4)_3$ model [89]. By using ^{14}N labeled ammonia it was concluded that a nitrogen donor bridges the two Mn ions in vivo; the most likely candidate is a lysine residue. Since only one nitrogen is detected, the remaining Mn ligands are believed to be oxygens.

An early EPR study of the superoxidized catalase from *L. plantarum* had a “multiline” signal indicative of a $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}(\mu\text{-O})_2$ core [90]. Consistent with this and the preceding study, a variable pH electron nuclear double resonance (ENDOR) analysis [91] of water and protein ligands of $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ *T. thermophilus* catalase and a comparison with model complexes containing N_4O_2 , N_3O_3 , and O_6 donor sets along with μ -oxo and μ -carboxylato ligands revealed O_6 or O_5N coordination environments.

The EXAFS studies of the $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ catalase from *L. plantarum* [82] reveal a 2.68 Å Mn...Mn separation. Since dinuclear Mn complexes that contain only two oxo bridges usually have Mn...Mn distances over 2.7 Å but less than 2.8 Å, the smaller 2.68 Å value suggests a triply bridging $(\mu\text{-O})_2(\mu\text{-carboxylato})$ formulation, and the presence of a di- μ -oxo subfragment is consistent with the ENDOR studies and supported by additional EPR and XANES data [82,90,92]. Importantly, the short Mn...Mn distance in the superoxidized state was detected without any interference from Mn...C scattering.

The assumed carboxylato bridge in the assignment is consistent with earlier proposals that in the oxidized $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ form the bridging is $(\mu\text{-O})(\mu\text{-carboxylato})_2$ [72,73,93]. Electron paramagnetic resonance spectroscopy shows that the



Mn ions are more strongly coupled in the $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ enzyme [3a,75] than in the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ or $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ form [75].

A very recent study [92b] has combined a variety of spectroscopic techniques to probe the interaction of $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ state of *L. plantatarum* with azide and cyanide. Azide binding caused no detectable changes in the XANES region and only minor changes in the EXAFS spectra, indicating that no significant variation in the metal coordination took place. In conjunction with ESEEM results, this is consistent with the azide binding at a locus on the protein close enough to perturb the Mn geometry. Although the superoxidized form of the enzyme is considered catalytically inactive, similarities in the binding of azide to the reduced and superoxidized forms indicate the same binding site. In addition to oxygen from bridging ligands, EXAFS data indicate that two histidines are coordinated to the Mn ions. However, only one histidine is detectable in the X-band ESEEM spectra [92b].

V.D. X-Ray Structure of Catalase

A preliminary x-ray structure of *T. thermophilus* manganese catalase (oxidized state) at ~ 3 Å resolution has been reported [80]. The original solution was obtained by multiple isomorphous replacement followed by phase improvement [80]. Recently both the reduced ($\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$) (Figure 10) and the oxidized

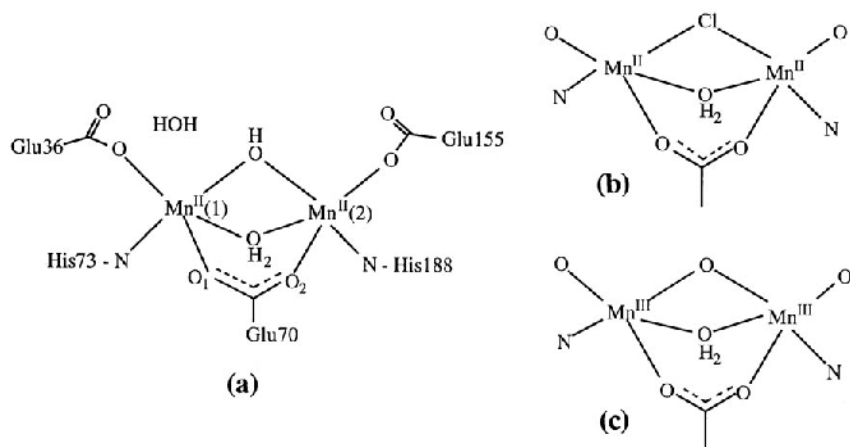


Figure 10 Representation of the dimanganese site of the (a) reduced, (b) chloride bridged, and (c) oxidized forms of *T. thermophilus* catalase. (After Ref. 84.)



(Mn^{III}Mn^{III}) forms have been refined to 1.6 Å and 1.4 Å resolution, respectively [84].

The dimanganese center is found in a hydrophobic area at the bottom of an 18 Å channel, a feature reminiscent of the active site environment in MnSOD. The Mn...Mn distance is 3.18 Å in the reduced form and 3.14 Å in the oxidized form (Table 2).

The manganese ions are bridged in both oxidation state levels by a single carboxylate (Glu70) and two nonprotein ligands, which have been modeled as μ-oxo and a water molecule in the oxidized form, and a μ-hydroxo and a water molecule in the reduced form, respectively. It appears that in the reduced form there are alternate sites approximately 2.0 Å apart, for the water binding to Mn(1). In the chloride form of the reduced enzyme the chloride ion has replaced the hydroxide.

V.E. Catalytic Mechanism

As mentioned, of the four potential oxidation levels known for the catalases (i.e., Mn^{II}Mn^{II}, Mn^{II}Mn^{III}, Mn^{III}Mn^{III}, and Mn^{III}Mn^{IV}), the Mn^{II}Mn^{II}/Mn^{III}Mn^{III} redox couple is the one effective in the disproportionation of H₂O₂ [95].

A proposed catalase mechanism, formulated before the x-ray structure of the reduced enzyme (Figure 10) had been elucidated, is shown in Scheme 1 [96].

Although the crystal structure revealed a single carboxylate bridge rather than two and coordinated OH and H₂O (not two H₂O) ligands, the overall mecha-

Table 2 Comparison of Mn...Mn Distances as Determined by X-Ray Diffraction and Scattering and Other Spectroscopic Techniques

	Mn ^{II} Mn ^{II}	Mn ^{III} Mn ^{III}	Mn ^{III} Mn ^{IV}
<i>L. plantatarum</i>	3.4 Å ^a		2.68 Å ^b
<i>T. thermophilus</i>	3.18 Å ^c	3.6 Å ^d	
	3.8 Å ^e	3.14 Å ^f	
	3.59 Å ^g		

^a EPR and microwave polarization [76].

^b EXAFS [82,92b].

^c Crystal structure, 1.4 Å resolution [84].

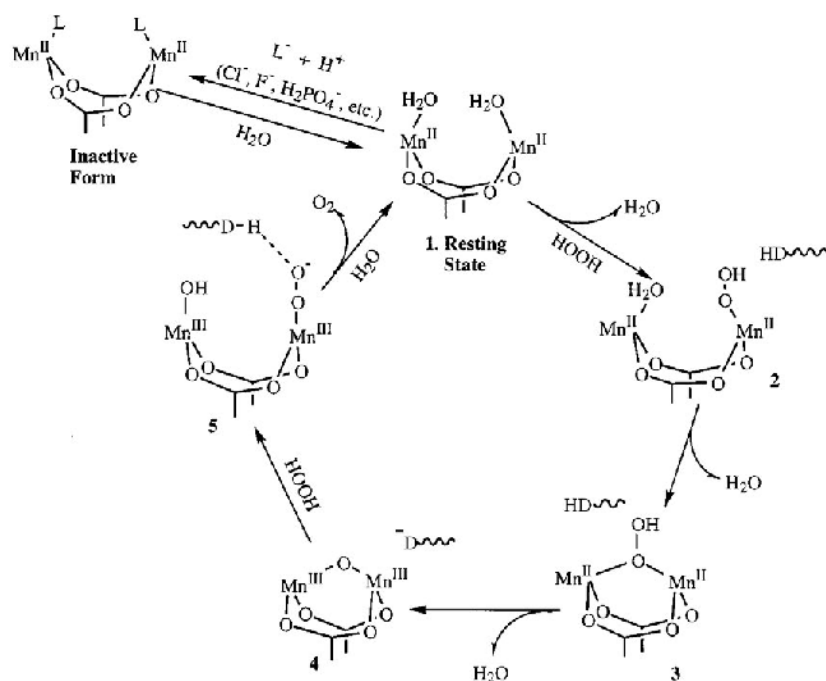
^d Crystal structure, 3.0 Å resolution [80,84].

^e EPR [94].

^f Crystal structure, 1.6 Å resolution [84].

^g EPR [79].





Scheme 1 Catalase mechanism showing schematically the bis(carboxylato) bridged dinuclear metal centers. DH/D⁻, hydron donor/acceptor species. (After Refs. 7, 96.)

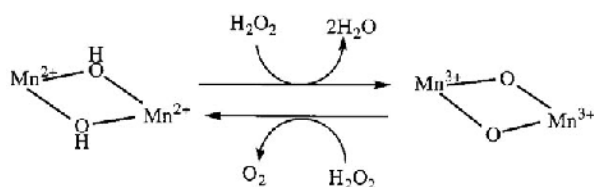
nism remains plausible. For example, the OH ligand may be replaced by the peroxide group instead of a H₂O molecule, as shown in Scheme 1.

The resting diaqua state of the enzyme (1), which can be inactivated by the coordination of anions (L⁻), loses one of the H₂O upon dihydrogen peroxide binding in the presence of a hydron acceptor D⁻. The resulting terminal hydroperoxo group (2) switches into a bridging mode (3), releasing the second Mn-bound H₂O molecule. Hydron donation from HD and Mn oxidation that follows give a μ -oxo oxidized complex (4), which upon binding of a second H₂O₂ yields a terminal hydroxo peroxo intermediate (5). Subsequent reduction and hydronation of this hydroperoxo complex release O₂ and the resting state reforms upon H₂O binding.

An alternative hypothesis assumes that the basis of the catalytic mechanism is the interconversion of a bis(μ -hydroxo) unit, [Mn^{II}(μ -OH)₂Mn^{II}], to bis(μ -oxo), [Mn^{III}(μ -O)₂Mn^{III}] (Scheme 2) [76].

Carboxylate groups are postulated to play a catalytic role, in addition to a





Scheme 2 Catalase mechanism showing schematically the bis(oxo/hydroxo) bridged dinuclear metal centers. (After Ref. 76.)

likely structural one. Thus, it has been suggested [97] that the role of the μ -carboxylato bridge in catalases (and other dimetalloproteins where two-electron redox chemistry is observed) is to prevent the formation of mixed valence species.

In general, as can be seen from the actual parameters for dihydrogen peroxide decomposition (Table 3), manganese catalases are less active than their heme iron counterparts. Catalytic rates comparable to these are a target for functional models of the Mn enzyme.

V.F. Model Compounds

Many manganese complexes decompose dihydrogen peroxide, but we limit our discussion to the functional dinuclear ones; the catalase activity of bis-dinuclear (tetranuclear) photosystem II (PSII) models is discussed later. Furthermore, mainly model complexes reported in the last 5 years are discussed in detail since previous work is covered in several excellent reviews [1,2b,3a,5,8–12].

The synthesis of a large number of dimanganese complexes containing $[(\mu\text{-O})_x(\mu\text{-RCOO})_y]$ bridging ligands has been stimulated by indications that this structural motif is present in Mn catalases [82] and possibly ribonucleotide reductase [99]. Such complexes have been used extensively for spectroscopic compara-

Table 3 Steady-State Kinetic Parameters of Dihydrogen Peroxide Decomposition by Mn Catalases

Mn ₂ (II,II) protein	K_{cat} (sec ⁻¹)	K_m (mM)	k_{cat}/K_M (M ⁻¹ sec ⁻¹)	Reference
<i>T. thermophilus</i>	2.6×10^5	83 ± 8	3.1×10^6	97
<i>T. plantarum</i>	2.0×10^5	350	5.7×10^5	3a
<i>T. album</i>	2.6×10^4	15	1.7×10^6	98

From Ref. 7.



tive purposes. In addition, many dinuclear manganese compounds decompose dihydrogen peroxide and thus have been presented as catalase functional models.

Dinuclear Mn complexes in various oxidation states include those in the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ state [79,94,96,100], $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ state [78,94,100r,100x,101], $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ state [73,100b,100w,100x,102], $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ state [40,78,94,100x,102l,103], and $\text{Mn}^{\text{IV}}\text{Mn}^{\text{IV}}$ state [102l,104].

Perhaps the most pertinent structural parameter of model complexes is the biologically relevant Mn...Mn distance. This distance has been found to be determined by the type of bridging ligands and the Mn oxidation states [11]. Specifically, a detailed analysis [82] of available x-ray structures reveals that $(\mu\text{-O})_2$ bridges promote Mn-Mn ~ 2.7 Å separations, $(\mu\text{-O})(\mu\text{-OH})$ bridges ~ 2.8 Å, and $(\mu\text{-O})(\mu\text{-carboxylato})_2$ bridges $\sim 3.0\text{--}3.3$ Å. Longer Mn-Mn separations are found whenever combinations of water and carboxylates, hydroxide and carboxylates, or just carboxylate ions bridge the two metal centers.

The synthesis of triply bridged dinuclear Mn^{III} complexes exhibiting the $[\text{Mn}_2(\mu\text{-O})(\mu\text{-carboxylato})_2]^{2+}$ core and a variety of terminal ligands has been driven by the belief that this core is present in oxidized catalases [102f]. The magnetic properties of the models have been compared with the antiferromagnetically coupled $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ site of the enzyme. Interestingly, the complexes $[\text{Mn}_2(\mu\text{-O})(\mu\text{-O}_2\text{CR})_2\text{X}_2(\text{bpy})_2]$ ($\text{X} = \text{Cl}, \text{H}_2\text{O}, \text{N}_3$) are found to be weakly antiferromagnetically ($\text{Cl}, \text{H}_2\text{O}$) or ferromagnetically (N_3) coupled (J ranging from -4.1 to 9.0 cm^{-1}), thus suggesting that the terminal ligand needs to be considered in magnetostructural correlations and future modeling work.

In the case of $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ complexes, both the ancillary and bridging ligands were varied in a series of di- μ -oxo μ -carboxylato structures such as $[\text{Mn}_2\text{O}_2(\text{cyclam})_2]^{3+}$, $[\text{Mn}_2\text{O}_2(\text{bpy})_4]^{3+}$, $[\text{Mn}_2\text{O}_2(\text{phen})_2]^{3+}$, $[\text{Mn}_2\text{O}_2(\text{OAc})(\text{tpen})_2]^{2+}$, and $[\text{Mn}_2\text{O}_2(\text{OAc})(\text{bpea})_2]^{2+}$. A comparison of their electronic and MCD spectra with those of superoxidized *L. plantarum* catalase and its azide derivative indicated the presence of a triply bridged di- μ -oxo $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ dinuclear core in superoxidized manganese catalase, consistent with EXAFS [82] and EPR [78] studies.

V.G. Functional Models

Although catalase cycles only between the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}/\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ states, manganese compounds in other oxidation states can also catalyze the disproportionation of H_2O_2 (Scheme 3) [100g].

The first example of a functional model of catalases were the compounds $[\text{Mn}_2(\text{L3})\text{Cl}_3]$ **22**, and $[\text{Mn}_2(\text{L3})(\text{OH})\text{Br}_2]$ **23**, ($\text{L3} = N,N,N',N'\text{-tetrakis}(2\text{-methylene-benzimidazole})\text{-1,3-diaminopropan-2-ol}$) (Figure 11) [100a].

The ligand contributes a bridging alkoxide and the metal coordination sphere is completed with one bridging and two terminal chloride anions. Incorporation of a carboxylate moiety in the system resulted in new compounds,



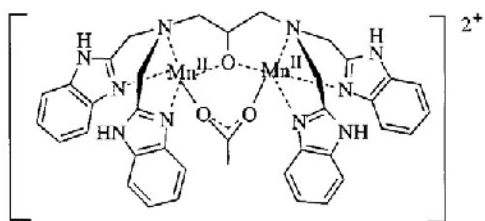


Figure 12 The cationic core of $[\text{Mn}^{\text{II}}(\text{L3})(\mu\text{-X})\text{Mn}^{\text{II}}]\cdot\text{Y}_2$, ($\mu\text{-X} = \text{CH}_3\text{CO}_2^-$, $\text{ClCH}_2\text{CO}_2^-$, $\text{Y} = \text{ClO}_4^-$, BPh_4^- , or CH_3CO_2^-). (From Ref. 100j.)

$(\text{CH}_3\text{CO}_2)(\text{butanol})](\text{ClO}_4)_2$ **27**, contain both 5- and 6-coordinate Mn ions. Crystallization from butanol results in a complex in which Mn(1) is 5-coordinate, but the coordination sphere of Mn(2) is expanded to accommodate a butanol molecule. Three electrons can be removed sequentially to yield four oxidation states ranging from $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ to $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$, thus matching the known catalase oxidation states [100j]. The EPR spectra of the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ and $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ states are comparable to that of the enzyme, thus suggesting similar electronic ground states, Mn^{III} electron orbital configurations, ^{55}Mn hyperfine splittings, and Heisenberg exchange interactions. Interestingly, the assignments hold despite the fact that the number and type of the bridging ligands are different from those in the enzyme (see Figure 12) but consistent with their common 5-coordinate geometry.

A study of the kinetics and mechanism of peroxide disproportionation by the complexes of L3 (Figure 12) probed anion inhibition pathways [96]. The disproportionation in water was found, as expected, to proceed by two consecutive two-electron steps ascribed to the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}} \leftrightarrow \text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ redox changes. The disproportionation in methanol, on the other hand, occurs after an initial lag time, which can be reduced upon addition of water prior to the addition of H_2O_2 . This observation, along with electronic spectral changes, suggests that μ -carboxylate displacement and/or H_2O binding at the active site is necessary for catalyst activation. Although the μ -carboxylate catalysts have high stability and are five times faster than the HO^- or Cl^- bridged species, their H_2O_2 decomposition rate (k_{obs}) is still $\sim 10^7$ lower than that of the enzyme.

The Mn complexes of dicarboxylic acids are efficient catalase mimics. The crystal structure of $[\text{Mn}^{\text{II}}(\eta^1\eta^1\text{-L4})(\text{phen})_2]$ **28**, ($\text{L4} = \text{cis-5-norbornene-endo-2,3-dicarboxylic acid}$ and $\text{phen} = 1,10\text{-phenanthroline}$) (Figure 13) shows that this complex is a water linked dinuclear compound in solid state [100n].

This complex disproportionates almost 900 molecules of H_2O_2 in the first minute in the absence of base and three times as many when imidazole is added. The role of the base in this case is not clear, but in catalytic Mn porphyrins the addition of imidazole accelerates the peroxide O—O bond homolysis [105].



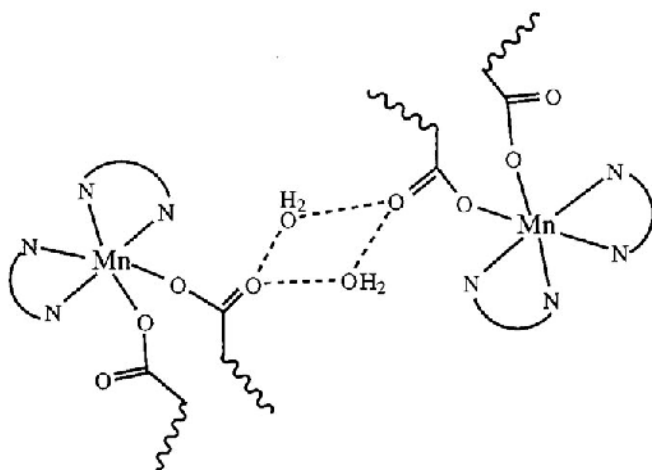


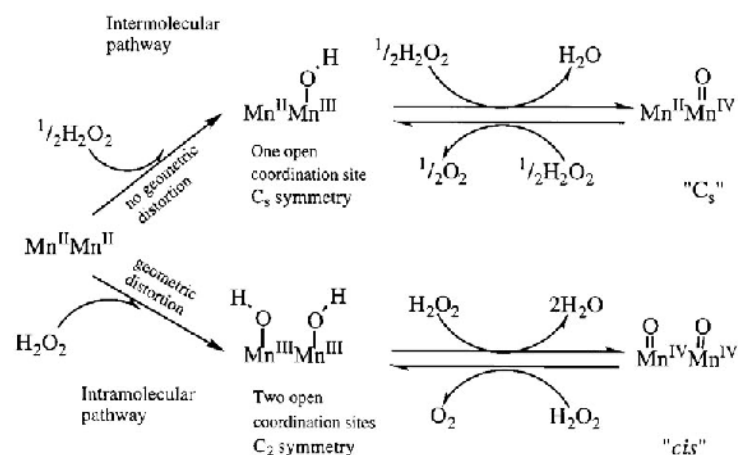
Figure 13 The Mn^{II} environment in complex **28**. Only the coordinating nitrogens of the phenanthroline are shown. (After Ref. 100n.)

Many variations of dinuclear μ -phenoxo-bis(μ -carboxylato)dimanganese(II) complexes of L5 [100e] show catalase-like activity (more than 1000 catalytic turnovers based on the measurement of evolved dioxygen) via the mechanism shown in Scheme 4 ($\text{R}_1 = \text{R}_2 = \text{CH}_3 = \mathbf{29}$).

The fast catalytic activity is associated with the $\text{cis-}[\text{Mn}^{\text{IV}}(\text{=O})]_2$ product, formed perhaps via an intramolecular pathway when the R groups are small. A similar pathway may be operational for catalase activity. On the other hand, when the active site is sterically hindered (e.g., when the methyl R_1 and R_2 groups (Figure 14) are replaced with bulkier pyridyl groups in **30**), the $[\text{Mn}^{\text{IV}}(\text{=O})\text{Mn}^{\text{II}}]$ product is formed. This complex cation, which has C_s symmetry, still has two vacant sites in cis positions, but one of them is sterically hindered, leaving only one Mn to form a $\text{Mn}^{\text{IV}}(\text{=O})$ group. Since only one coordination site is available, H_2O_2 decomposition requires two dinuclear complexes (intermolecular mechanism). For the "intramolecular pathway" an initial geometric distortion leads to two, trans open sites. A slow trans-cis isomerization, responsible for an induction period, allows the eventual formation of the $\text{cis-}[\text{Mn}^{\text{IV}}(\text{=O})]_2$ species. Compared to its monomanganyl counterpart, this bismanganyl species is much more efficient in disproportionating H_2O_2 . Thus, in the case of hindered ligands, since a trans to cis isomerization cannot take place, only a peroxo-bridged dinuclear intermediate can form, leading to lower activity.

Further reactions of all manganyl species mentioned with H_2O_2 regenerate the (OH) species and release dioxygen. These suggest why complexes with C_2





Scheme 4 Proposed mechanism of H_2O_2 disproportionation by L5 complexes. (After Ref. 100e.)

symmetry exhibited high O_2 evolution rates whereas those with C_s symmetry showed little activity [100d].

Complexes synthesized from asymmetric dinucleating variations of L5, where the alkyl groups are methyl or ethyl [100h,100i], also disproportionate H_2O_2 via a pathway mediated by oxomanganese(IV) species. It should be noted, however, that although Mn^{IV} is present in these complexes, Mn^{IV} so far has not been shown to be involved in the catalytic mechanism of the enzyme.

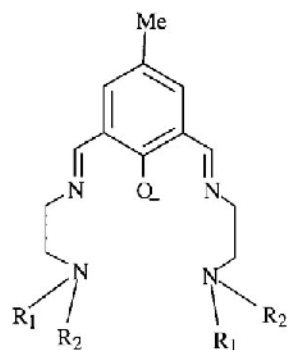


Figure 14 Ligand L5.



Replacing the carboxylate bridges with benzoates to form $[\text{Mn}^{\text{II}}_2\text{L5}(p\text{-XC}_6\text{H}_4\text{CO}_2)_2(\text{NCS})(\text{MeOH})]$ ($\text{X} = \text{H}$ **31**, Cl **32**, Me **33**, or NO_2 **34**) [100o] increases the rate constant in the order $\text{X} = \text{H} < \text{Cl} < \text{Me} < \text{NO}_2$, proportional to the bulkiness of the *para*-substituent X . This is consistent with the specified mechanism [100o].

All three complexes of the L6 ligand system, $[\text{Mn}_2(\text{L6a,b})(\text{RCO}_2)](\text{BPh}_4)_2$ ($\text{L} = 6\text{a}$ **35**, $\text{R} = \text{Ph}$; 6b **36**, $\text{R} = \text{Me}$, and $[\text{Mn}_2(\text{L6c})](\text{BPh}_4)_2$ **37** (Figure 15), disproportionate H_2O_2 in DMF at 0°C ; complex **37** has the highest catalytic activity [100q].

The x-ray structure of complex **37** reveals a di(μ -pyrazolato) core with each Mn ion coordinated octahedrally by two pyrazolate and two amino nitrogens in the equatorial plane and two axial pyridyl nitrogens. Spectroscopic evidence indicates that a pyrazolate and carboxylate bridge the two Mn in complexes **35** and **36**. For the two μ -pyrazolato μ -carboxylato di-Mn(II) complexes, the initial rate of dioxygen evolution is first order in [complex] and $[\text{H}_2\text{O}_2]$ ($k = 1.45 \text{ dm}^3 \text{ mol}^{-1} \text{ sec}^{-1}$ for complex **35**). The kinetics are consistent with the catalase mechanism of Scheme 4. Additional support for this mechanism comes from the observation of charge transfer bands characteristic of $[\text{Mn}^{\text{IV}}=\text{O}]$ chromophores. For the di(μ -pyrazolato) complex, kinetic studies indicate a yet unknown reaction pathway. For this complex the initial rate is first order in complex and second order in H_2O_2 ($k = 29 \text{ dm}^6 \text{ mol}^{-2} \text{ sec}^{-1}$).

Four dinuclear $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ complexes $[\text{Mn}_2(\text{L}^{7m,n})(\text{RCO}_2)_2]$ with $(m, n) = (2, 3), (2, 4), (3, 3)$ ($\text{R} = \text{CH}_3$ or C_6H_5) and one $[\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}(\text{L}^{3,3})(\text{RCO}_2)\text{Br}_2]$ **38** could be symmetrical ($m = n$) or unsymmetrical ($m \neq n$) (Figure 16) [100r].

All five complexes show moderate catalytic activity with the rate proportional to complex concentration and dependent upon its symmetry. Specifically, the O_2 yield is nearly 100% for the symmetrical complex **38** but lower for the

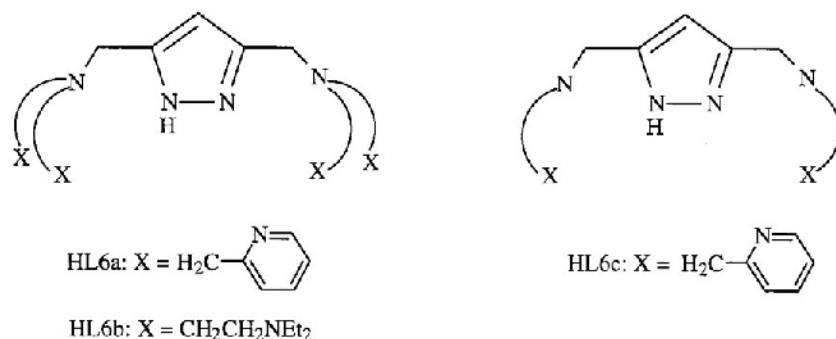


Figure 15 Hydronated forms of ligands L6.



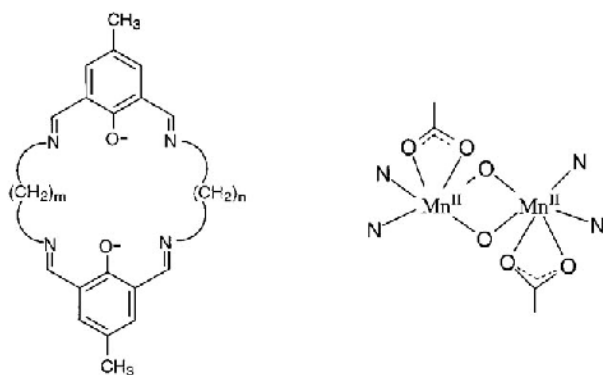


Figure 16 The $L7^{m,n}$ ligand and the coordination environment of the Mn L7 complexes.

unsymmetrical ones. Although in the native enzyme, UV-visible and EPR studies indicate that the $Mn^{II}Mn^{II}/Mn^{III}Mn^{III}$ cycle is involved in the catalytic process, the mixed valence $Mn^{II}Mn^{III}$ model is also catalytically active. This difference may be attributable to the presence of phenoxy bridges in the model complex but not in the enzyme.

Lengthening the side chain of L7 and adding a coordinating nitrogen to it yield L8 (Figure 17), which forms singly bridged $Mn^{II}Mn^{II}$ complexes $[Mn_2(R^1-L8)(R^2-CO_2)](ClO_4)$ [100f,100m].

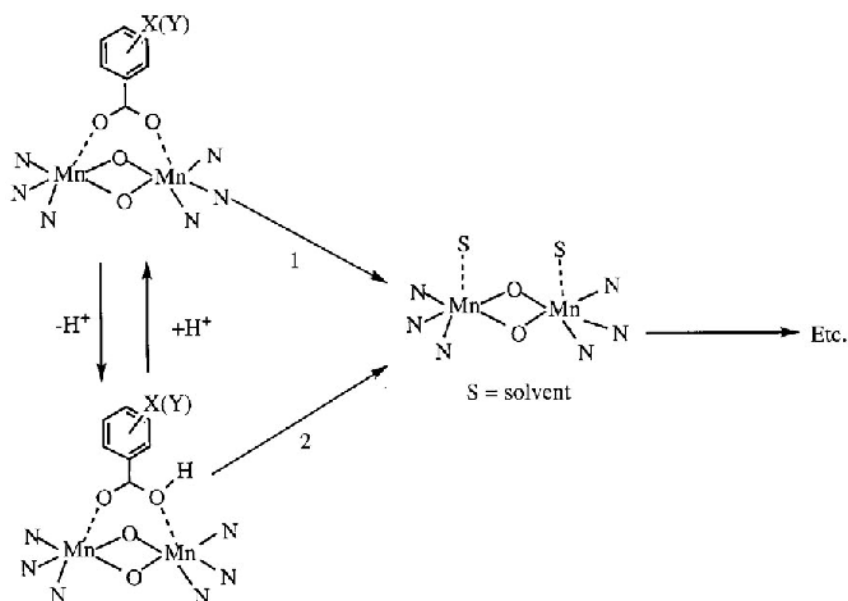
Systematic variation of the R^1 and/or R^2 groups (Figure 17) induces redox potential changes, an effect that was considered useful for “fine-tuning” the redox properties of functional model complexes, and maybe, for enhancing their catalytic activity [100f]. However, a comparison of complexes with the same oxidation potentials showed [100m] that variation in the Mn coordination had a more dramatic effect. Replacement of the carboxylate bridges with bridging ligand, Y, in $[Mn_2(R^1-L8)(R^2-CO_2)](ClO_4)$ ($R^1 = CH_3, Br$) (Figure 17), $Y = Cl, N_3, SCN, MeCO_2, CF_3CO_2$, or CCl_3CO_2 anions, results in enhanced catalase activity (V_{max} increased from 0.20 to 4.47 sec^{-1}), which parallels the increasing acidity of the species from which the bridging group was derived. This result suggests that the rate-determining step is dissociation of Y^- .

In a further study [100s] of the complexes $[Mn_2(L8)(RCO_2)](ClO_4)$, $[R = Ph, XC_6H_5(X = 2 \text{ or } 4-MeO, Me, Br, Cl, F, \text{ or } NO_2 \text{ and bridging ligands } XY, (XY = 2,4-(NO_2)_2, 3,4-(NO_2)_2, 2,4-(NO_2)_2)]$, the catalytic activity also increased with the acidity of the bridging group but only in the presence of 2,4,6-trimethylpyridine. When the base was absent the opposite was true. These results were taken as evidence for two possible reaction pathways for the reaction (Scheme





Complexes $[\text{Mn}_2(\text{L9})\text{X}_2(\text{NO}_3)_2(\text{H}_2\text{O})_2]$, which contain N-donor didentate



Scheme 5 Proposed dissociative mechanisms of formation of a bis(μ-oxo) active catalyst from $[\text{Mn}_2(\text{L8})(\text{PhCO}_2)](\text{ClO}_4)$ **49**. (After Ref. 100s.)

terminal ligands such as bpy, 4,4'-Me₂bpy, or phen, disproportionate H_2O_2 slowly ($V_{\text{max}} = 2.2 \times 10^{-2}$ to $7.8 \times 10^{-2} \text{ sec}^{-1}$). A related complex $[\text{Mn}_2(\text{L9})(\text{NO}_3) \times (\text{CH}_3\text{OH})_4(\text{H}_2\text{O})_2](\text{NO}_3)$ **50**, and perhaps other model complexes as well, however, decompose to give MnO_2 , which is known to have catalase activity and could mask the catalase activity, if any, of the model. The dinucleating L9 ligand

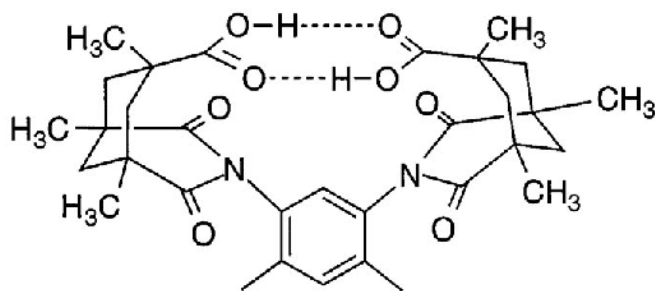


Figure 18 Ligand L9.



seems suited as a synthetic platform for the production of complexes exhibiting intermetallic separations ranging from 3.2 to 4.9 Å. Its ability to support metal centers in various oxidation states and coordination environments makes it valuable for biomimetic chemistry.

The manganese(III) complex $[\text{Mn}_4(\text{L10})_2(\text{CH}_3\text{O})_4(\text{CH}_3\text{OH})_4](\text{ClO}_4)_2 \cdot (\text{CH}_3\text{OH})_4$ **51** (L10 = 3,5-bis((salicylideneamino)methyl)pyrazole) [102c] shows high catalytic activity in dimethyl formamide (dmf), where it has a dinuclear structure rather than the tetranuclear structure observed in solid state (Figure 19). Such dinuclear-tetranuclear equilibria need to be considered when exploring the catalase activity of PSII models (discussed later).

Extensive use has been made of ligand L11 (L11 = 1,3-bis(salicylidene-amino)-2-propanol) for the preparation of complexes exhibiting catalase activity [11,100x,102o,106]. In particular, the dinuclear $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ complex of L11 **52** (Figure 20) is claimed to be the first functional catalase molecule for which both the oxidized and reduced forms have been isolated [102o].

Complex **52** is similar to Mn-catalase in that it is azide insensitive and forms a $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$ species on addition of hydroxylamine and a $\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ catalytically inactive form with an EPR spectrum closely resembling that of the enzyme. Importantly, the complex maintains its dinuclear structure in solution, while cycling between the $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$, $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ oxidation states and shows a good catalytic rate ($k_{\text{cat}} = 13 \pm 1 \text{ sec}^{-1}$) and stability (>1000 turnovers). Catalases, however, are approximately 3000 times more efficient.

Recently [100x] ring-substituted L11 ligands L11X, (L11X = (2-HO-(Xsal)pn, X = H, 5-Cl, 3,5-Cl₂, 5-NO₂) have been used to synthesize the $[\text{Mn}_2(\text{L11X})_2]^{-2,-1,0,+1}$ series whose oxidation levels parallel the known oxidation states of catalase. The interesting structural feature is that the Mn...Mn separations differ by no more than 0.11 Å irrespective of their oxidation states: $\text{Mn}^{\text{II}}\dots\text{Mn}^{\text{II}} = 3.33 \text{ Å}$, $\text{Mn}^{\text{II}}\dots\text{Mn}^{\text{III}} = 3.25 \text{ Å}$, $\text{Mn}^{\text{III}}\dots\text{Mn}^{\text{III}} = 3.36 \text{ Å}$, and $\text{Mn}^{\text{III}}\dots\text{Mn}^{\text{IV}} = 3.25 \text{ Å}$. These complexes also show catalase activity, a property that may be related to the ability of the ligand to induce the formation of either

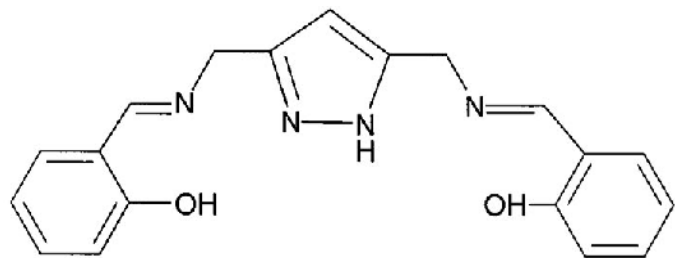


Figure 19 Ligand L10.



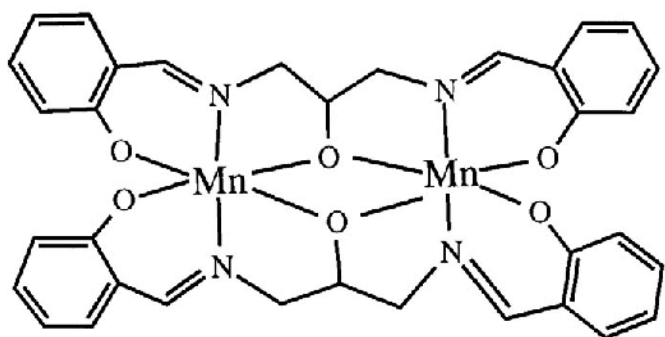


Figure 20 Complex 52 $[\text{Mn}^{\text{III}}(\text{L11})]_2$. The hydrogens on carbon atoms have been omitted for the sake of clarity.

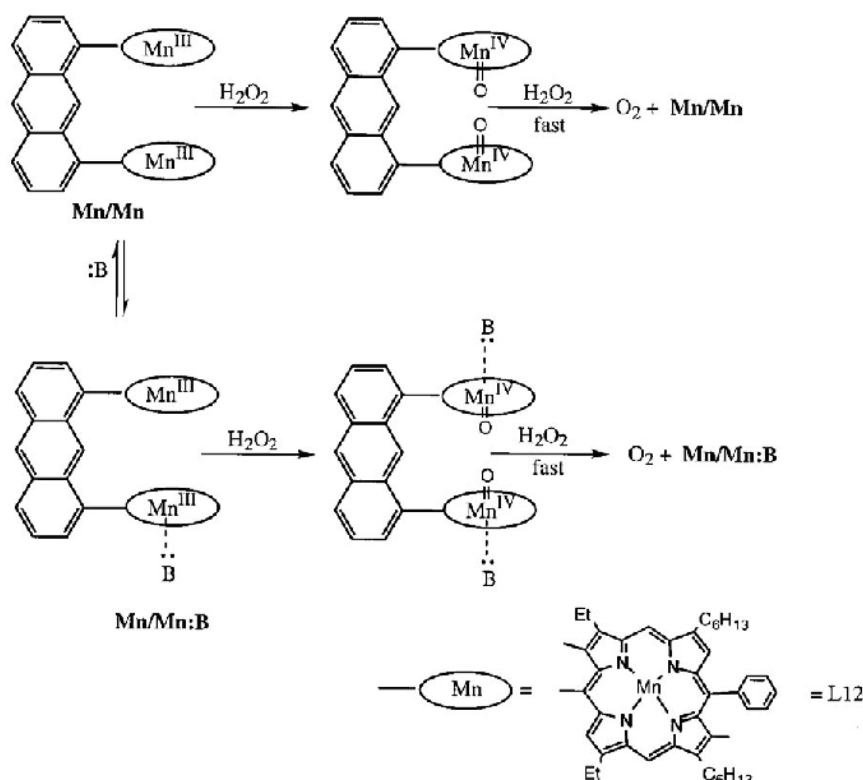
mono- or bisalkoxide bridged species. In this respect, the change in ligand coordination mode resembles the known carboxylate shift [107]. These complexes are the most active functional models of manganese catalases to date.

A completely different catalase mimic that, however, does not contain catalase-type ligands has been developed from rigidly linked Mn^{III} porphyrin dinuclear compounds (L12, Scheme 6) in order to produce a semirigid, “earmuff” complex [102d,102g,102j,102k,108]. This strategy turned out to be fruitful, particularly with anthracene-linked complexes. One representative, $[\text{Mn}^{\text{III}}_2(\text{L12})\text{Cl}_2]$, **53**, has an oxygen-evolving rate of 325 moles of O_2 per mole of catalyst per minute and high turnover numbers (maximum 1.5×10^4), in the presence of a base, 1-methylimidazole. The effect of the base, B, is depicted in the proposed mechanism shown in Scheme 6 [102g].

An O_2 evolution rate enhancement (6.6-fold larger than general-base catalysis) is observed when axial coordination of the base occurs [102k]. Unlike for catalase, however, the rate determining step seems to be the formation of a high-valent $(\text{Mn}^{\text{IV}})_2$ complex [102k]. A correlation between Mn...Mn separation and catalase activity of manganese porphyrin dinuclear compounds linked by various spacers revealed that the highest activity occurs when the separation is $\sim 4 \text{ \AA}$. This suggests that the highest turnover rate (2790 min^{-1}) is observed when both Mn ions can interact cooperatively with the H_2O_2 substrate in the catalyst cavity [102j]. Interestingly, the biphenyl-linked complex showed no catalase activity even though the $3.8 \text{ \AA}$ Mn...Mn spacing is similar to the $3.6 \text{ \AA}$ value observed for $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ -catalase of *T. thermophilus* (x-ray diffraction).

The role of bases was further probed by measuring the catalase activity of a series of *m*-phenylene-linked complexes. As expected, an increase in activity with the basicity and the concentration of added nitrogen bases was noted [102d].





Scheme 6 The effects of bases on O—O bond cleavage by 53. (After Ref. 102k.)

This dependency underscores the role of the bases in catalase activity (Figure 21): tuning the metal center (see also Scheme 6), assisting in H_2O_2 dehydrogenation (and hence its binding to Mn) and facilitation, via electronic inductive effects, of O—O splitting in $\text{Mn}(\text{H}-\text{O}-\text{O}-\text{H})$ intermediates.

The importance, for catalytic activity, of metal sites exhibiting different coordination environments has been evaluated by using asymmetrically coordinated dinuclear species [103b]. A mixture of 1,4,7-trimethyl-1,4,7-triazacyclononane (Me_3tacn) and bpy yielded bis(μ -oxo) μ -carboxylato [$\text{Me}_3(\text{tacn})\text{Mn}^{\text{III}}\text{Mn}^{\text{IV}}$ (bpy)], **54**, the first such complex. This was indeed catalytically active, but still at the same low level (10^5 slower than catalase) as other symmetrical models. Nevertheless, the requirement for two metals for catalysis was confirmed since decomposition of the dinuclear species yields inactive, but similarly coordinated mononuclear complexes.



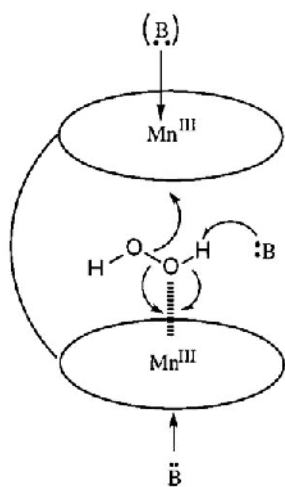


Figure 21 Possible roles of N bases in the reaction of H_2O_2 with complexes of L12. (From Ref. 102k.)

Other di- μ -oxo $Mn^{III}Mn^{IV}$ complexes, also models for the inactive, super-oxidized Mn catalase, show catalase activity. They may be only precursors for other species with different oxidation states. For example, a series of di- μ -oxo $Mn^{III}Mn^{IV}$ complexes of L13 (L13 = substituted bis(pyridylmethyl)ethylenediamine ligands) [103d] efficiently catalyze the disproportionation of H_2O_2 in neutral aqueous media. The activity increases with increasing electron-withdrawing character of the substituents but decreases with the steric bulkiness of substituent X, reaching zero for complex **62** (Figure 22).

Another dinuclear, high oxidation state complex, $[Mn^{IV}(salpn)(\mu_2-O)]_2$, **63**, contains a $Mn^{IV}Mn^{IV}$ center, an oxidation state level even higher than those known for catalases. Nevertheless complex **63** has catalase activity (1000 turnovers without decomposition) and follows a catalase pathway (Scheme 7) similar to that of *L. plantatarum* catalase (Scheme 1) [109].

The mechanism was elucidated by using ^{18}O isotope labeling, which showed that an exchange of the bridging oxo ligands occurs during the reaction, and that the resulting μ_2 -oxo atoms originate from the same peroxide molecule. As H_2O_2 is oxidized to O_2 the initial $Mn^{IV}Mn^{IV}$ complex is reduced to two Mn^{III} complexes. The newly formed mononuclear Mn^{III} species reacts with a second H_2O_2 molecule, and a (labeled) oxygen bridged dinuclear complex is formed to complete the catalytic cycle.



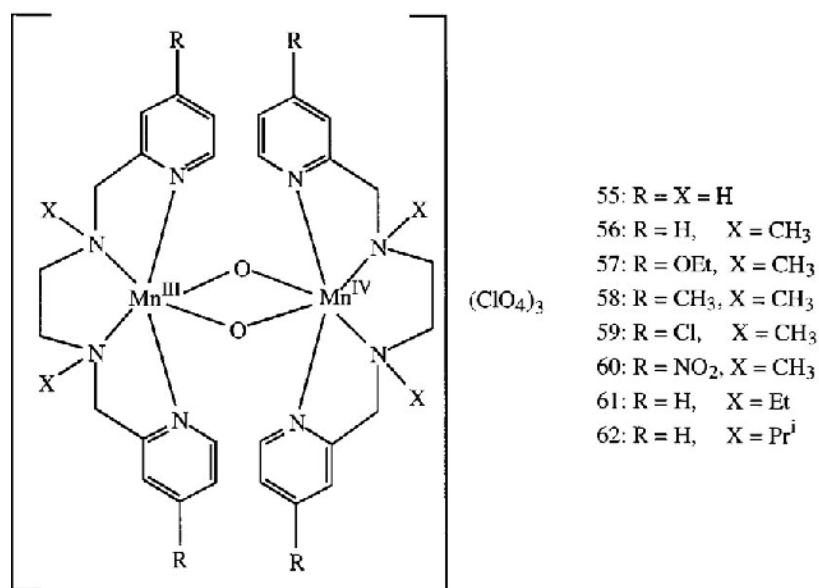
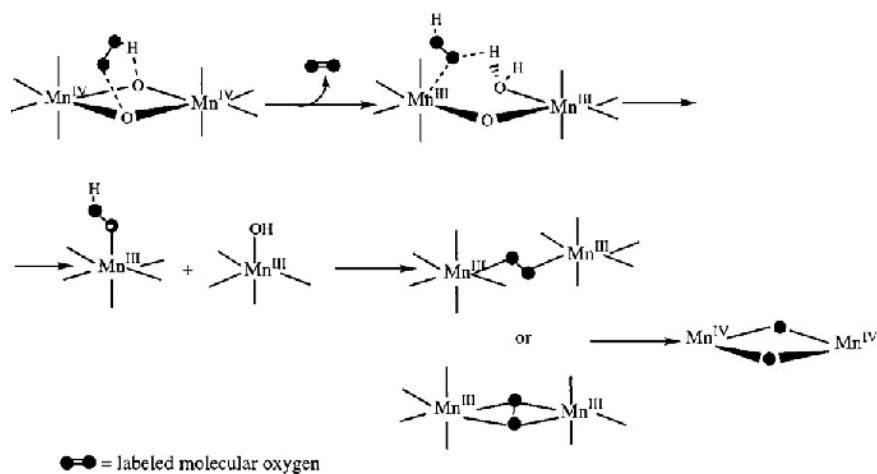


Figure 22 Schematic representation of complexes of L13. (From Ref. 103d.)



Scheme 7 A minimal pathway for the decomposition of H₂O₂ by [Mn^{IV}(salpn)(μ₂-O)]₂ **63**. (From Ref. 109.)



V.H. Conclusions

The correlation of catalase activity of higher oxidation state complexes with structural parameters stresses the importance of accessible active sites and correct electron densities for facilitating O—O bond splitting. The same trends are valid for truly biomimetic $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ and $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ models, but it seems that catalase activity can be successfully achieved with complexes exhibiting a variety of Mn coordination environments, bridging and terminal ligands, and even oxidation state levels that are either inactive or unknown in catalases. Thus a variety of “tuning” parameters are available to the biomimetic chemist. Yet, despite the extensive synthetic explorations of this parameter space, functional models of catalases exhibiting reaction sites similar to that of the enzyme and exhibiting comparable catalase activity remain to be prepared.

Perhaps even more challenging is the regulation of the catalytic activity in structurally related complexes. Nature has already accomplished this task, for example, in turning off the catalase activity of the Mn dinuclear center of arginases.

V.I. Catalase-Type Activity of Arginases

Arginases are ancient enzymes present in a variety of organisms ranging from bacteria to humans [110]. Along with phosphodiesterase and urease, arginases belong to the group of metal-dependent hydrolases. The primary role of arginases (L-arginine amidinohydrolase, EC 3.5.3.1), which contain two manganese centers at the active site, is to catalyze the conversion of L-arginine to ornithine and urea (Figure 23).

However, arginases also exhibit very weak catalase activity [111] ($k_{\text{cat}}/K_M = 11 \text{ M}^{-1} \text{ sec}^{-1}$; cf. catalases Table 3), not unexpectedly, considering their catalase-like active site.

Spectroscopic studies suggested the active site of arginase contains two Mn^{II} ions coordinated predominantly by oxygen groups [97]. On the basis of ENDOR and mutagenesis studies a small number of histidine ligands were also believed to be bound to Mn [97].

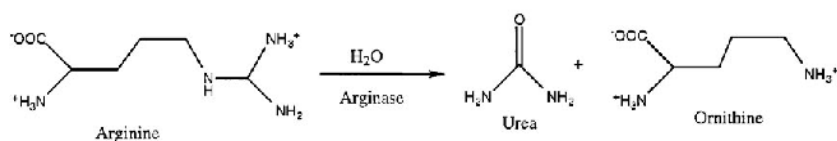


Figure 23 Arginine hydrolysis by arginase. (From Ref. 7.)



The 2.8 Å resolution crystal structure of the arginase from the thermophilic bacterium *Bacillus caldevelox* [112] reveals a hexameric structure with at least one Mn^{2+} bound per subunit. The 2.1 Å resolution structure of rat liver arginase, reported recently [81], reveals it to be trimeric, with the overall fold of the arginase monomer belonging to the α/β protein class. The $\text{Mn}^{\text{II}}\text{Mn}^{\text{II}}$ aggregate, shown in Figure 24, is found at the bottom of a 15 Å active-site cleft.

$\text{Mn}(1)$, which is more deeply buried in the cleft, has square pyramidal geometry, being coordinated by three oxygen [Asp124 (O1), Asp128 (O1), Asp232 (O1)] donors, one nitrogen (His101) donor, and one bridging water molecule. The second manganese, $\text{Mn}(2)$, is coordinated in a distorted octahedral manner by one nitrogen (His126), four oxygens [Asp 124 (O2), Asp232 (O1), Asp234 (didentate O1 and O2)], and the bridging water molecule. This water molecule is also hydrogen bonded (O—O distance of 2.8 Å) to Asp128. The metal centers are 3.3 Å apart, slightly below the 3.4–3.6 Å range calculated from the zero-field splitting observed in the EPR spectrum [79]. The stability of the metal binding site is enhanced by additional hydrogen-bonding interactions between the metal ligands (except Asp 128) and other protein residues [113].

Although mammalian liver arginases can be activated by Co^{2+} , Ni^{2+} , Fe^{2+} , and Cd^{2+} , only Mn^{2+} is considered [114] to be the physiological activator. Mn^{2+} is specifically required to activate the arginases from *Neurospora crassa* [115], *Rhodobacter capsulatus* [116], and the *Agrobacterium* TiC58 plasmid. This selectivity is attributed to the unique ability of Mn to orient the bridging water molecule properly for catalysis [81,117].

Not surprisingly, both manganese ions are necessary for the catalase activity of arginase, as confirmed by metal replacement experiments in a mutant arginase. Indeed, whereas the hydrolytic reaction was restored upon binding one Cd^{2+} ion to form a heteronuclear MnCd enzyme, catalase activity was not [111].

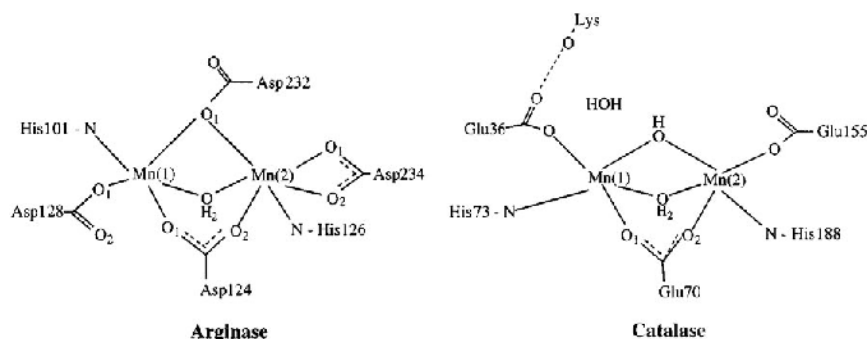


Figure 24 The first coordination spheres of $\text{Mn}(\text{II})$ in arginase and catalase.



A comparison of arginases and catalases reveals that these two dinuclear Mn enzymes, which have similar but not identical metal coordination environments, exhibit catalase activity that differs by several orders of magnitude (Table 3). The lower catalytic efficiency of arginase has been attributed [7] to a high Mn oxidation barrier induced by the overall weaker σ -ligand field, lower binding affinity for H_2O_2 or HO_2^- , or lack of hydron donors or acceptors at the active site. In contrast to the arginase/catalase case, their models, though exhibiting wider coordination variability, do not provide similar reactivity variability. This observation confirms that mimicking only the first coordination spheres of the enzyme's metal active site is a necessary but not sufficient condition for biomimetic catalysis.

VI. MANGANESE RIBONUCLEOTIDE REDUCTASE (MnRNR)

Another dinuclear Mn enzyme with quite a different functionality is MnRNR. Cell replication and deoxyribonucleic acid (DNA) formation require the reduction of the 2 hydroxyl group of a ribonucleoside phosphate [13a].

This reaction is accomplished by several types of iron ribonucleoside reductase (RNR) [1,5,7,13,118]. One of the best-characterized RNRs (from *E. coli*) contains two homodimeric protein components, R1 and R2. The R2 protein comprises an oxygen bridged dinuclear Fe(III) in its oxidized form [119]. All RNRs promote the formation of a stable organic radical, which, eventually, leads to the abstraction of a hydrogen atom from the ribose. In the case of *E. coli* RNR, the latter is accomplished by the R1 protein, specifically by a cysteinyl residue; a redox active cystine, also part of R1, provides the required reducing equivalents (Figure 25).

The transient cysteinyl radical on the R1 protein is produced by a stable but remote tyrosyl radical on the R2 protein. The role of the dinuclear metal

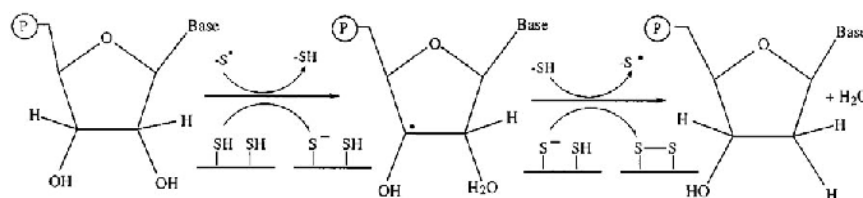


Figure 25 Proposed mechanism for ribonucleoside reduction. (After Ref. 13a.)



center is to produce this radical (see Figure 7 in Chapter 10), which is stabilized within a hydrophobic pocket.

The metal bridging μ -oxo group is derived from molecular oxygen, not from a water molecule, thus suggesting a peroxo intermediate. The introduction of this oxo group is accompanied by a "carboxylate shift" [107a] of a glutamate residue. The transition from the reduced R2 to the final oxidized form is believed to proceed via a high-valent, dinuclear intermediate [120], which produces the stable Tyr122 radical. Further details about RNR can be found in Chapter 10 of this monograph.

It is interesting to note that the R2 reduced structure was initially proposed on the basis of a R2 for which the Fe^{II} ions have been replaced by Mn^{II} [121].

The first bona fide MnRNR, isolated from *Corynebacterium* (formerly *Brevibacterium*) *ammoniagenes*, was reported about 10 years ago [122]. The MnRNR R1 protein is monomeric (dimeric in *E. coli*) [123]. A dinuclear $\text{Mn}^{\text{III}}\text{—O—Mn}^{\text{III}}$ unit was believed to be present at its active site (cf. oxidized *E. coli* RNR), and its sensitivity to hydroxyurea (a radical scavenger) suggested a mechanism similar to that of its iron counterpart. However, no tyrosyl radical EPR signal was observed from the MnRNR, casting doubts about the redox role of the manganese center [118]. Very recently, however, a stable EPR signal, which is inhibited by hydroxyurea and correlates directly with enzymatic activity, has been detected [124].

Whether the oxo-manganese center of MnRNR functions similarly to the oxo-iron center of FeRNR remains to be established. However, it is likely that the dinuclear Mn center plays a redox and, perhaps, an H abstraction role.

It is interesting to note that a manganese-organic radical system critical for catalysis is not unique. A related Mn-tyrosyl radical(s) ensemble is known to operate in Photosystem II (discussed later).

VII. PHOTOSYSTEM II

VII.A. Introduction

The process of photosynthesis is the primary source of energy and carbohydrates to most life on Earth and was also responsible for the transformation of Earth's atmosphere from reducing to oxidizing. Light-induced production of biological energy may or may not generate dioxygen, processes termed oxygenic and anoxygenic photosynthesis, respectively. Anoxygenic photosynthesis, encountered in some bacteria, does not use water as a source of electrons (needed to reduce CO_2), but other sources, such as dihydrogen gas, dihydrogen sulfide, or organic molecules.

Oxygenic photosynthesis, which does use water as a source of electrons,



emerged probably in aquatic cyanobacteria about 3×10^7 years ago, but an O_2 -rich atmosphere did not appear until 2×10^7 years ago, after the oxygen scavenging Fe^{2+} , present in oceans, was oxidized to Fe^{3+} .

Most photosynthetic oxygenic organisms possess two interacting apparatuses known as photosystem I (PSI) and photosystem II (PSII). These extract electrons from water and generate adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and reduced nicotinamide-adenine dinucleotide phosphate (NADPH) from $NADP^+$. The latter reaction is performed by PSI, a collection of light absorbing antennae and electron carriers capable of producing the strongest biological electron reductants known so far, thus making the reduction of $NADP^+$ thermodynamically favorable (see Ref. 125 for a recent review). Photosystem II, like PSI, is a complex apparatus that comprises at least 24 subunits, which include light-harvesting proteins, various polypeptides, and redox components, the latter including a Mn aggregate. Among these components the chlorophyll P-680, part of the PSII reaction center, can be photo-oxidized to $P-680^+$, which has an unusually high potential of at least +1.1V (the highest value known in biology), sufficient to drive the extraction of electrons from water (midpoint potential +0.82V). The electrons are removed from H_2O by the Mn center, transferred to a tyrosine radical cation and then to $P-680^+$ (see later discussion).

The formation of dioxygen from two water molecules is perhaps the most important but least understood of all bioinorganic reactions catalyzed by Mn:



This four-electron oxidation of water is accomplished by a tetranuclear manganese oxo center called the "water-oxidizing complex" (WOC) or "oxygen-evolving complex" (OEC) [4,8,126]. Inorganic cofactors such as Cl^- and Ca^{2+} ions are catalytically required in addition to organic cofactors such as tyrosyl radicals. The Mn and the cofactors assemble to form a complicated supramolecular catalyst, whose reaction mechanism is gradually being revealed.

Several major reviews [6,8,9,14] have recently surveyed this field; this chapter will review only briefly the current status of our understanding of the O_2 evolving process and will focus on the role of the Mn centers in O_2 evolution, in both OEC and model complexes.

It is interesting to note at the onset that, in contrast to the previously discussed mono- and dinuclear Mn enzymes, which seem to cycle between Mn^{II} and Mn^{III} oxidation states, the structurally more sophisticated Mn center of OEC takes advantage of higher Mn oxidation states to perform its function, although MnRNR may turn out to be an exception.

It is generally accepted that photosynthetic water oxidation involves five steps, termed the *S states*; each of these holds one of the oxidizing equivalents found in the OEC. This notion stems from the observation [127] that dark-adapted spinach chloroplasts subjected to short flashes of light exhibited a consistent pat-



tern of O_2 release after each fourth flash. Schematically (Figure 26), two water molecules enter a cycle (the Kok cycle) and through the intermediate S_0 to S_4 steps, formed by successive one-electron oxidations of the OEC, a molecule of dioxygen and four hydrons are released.

The S_0 to S_4 states of the OEC are generated by electron transfer to a P-680 via a redox-active tyrosyl radical, $Y_z^\bullet$. The manganese complex in the OEC is thought to function as a charge accumulator, extracting four electrons from two oxide ions in a single well-defined step that connects the S_4 and S_0 states. O_2 release accompanies this $S_4 \rightarrow S_0$ transition. The S_0 and S_1 states are stable (in the absence of light), but the metastable S_2 and S_3 states decay to S_1 . The S_4 state is also unstable but advances preferentially to S_0 as opposed to decaying to the S_3 state.

VII.B. A Structural Model for the Oxygen-Evolving Complex

A combination of x-ray absorption spectroscopy, EPR, and other techniques has been used to shed light on the Mn center of OEC. Specifically, three major well-resolved peaks are visible in the EXAFS spectra of the S_1 state of PSII [128]. The first peak is best fitted by two shells of O or N atoms with the first shell containing ~ 2 O or N ligand atoms per Mn at ~ 1.82 Å. This shell was assigned to O atoms bridging Mn atoms, consistent with the 1.8 or 1.9 Å Mn—O distances found in μ_2 or μ_3 -oxo bridged complexes [3c,72]. The second shell was fitted using O or N ligand atoms located 1.95–2.15 Å from the metal center. These distances are comparable to those of Mn—N and Mn—O ligands in multinuclear Mn complexes [3c,72] and are slightly biased toward an O-rich environment.

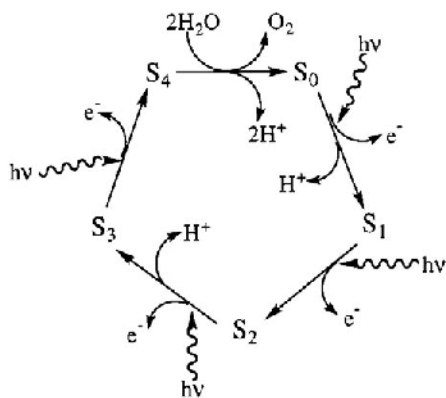


Figure 26 Schematic representation of the Kok cycle.



Although carboxylate ligands are strong candidates for providing O coordination (apart from water derived species), it should be noted that metal coordination by hydrogen carbonate ions was recently proposed [129].

The second peak was modeled by a single shell in which each Mn has ~ 1.25 Mn neighbors 2.72 Å away. Mn^{III} and Mn^{IV} complexes exhibiting this distance are common for two μ_2 -oxo [130] or μ_3 -oxo bridges for which the Mn—Mn distances range between 2.6 and 2.8 Å [131]. These distances do not change significantly upon the addition of a μ_2 -carboxylato bridge [130a–c]. The addition of a third, μ_2 -oxo or μ_2 -peroxo, bridge shortens these separations to 2.3 Å and 2.53 Å, respectively [132]. The third peak, observed at ~ 3.3 Å, is more difficult to fit: it may correspond to manganese alone or calcium at a longer distance or a combination of both.

Mn K-edge XANES has been used to probe both the symmetry and the oxidation level(s) of the S states. Mn oxidation state increases were detected in going from S_0 to S_1 [133], and from S_1 to S_2 [134] a decrease was noted for the S_3 to S_0 transition, but no change was observed for the S_2 to S_3 transition [135]. By comparing the Mn—K edges in OEC and Mn complexes, the oxidation level of Mn in the S_1 state was assigned as Mn^{III}_4 or $\text{Mn}^{\text{III}}_2\text{Mn}^{\text{IV}}_2$ (with a slightly better fit for the latter) [3d,134]. These data support the proposal that in the S_1 state one of the di- μ -oxo centers contains $\text{Mn}^{\text{III}}\text{Mn}^{\text{III}}$ and the other one is at the $\text{Mn}^{\text{IV}}\text{Mn}^{\text{IV}}$ oxidation level. The oxidation levels of the metal ions in the S_2 state are predicted by EPR to be $\text{Mn}^{\text{III}}_3\text{Mn}^{\text{IV}}$ [136].

Historically, EPR played and continues to play a major role in understanding the OEC. The S_2 state was the first one to produce characteristic multiline signals: 16 or 19 lines ($g = 2$) arising from $S = 1/2$ states [137] or an unresolved $g = 4.1$ signal originating from a higher-spin state [138]. The signals described may arise from an odd-electron, even-nuclearity cluster. Reduction of the S_2 state by one electron gives the even-electron, still paramagnetic (non-Kramers) S_1 , state. This state is EPR-active [139] but only if spectra are recorded in the unconventional parallel polarized mode. The S_0 state, though expected to be a Kramers doublet, was not observed until very recently [140], when 24 lines were detected ($g < 2.0$). Simulations [140c] of these S_0 states data using tetranuclear Mn assemblies are unable to distinguish between $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}_3$, $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}\text{Mn}^{\text{II}}_2$, or $\text{Mn}^{\text{III}}_3\text{Mn}^{\text{IV}}$ combinations. The new S_0 EPR signal, though resembling the S_2 , is wider, about an order of magnitude more stable (longer lived), and, importantly, it originates from a catalytically competent Mn complex [140b]. Furthermore, this signal has been ascribed to a magnetically coupled $\text{Mn}^{\text{II}}\text{—Mn}^{\text{III}}$ redox active subunit of the tetranuclear Mn aggregate, thus favoring formulations of the S_0 state that contain Mn^{II} . The overall OEC oxidation state increase in the S_2 to S_3 transition could be reconciled with the lack of Mn oxidation only if oxidation of an organic residue is considered. Indeed, a split EPR radical signal observed in a partially inhibited S_3 state has been explained by considering the interaction of an organic radical



with the S_2 state. The leading organic residue candidate is a histidine [3f,135,138g,141] (or a tyrosine), which is photo-oxidized in Ca^{2+} depleted [142] or Cl^- depleted (or F^- substituted) PSII samples [138g].

Calcium ions, which are essential for catalytic activity [3e], are believed [143] to be linked by a carboxylic acid residue to the Mn aggregate [144], either 3.3 Å [128,145] or at least 3.6 Å [146] from a Mn. Ca^{2+} ions are also required in the assembly of the Mn aggregate, at $\text{Mn}^{\text{II}}_x\text{Mn}^{\text{III}}_y$ oxidation level [147]. Ca^{2+} depletion or replacement by Sr^{2+} alters the magnetic interactions within the Mn aggregate as evidenced by variations in the S_2 multiline EPR signal, which loses its hyperfine structure ($g = 4.1$ signal) [142a,148].

The structural basis for the specified variability in OEC reactivity and spectroscopy is not understood, but possible functional and structural roles for Ca^{2+} ions in OEC include (1) assistance in dehydratation of Mn-bonded $-\text{OH}_x\text{O}$ groups that may form in OEC and that may [100v,149] (or may not [150]) yield dioxygen (assisted dehydratation favors O—O coupling because of oxidation potential depression estimated at 0.26 V/ H^+ for H_2O [3,151]); (2) stabilization of developing negative charges on dehydratated oxo species, which may encourage and/or prevent advanced oxidation; (3) “gate keeping” functions [142c], i.e., regulation of water access to Mn active sites.

Although it is clear that chloride is required for O_2 evolution [152], the catalytic role of the chloride ions is not clear, and neither is their exact location with respect to the Mn-oxo aggregate. Today there is no definitive direct evidence to decide whether Cl^- (and other halogens) binds directly at the Mn center or near it [8]. The EXAFS data for Cl^- or Br^- substituted samples from *Synechococcus* sp. suggest one terminal halide back-scatterer per four Mn [128], consistent with EPR change in the magnetic coupling [134d,138h].

A functional role for Cl^- as a “gate keeper” has been suggested [153], namely, to prevent HO^- binding at the Mn center, a process that would favor a $2e^-$ oxidation process leading to H_2O_2 formation, instead of the normal $4e^-$ oxidation required to produce O_2 . The preceding hypothesis implies that the site of O_2 evolution is the same as that of chloride binding: i.e., Cl^- binds to the Mn center.

The preceding spectroscopic data led to the structural proposal shown in Figure 27.

In addition to charge accumulation, the Mn-oxo aggregate may play a role in hydrogen abstraction [154]. Oxygen-evolving complex can be viewed as a supramolecular $[\text{Mn}_4\text{-Y}_z\cdot]$ center in which $\text{Y}_z\cdot$ abstracts hydrogens from the two terminal water molecules bound to the Mn_4 cluster (Figure 28) [126c,154a].

VII.C. Catalase Function of the Oxygen-Evolving Complex

The normal function of the OEC is the four-electron oxidation of H_2O , which generates O_2 . Besides this function O_2 can also be generated from H_2O_2 by a



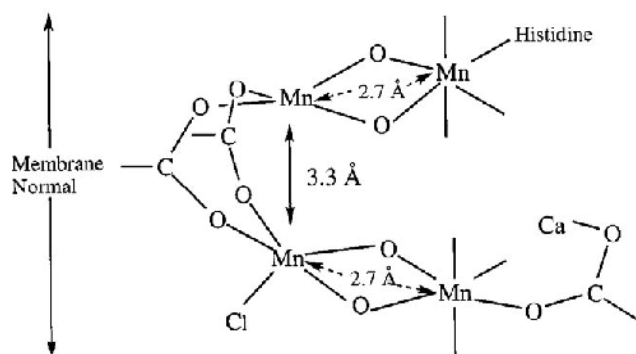


Figure 27 Proposed EXAFS model for the Mn complex in PSII. (From Ref. 128.)

catalase-like two-electron oxidation [155]. Such a functionality is not very surprising considering that, from a structural point of view, the Mn_4 center of OEC can be viewed as a sum of two dinuclear Mn centers (not necessarily identical), of the type present in catalases (discussed earlier). Although, in principle, any $S_x \rightarrow S_y$ ($y - x = 2$) pair of the five S states can perform the catalase reaction, only the *lowest* oxidation pair S_0, S_2 is apparently competent to evolve O_2 from H_2O_2 . This is in contrast with the evolution of O_2 from H_2O , which involves the *highest* oxidation state, S_4 , but consistent with the observation that higher oxidation states of catalases are inactive (discussed earlier).

The O_2 evolution from two water substrates raises the interesting question of when O—O bonds form or split. An elegant oxygen labeling study [155c] has established that the labeled, S_2 bound peroxide oxygens do not exchange with unlabeled water oxygens, and that only the latter type of oxygen is evolved upon illumination, consistent with the notion that the oxidation takes place in a single, four-electron step at the S_4 level.

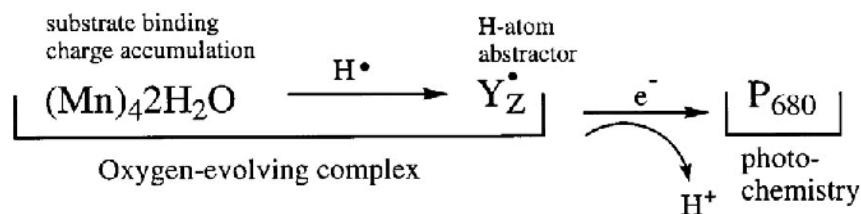


Figure 28 Proposed role of the Mn_4/Y_2^+ center in water oxidation. (From Ref. 154a.)



Although the study of the catalase-like function of OEC seems relevant to the structure and, indirectly, to the function of PSII, it should be noted that the OEC catalase activity has been recently challenged [156].

VII.D. Tetranuclear Mn Complexes and Other Mn Complexes That Evolve Dioxygen from H_2O_2

VII.D.1. General

A large number of biomimetic Mn models have been prepared, and their spectroscopic and structural properties, which played an important role in linking OEC spectroscopic features to structural topologies, have been reviewed recently [9]. Consequently, this review will be limited to synthetic biomimetic models that are directly catalytically relevant, i.e., that form, contain, or split O—O bonds.

VII.D.2. Mn-Peroxo Complexes: Structural Models for the Oxygen Bound S_2 and S_4 State

To date there are no firmly established tetranuclear Mn-peroxo complexes, but this does not exclude the possibility that a μ_4 -Mn peroxo or a μ_2 -Mn peroxo complex may be present in the H_2O_2 induced S_2 state or the normal S_4 state of OEC. Peroxo groups bridging two metal centers are more common, and perhaps more likely to be biologically relevant.

The possibility of formation of an O—O bond at a single Mn site of the OEC, on the other hand, seems remote, but nevertheless, two mononuclear model Mn-peroxo complexes, a peroxo-Mn^{III} porphinato **64** [157] and a peroxo-Mn^{III} pyrazolylborato complex **65** [158], have been structurally characterized. Both complexes contain side-on peroxo groups. Complex **65** has been crystallized in two forms, one brown and one blue. A peroxo-H—N (pyrazole) hydrogen bond is found only in the blue form (Figure 29).

The pyrazolylborate complex decomposes spontaneously to form bis(μ -oxo) complexes. Bridging peroxo groups are encountered in two other complexes. A μ_3 -oxo trinuclear Mn complex **66** [159] was reported several years ago (Figure 30). The second complex, **67**, [132] exhibits a bis(μ -oxo)(μ -peroxo) dimanganese(IV) core (Figure 31). Interestingly, although the peroxo group of **67** is stable in acetonitrile, O_2 is evolved in aqueous solutions containing chloride ions. Furthermore, this complex assembles from Mn^{II} and dioxygen (air) and decays to Mn^{III} upon O_2 release, thus providing a viable model for the S_4 to S_0 transition.

VII.E. Functional Models

Only a very limited number of authentic tetranuclear Mn complexes are known to have catalytic activity. Supramolecular Mn_4/Ca_2 , Mn_4/CaBa , and Mn_4/Ba_2



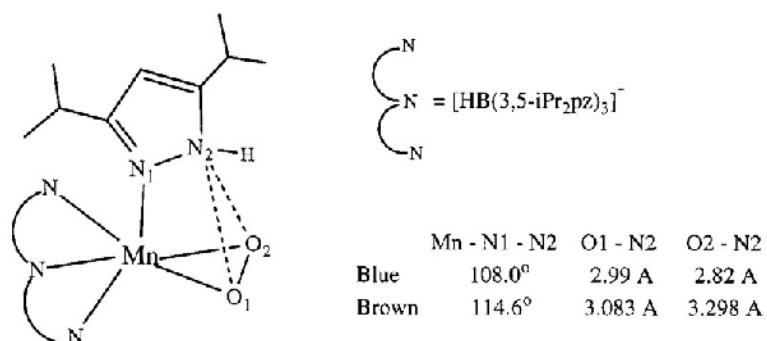


Figure 29 Schematic representation of complex **65** for which two isomeric forms exist. (After Ref. 158.)

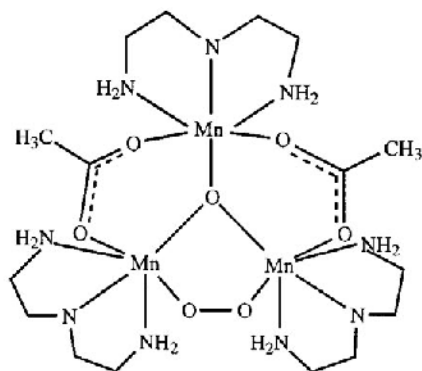


Figure 30 Schematic representation of complex **66**.

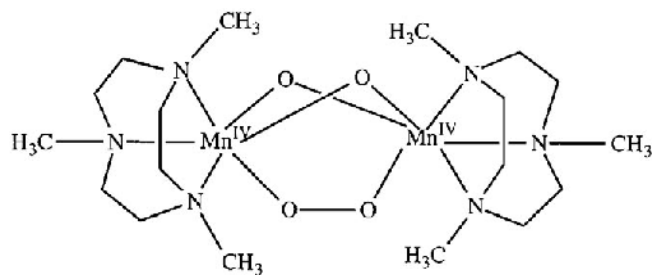


Figure 31 Schematic representation of complex **67**.



assemblies, models for the Mn_4/Ca OEC aggregate, have been shown to evolve O_2 from H_2O_2 at rates that exceed those obtained with MnO_2 or equimolar amounts of mononuclear Mn complexes [149a,b]. The low oxidation states of the Mn ions, $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}$, suggest that these complexes may be structural and functional models for the S_0 state of OEC. Interestingly, similarly to PSII, the initial rates of O_2 evolution are alkaline–earth metal-dependent even if the Mn moiety topology is similar. Recently, a tetranuclear complex with even lower Mn oxidation states, Mn^{II}_4 , has been shown to decompose H_2O_2 [100v]. Since all Mn ions are in +2 oxidation state, which formally matches a S_{-3} superreduced state of the OEC (assuming S_0 level is $\text{Mn}^{\text{II}}\text{Mn}^{\text{III}}_3$), the catalytic activity may correspond to a yet unobserved $S_{-3} \leftrightarrow S_{-1}$ transition.

Although the preceding catalase biomimetic reactions are important, the true biological role of the OEC is to evolve O_2 from H_2O . Only a few complexes evolve O_2 from H_2O and even fewer contain Mn (see complex **67**). Perhaps the best known case is the catalytic oxidation of water by the dinuclear ruthenium complex $[(\text{bpy})_2(\text{H}_2\text{O})\text{RuORu}(\text{H}_2\text{O})(\text{bpy})_2]^{4+}$ **68**, which has been well documented [9,160] and will not be discussed here.

A dinuclear Mn^{III} porphyrin complex linked by an *o*-phenylene bridge (L14) (Figure 32) [161] seems to be the first reported structurally well-defined manganese compound to oxidize H_2O by a four-electron process.

Isotope labeling studies showed that O_2 is evolved when an aqueous acetonitrile solution (1:20 $\text{H}_2\text{O}:\text{CH}_3\text{CN}$) containing *n*- Bu_4NOH is electrolyzed at potentials ranging from 1.2 to 2.0 V (vs. Ag/AgCl). In the absence of these catalysts

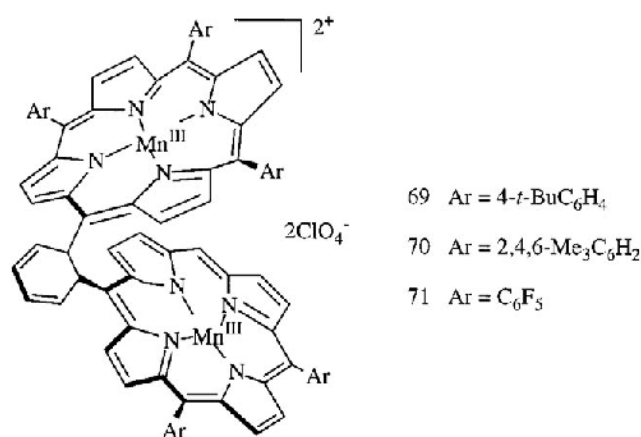


Figure 32 The bis(porphyrin) L14 dinuclear complexes **69**, **70**, and **71**. (From Ref. 161.)



a 2.3 V potential is required for O₂ evolution. Importantly, this seems to be a four-electron process (3.7 electrons were measured using a rotating disk electrode), that would support the notion that this reaction is a true water oxidation, despite the fact that the same complexes have catalase activity. Although this complex is not structurally biomimetic (there are no heme ligands to the Mn in OEC), the biomimetic activity emphasizes the importance of bringing two Mn in close proximity (the corresponding mononuclear complexes are inactive) and facilitating stabilization of high Mn oxidation states via electron delocalization.

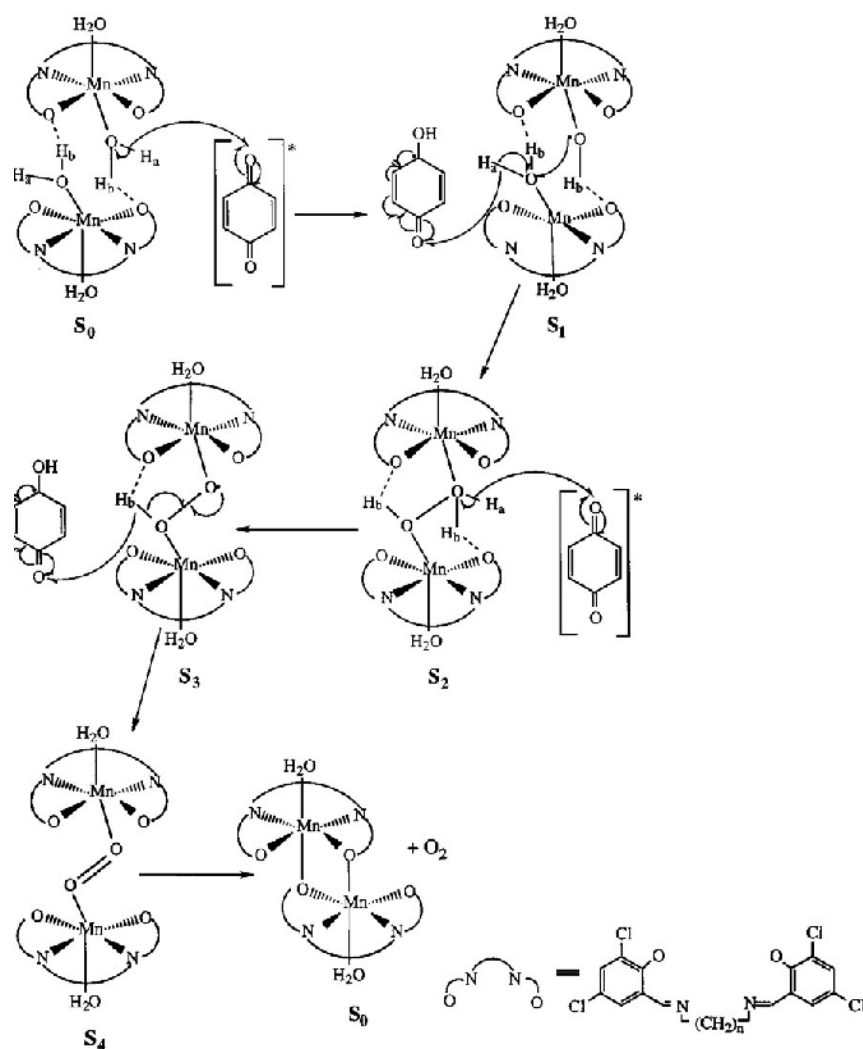
Among the Mn complexes of more biologically relevant ligands, [Mn(dpa)₂][−] **72** (dpa = dipicolinate) reacts with potassium peroxydisulfate (oxone) to evolve O₂ and form MnO₄[−], via a green dinuclear Mn^{III}Mn^{IV} intermediate, which has been identified by UV-Vis and EPR spectroscopy [162]. The evolution of O₂ declines with the formation of MnO₄[−], but at pH 3.5 two turnovers are observed. Replacement of dpa by other ligands, such as bpy **73**, phen **74**, and terpy **75**, produces the same active Mn^{III}Mn^{IV} dinuclear species. Interestingly, complex **73** shows no dioxygen evolution, whereas complex **75** is active (>50 turnovers) and stable. Complex **73** presumably dissociates too quickly to be catalytically active, but complex **75** both has open sites for solvent coordination and is also more resistant to ligand dissociation. Consequently, MnO₄[−] builds up, leading to decline in O₂ evolution for **73** but not for **75**. The origin of O₂ has been ascribed to H₂O (via coupling of two Mn=O fragments) and not to HSO₅[−] on the basis of the observation that the Mn-terpy complexes evolve O₂ from dimethyl dioxirane, a single oxygen donor. Thus, a biomimetic, dinuclear Mn-based oxygen-oxygen coupling mechanism is being proposed.

Recently [163], dinuclear manganese (III) complexes of Schiff bases such as L11 have been shown [163b,164] to evolve O₂ photolytically from water in the presence of *p*-benzoquinone. By using variable di-, tri-, or tetramethylene backbone linkages (Scheme 8), and a fixed N₂O₂ donor set, it was found that the trimethylene complex exhibits the greatest activity, suggesting that the *n* = 3 backbone most closely mimics PSII. An x-ray crystal structure analysis of a manganese complex of L11C (L11C = Cl-salen), a dichloro analog of L11, supports the previously suggested [165] catalytic pathway (Scheme 8).

Specifically, two μ-aqua-bridges located in the cleft of the dimer have one hydrogen pointing outward from each side, thus allowing the photo-excited *p*-benzoquinone (labeled* in Scheme 8), but not a bulky 2,5-*t*-Bu-*p*-benzoquinone, to enter the cleft and abstract a H• radical. This mechanism is consistent with the proposed role of the tyrosyl Y_z• radical as H• abstractor from water [165]. The reaction, however, is not catalytic, because of the irreversible formation of hydroquinone.

A dinuclear metal complex, **76**, in a more rigid framework, which forces two manganese centers into close proximity within a single macrocycle (Figure 33), also evolves O₂ from H₂O [163a,164].





Scheme 8 Proposed dioxygen evolution mechanism for Mn^{III} complexes of L11C and formal analogy to Kok states. The (CH₂)_n group is the variable backbone link referred to in the text. (From Ref. 163b.)



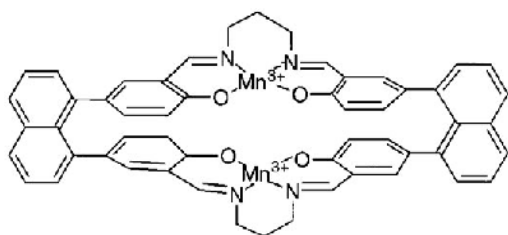


Figure 33 Structure of the Mn Schiff base macrocyclic complex **76**. (From Ref. 163a.)

This complex retains the previously determined optimal coordination parameters, i.e., a N_2O_2 donor set and a C_3 backbone (see Scheme 8), but the O_2 evolution in the presence of *p*-benzoquinone is slower than that of the unconstrained molecule. The reasons for this difference are not entirely clear, but it should be noted that this complex, unlike the unconstrained one, is water insoluble.

Hexanuclear manganese complexes of polyhedral silsesquioxanes, supported on SiO_2 , C, or Al_2O_3 , evolve O_2 from H_2O when oxidized by $\text{Ru}(\text{bpy})_3^{3+}$ [166]. The Mn ions (Figure 34) form a sandwiched ring structure and are octahedrally coordinated by four equatorial μ_2 -siloxo groups, one axial μ_6 -chloride ion, and one O from H_2O or ROH.

The reaction mechanism for this interesting system has not yet been reported. The dimanganese complex $[(\text{bpy})_2\text{Mn}^{\text{IV}}(\mu\text{-O})_2\text{Mn}^{\text{IV}}(\text{bpy})_2]^{4+}$, **78**, is another interesting case [167]. Although inactive in solution even in the presence of Ce^{IV} , in the presence of chemical or electrochemical (electrodes) oxidants, **78** evolves O_2 when used as a slurry or when heterogenized into Kaolin or Nafion. The complex eventually decomposes to yield Mn^{VII} . A saturated aqueous solution of another complex, $[\text{Mn}(\text{salen})(\text{H}_2\text{O})_2](\text{ClO}_4)_2$, **79**, forms an ill-defined brown precipitate upon oxidation with $(\text{NH}_4)_2[\text{Ce}(\text{SO}_4)_3]$, and further evolution of dioxygen from water occurs at the expense of Ce [168]. Unlike the previous Ce induced O_2 evolution case, permanganate is not detected in solution. The identity of the catalyst remains to be established.

Another report describes the O_2 evolution catalyzed by a series of di- and oligonuclear structural models of PSII [169] and their uncharacterized hydrolysis products, in the presence of $\text{Ru}(\text{bpy})_3^{3+}$. The catalytic activity seems to be ligand independent (if the ligand is not oxidized) and boosted by dispersion of the catalyst in phospholipids. The exact nature of the catalyst is not known, but this work suggests the importance of creating lipophilic, membranelike catalyst environments. In the preceding cases $\text{Ru}(\text{bpy})_3$ is not covalently linked to the Mn centers, thus making an outer-sphere electron transfer pathway likely.



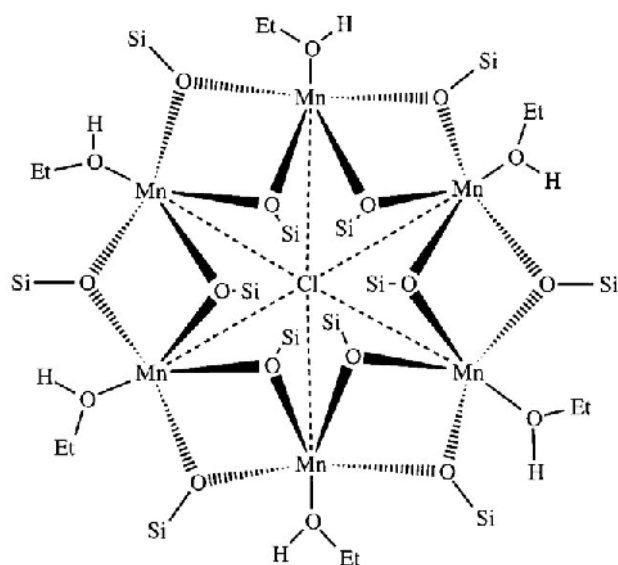


Figure 34 Schematic representation of the solvated Mn core of $\text{Na}[(\text{PhSiO}_2)_6\text{Mn}_6\text{Si}(\text{O}_2\text{SiPh})_6]\text{Cl}$, **77**. (After Ref. 166.)

Modeling the catalytic events that lead to O_2 evolution can be extended to cover the involvement of ligands and electron acceptors. For example, the P-680^+ of PSII has been functionally modeled [170] by a $\text{Ru}(\text{bpy})_3$ -type ion, covalently linked to a Mn^{II} through a bridging ligand (complex **80**) (Figure 35). Methylviologen (MV^{2+}) is the ultimate electron acceptor in this system. For a viable functional model, the electron transfer rate from the coordinated manganese should be higher than the rate of back transfer of an electron from methylviologen, the latter resulting in quenching of the $\text{Ru}(\text{bpy})_3$ photo-excited state. Intramolecular electron transfer was indeed detected from the manganese ion to the photo-oxidized Ru complex and its rate found to vary inversely with the through-bond $\text{Mn}\cdots\text{Ru}$ distance.

VII.F. Conclusions

Our current understanding of PSII indicates that the exact role of Mn , Cl^- , Ca^{2+} , and organic moieties in O_2 evolution, though more clear than several years ago, is still incomplete. Key questions such as, What is the structure of OEC?, Is O_2 formed at a Mn_2 subset of the Mn_4 aggregate?, What is the molecular nature of the multiple subunits involved in electron transfer?, What are the protection



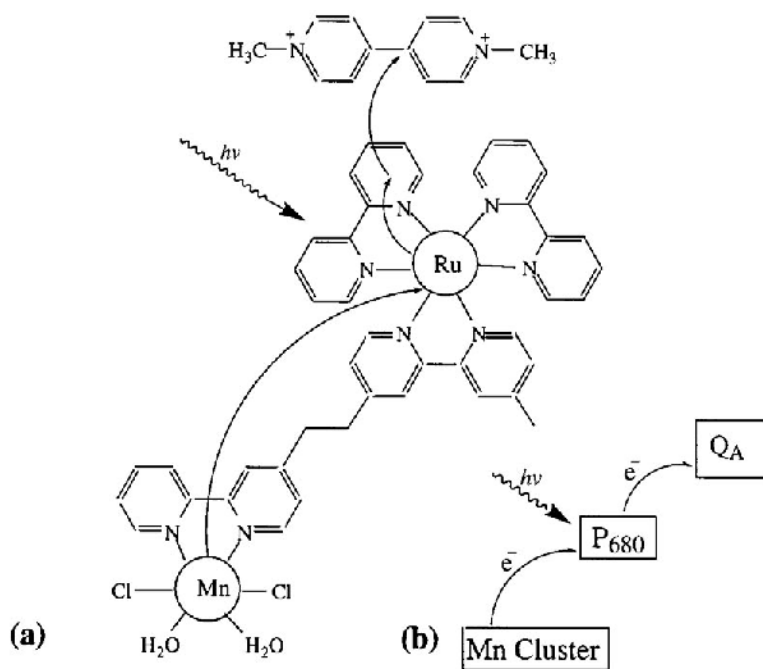


Figure 35 Photo-induced intramolecular electron transfer pathway of complex **80**, in the presence of methylviologen: (a) The Mn...Ru...MV²⁺ pathway; (b) the Mn...P₆₈₀...Q_A quinone pathway in PSII. (From Ref. 170.)

mechanisms that prevent inactivation of PSII given the presence of free radicals with high redox potentials?, remain unanswered. The combination of enzyme and modeling work described is testimony to the significant advance of our understanding of the OEC and suggests that most of the questions will be answered in the near future, even if an x-ray structure of PSII may not become available.

VIII. OTHER BIOLOGICAL MANGANESE SITES

VIII.A. Thiosulfate Oxidizing Enzyme

The bacterium *Thiobacillus versutus* has been shown to require manganese for both growth and oxidation of thiosulfate. This organism uses a multienzyme system that includes two colorless proteins, A and B, and two *c*-type cytochromes to oxidize thiosulfate to sulfate. Protein A is a thiosulfate-binding protein; protein



B contains significant amounts of manganese. The EPR data [171] suggest the presence in protein B of a spin-coupled dinuclear Mn^{II} cluster, perhaps of the type found in catalases and arginases. No additional information about the metal active site is currently available.

VIII.B. The Nonheme Mn Center of Filamentous Bacteria

Another nonheme Mn center is believed [172] to be present in photosynthetic green filamentous bacteria. The locus of the Mn ion in these bacteria is similar to that of the nonheme Fe^{II} in photosynthetic purple bacteria [173], i.e., between two quinones, along the pathway of electron transfer. Since the Fe center of purple bacteria does not seem to be involved directly in the electron transfer process (i.e., is not redox-active), the redox role of the Mn analog remains in question. This Mn may be redox-active, considering that (1) structural differences between the purple and green bacteria photosynthetic apparatus do exist [173] and (2) the green bacteria display different functionalities, such as CO_2 fixation, which does not occur via the classical Calvin or reverse Krebs cycle [174].

IX. CONCLUSIONS

The remarkable, versatile, and fascinating bioinorganic chemistry of Mn seems to be primarily centered around the seemingly opposite tasks of producing dioxygen by oxidizing O^{2-} but also rendering harmless the dioxygen reduction products, $\text{O}_2^{\bullet-}$ and O_2^{2-} , and, in the process, regenerating O^{2-} and (O_2).

Consistent with this task, Mn is found in “hard” oxygen and nitrogen-rich coordination environments, which seem to be sufficiently versatile to fine-tune the metal’s redox potential to cover the range required for the chemistry. The N and O based donor sets available for coordination along with the lack of ligand based electronic delocalization may limit the Mn oxidation state variability range. Perhaps not surprisingly, the higher degree of regulation of metal oxidation variability in the OEC case requires additional and different cofactors such as calcium and chloride. It seems, therefore, unlikely that Mn-S bonds will ever be found in enzymes directly involved in oxygen chemistry.

From a structural point of view, to date only Mn centers of nuclearity of 1, 2, and 4 are known in biology, consistent with the redox chemistry of oxygen:

- Nuclearity 1, redox chemistry of superoxide dismutation (1 electron)
- Nuclearity 2, redox chemistry of peroxide disproportionation (2 electrons)
- Nuclearity 4, oxidation chemistry of dioxygen formation (4 electrons)

It thus appears that **formally** there is only a one-unit oxidation state change per manganese, regardless of the nuclearity of the catalyst. This somewhat sur-



prising conclusion, considering the availability of Mn oxidation states ranging from +2 to +7, might be summarized in a formal “one metal–one electron” redox–nuclearity relationship for bioinorganic manganese involved in O₂ chemistry. If the “redox–nuclearity” relationship is valid, then there is little chance that a trinuclear or a pentanuclear (or higher nuclearity) Mn enzyme involved in O₂ oxidation or reduction will ever be found, thus limiting the biological Mn to the already known 1, 2, and 4 nuclearities.

Manganese environments, and consequently catalytic activity, are also highly regulated beyond the metal first coordination spheres. For example, related dinuclear manganese active sites present in arginase and catalase have catalase activity that is several orders of magnitude different; this difference is attributable, at least in part, to the shape and composition of the surrounding “pocket.” Another manifestation of nature’s coordination sophistication is that regulation of active sites results in an advanced degree of *hierarchical* compartmentalization of catalytic functionalities. One might expect higher nuclearity Mn centers also to have the functionality of the lower nuclearity ones, i.e., Mn catalases to exhibit superoxide dismutase activity and the OEC both catalase and superoxide dismutase activity. In reality, however, only the catalase activity of the OEC seems to be demonstrated, but not under normal operating conditions of the OEC catalyst. Even this reactivity, however, is in doubt. The understanding of catalytic mechanisms, especially that of OEC, is still incomplete, but it is likely that a combination of enzyme studies and modeling will provide the remaining answers in the not so distant future.

Biomimetic ligand design aimed at biomimetic functionality is also still far from perfect but has reached, occasionally, such a point of sophistication that, for example, one conformational isomer of a model exhibits zero SOD activity and the other one is very active.

The catalytic properties of Mn enzyme structural models are not limited to the natural substrates of the enzymes they mimic. One could classify this catalysis based upon the substrates as “biological mimetic catalysis” or “biomimetic catalysis” [175] and “biologically inspired catalysis” or “bioinspired catalysis” [176]. Unlike biomimetic catalysis, its bioinspired counterpart capitalizes on nature’s findings to change nonnatural substrates chemically and, perhaps, unravel novel chemistry.

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13

The Two B₁₂ Cofactors: Influence of the *trans* Nitrogen Ligand on Homolytic and Heterolytic Processes

Luigi G. Marzilli

Emory University, Atlanta, Georgia

I. INTRODUCTION

I.A. General

“Vitamin B₁₂” sounds familiar to both scientists and the general public [1]. However, it is not understood widely that the vitamin simply acts as a precursor to the biologically active coenzyme forms of B₁₂. These coenzymes contain a Co(III)—C bond, a type of bond first recognized in coenzyme B₁₂, Figure 1. All these species contain the corrin macrocyclic ring. The biochemistry of the vitamin and other related B₁₂ species resides in their active transport by a complicated system [2–4]. This transport provides humans with B₁₂ derivatives that can be converted to the biologically active cofactors. Such transport can be exploited to deliver drugs or diagnostic agents [5]. In reports of a 1996 conference, all such aspects of B₁₂ chemistry and biochemistry are described, including evidence that B₁₂ biosynthesis and porphyrin biosynthesis have interconnecting features [6]. It has been argued that B₁₂ systems predate porphyrins and that through evolution the corrin macrocycles were replaced by porphyrins [7]. As a result, porphyrin-based systems are now much more widely distributed (and much more widely studied) than B₁₂–(corrin)–based systems [8].

Indeed, only two enzymatic processes in humans are known to utilize B₁₂, although other organisms make wider use of B₁₂ compounds [6,9–12]. One may

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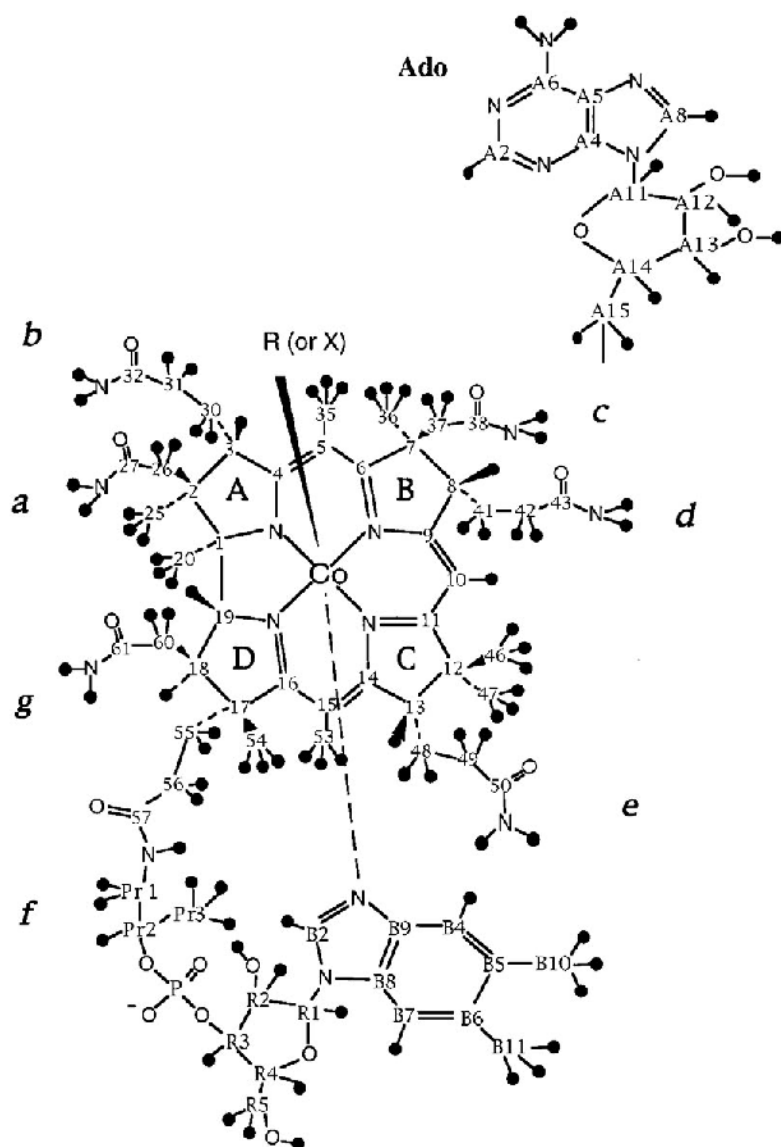


Figure 1 Schematic representation of the molecular structure, numbering of atoms, and designations of pyrrole rings of cobalamins. R = Me is methyl B₁₂; R = Ado is adenosylcobalamin (coenzyme B₁₂); X = CN is cyanocobalamin (vitamin B₁₂). Five-membered rings are labeled A–D, and the amide side-chains are labeled a–g.



argue that nature does not have such elaborate systems for the transport and activation of B₁₂ compounds for just a few biological roles. Therefore, one may speculate that B₁₂ must have as yet unrecognized functions. Alternatively, if we accept the convincing indications that B₁₂ predated porphyrins and that B₁₂-based systems have been replaced by more efficient porphyrin-based systems, then one may believe that the roles currently being filled by B₁₂ systems cannot be adequately filled by porphyrin systems. Thus, a comparative analysis of porphyrin and B₁₂ chemistry could provide clues into the elusive mechanisms of B₁₂-dependent enzymatic processes. Indeed, it may well be that the difficulties chemists have experienced mimicking most B₁₂ reactions have parallels in nature, where porphyrin-based systems have also failed to accomplish the same chemistry. It is not unexpected that the few remaining B₁₂-based biochemical transformations are simply very difficult to effect without an intimate role for B₁₂ or another metal species. Indeed, in some cases, the identical transformation can be effected, but only by other metalloenzymes [13–19].

The infinitely rich biochemistry and chemistry of B₁₂ systems would take several volumes to delineate adequately. (The now classic comprehensive two-volume work edited by Dolphin [1] should be consulted for an overview, along with the recent conference report [6]). Since the focus of this volume is biocatalysis involving metal species, I have selected two of the most relevant topics to describe in depth. Another criterion was used in this selection: I wished to illustrate the roles of the two B₁₂ coenzyme classes, methyl cobamides (MeCba's) and 5'-deoxyadenosyl cobamides (Cba's) (illustrated in Figure 1 by the cobalamins [Cbls]). The adenosyl coenzyme was the first recognized [1]; it is known to be involved in more enzymatic processes than methylCbl. Therefore, it is called *coenzyme B₁₂*, whereas the other important organocobalt coenzyme, methyl B₁₂, is usually simply called *methyl B₁₂* (MeB₁₂, MeCbl) [1].

The Co(III)—C bond in the natural coenzymes is resistant to cleavage in protic solvents. However, the bond length [20] is similar to that in models. Indeed, there appear to be no special corrin ring electronic properties necessary for such water-stable Co—C bonds; even Co(III)—CH₃ compounds with classical ligands such as ammonia or ethylenediamine have now been discovered [21]. Although such non-B₁₂-related systems are outside the scope of this review, I believe that the main reason that few such compounds are known lies in the paucity of synthetic routes. Since the Co—C bond, once formed, is relatively inert, such compounds could be used for multiple types of applications such as in molecular assemblies or devices [22]. The natural compounds and some models are photosensitive, however [23]. It is this photosensitivity that delayed the discovery of the coenzymes, leading instead to the isolation and characterization of the vitamin [1].

Cobamides consist of a cobalt corrinoid with a pendant nucleotide, which together occupy five of the six positions of an octahedral Co. The most important



cobamides are known as cobalamins; Cbls have α -ribazole as the pendant nucleotide. The most important difference in cobamides is the nature of the heterocyclic base of the pendant nucleotide. In Cbls, this base is 5,6-dimethylbenzimidazole (dmbim). However, in acetogenic bacteria such as *Clostridium thermoaceticum*, acetate biosynthesis involves a corrinoid/Fe-S protein [24–27]. The heterocyclic base in the methyl cobamide is 5-methoxybenzimidazole. This interesting enzyme system has attracted considerable attention because of the role of a Ni enzyme in the overall process forming acetate. Although the 5'-deoxyadenosyl axial ligand of 5'-deoxyadenosylCbl has an anomeric C with the usual β configuration, it is important to note that the anomeric carbon of the nucleotide, as the name α -ribazole implies, has the α configuration. Recent work in Toraya's laboratory indicates that the length and conformation of the nucleotide loop in Cbls may be optimal for binding of the dmbim moiety to Co via N3 [28–30]. The dmbim base is positioned for coordination on the "lower" α side at neutral to high pH (MeCbl in the base-on form), with the plane of the base along the "long" axis, i.e., C5—Co—C15. The corrin is bent upward in a butterfly manner with the "body" of the butterfly lying along the short axis (C10—Co midpoint of the C1—C19 bond). At low pH, the hydronated base-off species, MeCbl⁺, is formed (apparent pK_a [31] of the hydronated dmbim N3 is 2.9). The potential role of the axial nitrogen ligand has begun to receive considerable attention since evidence, including x-ray data [32,33], indicates that imidazole has replaced dmbim in some enzymes. The very long Co—N bond in the cofactors [20] prompted theoretical studies that suggest that the electronic character of the axial ligand could modulate Co—C bond homolysis/heterolysis. Recently there has been experimental support for these computations produced as part of a series of studies on axial base effects on the properties of adenosyl cobinamide [34–36].

The recent crystal structure determination of MeCbl bound to the cobalamin-binding domain of *Escherichia coli* methionine synthase [32] has revealed much new information. Specifically, the MeCbl was found to be in the (not-hydronated) base-off conformation, with the "nucleotide loop" important primarily in the binding of the cobalamin to the protein (Figure 2). Also, a histidine (His in the enzyme) is coordinated at the α -axial site. Likewise, an x-ray structure of a coenzyme B₁₂-dependent methylmalonyl-CoA mutase from *Propionibacterium shermanii* reveals that imidazole from the protein is bound, suggesting that this may be a common motif in cobalamin-dependent enzymes. Recent EPR evidence has shown that a nitrogenous ligand (probably the imidazole of an enzyme histidine residue) displaces the dmbim moiety of coenzyme B₁₂ in *P. shermanii* methylmalonyl-CoA mutase [37,38]. Additional spectroscopic methods for assessing the form of the bound cobalamins are needed [38].

Coenzyme B₁₂ plays a central role, not only in the B₁₂ field, but in several



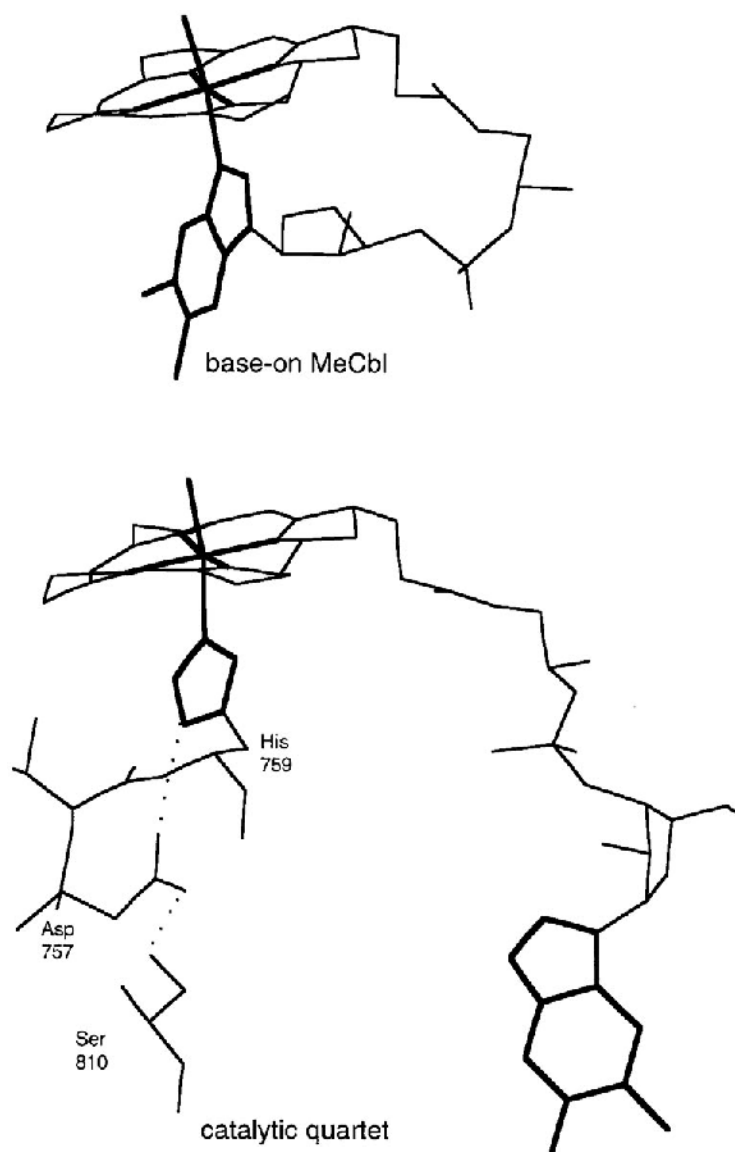


Figure 2 Comparison of the structures of MeCbl in the base-on form and in the B₁₂ binding domain of methionine synthase.



aspects of the development of biochemical [39,40] and physical methods. As an example, x-ray crystallography established the existence of the Co—C bond [41] in coenzyme B₁₂, although at the time this was a very complicated molecule for this method. Specifically, 5'-deoxyadenosine was attached through the 5'-carbon of the ribose moiety of adenosyl. About two decades later, x-ray crystallography confirmed the structure of the other coenzyme, MeB₁₂, only the second organoCbl to be so characterized [20]. Now many x-ray structural characterizations of organocobalt B₁₂ compounds have been performed [6,42–44].

A second and recent example of the role of B₁₂ systems in the evolution of physical methods involves heteronuclear 2D NMR. The nonpolymeric nature of B₁₂ serves as a special challenge for NMR since the NOE connectivities typically exploited for sequential assignments of oligomers or polymers are absent. Therefore, strategies were developed to use “proton framework” → “carbon framework” → “proton framework,” etc., approaches to assign signals sequentially using heteronuclear multiple bond coherence (HMBC) and heteronuclear multiple quantum coherence (HMQC) spectroscopy [45,46]. Such NMR methods have proved to be instrumental in assigning isomer configurations of B₁₂ analogs used to probe enzyme function since x-ray and neutron diffraction methods failed to resolve the issues [47]. These NMR methods have been the method of choice in a number of recent studies [6,48–50].

Another method in which B₁₂ studies played a very important role in the development of applications to metallobiochemistry is FT-Raman spectroscopy [51–57]. The Co—CH₃ stretching vibrational frequency can be correlated to Co—C bond strength in the ground state, but except in rare cases [25] this band is difficult to observe by the usual types of Raman spectroscopy because the organocobalt species are light sensitive. Recent elegant studies have shown that resonance Raman spectroscopy is a powerful way to elucidate the nature of the key M—C bonds in carbon monoxide dehydrogenase [25]. Early studies utilizing near-IR FT-Raman spectroscopy [52] showed for the first time that the Co—CH₃ stretching band is observable for solid-state MeCbl and organocobalt B₁₂ models [51,53]. The relatively low-excitation-energy (1.064 μm) laser used in this technique does not promote electronic transitions, thus eliminating potential problems associated with fluorescence and photolysis of the Co—C bond [52].

I.B. Role of Cobalt

As one might suspect, the different alkyl groups in the two classes of coenzymes lead to both different biochemical roles and different chemistry. In essence, coenzyme B₁₂-dependent enzymatic processes are radical (homolytic) processes—the chemistry of Co(II) and R'—whereas methyl B₁₂-dependent



enzymatic processes are nonradical (heterolytic) processes (the chemistry of Co(I) and R⁺).

There may be common themes in the role of protein–coenzyme contacts in these B₁₂-dependent enzymatic processes. In particular, these contacts could alter the relative stability of the Co(III)—R, Co(II), and Co(I) states to enhance reactivity. For coenzyme B₁₂-dependent enzymes, the deoxyadenosyl radical generates a substrate-derived radical, either directly or via a radical chain mechanism through the intermediacy of a protein-side-chain-based radical, such as S[•] of cysteine or O[•] of tyrosine. This protein-bound substrate-derived radical then undergoes rearrangement, possibly assisted by protein contacts. Thus, cofactor–protein contacts are probably very important in the activation of the Co—C bond, in altering the Co redox potentials, and in assisting in the rearrangements.

Unfortunately, the one x-ray structure of a coenzyme B₁₂-dependent enzyme reported has disorder problems and has a mixture of forms of the cofactor; thus, it is difficult to discern well the role of the protein [33]. The evidence that protein–cofactor contacts are important in the activation process is limited almost exclusively to a few of the coenzyme B₁₂-dependent enzymes. This evidence will be discussed later.

For coenzyme B₁₂ systems, the role of the inorganic center, i.e., the Co, may be limited to the key step of providing the initial organic radical [9,58–66]. This step is, nevertheless, fascinating and has attracted considerable attention. Recent evidence suggests that it is not so innocent [67]. Quite significant organometallic chemistry has resulted from attempts to utilize organocobalt derivatives to mimic the organic rearrangement reactions [9,68]. The role that Co may play in such rearrangements both in chemical systems and in the enzymatic cycle is the subject of great controversy. Interesting new chemistry will certainly emerge from investigations directed at elucidating the role of Co in the rearrangement process, and there is hope that we will gain further insight into the enzymatic processes from studies of putative organocobalt species derived from the substrates. With time the putative role of Co in the rearrangement steps has gone from being the focus of most mechanistic proposals to being generally accepted as unimportant. However, recent results provide tantalizing preliminary (and still unconfirmed) evidence that the substrate-derived radical must remain close to Co and that the cobalt center may play a (perhaps spatially distant) role in influencing the radical rearrangement. Of particular importance, the Co center must not react rapidly with the radical to form a Co—C bond [33]. Thus, the role of Co may lie between the two popular concepts: intimate involvement and no involvement. However, in view of the controversy, I will focus on the homolytic Co—C cleavage step and the factors influencing this step, in which the metal center is intimately involved. In contrast to the coenzyme B₁₂ mechanism, the biochemical processes utilizing MeB₁₂ intimately



involve the cobalt center. Therefore, I will discuss methyl transfer mechanisms in greater depth.

II. COENZYME B₁₂

II.A. Reactions Catalyzed

Coenzyme B₁₂ is utilized by enzymes known to catalyze a dozen rearrangement reactions [1,9,69] and, in addition, by B₁₂-dependent ribonucleoside triphosphate reductase (EC 1.17.4.2), commonly known as RTPR since the substrate is the nucleoside triphosphate [70,71]. The rearrangement reactions are given in Table 1. The most extensively studied enzyme is diol dehydratase (propanediol dehydratase, [DD]; EC 4.2.1.28) [12,28–30,39,72,73]. For example, in DD, broad signals assigned to low-spin cob(II)alamin and doublet signals assigned to a substrate-derived radical intermediate were observed with 1,2-propanediol, 1,2-ethanediol, glycerol, and *meso*-2,3-butanediol with the magnitude of their exchange interaction (j value) decreasing in this order [73]. A substrate with a smaller j value seems to be a more efficient mechanism-based inactivator. Since the j value decreases with the distance between radical species in a radical pair, these results were taken to suggest that a stabilizing effect of holoenzyme on radical intermediates during reactions decreases with the distance between Co(II) and a radical. It should be noted that DD does not have the sequence characteristic of the catalytic quartet and ¹⁵N labeling combined with EPR demonstrates that the dmbim is still bound [74].

A ribonucleoside diphosphate reductase (RDPR) (EC 1.17.4.1) also exists in a non-B₁₂-dependent form that relies instead on a nonheme dinuclear Fe center [14–19]. Although there have been a few recent reports on the B₁₂-dependent enzyme, the Fe system has been cloned, expressed, crystallized; its B₂ subunit characterized by x-ray crystallography; and extensively studied by both mechanistic and spectroscopic methods [14–19]. Since the study of the Fe system appears so promising, most recent effort has focused on it. There is very strong evidence for radical chemistry consistent with the preceding arguments that, typically, metal-based chemistry is required to substitute for B₁₂-based chemistry. The reader is referred to the recent literature [15,17–19] for details on these exciting systems.

II.B. Overall Mechanisms of Rearrangement Reactions

All rearrangement reactions in Table 1 can be categorized as an apparent intramolecular 1,2-shift of a hydrogen and an electronegative X group (OH, three examples; NH₂, five examples; C framework, four examples) [1,9,10].

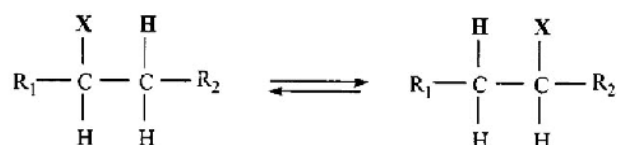


Table 1 Rearrangement Reactions Catalyzed by Coenzyme B₁₂ (AdoCbl)-Dependent Enzymes

OH shifts			
	diol dehydratase (DD)		+ H ₂ O
	diol dehydratase (DD)		+ H ₂ O
	glycerol dehydratase		+ H ₂ O
NH₂ shifts			
	ethanolamine ammonia lyase (EAL)		+ NH ₃
	D-ornithine mutase		
	L-β-lysine mutase		
	D-α-lysine mutase		
	L-leucine-2,3-amino mutase		
C-frame shifts			
	methylmalonyl-CoA mutase		
	isobutyryl-CoA mutase		
	glutamate mutase		
	α-methyleneglutarate mutase		

From Refs. 1, 9, and 10.





The rearrangement proceeds through a stepwise process (Figure 3) initiated by the key homolysis step (i). Of the two radicals formed, cob(II)alamin (B_{12r}) and 5'-deoxyadenosyl ($\text{Ado}^\bullet$), only B_{12r} is relatively long-lived. It is believed that, relatively soon after it is formed, $\text{Ado}^\bullet$ abstracts the H from the substrate (SH). The EPR studies [10,58 (see Ref. 12 therein),69] reveal that the radical, $\text{S}^\bullet$, remains close to the Co(II) center.

II.C. The Rearrangement Step: Is Co Involved?

If the $\text{S}^\bullet$ radical then rearranges with the help of the protein to the product radical, $\text{P}^\bullet$, then the role of the coenzyme is simply to initiate the reaction. (Such initiation is also thought to be the role of the coenzyme in B_{12} -dependent ribonucleotide reductase [16,69–71]). This possibility is labeled (a) in Figure 4.

On the other hand, five other possibilities exist: (b) the rearrangement is influenced by the Co through a loose electronic influence in the radical pair; (c) there is an electron transfer from the radical to Co(II) to produce Co(I) and a carbonium ion; and (d) there is an electron transfer from the Co(II) to the radical to produce Co(III) and a carbanion. In cases (c) and (d), the ionic substrate derivative would rearrange; (e) the Co(II) binds to the substrate radical to form a σ -bonded species, and then this new organocobalt species rearranges or generates

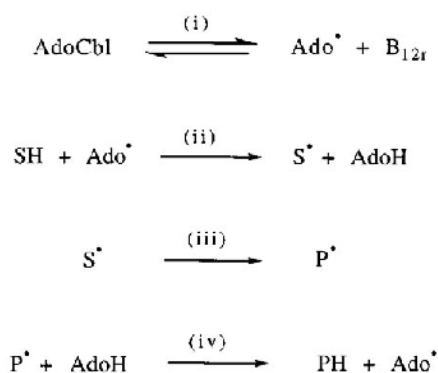


Figure 3 Possible mechanism for coenzyme B_{12} -dependent rearrangements.



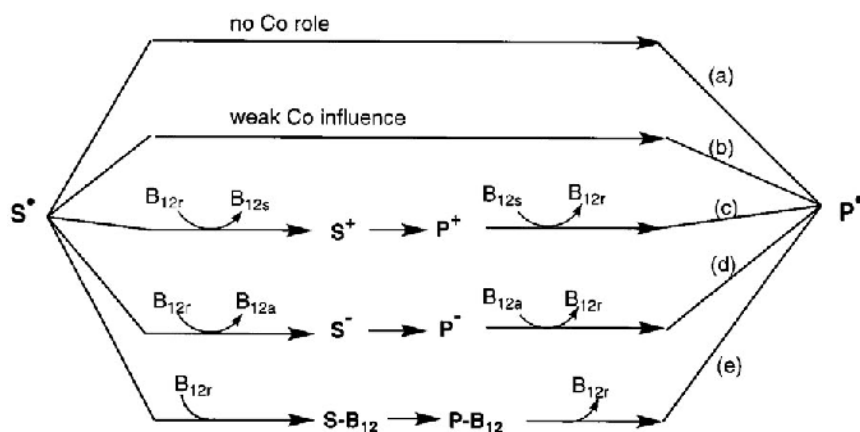


Figure 4 Possible pathways (a–e) for the conversion of the substrate-derived radical, S•, into the rearranged product radical, P•.

a species that rearranges. Finally, (f) a π -bonded Co intermediate could form (a variant of [e], not shown) and undergo rearrangement, instead of the σ -bonded species in (e).

There is some evidence that, when putative radical intermediates are generated, rearrangements will occur. For example, when the radical $\text{C}_2\text{H}_5\text{SC}(=\text{O})\text{C}(\text{CH}_3)(\text{COOC}_2\text{H}_5)(\text{CH}_2\cdot)$ was prepared by reaction of the bromo derivative with $(n\text{-C}_4\text{H}_9)_3\text{Sn}\cdot$ in the absence of Co, both unrearranged product and the product resulting from 1,2 migration of the thioester group were obtained (the latter in low yield) [66]. Since in related experiments with the $\text{C}_2\text{H}_5\text{SC}(=\text{O})\text{C}(\text{CH}_3)(\text{COOC}_2\text{H}_5)(\text{CH}_2^-)$ carbanion less selective (albeit faster) rearrangement was observed, formation of a substrate-carbanion was considered to be a much less likely pathway than the free-radical rearrangement process.

To the inorganic chemist, a process in which the Co center was intimately involved in the rearrangement reactions would be the most interesting and would point the way to new inorganic chemistry. Put differently, a process in which the rearrangement occurred exclusively through a protein-bound radical generated by some amino acid side chain radical (in a chain process)[16] would involve the Co center minimally and therefore would not reveal any new inorganic chemistry that was being exploited by nature. However, the imagination and insight of bioorganic and bioinorganic chemists have inspired several fundamentally interesting studies. The reader is referred to the previous edition of this volume for some discussion of this point [75].

The redox chemistry between the $\text{HOCH}_2\text{C}\cdot\text{HOH}$ radical and $\text{Cbl}(\text{II})$ that



would arise from electron transfer from the radical to Co(II) could be circumvented by a combination of rate effects controlled by substrate–protein contacts. These are (1) positioning of the radical from the Co(II) by a distance of $\sim 10 \pm 5$ Å and (2) acceleration of the rearrangement of the protein-bound substrate radical.

The one x-ray structure mentioned earlier [33] involves methylmalonyl coenzymeA mutase, combined with the partial substrate desulfo-CoA (substrate minus succinyl group and S atom). Although problems arise from the inherent difficulty of studying the large, fragile enzyme, some interesting features are clear. First, the cobalt atom is coordinated via a protein histidine, and the cofactor is base-off. (Binding of a protein nitrogenous ligand has been demonstrated by EPR [37].) Second, the Co—N(imidazole) bond is quite long (~ 2.5 Å). Although the exact value of the bond length must be considered to be uncertain, the existence of the long bond would be in accord with the idea that a weakly coordinated ligand is needed at the Co(III) state to promote homolysis (discussed later). Modulation of this length could influence the stability of the Co(II) state and influence the rate of the undesirable Co—C bond formation step (assuming that the most likely rearrangement process is in Figure 4). I postulate that the length of the Co—N(axial) bond is more readily controlled by the enzyme if the coordinating axial nitrogenous ligand is linked directly to the protein backbone (i.e., imidazole of histidine) than if it is part of the cofactor (such as the base-on cofactor). Third, the protein provides a channel for the entrance of the substrate and escape of the product. This channel leads to a pocket that protects the substrate-derived radical and maintains the radical's position close to the cofactor; this positioning could facilitate the remote influence of Co(II) on the radical rearrangement step such as indicated in (2).

Although work is in progress to explore further influence of Co on the rearrangement step, the remainder of this review will be devoted to B₁₂ areas in which the Co is clearly involved. However, before discussing in depth the important homolysis step, I turn to a brief consideration of the termination step.

II.D. The Termination Step

Little effort has been devoted to ascertain how the coenzyme is re-formed (the termination step). In the radical initiation step (ii, Figure 3), deoxyadenosine is formed. To re-form the Co-C bond to the 5' position of the nucleoside, a methyl group in the 5'-deoxyadenosine must be activated. Such activation requires either H atom abstraction, perhaps by the amino acid side chain radical or by a rearranged radical intermediate derived from the substrate (iv, Figure 3). This step is still not well understood. In the first edition of this volume [75], I expressed the hope that the steps that regenerate the coenzyme would become obvious once the rearrangement process was understood. Recent studies [67] to be described



in Section II.E now offer promise that the termination step might be elucidated from a better understanding of the homolysis step rather than the rearrangement process.

II.E. The Co—C Homolysis Step

II.E.1. The Co—C Bond Dissociation Enthalpies and Rate Acceleration

Estimates have been made that there is an increase of $10^{12\pm1}$ in the rate of the coenzyme B₁₂ Co—C cleavage in the holoenzyme (i, Figure 3) compared to cleavage of this bond in the absence of enzyme in solution [76]. In a limited review of some of the seminal experiments and key conceptual contributions in this field [77], the rate of Co—C bond homolysis was estimated to be increased by $\sim 10^5$ on binding of coenzyme B₁₂ to the protein and by a further factor of $\sim 10^5$ by substrate binding.

II.E.2. Enzyme Evidence for a Conformational Trigger

Careful studies involving structurally modified coenzyme and other alkylcobalt corrins strongly implicate a mechanochemical (conformational) mechanism for Co—C bond cleavage [10,12,28,78–82]. Structure–activity studies (mostly with DD [10,12] and ethanolamine ammonia lyase (EAL), (EC 4.3.1.7) [10,78]), combined with an assessment by EPR of the extent of the Co—C bond cleavage [79], demonstrate (1) that a fairly bulky alkyl linking group between the adenine ring and the Co is needed for activity (see especially Refs. [29,30,39,73]); (2) that the amide side chains of the corrin ring are important for enzyme binding; (3) that the dmbim moiety and its linkage to the corrin are important for activity; and (4) that the adenine moiety is also important. Typically, the addition of substrate to the holoenzyme greatly increases the amount of Co—C bond cleavage. Incompletely understood conformational changes occur in the B₁₂ systems when the coenzyme and its analogs bind to a buried pocket in the enzyme.

For EAL, rapid scan stopped-flow studies reveal that addition of substrate to holoenzyme generates steady-state percentages of the Co(II) form *in a few milliseconds*, whereas addition of coenzyme and substrate to apoenzyme requires *several seconds* [83]. These findings, along with the time course study of enzyme inactivation by some coenzyme analogs, suggest that the coenzyme and its analogues induce a conformational change in the enzyme.

Coenzyme analogs with simple-(CH₂)_n-links between Co and N9 of adenine are protected from photolysis when $n \geq 4$ and when bound to the buried pocket of EAL [80]. Large CD spectral changes accompany this EAL binding by 9-adeninylalkylCbIs containing propyl or longer chains but not by AdoCbl and 9-adeninylethylCbl [78,80]. It was suggested that the bulkiness of the latter



two alkyl groups prevented the conformational change (but the adenine [Ade] ring may have very different orientations in these Cbls). A conformational change would be facilitated by Co—C bond cleavage. For the longer-chain 9-adeninylalkylCbls, the conformational change can occur without Co—C bond cleavage. This reasonable explanation is in keeping with the stability toward photolysis of these species and their ability to deactivate EAL. However, there is presently no correlation between CD spectra and alkylCbl structure. Furthermore, it is not clear whether a bulky alkyl group in an alkylcobaltcorrin could resist a distorting force on the corrin, especially since we have recently observed Co—C bond lengths of 2.2 Å [84]. The CD studies were performed in the absence of substrate [80,85–87]. Presumably, enough driving force would exist in the presence of substrate for Co—C bond cleavage and a conformational change.

This evidence, along with studies on structure–activity relationships, suggests that the adenine ring interacts directly with the enzyme. However, in view of the fluxional character of the Ado group, we felt it was important to determine the relationship of the Ade to the corrin ring in the analogs. The structure of 9-adeninylpropylcobalamin (AdePrCbl), an 5′-deoxyadenosylcobalamin (AdoCbl) analog in which the ribose moiety of the Ado group has been replaced by a propylene chain, was determined by x-ray diffraction methods [44]. The general conformations of the corrin ring, benzimidazole, phosphate, and ribose in AdePrCbl are very similar to those of AdoCbl and MeCbl except for the amide side chains, which show some variability in the orientations of their amide groups. The Ade ring in AdePrCbl lies over the D ring of the corrin system, rotated about 120° clockwise from its position in AdoCbl. In addition to the Ade orientation found in the crystal structure, a second orientation, in which the Ade lies over the B ring of the corrin, is suggested by ¹H NOEs and by a comparison of the ¹H and ¹³C shifts of AdePrCbl to those of AdoCbl.

Our results suggest that Adealkyl groups in Cbls may have a highly fluxional character, permitting several orientations of the Ade. Previous studies have shown that binding to the AdoCbl-dependent enzymes, RNR and DD, is tighter for AdepentylCbl than for AdoCbl and the other AdealkylCbls [44]. We concluded that the flexibility of the alkyl chain, exhibited by the fluxional character of the Adealkyl group, and the orientation of the Ade ring could be responsible for the increased affinity of this analog for the enzyme. Differences in the orientation of the Ade and the fluxional character of the Adealkyl group, in addition to corrin ring flexibility, may also be useful in explaining the changes in the CD spectra of AdealkylCbls upon binding to EAL [10].

II.E.3. Other Aspects Concerning Cofactor Protein Contacts

Before discussing the mechanism of the conformational trigger, it is informative to review other data on Cbl–protein contacts. The role of the dmbim axial base



is less well defined. Although it remains coordinated in DD [74] and is necessary for binding to B₁₂-dependent RNR [88], the evidence is now clear that when the enzyme contains the catalytic quartet sequence, the ligand is an imidazole and that there may be no axial ligand in the methylated corrinoid iron-sulfur protein involved in CO dehydrogenase/acetyl-CoA synthase [25–27].

It is our belief that the evidence is overwhelming that there is a conformational trigger responsible for Co—C bond homolysis. The central issue is the nature of the mechanism. Prior to assessing possible mechanisms in more detail, we first briefly review information on Co—C bond dissociation enthalpies (BDEs) for B₁₂ derivatives and B₁₂ models in solution and relate this information to other well-established properties of these organocobalt species.

II.E.4. Co—C Bond Dissociation Enthalpies: Relation to Structure

Studies directed at determining relevant Co—C BDEs have attracted considerable interest [9,34–36,53,58–64,76,89–112]. At approximately the same time, Halpern [92] and later Finke showed that a reasonable estimate of the Co—C BDE can be obtained from $\Delta H_h^\ddagger$ (Co—C bond homolysis activation enthalpy) in solution of simple models. There is some controversy about the best procedures (cf. Refs. [110,112]), and the reader is urged to consult the original articles. However, the BDE estimates determined in different laboratories are similar enough for our purposes. For consistency, I have selected values from one of the two principal laboratories to discuss B₁₂ derivatives and models, since the most diverse compounds in each class have been studied by the Finke and Halpern laboratories, respectively.

When compared to all literature $\Delta H_h^\ddagger$ values (17–41 kcal mol^{−1}) for alkyl-cobalt bonds, the methyl-cobalt bond in methylB₁₂ and the adenosyl-cobalt bond in coenzyme B₁₂ and Ado-Cbi⁺ are relatively stable. Although methylB₁₂ exhibits the largest $\Delta H_h^\ddagger$ value reported [98] to date, 41 kcal mol^{−1}, coenzyme B₁₂ and Ado-Cbi⁺ both have large $\Delta H_h^\ddagger$ (33 and 37.5 kcal mol^{−1}, respectively) [76,102]. Therefore, removal of the trans-axial base strengthens the Co—C bond by about 4 kcal mol^{−1}, and coordination of the axial base to Co increases the rate of Co—C bond homolysis by a factor of 10².

The elegant evaluations of Co—C BDEs from the Halpern and the Finke laboratories require exhaustive and extensive experimental studies. The rates of decomposition of many alkyl Cbls have been evaluated in earlier, less rigorous studies in Schrauzer's and Pratt's laboratories [77]. The general features appear to be correct and the effects of steric factors on Co—C bond cleavage have been reviewed [77]. An abbreviated series relevant to our discussion is CH₃ < 5'-deoxyadenosyl < C₂H₅ < *i*-C₃H₇, CH₂CMe₃.

Since an essential, and perhaps the only, widely accepted role of coenzyme B₁₂ in the holoenzyme is the homolysis of the Co—C bond [9,58,61,66], compari-



son of the rates of thermally induced Co—C bond homolysis with Co—C bond lengths, Co—N bond lengths, and N-donor ligand dissociation rates of both models and Cbls is of special significance [63,64,84]. The factors influencing the Co—C(Ado) BDE and Cbl properties in general have been investigated extensively with model compounds such as those to be described next.

The most extensively studied models for B₁₂ coenzymes are cobaloximes (LCo(Hdmg)₂R), which contain two monodehydronated dimethylglyoximes (H₂dmg) in the equatorial Co(Hdmg)₂ plane and an axial, neutral, monodentate ligand L (Figure 5) [63,64,84,113–125]. The structural properties of cobaloximes in the solid state have been very useful in interpreting solution data [63,64,125]. The trend in structural data can be compared with Cbls on the one hand [20,41–44] and imine/oxime [55,126–134] and Schiff-base-type [57,135,136] models on the other hand [64]. The most common equatorial ligand system used in the imine/oxime models is the (Hdmg₂pn) ligand (Figure 5). These models have four N's coordinated to Co in the equatorial plane. In contrast, the Schiff-base systems have an N₂O₂ donor set, and the relatively useful saloph ligand (saloph = dianion of disalicylidene-*o*-phenylenediamine) is illustrated in Figure 5. In general, these

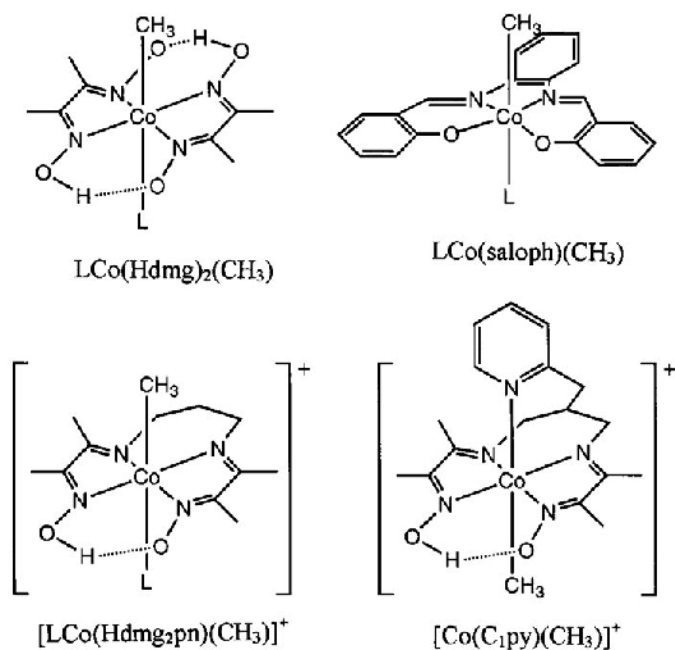


Figure 5 Structures of (Hdmg)₂ (cobaloxime), saloph, (Hdmg₂pn), and C₁py model compounds.



properties suggest the following trend: $\text{Co}(\text{Hdmg})_2 \sim \text{Co}(\text{Hdmg}_2\text{pn}) \ll \text{CbIs} \leq \text{Co}(\text{Schiff base})$. The Co—C bond energies decrease for closely analogous species following this general trend, although the differences are not large.

It is interesting and informative to compare the preceding trends with structural differences. For a given equatorial ligand, relatively little difference is found in Co—C bond lengths for a given type of R group. Rather, the length of the Co—C bond responds to the bulk of R and reflects roughly the relative Co—C cleavage rates given. A second important aspect of our findings [64] is that distortions in the equatorial ligand induced by the *trans* ligand L also lengthen and weaken the Co—C bond. In contrast to the similar Co—R bond lengths for a given R with different equatorial ligands, Co—N bond lengths are quite different, and these as well as L dissociation rates [63,64] indicate that Co—N bonding is weakest in the Schiff-base compounds. Furthermore, models show a distortion of the Co—C—C angle, although cobaloxime and imine/oxime models (for both, this angle is found to be 130°) are the only organocobalt complexes for which neopentyl compounds are sufficiently stable to be characterized by x-ray methods [114–116].

Other evidence that these are useful molecules with which to assess the properties of the B₁₂ cofactors comes from several in-depth FT-Raman spectral studies. In studies of cobaloxime B₁₂ models ($\text{LCo}(\text{Hdmg})_2\text{CH}_3$), the Co—CH₃ stretching frequency was found to be sensitive to the steric bulk of L (steric *trans* influence) rather than to the electronic *trans* influence [53]. The Co—C stretching band, *trans* axial ligand bands, and equatorial ligand bands underwent significant shifts between solution and solid states. Furthermore, the intensity of the Co—C stretching band in B₁₂ model compounds was found to be sensitive to the nature of the *trans* ligand. An ~6-fold increase in the Raman scattering intensity of the Co—C mode has been observed in methylcobaloximes [51] when going from $\text{H}_2\text{OCo}(\text{Hdmg})_2\text{CH}_3$ to $(\text{PPh}_3)\text{Co}(\text{Hdmg})_2\text{CH}_3$. Similarly, an ~10-fold increase was reported in the same mode when L was changed from H₂O to trimethylphosphane or 4-*tert*-butylpyridine in $[\text{LCo}(\text{bpb})\text{CH}_3]$, where bpb = 1,2-bis (2-pyridinecarboximido) benzene [56]. Intensity increases are also seen in derivatives of MeB₁₂ [54] and in Schiff-base models [57]. It is interesting that these properties, the actual frequencies, and even the Co—C bond lengths in these models closely reflect the same features of MeCbl.

In Section II.E.5, I shall discuss and correlate various factors that could be involved in promoting the Co—C bond homolysis in biological systems. First, I wish to focus on two structural features and their relationship to Co—C BDE. The first factor is the effect of ligand basicity and steric bulk on Co—C BDE. In a series of (pyridine) $\text{Co}(\text{Hdmg})_2\text{R}$ compounds, Halpern et al. [90] found that the Co—C BDEs (kcal mol⁻¹) increased with the basicity of the pyridine:

$$4\text{-CNpy} (17.9) < \text{py} (19.5) < 4\text{-CH}_3\text{py} (20.1) < 4\text{-NH}_2\text{py} \quad (21.1)$$

This trend can be understood as arising from the stabilization of the Co(III) oxida-



tion state relative to the Co(II) oxidation state by the better donors [90]. This dependence on N-donor ligands contrasts with other results from Halpern's laboratory but with L = P-donor ligands [91,94]. For such ligands, which are poor models for the axial dmbim, very little correlation between basicity and Co—C BDEs exists for cobaloximes, but this relationship does hold for the octaethylporphyrin (OEP) models (Table 2). Halpern interpreted these data with the suggestion that the porphyrin ligand is relatively inflexible; therefore, as found for cobaloximes with N-donor ligands, the Co—C BDEs primarily reflect stabilization of the Co(III) over the Co(II) oxidation state by the better electron donor ligands. In contrast, the cobaloxime complexes [63,64,114–123] experience butterfly bending (a distortion in which the two Hdmg ligands are displaced from the equatorial plane) away from the bulky ligand. When the axial ligand is an N donor (typically planar), there is no butterfly bending, even in cobaloximes. Therefore, when L = N-donor ligand for cobaloximes and even when L = N-donor ligand for porphyrin systems, L-electron donation is the only important factor influencing Co—C BDEs.

Such electron donation can also be modulated by the length of the Co—L bond. We find that when the planar N-donor ligand is bulky, e.g., 2-NH₂-py, the net result is a relatively long Co—N bond [119,120]. However, when the axial ligand is completely missing, such as in alkylCo(saloph) compounds [136], the Co—C BDE probably increases, as suggested by the relative stability of such compounds. Therefore, an ideal bond length must exist in Cbls, allowing for stability of the Co—C bond in the resting state of the enzymes and then facilitating Co—C cleavage when required [136]. For only the saloph series does evidence exist for both demonstrably weak Co—C bonds [93] and long Co—N bonds [135] when neither axial ligand is particularly bulky.

Pentacoordinate organocobalt(III) species have been suspected to exist either as reactive intermediates in ligand-exchange reactions [137–141] or, less commonly, as relatively stable species [140–142]. Clear-cut demonstrations of stable 5-coordinate Co(III) complexes are relatively rare [136,143]. One report contains two definitive examples of 5-coordinate complexes of the type Co(saloph)R (R = CH₃ and *i*-C₃H₇) [136]. Several interesting features emerge from these structures. First, the Co atom is displaced toward R. Second, the Co—C bond is shorter than in hexacoordinate compounds, but steric strain remains for *i*-C₃H₇, even in the pentacoordinate species.

The types of model systems in which 5-coordinate species have been invoked range from the relatively "electron-deficient" cobaloximes to the relatively "electron-rich" Schiff base complexes, where the corrin ring in cobalamins is replaced by two dioximato ligands or by a quadridentate N₂O₂ dinegative Schiff base ligand, respectively. These two classes of models, in fact, appear to "bracket" many characteristics of cobalamins since the cobaloximes are too electron-deficient and the Schiff base complexes are too electron-rich [136]. As such, an understanding of the differences in properties between these two types



Table 2 Influence of P-Donor Ligands on Co—C Bond Dissociation Energy (kcal mol⁻¹)

L	LCo(Hdmg) ₂ CH(CH ₃)(C ₆ H ₅) ^a	LCo(Hdmg) ₂ CH ₂ (C ₆ H ₅) ^b	LCo(OEP)CH ₂ (C ₆ H ₅) ^b
P(CH ₃) ₂ (C ₆ H ₅)	24	30.4	27.1
P(n-C ₄ H ₉) ₃	21	28.9	29.3
P(CH ₂ CH ₂ CN) ₃	20	—	—
P(C ₂ H ₅)(C ₆ H ₅) ₂	19	26.8	26.1
P(C ₆ H ₅) ₃	17	25.8	23.8
P(c-C ₆ H ₁₁) ₃	—	22.8	29.6

^a Data from Ref. 91.
^b Data from Ref. 94.

of systems could provide useful insight into the chemistry and biochemistry of cobalamins.

Ligand-exchange and structural data [144], together with estimates of the Co—C homolysis rate [93], support the notion that the Co(saloph) model system (Figure 5) contains a Co center that reproduces the Co center in cobalamins better than do most other models [93]. Although a Schiff base system, the saloph system (Figure 5) more closely reflects the properties of cobalamins than do other related systems that lack the electron-withdrawing phenylene ring [144].

The suspected 5-coordinate complexes, Co(saloph)R and “base-off” alkylcobalamins, share the behavior that Co—C bond homolysis is less facile than in the counterpart 6-coordinate complexes, LCo(saloph)R and “base-on” alkylcobalamins [93,141,145]. “Base-off” cobalamins (made by adding acid to hydronate the benzimidazole ring) and cobinamides (which are derived from cobalamins but lack 5,6-dimethylbenzimidazole) are believed to be 5-coordinate when R is a strong electron donor, but the evidence is primarily spectroscopic [141]. However, 5-coordinate species are generally more thermally stable than their 6-coordinate counterparts.

On the basis of arguments concerning Co—N bond distances in the solid [144], we suggested that the high ligand exchange rate of the saloph system most probably is both a ground-state effect (the consequence of the *cis* influence) and a transition-state effect (greater stability of the pseudo-5-coordinate, L—Co(saloph)R[‡], activated complex compared to L—Co(Hdmg)₂R[‡]). Our demonstration [136] of 5-coordinate saloph species in the solid clearly supports this suggestion.

Halpern's work on models demonstrates that poor electron donor ligands *trans* to R weaken the Co—C bond by destabilizing the Co(III) oxidation state [66,90,91,93]. However, removal of the *trans* ligand altogether stabilizes the Co—C bond. We demonstrated [136] that when R is bulky, the Co—C bond does get shorter in a 5-coordinate species. These steric effects may be important. For the ground state, the strength of axial coordination *trans* to R should have the effect shown at the left of Figure 6. For the activated complex, which we approximate as a cobalt(II) species, the dependence shown at the right of Figure 6 is likely. A 4-coordinate cobalt(II) is probably relatively unstable. Thus, the Co—C cleavage process would be most facile in a situation in which the Co—N bond changes from being a relatively long bond (weak donor) in the still 6-coordinate ground state to a relatively stronger bond leading to a 5-coordinate Co(II) species, as illustrated schematically in Figure 7. Line **a** indicates free energy changes expected in models and in cobalamins in the absence of enzymes. The minimum barrier would involve pair **b**, in which the 5,6-dimethylbenzimidazole supports formation of the 5-coordinate Co(II) species (B_{12r}) without stabilizing the 6-coordinate Co(III) relative to the 5-coordinate Co(III) species. In a 6-coordinate species with a weakly bound 5,6-dimethylbenzimidazole, the Co is



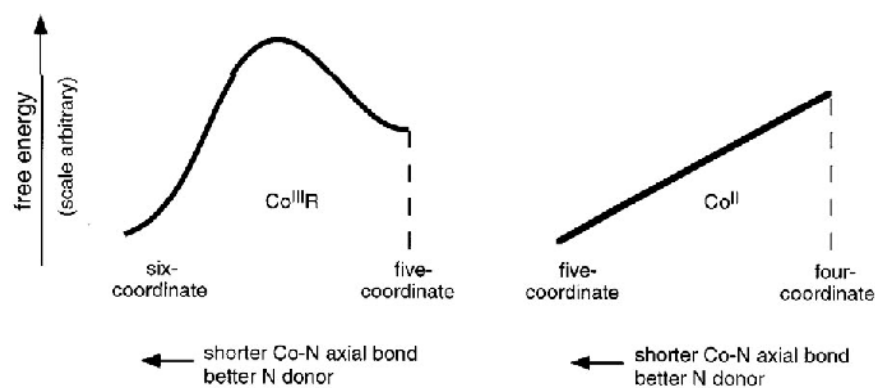


Figure 6 Approximate dependence of free energy on the nature of N donor as the donor strength increases for Co(III)R (left) and Co(II) (right). Eventually, the bond is broken, giving a species of lower coordination number.

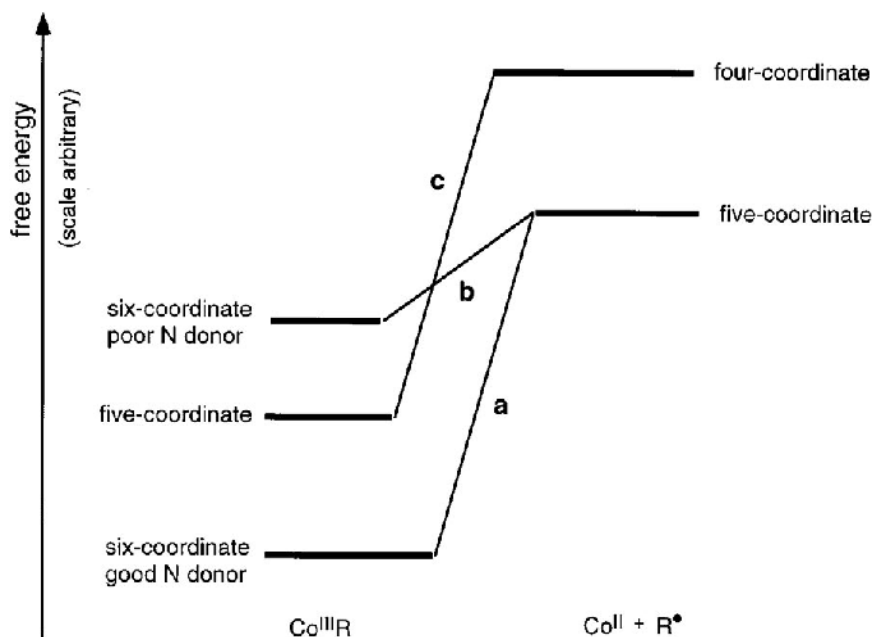


Figure 7 Hypothetical pairs of Co(III)R and Co(II) species. Since the transition state is expected to be only slightly higher in energy than the Co(II), such a scheme can act as a guide to indicate which pathways are preferred.



maintained in the plane defined by the four corrin N atoms, thereby maintaining steric repulsions between the 5'-deoxyadenosyl and the corrin. These steric interactions could be made more severe in the holoenzyme if the protein distorts the corrin [10,20,61,78,79,96,146]. Pair **c**, a 5-coordinate cobalt(III) and a 4-coordinate cobalt(II) species, would probably be joined by the most unfavorable pathway both because the 5-coordinate species is stabilized somewhat by the reduction in steric interaction and because a 4-coordinate cobalt(II) species should be relatively unstable.

The preceding qualitative picture requires further investigation of both the energetics and structural changes in models and cobalamins. However, if it represents a reasonable approximation of the interrelationships among structure, coordination number, and axial bond strengths, then the intermediacy of 5-coordinate $\text{Co}^{\text{III}}\text{R}$ species in the enzymatic process involving Co^{II} and radical chemistry of coenzyme B_{12} -dependent enzymes appears unlikely.

Somewhat longer $\text{Co}-\text{N}$ bonds are found in imine/oxime models than in the cobaloxime models. The propylene bridge in imine/oxime complexes alter the orientation of the planar N-donor ligands from lying over the six-membered $\text{Co}-\text{N}-\text{O}-\text{H}-\text{O}-\text{N}$ chelate ring as in cobaloximes to lying over the five-membered $\text{Co}-\text{N}-\text{C}-\text{C}-\text{N}$ chelate ring. This orientation is believed to introduce steric clashes with the equatorial ligand. Consequences of this steric clash appear to be longer than expected $\text{Co}-\text{N}(\text{axial})$ bonds, faster ligand dissociation rates, and relatively large butterfly bending even for planar nonbulky N-donor ligands (see Refs. [126–132]). We later studied a new model system, C_1py (Figure 5), in which the pyridine is connected to the equatorial (Hdmg_2pn) ligand via a one-methylene bridge. This linkage forces the pyridine moiety to lie over the six-membered $\text{Co}-\text{N}-\text{O}-\text{H}-\text{O}-\text{N}$ chelate ring (orientation in Figure 8a) [133]. This orientation of the pyridine moiety minimizes the butterfly bending (Figure 9 [55,133]). As mentioned, the influence of butterfly bending on $\text{Co}-\text{C}$ bond homolysis, as demonstrated in models, represents one of the most widely considered mechanisms for the homolysis process. Since the equatorial ligand moieties are very similar for the C_1py and (Hdmg_2pn) systems (Figure 5), the differences in butterfly bending between the two systems establish *for the first time* that an axial N donor ligand can induce significant butterfly bending in an organocobalt compound and that this effect is dependent on the orientation of the axial ligand (Figure 9). It is thus more reasonable to postulate butterfly bending in the mechanism for the conformational triggering of $\text{Co}-\text{C}$ bond homolysis, to be discussed next. These studies indicate that models should continue to play a central role in the development of methods for understanding B_{12} properties and biochemistry [55,147].

II.E.5. Mechanism of the Conformational Trigger

Although it is likely that the enzyme must use its cofactor-enzyme intrinsic binding energy [9] to weaken the $\text{Co}-\text{C}$ bond or otherwise “trigger” the $\text{Co}-\text{C}$



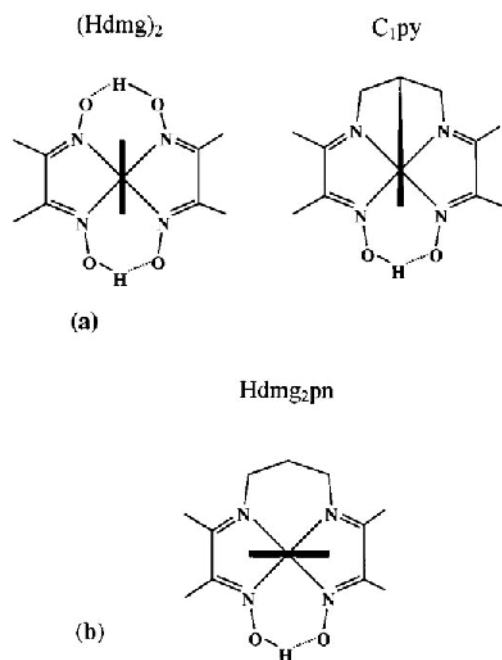


Figure 8 Eclipsed orientation of the pyridine plane in C₁py and (Hdmg)₂ derivatives (a) and staggered orientation of the pyridine plane in (Hdmg₂pn) derivatives (b), as established by many structural studies. (From Ref. 133.)

bond homolysis step, the manner in which the enzyme accomplishes this rate enhancement remains a mystery, despite the great effort that has been devoted to this problem. The molecular level details of this enzymatic process are of considerable interest. Mechanisms that have been considered to account for the enzyme-accelerated Co—C bond homolysis include [63,64] (1) a distortion in the corrin ring (the “butterfly” bending or upward conformational hypothesis just discussed for models); (2) a direct lengthening of the Co—C bond or separation of the Co and Ado moieties; (3) angular distortion of the Co—C bond; (4) an alteration in the position of the dmbim or the dmbim Co—N bond length; and (5) electron transfer.

The most frequently invoked molecular mechanism for the promotion of Co—C bond cleavage is *mechanism* (1), namely, a distortion in the corrin ring, increasing the steric interaction with the Ado moiety (Figure 10) [9,10,58–65,148]. For reasons mentioned briefly and elaborated later, we have emphasized an alternative suggestion involving control of the Co—dmbim bond length, a hypothesis that has gained both experimental support and adherents from other



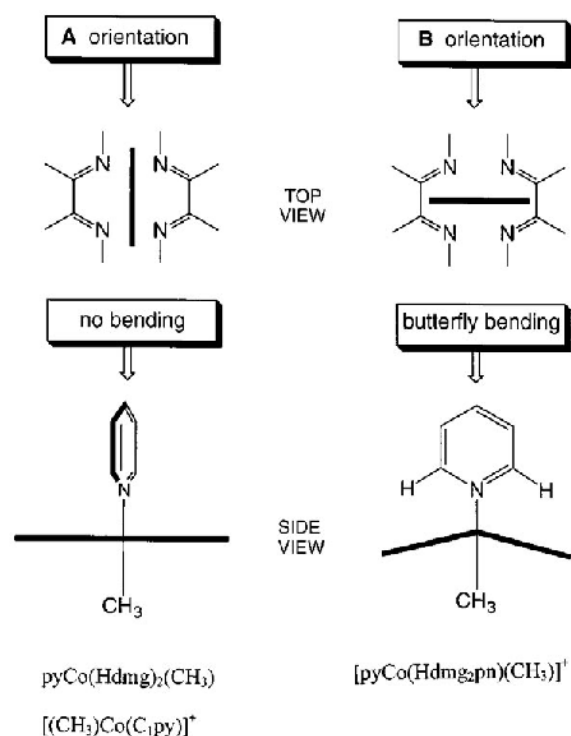


Figure 9 Schematic representations showing the explanation of why butterfly bending is observed in some models, but not in others.

laboratories [28,149]. The x-ray data [33] add a new dimension, the adjustment of this Co—N distance by protein control of the imidazole position.

Mechanism (1), first suggested almost three decades ago [150], continues to be the most invoked explanation of the role of the enzyme in promoting homolysis. Cleavage of the C—Co bond of sterically hindered alkylcobalamins (e.g., neopentylcobalamin) was markedly increased by diol dehydrase [72]. Such cobalamins do not function as coenzymes but convert to enzyme-bound hydroxocobalamin in stoichiometric first-order reactions. The strong competitive inhibition by AdoB₁₂ indicates that labilization occurs at the active site of the enzyme and is suggested to be caused by a steric distortion of the corrin ring.

Although conformational changes in the holoenzyme are implicated by numerous studies, there are interesting conflicting points regarding the feasibility of mechanism (1) between structural studies on alkylCbIs and models. Because



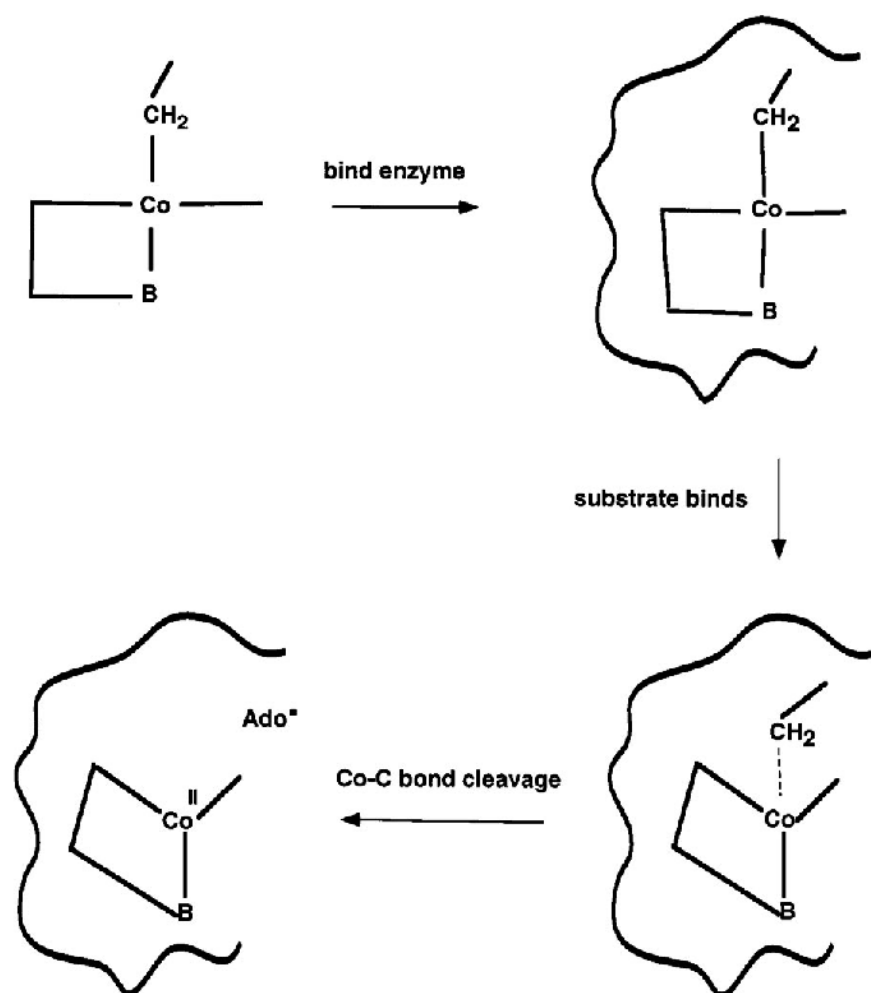


Figure 10 Schematic representations of how protein contacts could weaken the Co—C bond in bound AdoCb1.

the corrin ring is more flexible than the porphyrin ring, for example, it is widely believed that corrin distortions are the key cofactor distortions induced by protein–cofactor contacts. The work with the OEP models described [94] supports this view. The main structural support for this mechanism comes from studies of organocobalt compounds with sterically bulky P-donor ligands.

However, precise x-ray structural data on the methyl coenzyme and organo-



cobalamins [20,41–44] do not indicate large steric clashes, and the structure of Cbl(II), B_{12r}, also does not support this concept [149]. Specifically, we found that the cobalamin moiety, including the corrin ring, is very similar in methylCbl and coenzyme B₁₂ and that these structures are similar to that of vitamin B₁₂ [20]. Although we are comparing crystalline species in the solid state, one might have expected more different structures, if steric clashes are so important. In particular, the methyl group is relatively small compared to the Ado moiety.

The ultimate small axial ligand is no ligand at all. Such a situation exists for mechanism (2), namely, separation of Co and Ado radical. The observation that the reduction in size of the alkyl substituent from Ado to Me leads to little change in structure [20] also extends to B_{12r}, which lacks a substituent altogether. The Cbl parts of this Co(II) species are structurally similar to those in the Co(III) species [149]. Logically, since a change in oxidation state is involved, the structures should be different. However, the alkyl groups are excellent electron donors, and Marks [151] has suggested that the alkyl group places enough electron density on Co to make the Co(III) center essentially a (diamagnetic) “Co(II)” center. Under such circumstances, the very long Co—N bonds in organoCbIs are very similar to the bond in B_{12r}. The B_{12r} structure [149] led to the suggestion that the principal function of the cofactor protein contacts in the conformationally altered holoenzyme was to maintain separation of B_{12r} and Ado. This role appears to be more reasonable in view of the x-ray study [33].

Before discussion of mechanism (3) it is useful to consider fundamental studies on B₁₂ properties also relevant to both the (1) and the (2) mechanisms. Shepherd et al. [152] have suggested new implications of the close structural relationship of the Co(III)R and Co(II) B₁₂ species. They suggested that, since little reorganization of the corrin is needed and a lower activation barrier for reorganization would result, all alkylCbIs would have nearly independent structural and chemical behavior in the absence of coenzyme B₁₂—dependent apoenzyme. However, they found that the Co(III)/Co(II) reduction potentials ($E_{1/2}$) of alkylCbl decreased in a way that correlated almost linearly with the increase of the bulk of the R group (estimated by the Taft steric parameter E_s). In view of the similar nature of the Cbl portion of these molecules in the solid state, Shepherd et al. considered the possibility that the Cbl structure was more variable in solution. However, results of 2D NMR studies are inconsistent with such a change [44–46] and, therefore, other explanations were sought.

Shepherd et al. suggested that the changes in electrochemical properties reflected increases in Co—C bond length with bulk. A series of calculations were cited to rationalize these results. In particular, the MO calculations of Salem et al. [153] suggest that the two lowest LUMOs in alkylCbIs are a corrin center π -type orbital (π_8) and a metal-centered orbital (the antibonding combination of the d_z^2 and the alkyl σ orbitals). From the closeness in energy of these two LUMOs and the earlier calculations by Mealli et al. [154] that the energy of the



σ LUMO is sensitive to the length of the Co—C and Co—N bonds, Shepherd et al. argued that the dependence of the electrochemical data on R reflected the sensitivity of the σ LUMO to the Co—C bond distance and not to any significant differences in the Cbl part of the molecules.

The self-consistent field (SCF) calculations cited later [155] indicate that the overall energy of alkylCbls is not sensitive to axial bond distances and to puckering of the equatorial N donors. This finding reinforced the argument that the electron entered the σ LUMO and that the $E_{1/2}$ does not reflect the overall energy. No sterically induced change in the extent of corrin puckering appeared to be required to explain the electrochemical results. This information [152] prompted us to evaluate the NMR spectra in some detail, and we concluded that the ¹³C NMR shift data indicate a steric effect [49]. The data showed that the steric interaction between the Ado group and the corrin decreased Ado electron donation and simultaneously weakened the Co-dmbim binding. The net effect is that the overall structures of the two coenzymes are similar but that the Co center in the Ado B₁₂ coenzyme is slightly less electron-rich. These data account for the electrochemical trends [49]. Thus, although there is probably some distortion of the corrin ring and some steric interaction of the ring with Ado, these seem to be small effects.

This study [49], supported by related data [156], showed excellent correlations between B₁₂ and model compound shifts. Thus, the ¹³C NMR shifts also reflect the structure in solution since the model data show a strong correlation with structure [156]. This is strong evidence that the solution and solid-state structures are closely related. Results of recent x-ray and EXAFS studies [48], however, also agree with the NMR results from my laboratory [49] and call into question some of the early results arising from the application of x-ray edge and EXAFS methods. Again, a complicated and controversial area has involved B₁₂ compounds, and the reader is advised to examine the original literature on EXAFS studies, both the studies supporting and those contradicting the NMR and x-ray data.

An important aspect of such investigations into “butterfly bending” is the clear evidence for good relationships between such properties of models and the related cofactor derivatives. We next consider other hypotheses for the manner in which the Co—C bond could be weakened.

Another class of hypotheses (mechanism [3]) involves bending of bond angles (Figure 11). Such bending is a possible avenue through which coenzyme–enzyme binding energy could weaken the Co—C bond and thereby facilitate B₁₂–dependent reactions [77,157]. Computations predict that ϕ bending (bending of the Co—C—C angle, [(Figure 11)] away from tetrahedral is more important than θ bending in weakening the Co—C bond. Experimental tests for ϕ bending have been possible for some time, through the use of neopentyl derivatives, which are known to have weak Co—C bonds (see later discussion). Compounds we made



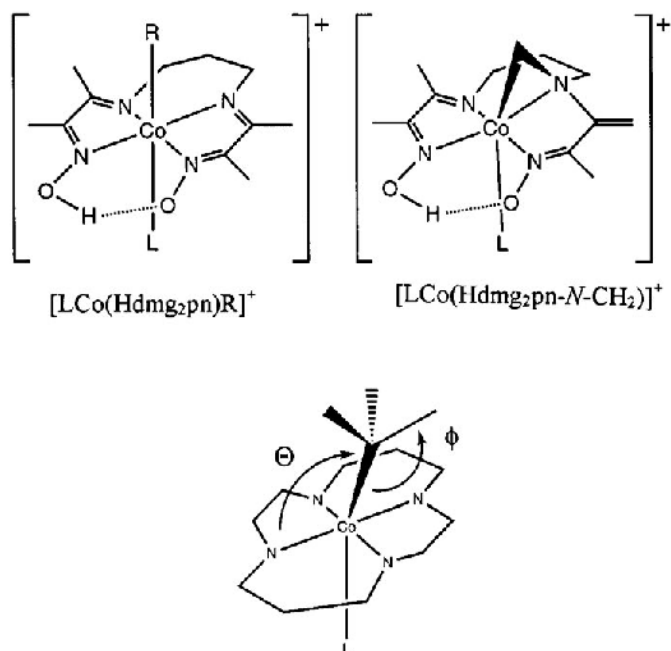


Figure 11 A typical imine/oxime model (upper left) that converts to an unusual Co—C—N metallocycle (upper right) on treatment of the BrCH₂ derivative with base. The acute angles make the metallocycle a good model for θ bending (bottom). The figure also shows ϕ bending.

in recent studies [158,159] provide the first opportunity to gain experimental insight into the likely consequences of θ bending on the Co—C bond. We found that BrCH₂-derivatives of some imine/oxime compounds readily formed compounds with a Co—C—N metallocycle (an η^2 -aminomethylene moiety, Figure 11).

Our analysis [158] shows that the distortion needed for Co—C—N ring closure occurs readily. Computations suggest that, in B₁₂ compounds, Co—C bending (θ bending angle [157], [Figure 11]) will foster an attractive interaction between the axial C and an equatorial N, leading to a lengthening of the Co—N bond [157]. The facile formation of the metallocycle supports the attractive interaction. The computations [157] suggest that θ bending even as great as we found will have a small effect ($\sim 15\%$ decrease in total overlap population of the Co—C bond).

We evaluated Co—C bond weakening both by consideration of the struc-



ture and by assessment of the ease of Co—C bond cleavage. It is clearly established that weak Co—C bonds are long [63,123,127,132]. However, the Co—C bond length of a typical model with a η^2 -aminomethylene moiety is significantly *shorter* than in undistorted related organocobalt compounds [127]. Qualitative observations under conditions for which Co—C bond cleavage normally is observed suggest that the metallocycles are relatively inert. The Co—C bond in ICo(Hdmg₂-pn-*N*-CH₂) did not thermally cleave after 2 days in conditions under which the imine/oxime analog with the ϕ -bent neopentyl ligand exhibited complete Co—C bond cleavage after only 1 day. Thus, the Co—C bond in the η^2 -aminomethylene moiety is relatively strong.

The other bending hypothesis, angular distortion [77] of the Co—C—C bond angle by the protein, has previously received more attention but is relatively difficult to assess. For example, the opening of the Co—C—C bond angle to $\sim 125^\circ$ is postulated to produce changes in hybridization of the carbon attached to Co [160]. Later computational studies by Zhu and Kostic [157] employed a simplified model with the Co attached to axial imidazole and methyl ligands in addition to the corrin ring. Fenske-Hall iterative molecular orbital calculations on this model indicated reduction of overlap in the Co—C bond by 43% and a consequent reduction of Co—C bond strength.

Probably the best evidence for the angular distortion model is the crystallographic results on model compounds with the neopentyl ligand [114–116,127]. These contain relatively large Co—C-bond angles. NeopentylCbl and neopentyl model complexes have been shown to be unstable [93]. Estimated BDEs (in kcal mol⁻¹) for [pyCo(saloph)R] follow the trend neopentyl (18) < isopropyl (20) < benzyl (22) < *n*-propyl (25) [93]. Furthermore, the homolysis rate of neopentylCbl is $\sim 10^6$ times faster than that of AdoCbl [111]; i.e., it is the best model to date since it can account for roughly half of the $10^{12\pm 1}$ enzymatic activation of the Co—C bond of AdoCbl [110]. There are no crystallographic data on neopentylCbl; however, studies in our laboratory demonstrate that the redox potential of neopentylCbl fits into a linearly related series with the new model system C₁py (Figure 5) that we have been studying [132]. The redox properties of C₁py and (Hdmg₂pn) complexes are also related [132]. In a neopentylCo(Hdmg₂pn) compound [127], the Co—C—C bond angle is large (130°). It is interesting that, on the basis of the redox properties, Shepherd et al. [152] have suggested that the Ado ligand is actually larger than the neopentyl ligand (i.e., Ado would have a higher Taft steric parameter).

A complication of the preceding analysis is that the Co—C bond length also increases in neopentyl derivatives [114–116, 127]. For isopropyl derivatives the Co—C bond length increases [161,162], simulating a direct lengthening, and as expected, the compounds are relatively unstable. However, there is no evidence that enzymes can induce such lengthening.

We concluded that ϕ bending is more likely than θ bending to be an impor-



tant means of Co—C bond weakening in B₁₂ reactions. However, the coordination sphere of organocobalt species appears to be readily distorted. The facility of the θ bending suggests potential biological functions. The θ bending could allow favorable cofactor–protein contacts or favorable stereochemistry during turnover.

A fourth possibility, which arose from our collaborative structural work on both models and cobalamins, is mechanism (4), an alteration in the dmbim position or Co—N (dmbim) bond length [119]. UV spectral data have been used to suggest that the Co—dmbim bond is broken during catalysis, giving the so-called base-off (benzimidazole-unhydrated) form of the coenzyme [163]. Furthermore, some unconfirmed evidence exists that axial base-free Ado-Cbi⁺ is still a partially active cofactor [76].

The role of the benzimidazole ligand is the most uncertain aspect of the involvement of the Cbl component in Co—C bond homolysis. Clearly, the dmbim is needed to promote homolysis, but this effect could be limited to enhancing steric effects by keeping the Co in-plane. As discussed, the exceptionally long Co—N bonds in organoCbIs led us to speculate that the dmbim is a weak donor that serves primarily to keep the Co in the plane in the Co(III) state and to stabilize the Co(II) form after Co—C bond homolysis. In effect, the requirement for a relatively weak donor is fulfilled by dmbim. The later discovery that the axial Co—N bond would also be long in B_{12r} [149], as we predicted [136], supports this speculation. Calculations by Christianson and Lipscomb [155] appear to be in conflict with this conclusion because the SCF energy shows soft minima as the Co—C and Co—N bond distances vary. Other theoretical studies [154] seem to support our view and add the feature that the presence of dmbim favors homolysis over heterolysis.

In a series of excellent studies on DD, Toraya and coworkers [12,28,74] have investigated a number of analogs with systematic variations in the nucleotide loop. This laboratory has synthetically replaced the α -D-ribofuranose ring at the nucleotide loop with a methylene chain, $-(CH_2)_n^-$ where $n = 2, 3, 4$, and 6 . They also made an analog with $n = 3$ and imidazole in place of dmbim. When the axial ligand was CN, the base-on form of the tetramethylene analog was the most stable of the dmbim derivatives with a pK value for dmbim hydration of 2, compared to a value of 0.1 for CNCbl. The tri- and hexamethylene analogs had pK values close to 3. Thus, the length of the chain (four) present in the natural Cbl appears optimal.

A reverse order in activity was found for DD activated by the Ado form of the $n = 3$ and $n = 4$ analogs, as assessed either by k_{cat} or k_{cat}/K_m . There thus appeared to be no correlation between the strength of the Co-dmbim interaction and activity. However, the base-on to base-off equilibrium was measured for the free derivatives. If, instead, an analysis of the equilibrium is made with λ_{max} values (~ 475 nm), then a case could be made that the trimethylene analog exists in the



base-on form in the “holoenzyme.” The K_m value is greater by an order of magnitude for the tetramethylene analog, although K_m for the trimethylene analog is comparable to that for AdoCbl.

These results can be interpreted to suggest that a repositioning of the nucleotide loop is required for activity. The energy required diminishes K_m for AdoCbl. The repositioned dmbim may correspond to the position of dmbim in the trimethylene analog, which consequently has a fairly large k_{cat} . It is not clear, however, whether such repositioning increases steric clashes with the corrin, enhancing or facilitating the corrin conformational distortion.

It is noteworthy that the imidazolyl analog inactivates the enzyme (but without inactivation of the apoenzyme). Deoxyadenosine and a B_{12r}-like molecule were formed, suggesting that a suicide inactivation involving one of the intermediates in the normal catalytic cycle occurred. It was concluded that the dmbim has an essential role in preventing inactivation during the catalytic process. This conclusion is in keeping with our suggestion that the interaction of the dmbim with Co may be tuned to stabilize the Co(II) state during the catalysis. At the same time, the dmbim group is not a sufficiently strong electron donor to stabilize the Co(III) state so much as to prevent homolysis.

Adenosylcobinamide methyl phosphate, a novel analog of adenosylcobalamin lacking the nucleotide loop moiety, did not show detectable coenzymatic activity with DD and was a strong high-affinity competitive inhibitor against AdoCbl [29]. The Co—C bond of this analog was almost completely and irreversibly cleaved in the presence of substrate with stoichiometric formation of 5'-deoxyadenosine. The adenosylcobinamide methyl phosphate seems to act as a pseudocoenzyme or a potent suicide coenzyme. Since adenosylcobinamide neither functions as a coenzyme nor binds tightly to apoenzyme, the authors concluded that the phosphodiester moiety of AdoCbl is essential for tight binding and for subsequent Co—C bond cleavage and for the normal progress of the catalytic cycle.

In another novel loop analog of AdoCbl, the 5,6-dmbim and D-ribose moieties of the nucleotide loop were replaced by pyridine and the trimethylene group, respectively [30]. The coordination of pyridine was stronger than that of dmbim in the corresponding homolog. The adenosyl form of the pyridyl analog was a better coenzyme for DD than the 5,6-dimethylbenzimidazolyl or imidazolyl counterpart. However, there was a concomitant inactivation during catalysis. Therefore, it was concluded that the dmbim moiety of AdoCbl is important for protecting the reactive intermediates from side reactions.

Finke [34,36] believes that the nearly strain-free 19-membered ring conformation places the dmbim base in a position to form a nearly ideal Co—N bond length, a conclusion consistent with our earlier suggestions based on synthetic and theoretical models [154]. In particular, studies from the Finke laboratory on varying the axial base in a series of AdoCbi⁺ models suggest that optimal kinetics



with Co—C bond homolysis favored over heterolysis is achieved in AdoCbl. In his analysis, Finke suggests that the acceleration in Co—C bond homolysis rate by the enzyme is mostly due to interactions of the corrin with the protein (the widely accepted view) [34]. Also the whole ribose ring is ideal because, as shown by Grissom's laboratory, the ribosyl ring oxygen is crucial for a high rate of cage recombination [164].

Much of the basis of the preceding analysis relies on studies in solution, outside the enzyme, and Finke cautions that more studies are needed. He downplays the role of the dmbim in facilitating faster cleavage since AdoCbi⁺ rate outside the enzyme is only $\sim 10^2$ times slower than for AdoCbl. The Toraya studies with adenosylcobinamide methyl phosphate show that the dmbim is not essential for cleavage [29]. However, I do not believe that the AdoCbi⁺ vs. AdoCbl comparison is necessarily contradictory to our suggestion that a weak axial base is needed. It is quite possible that AdoCbi⁺ has axially coordinated water, a ligand that can take the role of the dmbim if the other loop components are present to allow cofactor binding to the apoenzyme. The studies in the Finke laboratory with an axial *N*-methylimidazole support the preceding suggestion that the enzyme can modulate N donation, but it is suggested that this modulation is principally directed at minimizing heterolysis [35].

III. METHYL B₁₂—THE OTHER COENZYME

III.A. Cobamide-Dependent Methyl Transferases

In contrast to coenzyme B₁₂, where the alkyl moiety serves purely in a catalytic role, the alkyl group of methyl cobamides (MeCba's) is utilized as a reagent by MeCba-dependent enzymes; it is only the cobamide portion of the coenzyme that is catalytic. The cobamide-dependent methyl transferases have been reviewed [11,24–27,165]. Three cobamide-dependent methyl transferases have been studied; in some cases, more than one protein is required. The B₁₂ proteins include methionine synthase (officially called 5-methyltetrahydrofolate-L-homocysteine-S-methyltransferase [HCM] EC 2.1.1.13); MeCba-dependent enzyme from *Methanobacterium thermoautotrophicum* (MT₁); and the corrinoid/Fe-S protein from *Clostridium thermoautotrophicum*.

Both HCM and the little-studied MT₁ are base-off in the Co(I) state. As mentioned, in HCM a protein imidazole is coordinated to the Co(III)CH₃ form [32]. In contrast, the corrinoid/Fe-S protein utilizes a base-off Cba with 5-methoxybenzimidazole in place of dmbim. Formation of the base-off form is believed to lower the Co(II)/Co(I) couple to approximately -0.5 V, compared to the value of about -0.64 V as predicted from the free Cbl potential.

As its name implies, HCM is required in human metabolism to synthe-



size methionine. The substrate is homocysteine. Like HCM, MT₁ transfers CH₃ to a thiol acceptor, i.e., 2-mercaptoethanesulfonic acid. For the transfer to occur, a methyl transferase, MT₂, is required. However, CH₃-tetrahydrofolate (CH₃-H₄folate) is the CH₃ donor for MetH and the corrinoid/Fe-S protein, whereas CH₃OH is the donor for MT₁. *Methanosarcina barkeri* converts CH₃OH, CO₂ and H₂, and acetate to methane, with the possible involvement of corrinoid proteins. It is believed that, in these little-understood systems, the initial methyl donor may, in fact, be CH₃-tetrahydromethanopterin.

The corrinoid iron-sulfur protein from, for example, the anaerobic bacterium *Cl. thermoaceticum* participates in the Wood pathway for fixation of CO₂ in acetate biosynthesis. A methyl transferase converts CH₃-H₄folate and the corrinoid iron-sulfur protein to CH₃-corrinoid iron-sulfur protein. The latter protein then transfers the CH₃-group to carbon monoxide dehydrogenase. As mentioned, the transfer of the CH₃-group from the methylated MT₁ requires MT₂.

The requirement of a second noncorrinoid protein in these two systems from *M. barkeri* and *Cl. thermoaceticum* reemphasizes the intriguing mix of similarities and differences in these three Cba-dependent methyl transferases. Thus, HCM is unique among the three in not requiring a second protein. Of course, the corrinoid iron-sulfur protein is unique in possessing a 4Fe-4S cluster.

In the earlier version of this chapter [75], comparison of the HCM enzyme (then thought to utilize the dmbim base-on form) with the corrinoid/Fe-S protein utilizing the base-off form led me to suggest that axial N donor coordination deserves more study. This proved to be an excellent prediction since it predated the discovery of the imidazole coordination. I reasoned that the dissociation of the axial N donor moves the Co(II)/Co(I) potential to more positive values and also facilitates demethylation by thiols. On the other hand, the presence of an axial ligand bound to the Co could facilitate nucleophilic attack of Co(I) on CH₃-H₄folate. Unless the enzyme conformations are cycling back and forth to effect the base-off/base-on interchange, it is not possible to facilitate both donation and acceptance of the methyl group. A neutral imidazole is electronically similar to dmbim and for this reason the involvement of imidazole escaped experimental detection. Thus, all the analyses of axial base effects of this type are still valid. However, new dimensions for modulating the electron donation have been added since the imidazole ring can be partially dehydronated (via H bonding) or fully dehydronated. Likewise the Co—N distance can be controlled by the protein.

It has been suggested that interaction of the dmbim pulls the Co into the corrin plane, sterically inhibiting nucleophilic displacement at the axial methyl [166]. A bound imidazole would play the same role. Therefore, release of this steric strain in base-off Cbls and in MeCbi⁺ should facilitate dealkylation; base-off species dealkylate more rapidly [166], but different products are observed. It is essential to assess the relative importance of steric and electronic effects on



demethylation. Compared to Cbl(I), the resultant Cbi(I) should be a weaker nucleophile and less susceptible to oxidation. Consequently, any Cbi(I) product should be readily detected.

III.B. Methionine Synthase

All evidence strongly suggests that B₁₂-dependent methionine synthases (MetHs) from both prokaryotic and eukaryotic sources are similar [11,165,167]. Studies on bacterial enzymes are likely to provide insight into the catalytic pathways in both systems. The enterobacteria, *E. coli* and *Salmonella* spp., have two enzymes that promote the formation of methionine from homocysteine. The 133-kDa [168] Cbl-dependent enzyme (the *metH* gene product) is much more efficient than the Cbl-independent enzyme (the *metE* gene product) [165]. The Cbl-independent enzyme requires the polyglutamate form of CH₃-H₄ folate, whereas the B₁₂ enzyme can also utilize the monoglutamate form and has an absolute requirement for a reducing system and AdoMet. A review [165] compares the two enzymes, but our focus here is on the *metH* product, which has been cloned, overexpressed, and sequenced.

Now MetH is one of the best understood B₁₂ enzymes [11,32,169–173]. The overall mechanistic scheme (Figure 12) was suggested by early work in

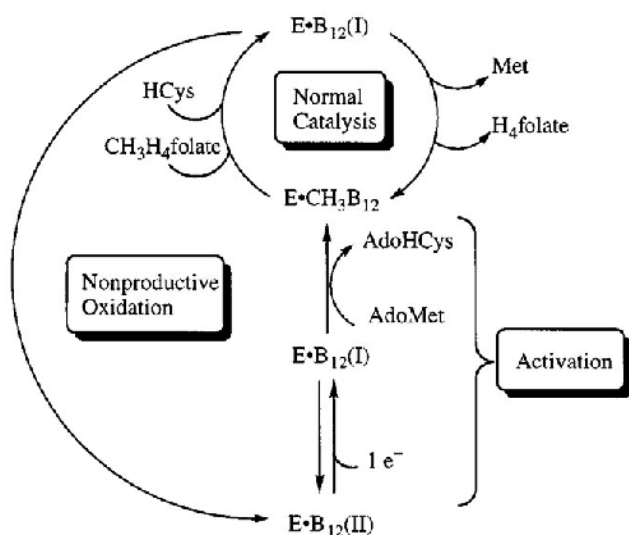


Figure 12 Postulated reaction mechanism of methionine synthase (MetH) showing the interconversion of the various oxidation states of enzyme-cofactor species, E•B₁₂.



several laboratories. In the scheme, the Cbl cycles between the Cbl(I) and MeCbl(III) forms. The more recent work from the Matthews lab has established the kinetic competence of the Cbl(I) form as an intermediate in the catalysis [174]. Transfer of the methyl group to homocysteine from CH₃-H₄ folate proceeds with retention, consistent with two consecutive nucleophilic displacements at the transferred carbon [165].

This mechanism can now be discussed in the context of the recent crystal structure determination of *E. coli* methionine synthase [32]. Specifically, the MeCbl was found to be in the (not-hydrated) conformation, with the “nucleotide loop” important primarily in the binding of the cobalamin to the protein (Figure 2). The histidine (His759 in the enzyme) coordinated at the α -axial site of “base-off” MeCbl bound in turn to the cobalamin-binding domain has been postulated to be part of a “catalytic quartet.” This quartet includes the Cbl and the triad, His759, Asp757, and Ser810. In this hypothesis, a hydrogen-bonding network connects the four components of the quartet. The Asp757 carboxylate attracts the imidazole hydron of His759, weakening the NH bond. This interaction gives the imidazole imidazolate character, making it a better ligand, which could stabilize the hexacoordinate methylcob(III)alamin species. In other phases of the catalytic cycle, hydron uptake by the quartet makes imidazole a weaker ligand and thus favors the square-planar cob(I)alamin (B_{12s}) species. Such stability would facilitate transfer of the methyl group to homocysteine in the methionine-forming step [32].

Cbl(I) is occasionally oxidized to Cbl(II). Cbl(II) is rereduced to Cbl(I) in a coupled electron-transfer reaction driven by the exergonic reaction with AdoMet (a process with an overall free energy decrease of 9 kcal mol⁻¹). Nucleophilic attack of Co(I) on the S—CH₃ group of AdoMet is reasonable, and MeCbl is formed from AdoMet under reducing conditions in nonenzymatic processes. The standard reduction potential for Co(II)/Co(I) measured spectroelectrochemically by monitoring the EPR signal of the base-on Co(II) form was reported as -0.526 V vs. NHE. Although made more positive by conformational changes than the base-on B₁₂ potential of the free cofactor in pH 7 solution (-0.61 V), Co(I) could still not be effectively produced by typical reductants (-0.3 to ca. -0.46 V) used in vitro. In the presence of AdoMet, the reduction of the enzyme-bound form was not reversible. However, even at relatively low applied potentials (ca. -0.1 V), MeCbl was fully formed. Thus, the exergonic formation of MeCbl from AdoMet drives the endergonic Co(II) to Co(I) reduction at potentials provided by the reductants used in in vitro activation (e.g., *threo*-1,4-dimercapto-2,3-butanediol [DTT], -0.32 V); this activating system is needed infrequently. Once it is activated, the catalytic interconversion between Cbl(I) and MeCbl has been shown by rapid reaction kinetics to occur by an ordered sequential mechanism [174]. Thus, Cbl(I) is regenerated in the catalytic cycle by demethylation of MeCbl rather than by reduction. No special enzymatic properties have been invoked for



this demethylation since the feasibility of the dealkylation of MeCbl has been accepted on the basis of evidence from model studies [166,175]. In the following paragraphs, we consider the feasibility of the dealkylation and the validity of the evidence.

Although assessment of the electron distribution in organocobalt compounds is difficult, the smooth variation of spectral and reactivity patterns clearly demonstrates that the axial alkyl ligands are simply excellent carbanionic donor ligands [137,176,177]. Brown has questioned the reasonableness of a nucleophilic attack at such Co—C centers by the RS^- nucleophile [178]. Furthermore, the reported result that thiol-promoted Co—C bond cleavage of organocobaloximes occurred under neutral to acidic conditions [179–181] could not be reproduced [182–184]. Treatment of MeCbl with the thiolate form of β -mercaptoethanol resulted in methylation of the thiolate and Co—C bond cleavage [175]. The ultimate product of dealkylation is not the predicted Co(I), but Co(II), an observation attributed to catalytic Co(I) oxidation by disulfide impurities [166]. Another reaction involving methylation of a thiolate by an organocobalt compound was reported [185] for the 5-coordinate model, $CH_3Co(III)Pc$ (Pc = phthalocyanine). Thiolate coordination *trans* to the alkyl group has been invoked in several instances for MeCbl and organocobalt B_{12} models, although no characterized isolated complexes of this type have been reported. It was suggested that CH_3 transfer from $CH_3Co(III)Pc$ was inhibited by the formation of the inactive thiolato complex, $CH_3Co(III)Pc(SPh)$ [185]; however, this complex was not isolated. In contrast, it was suggested that thiolate coordination did not occur in MeCbl (thus replacing the 5,6-Me₂dmbim) since no change was observed in the ^{13}C NMR spectrum of a sample of MeCbl treated with the thiolate form of dithiothreitol [175], whereas others suggest that thiols interact strongly enough to bind to even the small percentages of base-off Cbls [186]. Thus, elucidating the interaction of thiolates with Co(III) complexes is of considerable importance for understanding MeCbl function.

We recently used model systems to address various aspects of thiolate B_{12} chemistry, including three types of reactions of thiolates with organocobalt compounds (Figure 13): (1) attack at the organometallic carbon to form a thioether; (2) reaction (1) followed by cleavage of the Co—C bond; or (3) coordination at the Co *trans* to the alkyl group (thiolate coordination) [124]. Reaction (1) is feasible when the Co-bound carbon has a leaving group such as Br. However, no reaction was observed at this carbon, consistent with the carbanion nature of the carbon. The second reaction (reaction [2]) is the methyl transfer reaction mentioned as part of the HCM catalytic cycle. However, our latest studies [124] confirm the studies with most models [182–184] that treatment with thiolates does not lead to a thioether. Thiolate coordination (reaction [3]) is discussed in the following paragraphs.

Past studies have shown that in general the Co—C bond is not very suscep-



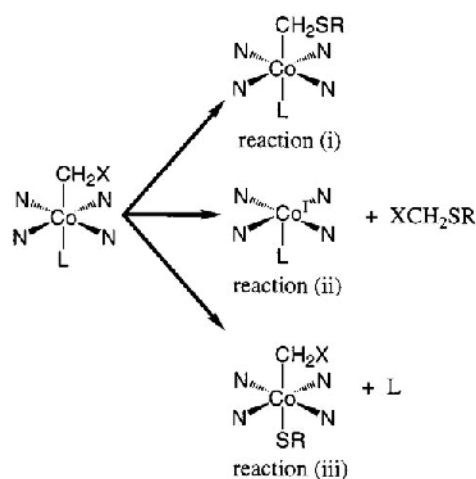


Figure 13 Possible reactions of thiolates with organocobalt compounds.

tible to attack by the thiolate nucleophile. We now consider ways by which this reaction can be promoted. One potential mode for activating the Co(III)—C bond for demethylation is to replace the ligand *trans* to the alkyl group with a better donor [32,54,55]. Examination of likely candidates would involve the thiolate ligand, the imidazolate ligand, and another methyl group. I do not believe all three of these ligands are equally likely prospects, but they are conceivable in the biological system. Furthermore, it is not likely that any ligand short of the best donors could weaken the Co—C bond since our FT-Raman study of the influence of the *trans* ligand on the Co—C stretching frequency suggests that significant Co—C bond weakening will occur only if the *trans* ligand is a very strong donor, e.g., an alkyl ligand [55]. However, this pathway cannot be excluded *a priori*.

One possible *trans* influence ligand we considered was thiolate. We found the alkyl thiolate ligand is comparable to alkyl phosphanes in its affinity for axial coordination to B₁₂ models [124]. It is a much stronger ligand than biologically relevant N heterocycles such as benzimidazole and imidazole. The principal reaction following addition of thiolates to organocobalt complexes is S-coordination reaction (3), not Co—C bond cleavage reaction (2). The structure of [AsPh₄][EtSCo(Hdmg)₂CH₃] revealed that the Co(III)—S bond *trans* to the Co—C bond is unusually long compared to other Co(III)—S-containing alkyl thiolate complexes. The influence of the thiolate ligand on the length of the *trans* Co—C bond was shown to be small in this first crystallographic determination of the structure of this type of compound. Although relevant structural information is



limited to this compound and additional x-ray data are needed to confirm this result, NMR shift trends and the FT-Raman data indicate that thiolates have a moderate *trans* influence. It is thus likely that thiolates will not significantly influence the Co—CH₃ bond in MeCbl. Therefore, thiolate coordination by homocysteine is unlikely to be an important step in the enzymatic processes involving methionine synthase. In fact, the protein may act to prevent thiolate coordination since the results presented here and in the studies with CH₃Co(III)Pc [185] suggest that such coordination may impede attack at the Co—C bond.

A second possible *trans* influence ligand is imidazolate. Methylcobinamide (MeCbi⁺) derivatives are well suited for studies of imidazole substitution at the *trans* α site such as occurs in the binding of the cofactors to human B₁₂ enzymes. The MeCbi's contain the important corrin ring and the axial methyl group but lack the nucleotide loop; this loop favors dmbim coordination and thereby inhibits coordination by other ligands. Thus, MeCbi⁺ will add a histidine ligand, producing a species that is a good initial structural model of the Co coordination environment in the enzyme system. Imidazolate MeCbi complexes could be formed under strongly basic conditions. An ~ 10 nm red shift in the 527 nm β -peak in the UV/vis spectrum of imidazole and NAcHis MeCbi complexes was observed when the solution basicity was increased to 1M NaOH, but the N-MeIm complex did not show the shift; these results provide strong evidence for the formation of imidazolate complexes. Before this study [54], there was essentially no evidence for imidazolate bound to organocobalt corrinoids. The replacement of dmbim by imidazole and imidazolate has only small effects on Raman bands of both the axial bond and the corrin. These spectral results suggest that the reason that imidazole is substituted for dmbim does not involve the methylCo(III) corrinoid ground state species. Instead, the properties of imidazole, such as its ability to form a strong imidazolate-like donor or its small steric size, could be important for an intermediate or an activated complex in the catalytic cycle.

A third possible *trans* influence ligand is methyl (i.e., a second methyl, but bound at the *trans* α site). As mentioned, the strongest *trans* influence ligand that can be imagined in a biological system is another alkyl group. The FT-Raman data show such groups have the greatest influence in lowering the Co—C stretching frequency [55]. However, we found that even the dialkyl complex, Co(Hdmg₂pn)(CH₃)₂ with the strongly directing *trans* ligands, did not react with thiolates. As an aside, in this study [124] even the metallocycle complex with a highly distorted alkyl carbon sterically exposed to attack by the thiolate was not reactive. In summary, relatively few model systems are capable of reacting with thiolates to produce thioethers cleanly and readily.

Within the normal conditions of biological systems, thiolates are disfavored compared to thiols. Thiols are even weaker nucleophiles than thiolates. For methionine synthase this problem is overcome by having a Zn binding site for homocysteine [171]. The relative reactivity should be RS[−] > RSM > RSH.



IV. CONCLUSION

The study of B₁₂ systems has involved research in inorganic/organometallic syntheses, bioorganic chemistry, biosynthetic chemistry, electrochemistry, theoretical chemistry, and spectroscopy in addition to the typical studies associated with enzymes (kinetics, cloning and expression, etc.). Of particular note, since the B₁₂ compounds are among the most complicated natural products, they often serve as a venue for studies with new methods and for the extension of methods from small molecules to macromolecules. X-ray diffraction methods have revealed that in both known types of human enzymes, the coenzyme binds in the base-off form with a protein imidazole side chain bound to the Co.

The cobalt center in MeCbl, one of the two important B₁₂ coenzymes, is clearly involved in key steps in catalytic methyl transfer processes. Here, the Co center cycles between Co(I) and Co(III)CH₃. In methionine synthase, the proposed mechanism involves direct nucleophilic attack on the C of the Co(III)CH₃ group. In model reactions, the thiolate most frequently simply binds *trans* to the alkyl group to give a product recently established by an x-ray study of a model system. The protein may block access to the Co, thus preventing this reaction common in models. It is likely that the reactive form of the bound cofactor is five-coordinate in the key point in the catalytic cycle. This reactive form will lead to a four-coordinate Co(I) species. The axial coordination of the cofactor by a protein imidazole allows for a finer tuning of the Cbl chemistry and may permit control of the coordination number. Thus, recoordination of Co in the Co(I) state may facilitate attack on methyltetrahydrofolate and re-formation of Co(III)CH₃.

One clear role of the Co center in biocatalytic processes requiring AdoCbl is Co—C bond homolysis, and a conformational mechanism probably triggers this homolysis. Recent evidence that the homolysis step is influenced significantly by the substrate and other evidence that the radicals formed stay close to the Co suggest that the Co may influence the rearrangement reaction; however, there is no clear evidence favoring the involvement of cobalt in the rearrangement steps.

Model studies have helped to clarify the potential involvement of Co in these processes and have been useful in the development of methods to estimate Co—C BDEs. Recently more emphasis has been placed on models with axial ligands bound to Cbi's. These are useful for modeling the possible consequences of the binding of the imidazole to the cofactors. For example, in studies aimed at assessing the possible involvement of the imidazolate group in weakening the *trans* CoCH₃ bond, the slight weakening observed indicates that this is not the role of the imidazole. In studies directed at understanding the role of the imidazole or dmbim in the homolysis process, AdoCbi⁺ derivatives were used; the results suggest that the imidazole must have the correct donating ability to minimize the undesirable heterolysis reaction.

The major new development in the field since the earlier edition of this



volume [75] is the discovery that an imidazole side chain in the protein can bind to the cofactor. Remarkably, both types of cofactors (each found in one of the two classes of enzymes present in humans) have this new type of binding. This finding has greatly expanded the types of modulation of cofactor reactivity by the B₁₂ enzymes. Understanding biocatalysis involving these enzymes has thus become more of a challenge. Nevertheless, it should not be forgotten that the dmbim-bound base-on cofactor is involved in other B₁₂ enzymes (not found in humans). Thus, considerations of B₁₂ involvement in biocatalysis that predate the discovery of imidazole coordination are still equally valid but are relevant to fewer types of B₁₂ enzymes.

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14

Formation, Structure, and Reactivity of Copper Dioxygen Complexes

Kenneth D. Karlin

The Johns Hopkins University, Baltimore, Maryland

Andreas D. Zuberbühler

University of Basel, Basel, Switzerland

I. INTRODUCTION

I.A. General Considerations

In addition to the known utility of the copper ion in both stoichiometric and catalytic oxidative transformations [1–4], this metal is also well established as a required trace element having a crucial role in proteins involved in electron transfer and dioxygen (O_2) processing [5–14]. Copper enzyme active sites possessing a diversity of coordination structures are involved in O_2 transport, mixed function oxidases (monooxygenases), dioxygen transferases (dioxygenases), and electron transfer oxidases (oxidases). The interaction of Cu(I) with dioxygen is an essential component of both chemical and biological processes, prompting considerable research efforts on fundamental aspects of O_2 reactivity with well-characterized low-molecular-weight copper complexes. It is the purpose of this chapter to summarize what is known about copper/ O_2 interactions in proteins and in chemical systems. We will emphasize biomimetic studies, focusing on advances in the reversible binding of dioxygen to Cu(I) complexes and in the related “activation” of O_2 , where monooxygenation of substrates occurs.

Compared to knowledge of copper and nonheme iron-mediated processes (see Chapter 10), there is a great deal of information concerning the binding and activation of molecular dioxygen by hemoproteins and porphyrin-containing



metal complexes. Considerable insights into structural, spectroscopic, and mechanistic aspects of the porphyrin–iron areas have been made through biomimetic studies using small molecule active site model compounds [15,16]. Our own endeavors in the copper area were initiated by the apparent diversity of highly interesting but somewhat poorly characterized (at the time) copper proteins involved in O₂ utilization, the paucity of established coordination chemistry of reduced Cu(I) possessing nitrogenous ligand environments, and the general lack of information on Cu(I)/O₂ interactions and reactivity. Model studies are intended to establish such coordination chemistry so that they can (1) provide a reasonable basis for hypothesizing biological structures and reaction intermediates, (2) determine the competence of such moieties toward reactivity patterns observed in metalloprotein chemistry, and (3) allow for exploitation of copper/O₂ chemical systems as practical O₂ carriers or oxidation catalysts.

As will be seen in the following descriptions, advances in the understanding of copper protein structures and chemistry confirm that a diversity of active site structures with varying copper ion nuclearity, coordination, and cofactors are involved in O₂ binding and activation; this is indicative that a variety of mechanisms have evolved for effecting copper-mediated substrate oxidation and O₂ reduction. Biomimetic chemical systems have also developed to the point of contributing significantly to our understanding of Cu(I)/O₂ reactivity, copper-dioxygen structures, and monooxygenation processes.

I.B. Copper Proteins and O₂ Processing

As the focus of this review is on copper-dioxygen chemistry, we shall briefly summarize major aspects of the active site chemistry of those proteins involved in O₂ processing. The active site structure and chemistry of hemocyanin (Hc, O₂ carrier) and tyrosinase (Tyr, monooxygenase) will be emphasized, since the chemical studies described herein are most relevant to their function. The major classes of these proteins and their origins, primary functions, and leading references are provided in Table 1. Other classes of copper proteins not included here are “blue” electron carriers [13], copper-thiolate proteins (metallothioneines) [17], and NO_x reductases (e.g., nitrite [NIR] [18] or nitrous oxide [19]).

Following earlier spectroscopic studies carried out on proteins including the multicopper oxidase laccase [20], copper ions have been historically classified as types 1, 2, and 3 [13]. Type 1 possesses cysteinate coordination, a high redox potential, small electron paramagnetic resonance (EPR) hyperfine splittings, and an intense blue color ($\lambda_{\text{max}} \sim 600$ nm) and occurs in electron carriers and multicopper oxidases. Type 3 originally referred to the dinuclear center in laccase (magnetically coupled, EPR-silent, 330 nm absorption), but the terminology has extended to any coupled dinuclear center, such as in hemocyanin and tyrosinase. Type 2 (“normal”) has normal extinction coefficients and EPR parameters, and reaction



Table 1 Major Classes, Origins, and Primary Functions of Copper Proteins

Protein	Source	Biological function	References
Oxygen carrier			
Hemocyanin	Molluscs and arthropods	O ₂ transport	6–11,21–25
Copper oxygenases			
Tyrosinase	Fungal, mammal	Tyrosine oxidation	6–11,22
Dopamine β -hydroxylase	Adrenal, brain	Dopamine $\rightarrow$ norepinephrine	33
Peptidylglycine α -amidating monooxygenase (PAM)	Pituitary, heart	Oxidative N-dealkylation	33,38
Methane monooxygenase (MMO)	Methanogenic bacteria	Methane $\rightarrow$ methanol	22,56
Copper dioxygenases			
Quercetinase	Fungal	Quercetin oxidative cleavage	59,60
Copper oxidases			
“Blue” oxidases			
Laccase	Tree, fungal	Phenol and diamine oxidation	22,61
Ascorbate oxidase	Plants	Oxidation of L-ascorbate	22,61
Cenuloplasmin	Human, animal serum	Weak oxidase activity	22,66,67
“Nonblue” oxidases			
Amine oxidase	Most animals	Elastin, collagen formation	33
Galactose oxidase	Molds	Galactose oxidation	33
Cytochrome c oxidase	Mitochondria	Terminal oxidase (hydron pump)	84–86
Phenoxazinone synthase	Streptomyces	Phenoxazinone formation	22
Other			
Superoxide dismutase (SOD)	Red blood cells, animals	O ₂ ⁻ detoxification	87,88





Figure 1 *Limulus* II deoxyhemocyanin and oxyhemocyanin Cu_2O_2 structures.

chemistry occurs at these sites. As such, the type 2 designation has been applied to the copper ions in dopamine β -hydroxylase, the “colorless” anion binding site in multicopper oxidases, galactose oxidase, amine oxidases, nitrite reductases, and superoxide dismutase. These designations are still widely used, but perhaps should not be, since advances now indicate a very large diversity of structural, spectroscopic, or active site cofactors, even within a given classification.

I.B.1. O_2 Transport: Hemocyanins

Hemocyanins are very large (M_r 4.5–90 $\times 10^5$ Da) dioxygen transporting proteins that function in the hemolymph of invertebrates belonging to several species of the phyla of Mollusca and Arthropoda [21]. These occur as highly cooperative multisubunits; molluscan hemocyanins consist of 10 or 20 subunits with each functional unit having a molecular weight of about 55,000 Da. Arthropodal hemocyanins are hexamers or multihexamers with larger subunits ($\sim 75,000$ Da). In both classes of carriers, O_2 binding occurs at a dinuclear copper center and spectroscopic similarities indicate a closely related active site structure and binding mode [22].

The deoxy forms of hemocyanins are colorless, as a result of their $3d^{10}$ dicopper(I) centers. Although chemical and x-ray absorption spectroscopic studies had shed considerable light on the nature of the deoxy-Hc dicopper binding site, there now exist two x-ray crystal structures, the first on the spiny lobster Hc, *Panulirus interruptus* [23], and a recent one of the horseshoe crab *Limulus* II protein [24]. The structures exhibit rather different active-site characteristics, and since the former was crystallized at low pH and possesses rather odd copper(I) coordination, the latter *Limulus* II structure is probably representative. It indicates that the two Cu(I) ions are 4.6 Å apart, each found in a trigonal-planar coordination environment with Cu–N^{His} bond distances of about 2.0 Å (Figure 1). Intersubunit O_2 binding cooperative effects are probably initiated and trans-

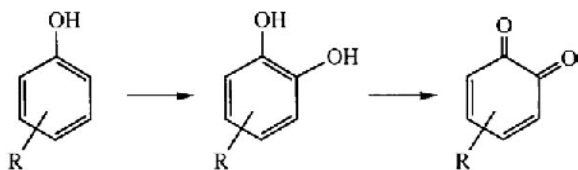


mitted as a result of the movement of histidine residue(s) and copper ion(s) upon O_2 coordination [24].

Binding of O_2 leads to intensely blue oxy-Hc, and resonance Raman studies show that the dioxygen is bound as peroxide (formally O_2^{2-} with $\nu_{O-O} \sim 750\text{ cm}^{-1}$, indicating an oxidative addition of O_2 to give peroxodicopper(II) [22]. This oxy site is EPR-silent and diamagnetic, reflecting strong magnetic coupling between the two Cu(II) centers. A recent x-ray structure reveals a side-on $\mu-\eta^2:\eta^2$ -peroxo coordination with $Cu...Cu = 3.6\text{ \AA}$ [21,25]. The electronic spectrum of oxy-Hc is distinctive and dominated by $O_2^{2-} \rightarrow Cu(II)$ ligand-to-metal charge transfer transitions at 345 ($\epsilon = 20,000\text{ M}^{-1}\text{cm}^{-1}$) and 570 ($\epsilon = 1,000\text{ M}^{-1}\text{cm}^{-1}$) nm, with an additional circular dichroic feature at 485 nm [22].

I.B.2. Copper Oxygenases

Tyrosinase (EC 1.14.18.1). This enzyme has been known for over 80 years and was one of the first to be discovered as an oxygenase on the basis of an $^{18}O_2$ labeling experiment performed by H. S. Mason et al. in 1955 [26]. It occurs widely in bacteria, fungi, plants, and mammals (see also Chapter 15). It functions in melanin pigment formation and in the well-known browning reaction observed in fruits and vegetables. Tyrosinases possess both monooxygenase and oxidase activity, ortho-hydroxylating monophenols to catechols (cresolase activity), and further oxidizing these to *o*-quinones (catecholase activity). As such, it is an internal monooxygenase with the diphenol hydroxylation product itself serving as cosubstrate, reducing Cu(II) back to Cu(I) to repeat the catalytic cycle (Scheme 1).



Scheme 1

Chemical and spectroscopic evidence [22] indicates that tyrosinases have a coupled dinuclear active site nearly identical to that found in hemocyanins, such that a stable oxy form (oxy-Tyr; $\lambda_{\max} 350\text{ nm}$, $\epsilon = 26,000\text{ M}^{-1}\text{cm}^{-1}$) can be generated either from O_2 or from reaction of met-Tyr plus dihydrogen peroxide. This latter observation is of interest in terms of being able to generate a Cu(II) hydroxylating reagent equally from Cu(I)₂ plus O_2 or from Cu(II)₂ plus H_2O_2 , as is also observed in a model hydroxylating system discussed later.



In addition to extensively studied probe-ligand-bound Tyr derivatives (e.g., azide), substrate analogs and inhibitors have been used to study tyrosinase; these investigations point to an active site that is highly accessible to exogenous ligands compared to Hc [22]. Sequence comparison and ligand binding studies using *Neurospora* tyrosinase also support this idea [27], to the extent that in a simplistic view, Hc's can be viewed as consisting of a Tyr protein with an additional protein sequence making up a domain that shields the active site, preventing binding of large ligand substrates.

The substrate analog and spectroscopic studies led Solomon et al. to suggest a mechanism for the Tyr cresolase activity (Figure 2) [22]. Here, a phenol substrate could bind initially to oxy-Tyr in an axial fashion, a possibility confirmed in model studies [28,29]. In this ternary Cu_2/O_2 /substrate complex, rearrangement through a trigonal bipyramidal intermediate could be accompanied by ortho-hydroxylation, followed by loss of water and coordination of the diphenol product. Such a catecholate dicopper(II) complex is known in model systems [30]. Intramolecular electron transfer would result in release of product *o*-quinone and the dicopper(I) produced could react with O_2 again to produce oxy-Tyr.

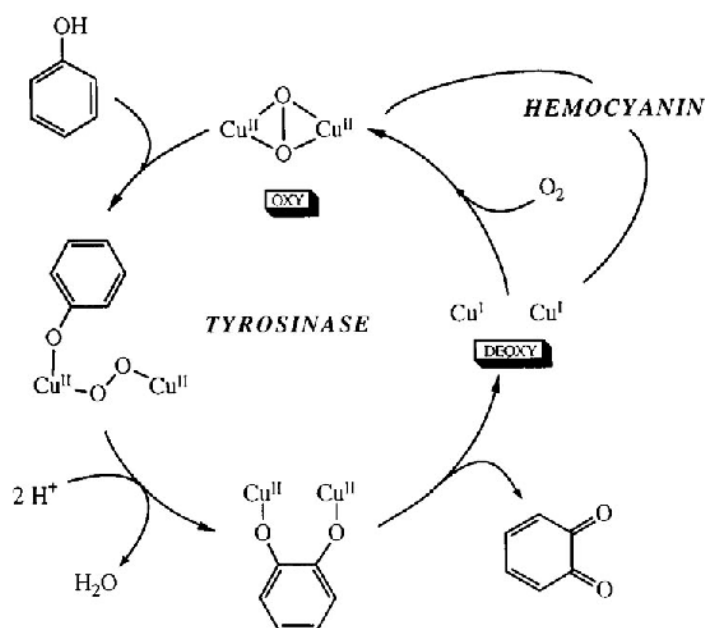


Figure 2 Tyrosinase phenol *o*-hydroxylation and relationship to hemocyanin Cu_2O_2 chemistry.



The importance and role of H^+ and their transfer should be considered in a further understanding of the O_2 -activation (e.g., hydroxylation) process. Questions to consider include the followings: (1) Is a reactive copper hydroperoxide involved, formed by hydron (H^+) transfer (from phenol substrate?) to bound peroxide? (2) Does an “endogenous” hydroxide aid in the activation step, accepting a hydron, liberating water, and causing coordination changes? (3) Can a $\mu-\eta^2:\eta^2$ -peroxo group itself attack the phenol substrate, either directly or via an initial O—O bond cleavage process? Model studies are discussed here to address some of these matters; they also suggest that the phenol hydroxylation is essentially an electrophilic attack, in line with earlier observations [22]. The coordination chemistry studies suggest that an $\eta^2:\eta^2$ -peroxo group acts as an electrophile and this is supported by theoretical studies [31]. Model studies also have prompted the recent suggestion that phenol oxygenation may lead directly to quinone, bypassing the presumed catechol initial product [32]. This will be discussed further later.

Dopamine β -Hydroxylase (EC 1.14.17.1). The mammalian enzyme dopamine β -hydroxylase (D β H) is found in the adrenal medulla, where it catalyzes the ascorbic acid-dependent stereospecific benzylic hydroxylation of phenylethylamines such as dopamine to the neurotransmitter norepinephrine [33]. A stoichiometry of two coppers per catalytic unit is indicated, although the lack of any magnetic interaction between Cu(II) centers has precluded suggestions for dinuclear copper/ O_2 reactions. Some studies [33] suggest that the two copper ions have different roles, one binding ascorbate (or another competent one-electron donor) and accepting electrons; the other site carries out O_2 and substrate binding prior to hydroxylation. Previously, the two copper centers have been spectroscopically indistinguishable, but an important advance demonstrates their inequivalence, since only one site binds CO in competition with O_2 [34].

A recent reaction mechanism [33,35] (Figure 3) involves an initially fully reduced and catalytically competent enzyme. After O_2 and substrate binding, electron transfer to dioxygen generates a mononuclear copper-hydroperoxide (i.e., Cu(II)—OOH); a hydron is required for catalysis. Intricate ^{18}O kinetic isotope experiments [35] suggest that the activation step involves reductive cleavage of the O—O bond prior to attack of substrate. This result is rationalized (Figure 3) by the proposed involvement of a nearby tyrosine residue, leading to the production of water, a tyrosyl radical, and a copper-oxo-radical oxidant. Hydrogen abstraction leads to substrate benzylic radical formation, and H-atom transfer back to the tyrosyl radical, followed by copper-oxygen “rebound” to couple the radicals, then generates a copper(II)-alkoxide intermediate. The hydroxylated product is eliminated upon hydronation and electron transfer (from the other copper site), regenerating the reduced active site.

Primarily through the use of extended x-ray absorption fine structure



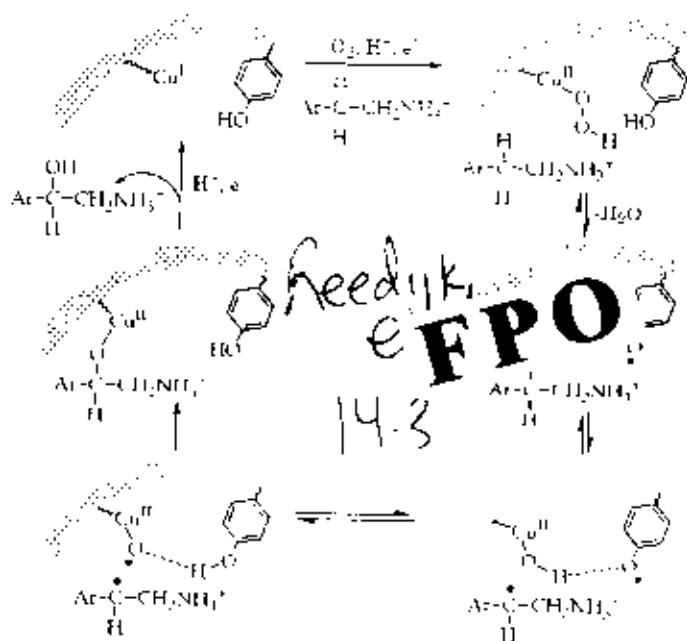


Figure 3 A proposed mechanism for dopamine- β -hydroxylase.

(EXAFS) and edge absorption spectroscopic studies [36,37], it is now generally agreed that the oxidized form of DBH contains 2–3 histidine and 1–2 O/N ligands [33]. However, substantial reorganization in the reduced form leads to the suggestion that the site responsible for substrate hydroxylation (referred to as Cu_B) possesses a heavy atom scatterer, most probably a methionine residue, one that is also conserved in the peptidylglycine α -amidating mono-oxygenase (PAM) [33,37], discussed later. Results of infrared spectroscopy of CO bound to this reduced Cu_B site are also consistent with the presence of a methionine ligand [37]. From enzyme or perhaps model studies, it will be of great interest to learn how oxygen activation chemistry can occur at a metal center possessing a sulfur ligand, and how the two copper ions, each in a different coordination environment, participate in the enzyme reaction.

Peptidylglycine α -Amidating Monooxygenase (EC 1.14.17.3). Many physiological secretory polypeptide hormones and neuropeptides (e.g., gastrin, calcitonin, vasopressin) are COOH-terminal amides and are known to be enzymatically generated from prohormone peptides containing a glycine extended



sequence. A copper ion-, ascorbate-, and dioxygen-dependent enzyme effecting this oxidative N-dealkylation reaction was identified by Bradbury et al. [38]. The enzyme appears to be bifunctional, carrying out two reactions in sequence. The first generates a hydroxyglycine intermediate, which is followed by a second dissociation (lyase activity) to give glyoxylate and carboxamide products [33,39] (Figure 4). May [40] has shown that peptidylglycine α -amidating monooxygenase (PAM) can support monooxygenation of alternate substrates, (i.e., in sulfoxidation, amine N-dealkylation, and O-dealkylation reactions). A number of chemical model systems for PAM have been described [41–46]. The similarity of requirements for enzyme activity of PAM and D β H is striking.

Insight into the active site structure has come from enzyme biochemical and biophysical investigations (i.e., EPR, CO binding and infrared spectroscopy, and Cu x-ray absorption spectroscopy) [33,47,48]. As for D β H, a dicopper moiety with inequivalent metal ion centers is suggested. A methionine ligand is implicated for the active site Cu_B, along with two histidine ligands, and the reduced Cu_A site environment is suggested to have three histidine ligands. Confirmation of the unsymmetrical Cu environment and its coordination has very recently come from an x-ray structure determination (PHM mono-oxygenase domains of PAM) of an oxidized form. The Cu_A site with three protein histidine ligands, and the Cu_B including two histidine and one methionine ligand, are separated by ~ 11.0 Å [49]. Confirmation of this unusual coordination by methionine and the organization of the active site dicopper ions will provoke a new phase of inquiry concerning the mechanism of monooxygenation in this enzyme and in D β H.

Phenylalanine Hydroxylase (EC 1.14.16.1). In arene hydroxylation of phenylalanine to produce tyrosine, mammalian phenylalanine hydroxylase (PAH) requires nonheme iron and tetrahydropterin cofactor. The role of the metal is

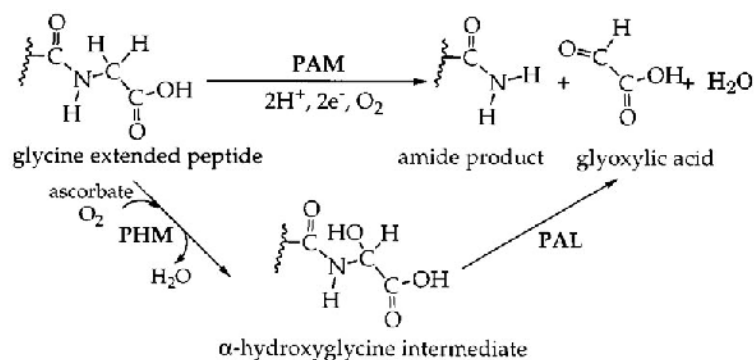


Figure 4 Reaction mediated by peptidylglycine α -amidating monooxygenase (PAM).



thought to be the stabilization or activation of a peroxypterin intermediate [50–52]. Earlier work and extensive investigations by the group of Benkovic in the last 10 years suggested that *Chromobacterium violaceum* PAH was a copper-dependent monooxygenase [50,51]. However, their more recent studies [51,53] suggest that this is not the case. More work is needed to clarify the situation for this bacterial PAH.

Methane Monooxygenase. The enzyme methane monooxygenase (MMO) is found in methanotrophs and has primarily been studied in its soluble form, as a nonheme μ -oxo diiron-dependent protein [54,55]. There is also a membrane-bound form that exhibits a large fraction of the activity (methane $\rightarrow$ methanol). Characterization on the *Methylococcus capsulatus* (Bath) protein is still at an early stage, but the enzyme is copper-dependent, suggesting its classification as a copper monooxygenase (Table 1). Chan, collaborators, and coworkers [56] suggest that the enzyme may contain several types of copper active site clusters, including trinuclear centers; DiSpirito [57] describes a spectroscopically detectable required nonheme iron and proposes an active site with both iron and copper.

Copper Dioxygenases. Dioxygenases incorporate both atoms of O_2 into a substrate. Keevil and Mason [58] have listed three copper dioxygenases: 2,3-dihydroxybenzoate 2,3-dioxygenases that effect 1,2-cleavage of aryl 1,2-diols; indole dioxygenases; and quercetinases (2,3-dioxygenases that cleave quercetin to give 2-protocatechuoyl phloroglucinol and carbon monoxide). In the latter case, the copper ion is thought to bind at 3-hydroxy and 4-carbonyl groups. Copper may serve to activate the substrate, allowing O_2 attack to give an organic peroxide that then cleaves to give product [59,60].

I.B.3. Copper Oxidases

Effecting either the one- (e.g., ascorbate, phenols) or two-electron (e.g., amines, galactose) oxidation of substrates, copper oxidases react with dioxygen, reducing it to either dihydrogen peroxide or water. Not discussed here, copper nitrite (NIR) and nitrous oxide reductases [5,18,19] possess active site structures similar to those found in many O_2 -processing copper proteins, also effecting the formal two-electron reduction of their substrates. Nitrous oxide reductase also has several biological and spectroscopic features that are similar to those in cytochrome-c-oxidase.

“Blue” Multicopper Oxidases. These include laccases, ascorbate oxidase, and ceruloplasmin [22,61], which along with cytochrome c oxidase (CcO; with Fe and Cu) can couple the one-electron oxidation of substrates (e.g., ascorbate, diamines, monophenols; Fe^{2+} for ceruloplasmin; cytochrome c, for CcO) to the full reduction of dioxygen to water (i.e., $O_2 + 4e^- + H^+ \rightarrow 2H_2O$).

Whereas the type 1,2, and 3 designation came from studies on laccases,



elegant spectroscopic investigations by Solomon [62,63] suggested that a trinuclear copper ion cluster must be present in laccase, a finding now supported by x-ray crystal structures on ascorbate oxidase, including oxidized, fully reduced, and other derivative (peroxide, azide) forms (Figure 5) [64,65]. Ascorbate apparently serves to “feed” electrons to the type 1 (T1) copper, which passes electrons through a Cys–His protein pathway to the trinuclear center, where dioxygen reduction occurs [22,61]. For laccase, important advances have occurred in the elucidation of the reaction mechanism of reduced enzyme with dioxygen; Solomon’s group has characterized both two- and four-electron reduced dioxygen intermediates, a hydroperoxo complex, and a species with cleaved O—O bond [22]. Low-resolution but highly informative x-ray structures of ceruloplasmin have recently been published [66,67], revealing two isolated sites of the type 1 variety, plus a trinuclear cluster with associated type 1 “blue” site similar to that found in ascorbate oxidase. Model studies of $\text{Cu(I)}_3/\text{O}_2$ reactivity have begun to appear [68,69].

“Nonblue.” These include galactose oxidase (GO) and amine oxidases (e.g., plasma amine oxidase, diamine oxidase, lysyl oxidase), which produce dihydrogen peroxide by the two-electron reduction of O_2 [33]. For GO (stereospecific primary alcohol oxidation), spectroscopic studies by Whittaker [70,71] suggest that the two-electron oxidation carried out by a mononuclear copper center is aided by a stabilized ligand-protein radical (i.e., $(\text{L})\text{Cu(I)} + \text{O}_2 \rightarrow (\text{L}^{\bullet+})\text{Cu(II)} + \text{H}_2\text{O}_2$), obviating the need to get to Cu(III) in the catalytic cycle. Protein x-ray structures [33,72] reveal a novel copper protein cofactor, which would seem

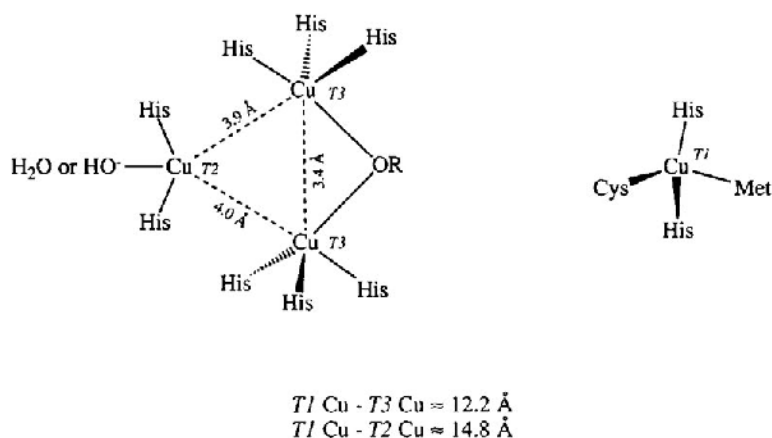


Figure 5 Tetranuclear copper cluster of the ascorbate oxidase active site.



to be set up for stabilization of a radical-cation species. Here, a thioether bond links a Cys to a Cu-coordinated Tyr ligand, and the latter also undergoes a stacking interaction with a Trp residue (Figure 6). Interesting synthetic models for this active site and its chemistry, both structural and functional, have recently been described [73–80].

Amine oxidases (oxidative deamination of amines) have been long known to possess a carbonyl-containing cofactor (CoF), which at various times has been thought to be pyridoxal phosphate and pyrroloquinoline quinone (PQQ). Klinman et al. [33] first showed that for bovine serum enzyme, the cofactor is a peptide-bound quinone derived from a protein tyrosine, topaquinone (TPQ) (Scheme 2). Two recent protein x-ray crystal structures (from *Escherichia coli* [81] and eukaryotic pea seedling [82]) confirm this conclusion, while also verifying the spectroscopically deduced coordination environment, with three histidine ligands (Scheme 2). The TPQ cofactor lies close to the copper ion, but is not coordinated, and it is deduced that this group is conformationally flexible. The enzyme reaction involves initial Schiff-base formation of amine substrate with quinone [33]. The role of copper is to facilitate reoxidation by O_2 of the reduced cofactor (after the substrate reactions); a Cu(I)-semiquinone intermediate (in equilibrium with Cu(II)-quinone) has been detected and is presumed to interact directly with O_2 [33]. Another aspect of considerable interest is the self-processing of TPQ, which is derived from a protein tyrosine precursor. Copper ion is implicated in this cofactor biogenesis and mechanistic schemes involving copper-dioxygen chemistry have been presented [33,83].

Cytochrome-c Oxidase. Readers are referred to a comprehensive review [84] on this enzyme. Considerable advances are from two recent protein x-ray structures [85,86], now detailing the general picture previously provided by biochemical and spectroscopic studies. A cysteine-bridged dinuclear Cu_A center

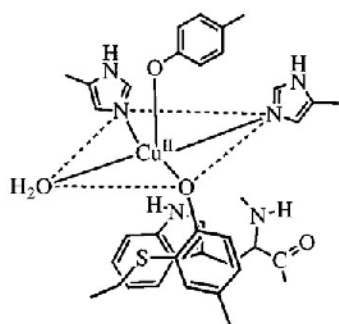
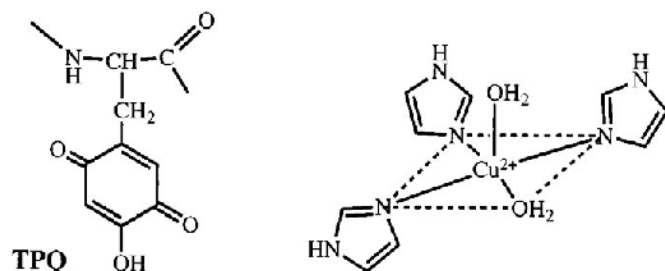


Figure 6 Galactose oxidase active site with its coordinated redox active cofactor.





Scheme 2

functions as the initial site of electron entry and transfer. A heme- α_3 /Cu_B center (Cu . . . Fe = 4–5 Å; Cu_B with three histidine ligands) is the site of O₂ binding, reduction, and O—O cleavage to water. Iron(II)-oxy (analogous to oxy-hemoglobin), peroxy, and ferryl Fe=O intermediates have been detected [84].

Other Copper Oxidases. A number of additional multicopper oxidases have been detected [22], including phenoxazinone synthase (Table 1). This enzyme catalyzes the overall six-electron oxidative coupling of 2-aminophenols to form 1-aminophenoxazinone, the final step in the bacterial (*Streptomyces*) biosynthesis of the antineoplastic agent actinomycin.

I.B.4. Superoxide Dismutase

Cu—Zn superoxide dismutases (SODs) [87,88] are abundant in eukaryotic cells and may serve to protect cells against the toxic effects of superoxide or deleterious oxy-products derived from O₂^{•−}. The active site copper and zinc ions are 6.3 Å apart and are bridged by a histidine imidazolate. In the oxidized form Cu(II) is roughly pentacoordinate, with four His N's and a water molecule. A highly conserved Arg residue is thought to stabilize Cu(II)-bound anions (e.g., Cu(II)—O₂^{•−}); a redox reaction releases O₂, generating Cu(I), which can reduce more O₂^{•−} substrate to give peroxide and Cu(II).

Genetic mutations in Cu—Zn SOD have been linked to the neurodegenerative disease amyotrophic lateral sclerosis (ALS), Lou Gehrig's disease [89].

II. DISCRETE Cu_nO₂ COMPLEXES

II.A. Earlier Suggestions

Unstable copper dioxygen intermediates have been commonly postulated in mechanistic schemes describing the auto-oxidation of Cu(I) complexes or the



role of copper ion in catalytic oxidations of organic substrates [1–4]. In many cases, such formulations were based on little more than the assumption that Cu(I)/Cu(II) redox reactions are generally expected to proceed via an inner sphere mechanism rather than outer sphere electron transfer. In other cases, kinetic evidence has been somewhat more substantial. For instance, effective competition between one- and two-electron reduction paths, the latter necessarily involving a primary 1:1 adduct Cu_2O_2^+ , have been implicated from detailed kinetic studies (using a series of substituted imidazoles as supporting ligands [90,91]).



$$-\frac{d[\text{O}_2]}{dt} = \frac{k[\text{Cu}_{\text{aq}}^+][\text{O}_2](1 + k'[\text{H}^+])}{1 + k''[\text{H}^+]} \quad (3)$$

Also, the hydron-mediated auto-oxidation of the aqua ion $(\text{Cu}_{\text{aq}})^+$ cannot be rationalized without the primary formation of a dioxygen adduct according to Equation 1 [8,92]. Although there is little doubt that, on this basis, unstable Cu_2O_2^+ are a standard species in Cu(I) auto-oxidation, it is equally clear that no details whatsoever regarding their stability and structural properties were known at the time. In fact, all early reports on spectroscopic observation of Cu(I) dioxygen complexes have been found to be based on some kind of misinterpretation. The only hard data concerning such fleeting species have been the bimolecular rate constant of formation starting from $(\text{Cu}_{\text{aq}})^+$, namely, $9.5 \times 10^5 \text{ M}^{-1} \text{ sec}^{-1}$ [92]. The assumption that the dioxygen underwent an overall two-electron reduction to peroxide was also supported by the fact that dihydrogen peroxide could be detected in the reaction mixture of Cu(I) imidazole complexes and dioxygen in protic media [90,93].

Although reports on utilizing the CuCl/amine/ O_2 system for oxidative transformations of organic compounds started to appear more than a century ago, information on its active species and data on the mechanism of the reaction emerged only in the 1970s. Investigations by Bodek and Davies [94,95] suggested that so-called active oxo-Cu(II) species are formed in the course of the reaction of Cu(I) halide-amine complexes with molecular dioxygen in aprotic solvents (which are very basic and responsible for the oxidizing ability of the system). Subsequent kinetic and Raman spectroscopy studies [96,97] on the oxidation of $\text{L}_2\text{Cu}_2\text{Cl}_2$ ($\text{L} = N,N,N',N'$ -tetraethylethylenediamine) by dioxygen in methylene chloride revealed that a tetranuclear, mixed-valence peroxocopper complex is formed at low temperature. Solid mixed-valence copper dioxygen complexes ($\text{L}_3\text{Cu}_4\text{Cl}_4\text{O}_2$, where L is substituted pyridine) have been isolated on exposure of Cu(I) chloride-L complexes to dioxygen in diethyl ether suspensions [98]. These compounds are only stable in the solid state, and evidence for their peroxo character is based on the observations that (1) the dioxygen could be recovered



quantitatively by controlled thermolysis, (2) acidification results in the formation of dihydrogen peroxide, and (3) reaction with 9,10-phenanthrenequinone produces ring cleavage, probably through a typical peroxide mechanism [99].

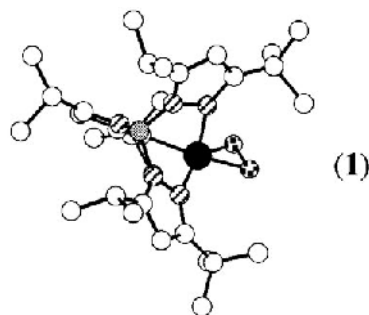
Despite the implication for Cu_nO_2 species from these studies and a few other initial reports of possible Cu_2O_2 complexes using polydentate ligands [3,6,100], it was not until the mid-1980s that well-characterized complexes (i.e., those having a definitive structure with intact O—O bonds, based on vibrational investigations or an actual x-ray structure) were described.

II.B. Structurally/Spectroscopically Characterized Cu_nO_2 Species

Summaries of Cu_nO_2 complexes and their properties have appeared [3,6,7,9,10]. Here, we will mention some aspects already covered but highlight newer results and insights, particularly for cases where crystallographic studies have elucidated Cu_2O_2 structure.

II.B.1. Mononuclear CuO_2 Complexes

Recent developments [101] are the generation and x-ray structural characterization of $\text{Cu}(\text{O}_2)(\text{HB}(3\text{-tBu-5-}i\text{Prpz})_3)$ (**1**; Scheme 3) ($\text{HB}(3\text{-tBu-5-}i\text{Prpz})_3 = \text{hy-$



Scheme 3

drido-tris(3-*tert*-butyl-5-*iso*-propyl)pyrazolylborate anion), with side-on bound $\eta^2\text{-O}_2^-$ ligand. This is formed by adding dioxygen to cold solutions of $\text{Cu}(\text{dmf})(\text{HB}(3\text{-tBu-5-}i\text{Prpz})_3)$ (dmf = dimethylformamide). Dioxygen binding is reversible as demonstrated by cycling experiments followed by UV-Vis spectroscopy (for **1**, $\lambda_{\text{max}} = 352$ ($\epsilon = 2330$), 510 (230), 660 (sh) nm). Other physical characteristics of interest are that **1** is diamagnetic (^1H NMR spectroscopy and magnetism), and it exhibits a $\nu(\text{O—O})$ at 1111 cm^{-1} (1062 cm^{-1} using $^{18}\text{O}_2$) in



resonance Raman spectra. Corresponding values of 1111 and 1060 cm^{-1} were seen in resonance Raman experiments. These properties are in accord with the superoxo-copper(II) formulation for **1**, and consistent with the observed O—O bond distance of 1.22 (3) Å. A previous report [102] that the ethylene complex $\text{Cu}^1(\text{C}_2\text{H}_4)(\text{HB}(\text{Me}_2\text{pz})_3)$ formed a superoxo species is called into question by these researchers [10,101], since the properties of that O_2 -adduct product match those of the structurally characterized μ -peroxo complex $[\text{Cu}(\text{HB}(\text{iPr}_2\text{pz})_3)]_2(\text{O}_2)$ (**5**), discussed later.

Other investigations involving reactions of O_2 with $[\text{LCu}(\text{I})]^+$ complexes, where L is a tripodal tetradentate ligand such as L = tmpa = tris[(2-pyridyl)methyl]amine, have resulted in significant advances in our understanding of Cu_2O_2 complexes, since x-ray structural analysis of $[\{(\text{tmpa})\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**3**) was possible. This will be discussed in some detail. Here, we mention that a kinetic study [14,103] of O_2 binding in this system reveals the presence of a primary mononuclear O_2 adduct $[(\text{tmpa})\text{CuO}_2]^+$, for which kinetic, thermodynamic, and electronic spectral parameters have been determined (see Section II.C.).

II.B.2. Dinuclear Cu_2O_2 Complexes

Structurally Characterized Cu_2O_2 Compounds.

A trans- μ -1,2-peroxo dicopper(II) complex. The first structurally characterized Cu_2O_2 species was $[\{(\text{tmpa})\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**3**), formed from the reaction of $[(\text{tmpa})\text{Cu}(\text{RCN})]^+$ (**2**) with dioxygen at -80°C in EtCN or CH_2Cl_2 solvent ($\text{Cu}:\text{O}_2 = 2:1$, manometry) (Figure 7) [104,105]. Complex **3** is intensely purple, with strong UV-Vis absorptions at 440 ($\epsilon = 2000 \text{ M}^{-1}\text{cm}^{-1}$), 525 (11,500), and 590 (sh, 7600) nm and an additional *d-d* band at 1035 nm [180].

Although strong binding occurs at reduced temperatures, the O_2 (and CO) coordination to $[(\text{tmpa})\text{Cu}(\text{RCN})]^+$ (**2**) is reversible; this is shown by the reactions interconverting complexes **2**, **3**, and $[(\text{tmpa})\text{Cu}(\text{CO})]^+$ or $[(\text{tmpa})\text{Cu}(\text{PPh}_3)]^+$. When a vacuum is applied to $[\{(\text{tmpa})\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**3**) in EtCN while heating briefly, the purple solution decolorizes and $[(\text{tmpa})\text{Cu}(\text{RCN})]^+$ (**2**, R = Et) is produced. Rechilling ($< -80^\circ\text{C}$) followed by introduction of O_2 regenerates **3** and this procedure can be repeated several times without severe decomposition. Dioxygen can also be displaced from **3** by reaction with either CO or PPh_3 (in EtCN) to give the adduct $[(\text{tmpa})\text{Cu}(\text{CO})]^+$ or $[(\text{tmpa})\text{Cu}(\text{PPh}_3)]^+$. In both cases, the O_2 evolved can be identified, and near-quantitative (95%) evolution of O_2 is observed by manometry for PPh_3 . Carbon monoxide can be used to effect repetitive ‘‘carbonyl cycling’’; in EtCN, O_2 is displaced from **3**, giving $[(\text{tmpa})\text{Cu}(\text{CO})]^+$, the CO is removed via vacuum/Ar-purge cycles at room temperature, providing $[(\text{tmpa})\text{Cu}(\text{RCN})]^+$ (**2**), and rechilling of the solution of **2** followed by oxygenation regenerates **3** [104,105].

Thermally and hydrolytically unstable crystals of $[(\text{tmpa})\text{Cu}_2(\text{O}_2)]$ -



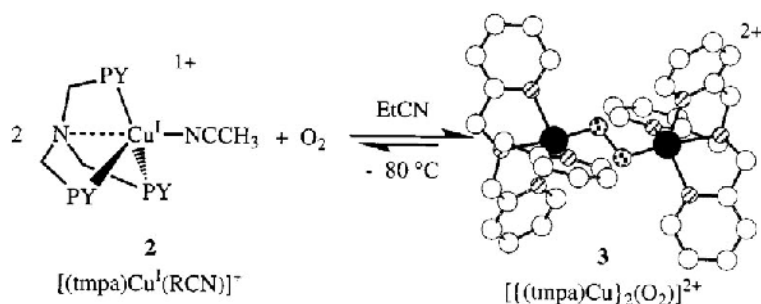


Figure 7 Reversible binding of O_2 to **2** to give structurally characterized species **3**, a *trans*- μ -1,2-peroxo dicopper(II) complex.

$(\text{PF}_6)_2 \cdot 5\text{Et}_2\text{O}$ (**3**) were obtained at -85°C in EtCN and x-ray data were obtained at -90°C . The centrosymmetric complex is best described as a peroxo dicopper(II) species. It contains a *trans*- μ -1,2- O_2^{2-} group (derived from O_2) bridging the two Cu(II) ions (Figure 7). The Cu atom is pentacoordinate with a distorted trigonal bipyramidal geometry and the peroxo oxygen (O1) atoms occupy axial sites. The Cu—Cu' separation is 4.359(1) Å and the O1—O1' bond length is 1.432(6) Å, which are structural parameters similarly found for peroxo-bridged dicobalt(III) complexes.

In spectroscopic and theoretical studies [106], both an intraperoxide O—O stretch (832 cm^{-1}) and a copper—oxygen stretch (561 cm^{-1}) have been identified using resonance Raman spectroscopy, and the three strong visible absorptions have been assigned as peroxo-to-copper charge transfer transitions. The 832 cm^{-1} stretching vibration fixes the oxidation state of the dioxygen moiety in $[(\text{tmpa})\text{Cu}]_2(\text{O}_2)^{2+}$ (**3**) as O_2^{2-} , consistent with the presence of a *d-d* band at 1035 nm, expected for Cu(II) but not Cu(I). Complex **3** is EPR-silent and exhibits a nearly normal ^1H NMR spectrum, suggesting that it is essentially diamagnetic. Magnetic susceptibility measurements performed on **3** confirm this assumption, with a finding that $-2J > 700\text{ cm}^{-1}$, based on $H_{\text{ex}} = -2JS_1 \cdot S_2$ [107]. These results indicate that a single bridging O_2^{2-} ligand can mediate strong magnetic coupling between Cu(II) ions even at 4.35 Å.

A side-on μ - η^2 : η^2 -peroxo dicopper(II) complex. A very important development in copper-dioxygen chemistry occurred in 1989 with the report by Kitajima et al. [10,108] that another Cu_2O_2 species could be prepared and structurally characterized by using copper complexes with a substituted anionic tris(pyrazolyl)borate ligand. This intensely purple compound, $\{\text{Cu}[\text{HB}(3,5\text{-iPr}_2\text{pz})_3]\}_2(\text{O}_2)$ (**5**), was prepared either by reaction of $\text{Cu}[\text{HB}(3,5\text{-iPr}_2\text{pz})_3]$ (**4**) with O_2 or by careful addition of aqueous hydrogen peroxide to the μ -dihydroxo



dimer complex $\{\text{Cu}[\text{HB}(3,5\text{-iPr}_2\text{pz})_3]\}_2(\text{OH})_2$ (**6**, Figure 8) [10,108]. Compound **5** is a stable solid at room temperature or even in solution at mildly reduced temperatures (e.g., -20°C).

The most important feature observed in the x-ray structure of **5** is the side-on $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo}$ coordination that holds the two $\text{Cu}[\text{HB}(3,5\text{-iPr}_2\text{pz})_3]$ units together (Figure 8). This is a rare example of such a peroxo coordination in *d*-block chemistry; there is a V complex [109] and other examples are known. The O—O bond distance of 1.412 (12) Å is typical of peroxide, and the Cu . . . Cu distance is 3.560 Å. The geometry about each Cu(II) ion is distorted square pyramidal with two shorter Cu—N bonds and a longer (2.258 Å) axial pyrazolyl interaction [10,108].

The physical characteristics reported for **5** are striking, especially in their seemingly close relationship to those observed for the protein oxy-Hc. The Cu...Cu distance in **5**, its diamagnetism (normal ^1H NMR; Evans susceptibility), and the electronic spectrum with 349 nm ($\epsilon = 21,000$) and 551 nm ($\epsilon = 800$) bands closely resemble the properties of oxy-Hc. The particularly low value (even for peroxide) of the O—O stretch of 741 cm^{-1} (resonance Raman) in **5** also matches corresponding values seen in oxy-Hcs ($\sim 750\text{ cm}^{-1}$); this latter property has been accounted for in theoretical studies [110], which show that the unoccu-

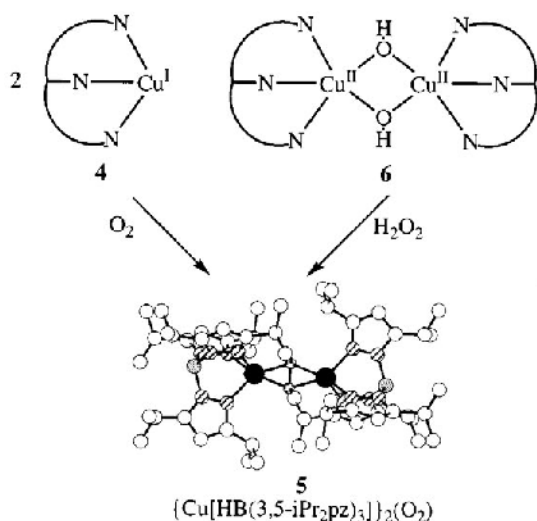


Figure 8 Reactions generating complex **5**, a structurally characterized $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo}$ dicopper(II) complex.



pied peroxide σ^* orbital can act as a π acceptor in a $\mu\text{-}\eta^2\text{:}\eta^2$ -peroxo coordination such as seen in 5.

Kitajima's structural elucidation and characterization of 5 have completely turned around our thinking about Cu_2O_2 structure and chemistry, because along with other information, the definite demonstration of the viability of a $\mu\text{-}\eta^2\text{:}\eta^2$ -peroxo coordination in copper chemistry strongly suggests that this moiety is present in proteins such as oxy-Hc and oxy-Tyr.

Other $\text{Cu}:\text{O}_2 = 2:1$ Complexes. A number of other Cu_2O_2 species for which x-ray crystal structures are not available have nevertheless been well characterized: Complex $[\text{Cu}_2(\text{XYL}-\text{O})(\text{O}_2)]^+$ (**8**, Figure 9, PY = 2-pyridyl) was one of the first. It was derived from a phenoxo-bridged dicopper(I) complex $[\text{Cu}_2(\text{XYL}-\text{O})]^+$ (**7**) that was structurally characterized by x-ray studies [111], and full resonance Raman [112] and EXAFS spectroscopic investigations [113] have been carried out. Species **8** is formed in dichloromethane solution as a deep purple compound at -80°C by reaction of O_2 ($\text{Cu}:\text{O}_2 = 2:1$, manometry). A resonance enhanced Raman band at 803 cm^{-1} fixes the oxidation state of the O_2 ligand as O_2^{2-} . This band shifts to 750 cm^{-1} when using $^{18}\text{O}_2$ to generate **8**. A copper-peroxide stretch is found at 488 cm^{-1} (464 cm^{-1} for $^{18}\text{O}_2$), and this absorption was used in the analysis of a mixed isotope experiment with $^{16}\text{O}-^{18}\text{O}$, which showed two components at 465 and 486 cm^{-1} . Since the EXAFS data provided a $\text{Cu}\cdots\text{Cu}$ distance of $3.31\text{ \AA}$ for **8**, a μ -phenolato- μ -1,1-peroxo coordination is ruled out, leaving either a $\text{Cu}-\text{O}-\text{O}$ -terminal binding to a single copper ion or an unsymmetrical bridging $\text{Cu}-\text{O}-\text{O}\cdots\text{Cu}$ as possibilities. A $\mu\text{-}\eta^2\text{:}\eta^2$ -peroxo coordination does not fit with either the spectroscopic data (i.e., more than 2 CT absorptions for bridging peroxo complex) or the vibrational data; this suggests that the peroxide O atoms are inequivalent.

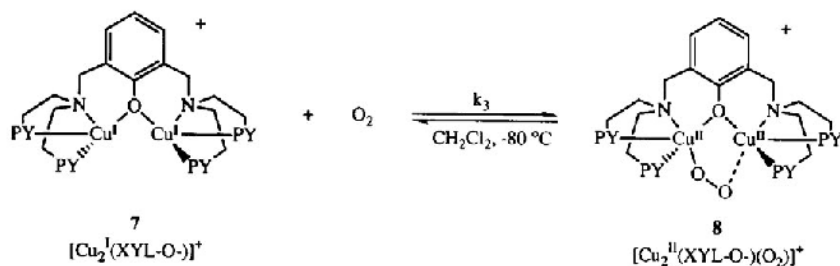


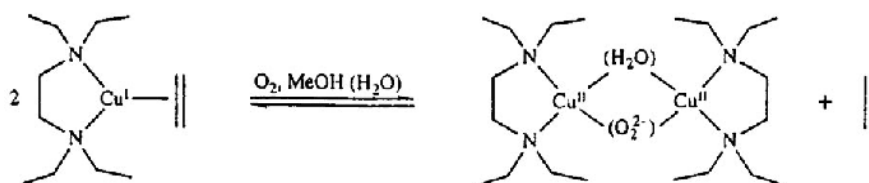
Figure 9 Reversible reaction of O_2 giving phenoxo-bridged complex **8**, with terminally (or unsymmetrically) bound peroxo ligand.



The resonance Raman enhancement profile studies allowed a UV-Vis band assignment, with strong 505 ($\epsilon = 6300$) and 610 ($\epsilon = 2400$) nm absorptions assigned to $\pi_{\sigma}^* \rightarrow d_{x^2-y^2}$ and $\pi_v^* \rightarrow d_{x^2-y^2}$ peroxide-to-Cu(II) LMCT transitions. For terminal peroxo coordination to a single Cu(II) ion, the presence of two such absorptions is expected on theoretical grounds. A structure with O_2^{2-} bridging the two copper ions would require equatorial peroxide coordination to a second Cu(II) in trigonal bipyramidal coordination, for example; analogous phenolate-bridged Cu(II) dinuclear complexes are known.

Sorrell [114] has prepared ligands similar to that found in **8**, but possessing pyrazole or mixed pyrazole/pyridine ligands. The corresponding phenoxo-bridged dicopper(I) complexes appear to bind O_2 in the same fashion; these O_2 complexes all exhibit a characteristic purple color with strong 500–510 and 610–630 nm bands that are presumed also to be peroxo-to-Cu(II) LMCT transitions.

After his early success in generating a superoxo-Cu(II) complex using commercially available ligands, Thompson also generated an interesting peroxo dicopper(II) complex using ethylenediamine derivatives [115,116]. Thus, when a Cu(I) ethylene complex $[Cu(teen)(C_2H_4)]ClO_4$ (teen = *N,N,N',N'*-tetraethylethylenediamine) is reacted with dioxygen, deep blue solutions develop and a blue powder can be isolated at reduced temperatures. Analytical and spectroscopic data suggest the formulation $[Cu_2(teen)_2(H_2O)(O_2)](ClO_4)_2$ (**9**; Scheme 4), a



Scheme 4

9

μ -peroxodicopper(II) complex. Species **9** is EPR-silent and the UV-Vis features are typical of square-planar teen-Cu(II) complexes; the 3570, 1670, and 1620 cm^{-1} bands indicate water coordination. Unpublished vibrational studies are also consistent with the peroxo assignment [3], since an 825 cm^{-1} infrared (IR) band shifts to 770 cm^{-1} when using $^{18}O_2$. Reactions of **9** with ethylene or CO give $[Cu(teen)(C_2H_4)](ClO_4)$ and $[Cu(teen)(CO)](ClO_4)$, respectively, and when **9** is allowed to stand under a dinitrogen atmosphere, a disproportionation reaction occurs, giving $[Cu_2(teen)_2(OH)_2](ClO_4)_2$. When **9** is allowed to stand at room temperature under a dioxygen atmosphere, hydroxylation of an ethyl substituent occurs [3,115]; this suggests that the peroxo ligand has a close proximity to the



ethyl group (unless activation of coordinated teen allows direct attack by free O_2).

Xylyl and Nn ligand complexes. In our own laboratories, we have extensively studied copper complexes containing dinucleating ligands where two bis[2-(2-pyridyl)ethyl]amine (PY2) units are linked to the amino nitrogen by a variable hydrocarbon moiety. This can be either a xylyl spacer group or one with a variable methylene chain $-(CH_2)_n-$, where $n = 3, 4$, or 5 . Both tricoordinate dicopper(I) compounds $[Cu_2(R-XYL-H)]^{2+}$ (**10**, Figure 10) and $[Cu_2(Nn)]^{2+}$ (**13**, Figure 11) react with O_2 in a reversible manner, producing $[Cu_2(R-XYL-H)(O_2)]^{2+}$ (**11**) and $[Cu_2(Nn)(O_2)]^{2+}$ (**15**), respectively; spectroscopic comparisons (e.g., the number of bands, their position and intensity) indicate they possess very similar peroxo-dicopper(II) structures. Oxyhemocyanin possesses qualitatively similar absorptions, and this, in part, led us to suggest that **11** and **15** may be reasonable spectroscopic and structural models for the protein active site [117,118].

The xylyl systems (Figure 10) were actually the first ones described in our laboratories, with the tricoordinate dicopper(I) complex $[Cu_2(R-XYL-H)]^{2+}$ (**10**, $R = H$) serving initially as a crude model for deoxy-Hc [119]. Further reac-

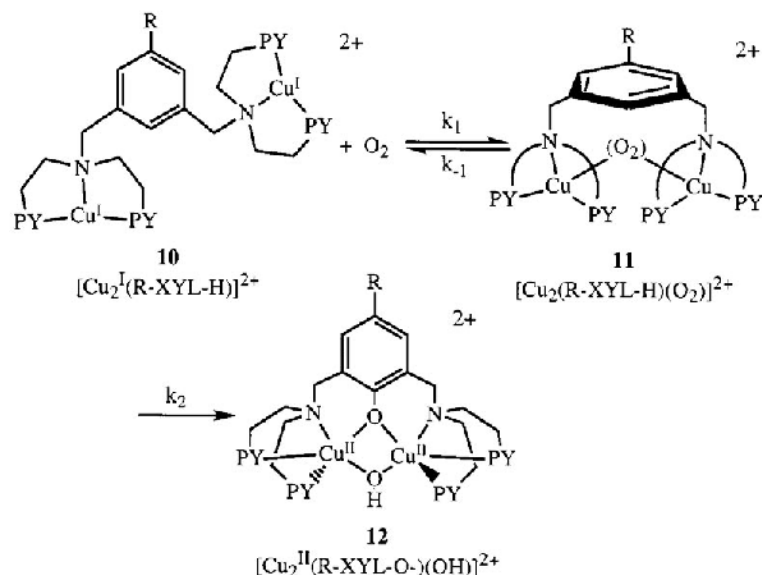


Figure 10 A tyrosinase model system: reversible oxygenation of xylyl dicopper(I) complex **10** to give **11**, followed by hydroxylation to give **12**.



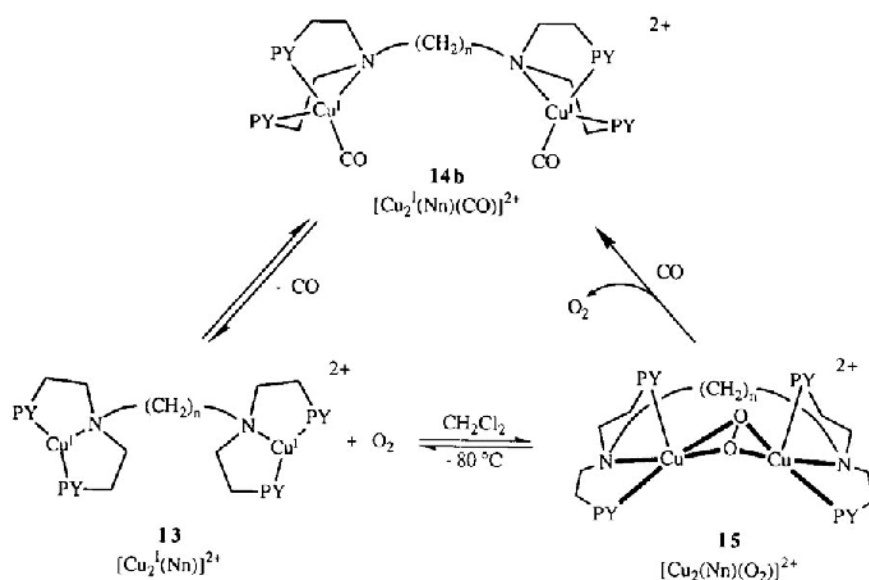


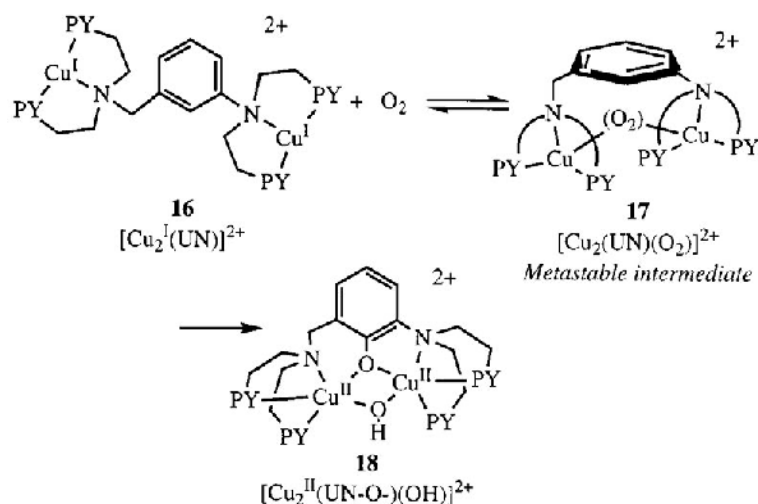
Figure 11 Reversible O_2 and CO binding to dicopper(I) complex **13** ($n = 3\text{--}5$). Complexes **15** possess $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo}$ coordination.

tion of $[\text{Cu}_2(\text{R}\text{---}\text{XYL}\text{---}\text{H})(\text{O}_2)]^{2+}$ (**11**) results in the hydroxylation of the xylyl moiety (Figure 10), which provides the phenolic ligand precursor for complexes **7** and **8**. This important tyrosinase model, reaction **11** $\rightarrow$ **12**, is described in Section III.B.1.

Low-temperature stopped-flow kinetic studies (see Section II.C.1) [14,120] provided the first evidence concerning the O_2 binding to $[\text{Cu}_2(\text{R}\text{---}\text{XYL}\text{---}\text{H})]^{2+}$ (**10**, $\text{R} = \text{H}$) (i.e., that the process is reversible and the $\text{Cu}:\text{O}_2$ stoichiometry is 2:1). Simultaneous spectral analysis ($\lambda > 360$ nm) revealed the very distinctive features attributable to complex $[\text{Cu}_2(\text{R}\text{---}\text{XYL}\text{---}\text{H})(\text{O}_2)]^{2+}$ (**11**), including a strong band in the range 435–440 nm ($\epsilon = \sim 3000\text{--}5000$) with weaker absorptions at lower energy. Supporting evidence comes from studies of synthetic analogs of the “parent” xylyl system, $[\text{Cu}_2(\text{R}\text{---}\text{XYL}\text{---}\text{H})(\text{O}_2)]^{2+}$ (**11**, $\text{R} = \text{NO}_2$, F , CN). For these derivatives, reactions with O_2 are reasonably fast (see Section II.C.1) and hydroxylation still occurs, but the latter process is slowed to the point that the $[\text{Cu}_2(\text{R}\text{---}\text{XYL}\text{---}\text{H})(\text{O}_2)]^{2+}$ (**11**) intermediates are stabilized (-80°C) and observable by classical spectroscopy [121]. The peroxo species all exhibit absorption bands similar to that of $[\text{Cu}_2(\text{Nn})(\text{O}_2)]^{2+}$ (**15**) (e.g., $\lambda_{\text{max}} = 350$ ($\epsilon = 20,000$) and 435 ($\epsilon = 5000$) nm for $\text{R} = \text{NO}_2$, and $\lambda_{\text{max}} = 360$ ($\epsilon = 10,000$) and



435 ($\epsilon = 3000$) nm for R = F). Stabilization of a relevant peroxo-dicopper(II) intermediate is particularly striking in a case where we synthesized an unsymmetrical analog $[\text{Cu(I)}_2(\text{UN})]^{2+}$ (**16**) [122]. Low-temperature oxygenation provides a species $[\text{Cu(II)}_2(\text{UN})(\text{O}_2)]^{2+}$ (**17**) ($\lambda_{\text{max}} = 360$ ($\epsilon = 11,000$) and 520 ($\epsilon = 1000$) nm) that is so stable that the O_2 can be removed and cycling between **16** and **17** is possible (Scheme 5). Warming to room temperature results in the high-yield



Scheme 5

isolation of a hydroxylated product $[\text{Cu(II)}_2(\text{UN—O—})(\text{OH})]^{2+}$ (**18**); a labeling experiment proved that the phenoxo oxygen atom is derived from dioxygen. Incidentally, complex **18** can be reduced to a dicopper(I) complex $[\text{Cu(I)}_2(\text{UN—O—})]^+$, which reacts to form a dioxygen adduct $[\text{Cu}_2(\text{UN—O—})(\text{O}_2)]^+$ having greater thermal stability than the analogous compound $[\text{Cu}_2(\text{XYL—O—})(\text{O}_2)]^+$ (**8**) (Figure 9) [123]. In addition, a 2:1 superoxo dicopper(II) complex $[\text{Cu}_2(\text{UN—O—})(\text{O}_2)]^{2+}$ can be obtained from one-electron oxidation of $[\text{Cu}_2(\text{UN—O—})(\text{O}_2)]^+$ (by Ag^+), or by reversible addition of O_2 to the mixed-valent species $[\text{Cu(I)Cu(II)}(\text{UN—O—})]^{2+}$ [124].

Another observation relating the structures of $[\text{Cu}_2(\text{Nn})(\text{O}_2)]^{2+}$ (**15**) and $[\text{Cu}_2(\text{R—XYL—H})(\text{O}_2)]^{2+}$ (**11**) comes from the examination of a 2-fluoro-substituted complex $[\text{Cu}_2(\text{XYL—F})]^{2+}$ (**19**), which does not hydroxylate when reacted with dioxygen. At -80°C , the reaction of **19** with O_2 generates a species formulated as $[\text{Cu}_2(\text{XYL—F})(\text{O}_2)]^{2+}$ (**20**), based on manometric measurements showing that $\text{Cu}:\text{O}_2 = 2:1$. The UV-Vis spectral characteristics for **20** also closely match those observed for $[\text{Cu}_2(\text{R—XYL—H})(\text{O}_2)]^{2+}$ (**11**) and



$[\text{Cu}_2(\text{Nn})(\text{O}_2)]^{2+}$ (**15**), in particular the N5 derivative, which also has a five-carbon chain connecting the tridentate PY2 units [125].

Either $[\text{Cu}_2(\text{Nn})]^{2+}$ (**13**, tricoordinate Cu(I)) or $[\text{Cu}_2(\text{Nn})(\text{CH}_3\text{CN})_2]^{2+}$ (**14a**, tetracoordinate Cu(I)) can be reacted with O_2 at -80°C in CH_2Cl_2 [126,127], producing deep brown or purple solutions of species $[\text{Cu}_2(\text{Nn})(\text{O}_2)]^{2+}$ (**15**). As indicated in Figure 11 (PY = 2-pyridyl), the binding of both O_2 and CO to **13** is reversible. Carbon monoxide binding to **13** is stronger than that of O_2 , since CO can displace O_2 from **15** to give $[\text{Cu}_2(\text{Nn})(\text{CO})_2]^{2+}$ (**14b**, Figure 11). The relative binding strength of CO versus O_2 to reduced copper parallels that observed for heme proteins and porphyrin-iron(II) complexes.

As already indicated, $[\text{Cu}_2(\text{Nn})(\text{O}_2)]^{2+}$ (**15**) possess striking UV-Vis properties, with multiple and strong charge-transfer absorptions. The position and relative intensities of these bands vary with the length of the polymethylene unit connecting the two PY2 donor groups [126,127], reflecting subtle changes in the mode of O_2 binding. The characteristic 350–360 nm band with $\epsilon = 16,000$ – $21,000 \text{ M}^{-1}\text{cm}^{-1}$ dominates; the presence of this distinctive intense absorption (also seen in **5**) in part provides indications for the possible close relationship of $[\text{Cu}_2(\text{Nn})(\text{O}_2)]^{2+}$ (**15**) and $[\text{Cu}_2(\text{R}-\text{XYL}-\text{H})(\text{O}_2)]^{2+}$ (**11**) to the Cu_2O_2 oxy-Hc chromophore, with its 345 nm ($\epsilon = 20,000$) feature.

Because $[\text{Cu}_2(\text{Nn})(\text{O}_2)]^{2+}$ (**15**) do not undergo the further reactions found for their xylyl relatives, stabilized solutions (i.e., -80°C in CH_2Cl_2) have been amenable to study by various other physical techniques. We have yet to obtain vibrational data for complexes **15**, but a variety of other evidence is consistent with a peroxo-dicopper(II) formulation. Species **15** possess low-energy weak *d-d* absorptions diagnostic of Cu(II), and x-ray absorption measurements carried out on two derivatives (N3 and N4) confirm the Cu(II) oxidation state (XANES, near-edge structure) [118]. The EXAFS data also allow the determination of the Cu...Cu distances, which vary between 3.3 and 3.4 Å, depending on *n*, similar to the distance found in **8**. To account for EXAFS outer shell multiple scattering caused by the pyridine ligands, we proposed a $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo}$ structure for $[\text{Cu}_2(\text{Nn})(\text{O}_2)]^{2+}$ (**15**), shown in Figure 11. The bent ‘butterfly’ structure was suggested in order to accommodate the EXAFS derived. Cu...Cu distances observed (while keeping reasonable Cu—O and O—O bond lengths), which are presumably caused by ligand constraints. Species **15** are EPR-silent, have normal ^1H NMR spectroscopic properties, and exhibit solution diamagnetism [107]; thus, it appears to provide another class of compounds where a single peroxo ligand bridges and strongly antiferromagnetically couples two Cu(II) ions. In light of the crystallographically described $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo}$ dicopper(II) complex of Kitajima et al., the analogous bent formulation in **15** now seems justified.

It should be noted that it is not necessary to have dinuclear complexes with dinucleating ligands Nn, in order to generate such Cu_2O_2 complexes. $[\text{Cu}(\text{RPY2})]^+$ (R = Me, PhCH_2 , Ph) are tricoordinate mononuclear complexes



and oxygenation at -80°C is slow (minutes to hours), but give dinuclear peroxo-dicopper(II) complexes $[\text{Cu}_2(\text{RPY}2)_2(\text{O}_2)]^{2+}$ with $\text{Cu}:\text{O}_2 = 2:1$ (manometry) and spectral properties very similar to those of $[\text{Cu}_2(\text{Nn})(\text{O}_2)]^{2+}$ (**15**) [43]. Analogous reactivity is reported for the corresponding imidazole complexes [128].

In continuing low-temperature kinetic studies of halo-Cu(I)-amine reactions with O_2 , Davies et al. have observed and partially characterized peroxo-copper(II) complexes for $\text{L}_2\text{Cu}_2\text{Cl}_2$ ($\text{L} = \text{teed}$) [96,97]. At room temperature, complete reduction of dioxygen occurs to give green, dinuclear $\text{LCu}(\text{Cl},\text{O},\text{Cl})\text{CuL}$, but at lower temperatures (i.e., $< -26^{\circ}\text{C}$) two forms of a tetranuclear mixed-valence peroxo Cu(II) complex exist in equilibrium. In particular, one of the forms is associated with 380 ($\epsilon = 1600 \text{ M}^{-1} \text{ cm}^{-1}$) and 650 ($\epsilon = 650$) nm electronic spectral absorptions and a resonance Raman $\nu_{\text{O}-\text{O}}$ band at 822 cm^{-1} .

Kida et al. [129] have structurally characterized a dicopper(I) complex using a novel macrocycle, $[\text{Cu}_2(\text{tpmc})]^{2+}$ ($\text{tpmc} = 1,4,8,11\text{-tetrakis}(2'\text{-pyridylmethyl})\text{-}1,4,8,11\text{-tetra-azacyclotetradecane}$). This species contains two tricoordinate Cu(I) ions, which are $6.74 \text{ \AA}$ apart. Oxygenation in solution below -60°C quasi-reversibly affords a mixture of peroxo products, based on resonance Raman studies ($\nu_{\text{O}-\text{O}} = 820$ and 771 cm^{-1} with $\nu_{\text{Cu}-\text{O}} = 529$ and 491 cm^{-1}). Interestingly, warming this solution leads to the isolation of bis(pyridine-2-carboxylato)-copper(II), indicating ligand oxidation and C—N bond cleavage.

A Cu_2O_2 complex with imidazole ligands. Although imidazole coordination from side-chain histidine residues pervades Cu-enzyme chemistry, none of the low-molecular-weight complexes that we have described is based exclusively on imidazole nitrogen donors. This is in spite of the reasonable amount of effort put into the synthesis of polyimidazole ligands and subsequent coordination chemical investigations. However, we have been able to accomplish this goal by employing low-temperature techniques along with a simple imidazole ligand [130]. Whereas the two-coordinate complex $[\text{L}_2\text{Cu}]\text{PF}_6$ ($\text{L} = 1,2\text{-dimethylimidazole}$) is unreactive toward O_2 , three-coordinate $[\text{L}_3\text{Cu}]\text{PF}_6$ (**21**, Figure 12) reacts readily at -90°C to give a brown solution of the peroxo-dicopper(II) complex $[\{\text{L}_3\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**22**) [UV-Vis: λ_{max} (ϵ , $\text{M}^{-1} \text{ cm}^{-1}$): 346 (sh, 2200), 450 (sh, 1450), 500 (1900) and 650 (600) nm]. Manometric measurements confirm the stoichiometry of O_2 addition. Several lines of evidence are consistent with the peroxo-dicopper(II) formulation. X-ray absorption spectroscopic studies performed on solutions of $[\{\text{L}_3\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**22**) (100 K) confirm a Cu(II) oxidation state assignment and provide two possible structural models for the Cu_2O_2 moiety in this species: a bent $\mu\text{-}\eta^2\text{:}\eta^2$ — or a planar *trans*- $\mu\text{-}1,2$ -peroxo structure (Figure 12). Introduction of *tmpa* to -90°C solutions of **22** causes its immediate conversion to the well-characterized complex $[\{(\text{tmpa})\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**3**), which may be an example of a dynamic “self-assembly” process utilizing a “preformed” Cu_2O_2 core.



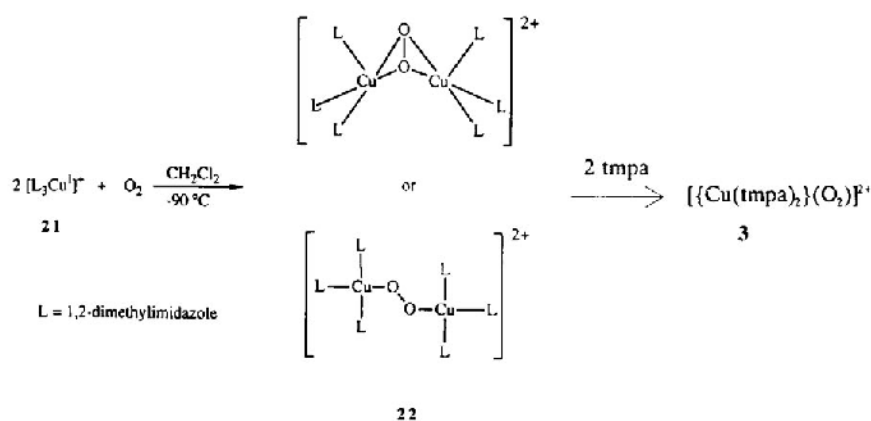


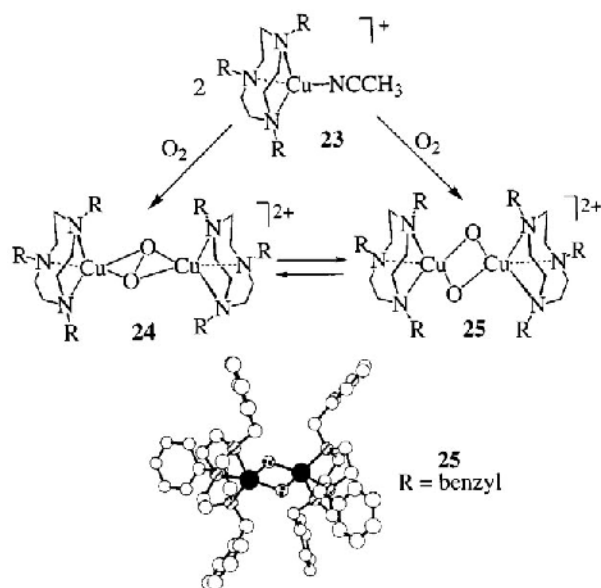
Figure 12 Formation of a peroxo dicopper(II) complex **22** containing a simple imidazole ligand. The “preformed” Cu_2O_2 moiety can be “transferred” by substitution of L donors by tmpa, giving known complex **3**.

In continued studies utilizing copper complexes for synthetically useful oxidative transformations, Capdevielle and Maumy [131] have reported the isolation and preliminary characterization of an oxidizing copper reagent with the raw formula CuO_2H . The EPR-silent species is isolated from the reaction of Cu(II) salts (e.g., $\text{Cu}(\text{NO}_3)_2$) with aqueous dihydrogen peroxide. The room-temperature stable species releases dihydrogen peroxide or dioxygen, depending on conditions, and it is capable of oxidation/oxygenation of substrates such as alcohols, primary amines, and toluene. In the absence of additional spectroscopic data, the authors prefer a $\text{Cu}(\text{III})(\text{O})(\text{OH})$, rather than a peroxyhydroxide $\text{Cu}(\text{II})_2(\text{O}_2)(\text{OH})_2$, description for this species.

Side-on peroxo-dicopper(II) species and a bis- μ -oxo dicopper complex. Since the characterization of side-on peroxo complexes with pyrazolylborate ligands (i.e., **5**) or Nn and xyllyl ligands (i.e., **11** and **15**), others have shown that $\mu\text{-}\eta^2\text{:}\eta^2$ -peroxo-dicopper(II) species also form with tris(1-R-4-R'-imidazolyl)phosphanes [132–134] or trisubstituted triazacyclononane (tacn) ligands. For the latter case, Tolman and coworkers [135,136] have also demonstrated an O—O bond scission reaction. Thus, oxygenation of **23** can give both $\text{Cu}/\text{O}_2 = 2:1$ adducts **24** or **25**. For $\text{R} = i\text{-Pr}$ in CH_2Cl_2 solvent at low temperatures **24** ($\lambda_{\text{max}} = 366 \text{ nm}$, $\nu_{\text{O-O}} = 722 \text{ cm}^{-1}$) is formed reversibly. When the oxygenation is carried out in tetrahydrofuran as the solvent, **25** is obtained. Its properties include UV-Vis features at 324 and 448 nm, and $\nu(\text{Cu}_2\text{O}_2) = 600 \text{ cm}^{-1}$ (580 using $^{18}\text{O}_2$). The equilibrium between **24** and bis- μ -oxo dicopper compound **25** is sensitive to the nature of the counteranion and solvent mixture. Spe-



cies **24** and **25** have been shown to be interconvertible (see Section II.C), an important finding, since the $\mathbf{25} \rightarrow \mathbf{24}$ reaction represents a unique O—O bond-forming reaction. The structure of **25** (R = benzyl) has been elucidated, revealing $\text{O}\cdots\text{O} = 2.287 \text{ \AA}$, with a short value of $\text{Cu}\cdots\text{Cu} = 2.794 \text{ \AA}$. Theoretical arguments suggest a $[\text{Cu}^{\text{III}}_2(\mu\text{-O}^{2-})_2]^{2+}$ assignment for **25** (Scheme 6).

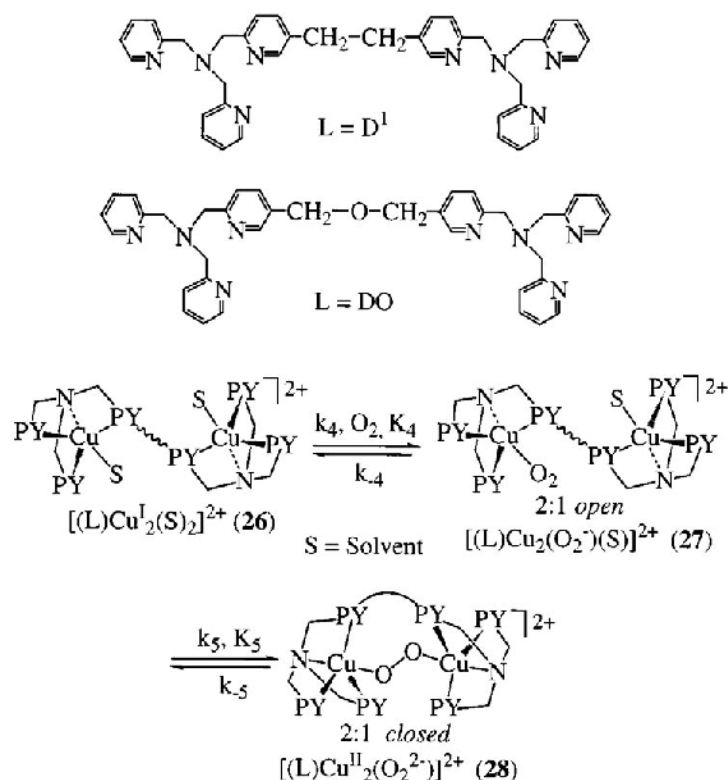


Scheme 6

Room-temperature stable $\text{Cu}:\text{O}_2 = 2:1$ complexes. Generation of solution room-temperature stable Cu_2O_2 adducts has been an elusive goal but was recently achieved by using special dinucleating ligands. The “bis-tmpa” ligand D^1 forms a peroxo-dicopper(II) species $[(\text{D}^1)\text{Cu}^{\text{II}}_2(\text{O}_2^{2-})]^{2+}$ (**28**, $\text{L} = \text{D}^1$) at -80°C , which is spectroscopically analogous to $[\{(\text{tmpa})\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**3**) [14,137]. Kinetic-thermodynamic analysis (Section II.C) reveals this complex to be strained relative to **3**, and warming leads to rearrangements giving a trinuclear species, $[\{(\text{D}^1)\text{Cu}^{\text{II}}_2(\text{O}_2^{2-})\}_3]^{6+}$, with *intermolecular* μ -peroxo moieties [14,137]. Acetone as solvent (compared to EtCN) confers considerable room temperature stability to **28** ($\text{L} = \text{D}^1$), $K_4K_5 > 4 \times 10^3 \text{ M}^{-1}$, and $t_{1/2}$ (decomp.) ~ 40 sec. The ligand DO appears to provide relief of steric constraints, and $[(\text{DO})\text{Cu}^{\text{II}}_2(\text{O}_2^{2-})]^{2+}$ (**28**, $\text{L} = \text{DO}$) fully forms at room temperature and possesses excellent stability, $t_{1/2}$ (decomp.) ~ 60 sec [138] (Scheme 7).

Use of the macrocyclic ligand MEPY22PZ also produces a room-tempera-





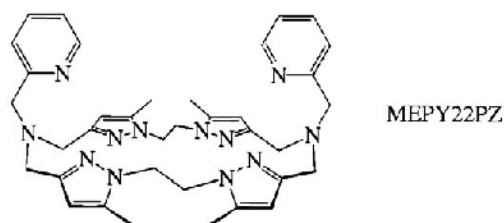
Scheme 7

ture stable Cu_2O_2 adduct $[Cu_2(MEPY22PZ)(O_2)]^{2+}$ (**29**), by oxygenation of dicopper(I) precursor $[Cu^I_2(MEPY22PZ)]^{2+}$ in CH_3CN ($t_{1/2}$ (decomp.) ~ 200 sec) or even in the protic solvent MeOH. Spectroscopic data (e.g., UV-Vis, resonance Raman) for **29** closely match those observed for $[{(tmpa)Cu}_2(O_2)]^{2+}$ (**3**), and thus **29** is similarly assigned a *trans*- μ -1,2-peroxo-dicopper(II) structure [139] (Scheme 8).

II.B.3. A Trinuclear $Cu_3(O)_2$ Complex in a $Cu(I):O_2 = 3:1$ Reaction

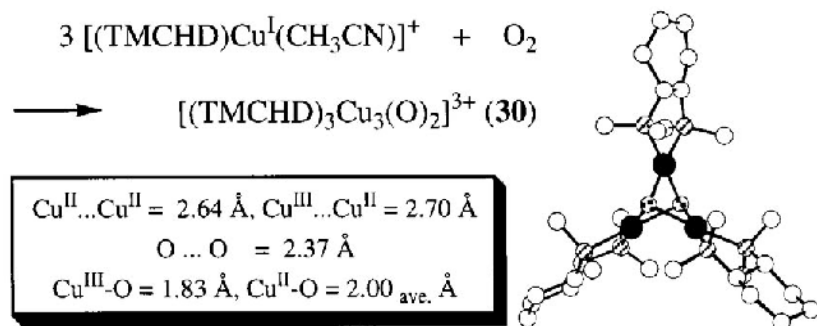
Recent studies [140] using $Me_4(1R,2R)$ -cyclohexanediamine (TMCHD) as ligand reveal a remarkable $Cu/O_2 = 3:1$ reaction of O_2 with the mononuclear complex $[(TMCHD)Cu^I(CH_3CN)]^+$. The product is the trinuclear cluster compound





Scheme 8

$[(\text{TMCHD})_3\text{Cu}^{\text{II}}_2\text{Cu}^{\text{III}}(\mu_3\text{-O}^{2-})_2]^{3+}$ (**30**) ($\lambda_{\text{max}} = 355 \text{ nm}$), with two μ_3 -oxide ligands and three copper ions. An x-ray structure reveals one of these to be unique, and the short Cu—O bond distances and x-ray absorption measurements and comparisons [141] indicate that this is a copper(III) ion, affirming the overall reaction to be a four-electron reduction and O—O cleavage of dioxygen. Varying the nature of the ethylenediamine-type ligand or conditions leads to bis- μ -oxodicopper(III) complexes $[(\text{ligand})_2\text{Cu}_2(\text{O})_2]^{2+}$ (**31**) (i.e., Cu/O₂ = 2:1), with structures similar to **25** [140] (Scheme 9).



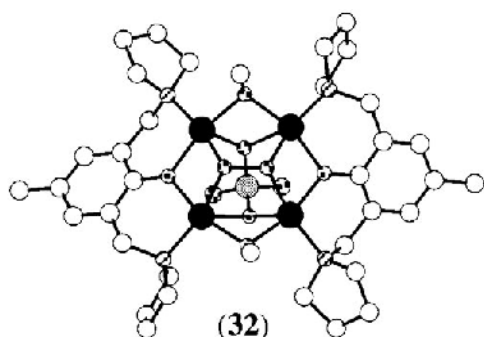
Scheme 9

II.B.4. A Tetranuclear Cu₄O₂ Complex

A novel peroxocopper complex with μ_4 -coordination is formed when copper(II) perchlorate is reacted with the tridentate ligand 4-methyl-2,6-bis(pyrrolidinomethyl)phenol (HL) in methanol solution, in the presence of air, triethylamine base, and 3,5-di-*tert*-butylcatechol [142]. The four copper(II) ions in $[\text{Cu}_4\text{L}_2(\text{O}_2)(\text{OMe})_2(\text{ClO}_4)]^+$ (**32**) form a near-planar rectangle, with alternate methoxo (Cu...Cu = 3.03 Å) and phenolato (Cu...Cu = 2.99 Å) bridging ligands.



Weaker axial coordination is completed by bridging perchlorato oxygen atoms. The peroxide ligand has a typical O—O bond length of 1.453(4) Å (Scheme 10).



Scheme 10

II.B.5. Conclusions and Relevance to Hemocyanin and Tyrosinase

We have seen a quantum leap in progress in the coordination chemistry of copper-dioxygen interactions, resulting in a complete change in thinking about Cu_2O_2 structure and related protein chemistry. The problems of copper ion lability, peroxide disproportionation and air/moisture sensitivity have been overcome, and it has been proved that low-molecular-weight Cu_nO_2 or $\text{Cu}_n(\text{O})_2$ complexes can be prepared, and that several different structural types exist. It is apparent that $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo}$ coordination is present in oxy-Hc and oxy-Tyr.

II.C. Kinetics and Thermodynamics of Formation of Cu_nO_2 Species

In addition to the O_2 -carrier Hc, interactions of O_2 with Cu(I)_n ($n = 1\text{--}3$) have been implicated in a number of other copper proteins, including monooxygenases and oxidases (Section I.B). Although there has been considerable progress in understanding the thermodynamics and kinetics of reversible reactions of O_2 with iron proteins (e.g., hemoglobin, myoglobin, hemerythrin) and synthetically derived iron(II) and cobalt(II) complexes [143–149], corresponding data for Hc have been limited [120,148,150] and no kinetic/thermodynamic data have been available for synthetic copper-dioxygen systems. The spectroscopically and/or structurally characterized copper-dioxygen species described here afford the opportunity to obtain such information [14,151].



This kind of knowledge is of critical importance in determining the contributions of environmental factors such as coordination, coordination geometries, and medium effects toward O_2 affinities and differential binding of O_2/CO . Elucidation of such factors is also indispensable in the development of (1) an understanding of biological dioxygen activation and utilization, (2) practical dioxygen carriers, and (3) the field of metal-catalyzed oxidation and oxygenation with O_2 .

II.C.1. Dinuclear $[Cu_2(L)(O_2)^{2+}]$ Complexes with bis-(2-Pyridylethyl)Amino Coordination

Pseudoreversible dioxygen binding in a $Cu:O_2 = 2:1$ manner has been kinetically studied for $[Cu_2(R-XYL-H)]^{2+}$ (**10**; $R = H, NO_2, tBu, F, CN, OMe$) [14,120,121,151], $[Cu_2(H-XYL-O^-)]^+$ (**7**) [14,120], and $[Cu_2(N4)]^{2+}$ (**13**) [14,152]. In none of the cases have superoxolike intermediates with O_2 bound to a single copper been observed. Kinetic and thermodynamic parameters for oxygenation of (**7**), (**10**), and (**13**) are given in Table 2.

The first system for which kinetic and thermodynamic parameters were obtained involved the oxygenation reaction of complexes $[Cu_2(R-XYL-H)]^{2+}$ (**10**; $R = H, NO_2, tBu, F, CN, OMe$). As discussed in Section II.B.2, these xylyl complexes form O_2 adducts $[Cu_2(R-XYL-H)(O_2)]^{2+}$ (**11**), which have been suggested to have the same $\mu-\eta^2:\eta^2$ -peroxo structure as proposed for $[Cu_2(Nn)(O_2)]^{2+}$ (**15**). The oxygenation of **10** effects the stoichiometric hydroxylation of the arene ligand to produce $[Cu_2(R-XYL-O-)(OH)]^{2+}$ (**12**, Figure 10), a process closely mimicking the action of the copper monooxygenase tyrosinase [153]. This latter aspect is discussed in Section III.B. Here we focus on the first part of the reaction, involving the reversible binding of O_2 to **10**, giving $[Cu_2(R-XYL-H)(O_2)]^{2+}$ (**11**).

Results for $R = CN$ and $R = OMe$ are missing from Table 2. Although in synthetic manipulations [121] both of these cleanly hydroxylate at room temperature to give corresponding products $[Cu_2(R-XYL-O-)(OH)]^{2+}$ (**12**), they could not be reasonably studied by stopped-flow kinetics. The cyano compound undergoes particularly strong temperature-independent photochemical interference; this was partly seen but accounted for in the other complexes. The methoxy complex gives neither a dioxygen adduct nor hydroxylates at low temperature, in spite of the fact that, on the basis of electronic considerations, it might be expected to undergo the fastest oxygenation reaction (Section III.B.1). As a possible explanation, low-temperature NMR studies [121,154] suggest that the copper chelating arms in $[Cu_2(R-XYL-H)]^{2+}$ (**10**) do not freely rotate in solution, but get locked in conformations that are apparently unfavorable for oxygen binding.

As can be seen from the data in Table 2, the rapid reaction with O_2 is characterized by low-activation enthalpies and large negative activation entropies.



Table 2 Kinetic and Thermodynamic Parameters for Oxygenation of Dinuclear Complexes $[\text{Cu}_2(\text{L})]^{2+}$ with bis(2-Pyridyl/ethyl)Amino Coordination

Complex	k_1 (223K) ($\text{M}^{-1}\text{sec}^{-1}$)	$\Delta H^\ddagger$ (kJ mol^{-1})	$\Delta S^\ddagger$ (JK $^{-1}$ mol^{-1})	k_{-1} (223K) (sec^{-1})	$\Delta H^\ddagger$ (kJ mol^{-1})	$\Delta S^\ddagger$ (JK $^{-1}$ mol^{-1})
10 (R = NO ₂)	281 (2)	6.4 (1)	-167 (1)	0.042 (1)	59 (1)	-8 (4)
10 R = H	1240 (10)	8.2 (1)	-146 (1)	0.072 (5)	70 (1)	50 (6)
10 R = t-Bu	1760 (30)	9.1 (3)	-140 (1)	0.11 (1)	83 (4)	110 (20)
10 R = F	331 (4)	29 (1)	-66 (1)	0.048 (4)	81 (3)	90 (10)
13 (n = 4)	8 (2)·10 ⁴	18 (2)	-70 (9)	—	—	—
13 (n = 4) ^a	1.4 (0.6)·10 ⁴	0 (3)	-162 (18)	—	—	—

	K_{eq} (223K) (M^{-1})	K_{eq} (298K) (M^{-1})	ΔH° (kJ mol^{-1})	ΔS° (JK $^{-1}$ mol^{-1})
10 (R = NO ₂)	1.4·10 ⁴	9	-53 (1)	-159 (4)
10 R = H	1.7·10 ⁴	3.9	-62 (1)	-196 (1)
10 R = t-Bu	1.6·10 ⁴	0.7	-74 (4)	-250 (20)
10 R = F	6.9·10 ³	5.8	-52 (3)	-156 (10)
7	2.7·10 ⁵	35	-66 (1)	-192 (2)
13 (n = 4)	8 (1)·10 ⁴	31	-58 (2)	-165 (8)
13 (n = 4) ^a	18(8)	0.4	-28 (3)	-101 (19)

^a Values for metastable end-on μ -peroxo intermediate.

ies. Among the four derivatives of **10** studied, there is a tendency toward compensation with higher enthalpies of activation coupled with more favorable activation entropies. The net result is very similar rate constants k_1 at the intermediate temperature of 223K. The higher $\Delta H^\ddagger$ observed for the fluoro derivative may be related to the hindered rotation discussed for the R = OMe derivative. Trends observed in $\Delta H^\ddagger$ for the release of O₂ from the Cu₂O₂ complex (i.e., k_{-1}) suggest that electron donating groups on the xylyl ring increase the copper-dioxygen bonding in [Cu₂(R—XYL—H)(O₂)]²⁺ (**10**), and, along with the $\Delta S^\ddagger$ values observed, it seems that the xylyl ring electronic structure has a direct influence on the process of dioxygen binding.

This is supported by the equilibrium parameters obtained for binding of O₂ to [Cu₂(R—XYL—H)]²⁺ (**10**), Table 2 [14,120,121,151]; here, the O₂-binding strength ΔH° directly correlates with xylyl substituent (R) inductive effects. All of the systems are characterized by large negative standard enthalpies and entropies. As is generally seen in O₂ binding by metal complexes [143,146,147], increasingly favorable (negative) enthalpies are compensated by larger negative entropies.

On the basis of simple coordination chemical considerations, and as copper ion redox potentials are nearly identical with R = H and R = NO₂ [154], one would assume the O₂-binding properties of [Cu₂(R—XYL—H)]²⁺ (**10**) to be rather independent of R. The experimental results are somewhat at variance with these expectations and the effects are as great or greater for O₂ displacement (i.e., k_{-1}) than for ring oxygenation (i.e., k_2 , Section III.B.1). We believe that the dicopper bound electrophilic peroxo group in [Cu₂(R—XYL—H)(O₂)]²⁺ (**11**) (as a $\mu\text{-}\eta^2\text{:}\eta^2\text{-O}_2$ group) is in very close proximity to the xylyl π -system, and this could provide an explanation for the R-group influence upon O₂ binding and release. However, a more likely explanation lies in the nature of the O₂-binding process as described by k_1 and k_{-1} , which are a composite of at least three steps: (1) preparation of a conformation of **10** suitable for binding O₂ to one copper ion, (2) binding of O₂ to one copper (intermediate), and (3) formation of the observable peroxide complex **11**. As the intermediate cannot be observed under any conditions, it must be quite unstable, and we have to assume that the transition state for the O₂-binding reaction is rather close (i.e., early) to the nonbonded form. Therefore, the observed trends for k_{-1} , for example, do not reflect actual O₂-binding strength in [Cu₂(R—XYL—H)(O₂)]²⁺ (**11**), but rather are due to differences of conformational stabilities in the precursor, [Cu₂(R—XYL—H)]²⁺ (**10**).

In summary, the thermodynamic data clearly indicate that the quasi-reversible dioxygen binding is entirely driven by enthalpy, with room temperature instability due to the large negative entropies. Xylyl group substituents affect the O₂-binding strength in a way that suggests some interaction with the arene ring. The compensating effects seen in ΔH° and ΔS° values lead to rather similar equilib-



rium constants for O₂ binding to complexes **10** at 223K, and this typical example further serves to caution against deriving conclusions concerning relative stabilities or reactivities based on data obtained at a single temperature.

For the oxygenation of the phenoxo-bridged dicopper(I) complex [Cu₂(XYL—O—)]⁺ (**7**) to give [Cu₂(XYL—O—)(O₂)]⁺ (**8**), the analysis of stopped-flow kinetic data [120] confirmed the synthetic-chemical manipulations [111], indicating the existence of a simple reversible system (Figure 9). The rate of reaction with dioxygen was too fast to be measured even at −100°C, and a lower limit for the oxygenation rate was determined to be $k_3 > 10^6 \text{ M}^{-1}\text{sec}^{-1}$. Thus, the presence of a bridging phenoxo group has the effect of preorganizing the dicopper(I) complex (lower activation barrier), allowing for a rate of oxygenation of [Cu₂(XYL—O—)]⁺ (**7**) that is more than three orders of magnitude greater than that observed for the xylyl complex [Cu₂(H—XYL—H)]²⁺ (**10**), where $k_1 = 533 \text{ M}^{-1}\text{sec}^{-1}$ at −80°C [120]. Thermodynamic parameters could be obtained from an analysis of the temperature dependence of UV-Vis spectra of **7** and **8**, yielding values very similar to those observed for the xylyl complexes (Table 2).

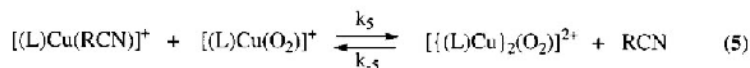
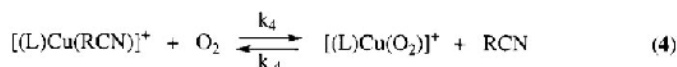
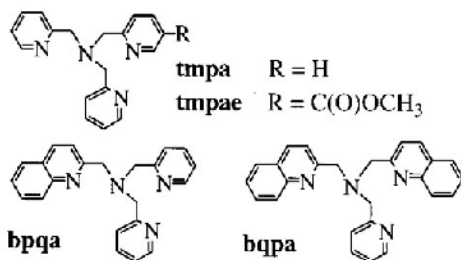
With [Cu₂(N4)(O₂)]²⁺ (**15**) in which the *m*-xylyl spacer is substituted by a more flexible tetramethylene bridge, both rate of formation and thermodynamic stability are increased relative to [Cu₂(H—XYL—H)(O₂)]²⁺ (**11**), but not dramatically so. Again, the activation enthalpy is low and activation entropy strongly negative. A considerable binding strength (−58 kJ mol^{−1}) for the μ-η²:η² peroxo complex is compensated by a reaction entropy of −165 JK^{−1}mol^{−1} (for **10**, R = H: Δ*H*^o = −62 kJ mol^{−1}, Δ*S*^o = −196 JK^{−1}mol^{−1}). Most interestingly, at low temperature, formation of the side-on complex is preceded by the rapid formation of a less stable isomer with spectroscopic properties of an end-on μ-peroxo species [152]. End-on to side-on peroxo conversion is likely to induce a considerable shortening in the Cu...Cu distance from ca. 440 to 340–370 pm. Since the Cu...Cu separation is 460 pm in deoxyhemocyanin [24] but 360 pm in oxyhemocyanin [21,25], we can suggest that an analogous thermodynamically driven rearrangement may be the basis for initiating subunit–subunit cooperativity in hemocyanin O₂ binding.

II.C.2. Complexes with tris-(2-Pyridylmethyl)Amino Coordination

X-ray structural characterization of [{(tmpa)Cu}₂(O₂)]²⁺ (**3**) [104] has made a kinetic study of its formation from O₂ and [(tmpa)Cu(RCN)]⁺ (**2**) especially attractive. Along with tmpa, we have been studying analogs with quinolyl groups in the place of the pyridine donors [14,103,151], tmpa with one pyridyl substituted by carboxymethyl in 5-position, tmpae [103], as well as dinucleating ligands D¹ and DO (mentioned in Section II.B.2), where two tmpa moieties are



tethered by a $\text{—CH}_2\text{—CH}_2\text{—}$ or $\text{—CH}_2\text{—O—CH}_2\text{—}$ linker, respectively [14,137,138,155]. Contrary to the situation found for complexes with bis(2-pyridylethyl)amino coordinating units (see Section II.C.2), for tmpa and its derivatives, stepwise formation of discernible superoxo (O_2 bound to one copper only) and peroxy adducts ($\text{Cu}:\text{O}_2 = 2:1$) has been the rule rather than the exception. With tmpa, spectroscopic, kinetic, and thermodynamic parameters represent the first such data for a primary 1:1 copper dioxygen interaction [156]. All complexes react quasi-reversibly with dioxygen, conforming to Eqs. (4) and (5) for the mononuclear complexes [14] (Scheme 11).



Scheme 11

For the dinuclear complexes with the ligands DO and D^1 , the analogous reactions are given by the analogous scheme representing (26) $\leftrightarrow$ (27) $\leftrightarrow$ (28) (Scheme 7) (see Section II.B.2).

Since peroxy formation is an intermolecular process (Eq. 5) for mononuclear, but an intramolecular one for dinuclear starting complexes, corresponding rate constants have different reaction orders and are not directly comparable. We thus concentrate the discussion on a few activation and thermodynamic parameters.

As might be expected, superoxo formation (k_4) is quite analogous for the complexes studied. Activation enthalpies are around 30 kJ mol^{-1} , different overall rates being governed by activation entropies ranging from $+14$ (tmpa) to $-53 \text{ JK}^{-1}\text{mol}^{-1}$ (bpqa), the latter probably for steric reasons slowing solvent exchange. Dissociation of superoxo (k_{-4}) has considerably higher activation enthalpies (55 (D^1) to 66 (tmpa) kJ mol^{-1}) with considerable activation entropies ranging from



72 (bqpa) to 137 (tmpa) $\text{JK}^{-1}\text{mol}^{-1}$. In fact, combination of k_4 and k_{-4} to the equilibrium constant K_4 leads to practically identical thermodynamic parameters for all complexes ($\Delta H^\circ = -32$ to -35 kJ mol^{-1} , $\Delta S^\circ = -123$ to $-129 \text{ JK}^{-1}\text{mol}^{-1}$), including the much slower reacting bqpa. As discussed for XYL and N4 complexes in the previous section, room temperature stability is precluded by the strongly negative reaction entropies [14].

As peroxo complex formation is an intramolecular process with dinucleating ligands, one could hope to overcome at least part of the disfavorable entropies by using D^1 or DO instead of mononuclear tmpa analogs. This expectation has indeed been fulfilled [137]: with D^1 , the overall reaction entropy for peroxo formation is increased to $-89 \text{ JK}^{-1}\text{mol}^{-1}$ relative to values ranging from -170 (tmpae) to -220 (tmpa) $\text{JK}^{-1}\text{mol}^{-1}$. This very substantial stabilization is, however, to a large extent offset by a corresponding increase in reaction enthalpy from -81 kJ mol^{-1} for tmpa to -35 kJ mol^{-1} for D^1 . This unfavorable reaction enthalpy is directly reflected in the corresponding kinetic parameters as well as in the absorption spectra. In fact, steric strain in **28** is released by subsequent intermolecular aggregation to a trinuclear species, leading to a peroxo spectrum much more closely related to that of unstrained $[(\text{tmpa})\text{Cu}]_2(\text{O}_2)]^{2+}$ (**3**) and to thermodynamic parameters intermediate between those of (**3**) and (**28**). With respect to peroxo formation, bqpa considerably differs from the other ligands discussed in this section. Most likely because of steric hindrance increased by the two quinolyl groups, the stability of the peroxo complex is reduced by five orders of magnitude relative to tmpa at low temperature, and in fact the superoxo rather than the peroxo complex is the thermodynamically stable species under typical experimental conditions [103].

As mentioned, room-temperature stability of an intramolecular peroxo adduct based on a derivative of tmpa has nevertheless been obtained: By reducing steric strain with a more flexible $-\text{CH}_2-\text{O}-\text{CH}_2-$ linker, 70% formation at 298K is obtained in EtCN and even full formation has been achieved meanwhile by switching to the less Cu(I)-coordinating acetone [138].

The complex $[(\text{tmpa})\text{Cu}(\text{O}_2)]^+$ (**2'**) is generated with $k_4 = 1.8 \times 10^4 \text{ M}^{-1}\text{sec}^{-1}$ and $K_{\text{eq}} = k_4/k_{-4} = 1.4 \cdot 10^3 \text{ M}^{-1}$ at -90°C . These values can be compared with those observed for cobalt or iron complexes. The formation of $[(\text{tmpa})\text{Cu}(\text{O}_2)]^+$ (**2'**) ($k_4 \sim 10^8 \text{ M}^{-1}\text{sec}^{-1}$ calculated at 25°C) is faster than rates seen for most LCo(II) 1:1 oxygenation reactions, where $k \sim 10^3-10^6 \text{ M}^{-1}\text{sec}^{-1}$ (25°C). For heme proteins or porphyrin-Fe(II) model complexes, the O_2 on-rates ($k \sim 10^6-10^9$) are similar to those seen for formation of (**2'**). However, the off-rates for iron species appear to be much smaller, giving rise to large K_{eq} values in the range of 10^4-10^6 at 20°C [143-149].

The 2:1 O_2 binding by tmpa and bqpa complexes may also be compared to that seen for peroxo-dicobalt(III) compounds derived from mononuclear precursors, where the molecularity of reaction is analogous. Thus, k_{on} is calculated



to be $4.4 \cdot 10^5 \text{ M}^{-2}\text{sec}^{-1}$ at 25°C ($6.0 \cdot 10^7$ at -90°C), and this value is in the range seen for ligand-dependent values observed for the cobalt complexes. In terms of equilibrium binding parameters, Co_2O_2 complexes also exhibit large negative ΔS° values, but considerable room temperature stabilities ($\log K_{\text{eq}} \sim 6\text{--}15$) are derived from much larger negative $\Delta H^\circ_{\text{formation}}$ values (e.g., -120 to -150 kJ/mol) [143,145–149,156], probably as a result of stronger bonding by cobalt complexes with a larger 3^+ charge.

II.C.3. Complexes with Substituted Triazacyclononanes

The most prominent kinetic result obtained with $[\text{Cu}(\text{R}_3\text{-tacn})(\text{CH}_3\text{CN})]^+$ (**23**, $\text{R} = \text{---CH}(\text{CH}_3)_2$) has been the reversible interconversion between a $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo}$ complex (**24**) and the corresponding bis- $\mu\text{-oxo}$ isomer (**25**) (Section II.B.4) [135]. In fact, this extremely fast making and breaking of the O—O bond may have model character for quite a variety of copper enzymes and biomimicking reactions and may be of relevance to fields as remote as photosynthesis [157]. Although actual rate constants for the (**24**) $\leftrightarrow$ (**25**) conversion still are to be obtained and might need special techniques like low-temperature T-jump, kinetics for formation and decay have been obtained for a variety of ligands, including $i\text{Pr}_3\text{tacn}$ ($\text{R}_1 = \text{R}_2 = \text{R}_3 = \text{---CH}(\text{CH}_3)_2$) $\text{BziPr}_2\text{tacn}$ ($\text{R}_1 = \text{benzyl}$, $\text{R}_2 = \text{R}_3 = \text{---CH}(\text{CH}_3)_2$) as well as dinucleating ligands in which two $i\text{Pr}_2\text{tacn}$ units are tethered by a $\text{---CH}_2\text{CH}_2\text{---}$ [158] or $m\text{-xylyl}$ linker [159].

Kinetics of adduct formation differ considerably from the systems discussed in Sections II.C.1 and II.C.2, inasmuch as making the first $\text{Cu} \cdots \text{O}_2$ bond is essentially rate-limiting for all systems and the superoxo complex thus formed is but a steady-state species, if kinetically relevant at all. In addition, $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo}$ and/or bis- $\mu\text{-oxo}$ formation seems to be irreversible under most experimental conditions, precluding determination of thermodynamic data so far. Not surprising in view of the practically identical coordination sphere, rates for making the first $\text{Cu}\text{---O}_2$ bond and thus overall formation are rather similar: $\Delta H^\ddagger = 37$ to 39 kJ mol^{-1} , $\Delta S^\ddagger = -62$ to $-30 \text{ JK}^{-1} \text{ mol}^{-1}$ [135,158,159]. In line with the rate-limiting formation of the first $\text{Cu}\text{---O}_2$ bond, activation enthalpies are much higher than those obtained for the formation of other peroxo complexes, but similar to those obtained for superoxo formation with tmpa derivatives (cf. Section II.C.2).

Most of the low-molecular-weight dicopper-dioxygen adducts obtained so far can only be studied at low temperature because of the strongly disfavorable (negative) associated reaction entropies. Recently, however, major inroads have been made toward obtaining room-temperature stability of such adducts, by using unstrained dinucleating ligands and optimizing solvent properties [138,139], shifting emphasis of future research from attempts at further adduct stabilization to structure–reactivity relationships and eventually catalytic activity.



II.C.4. Comparison with Hemocyanin

With the characterization of low-molecular-weight copper-dioxygen complexes, their kinetic and thermodynamic properties [14] may be compared to those of the biological oxygen carrier. Reported binding enthalpies for hemocyanin are in the range of -46 to $+13$ kJ mol $^{-1}$ [160,161]; thus, the binding of O $_2$ by the model complexes is easily as strong as or stronger than in model compounds. In addition, the enzyme rates of O $_2$ binding ($\sim 10^7$ M $^{-1}$ sec $^{-1}$) can be achieved even at low temperature, at least for [Cu $_2$ (XYL—O—)(O $_2$)] $^+$ (**8**) (Table 2) [120]. Well-characterized room-temperature stable dicopper-dioxygen complexes in solution are limited and, as found for those studied thus far, this is due to the unfavorable entropy which is overcome by cooperative effects present in large multisubunit proteins. Nevertheless, quasi-reversible interactions of O $_2$ with Cu(I) complexes do generally occur and can be studied in detail.

III. REACTIVITY OF Cu $_n$ O $_2$ COMPLEXES

With access to a number of types of dinuclear copper-dioxygen complexes, it has been of interest to survey their reactivity with organic and inorganic substrates in order to explore the possibility of using them as stoichiometric oxidants or as oxidation catalysts. Such studies are also aimed at the further characterization and comparison of these Cu $_2$ O $_2$ species to provide additional insights into their chemical nature and to elucidate principal reactivity features of the peroxo moiety in these complexes. Structure–reactivity correlations will of course be important in deducing relevance to biological or catalytic chemical systems. In cases where the exact coordination mode of a dioxygen ligand to copper cannot be determined by a direct x-ray study or cannot be deduced unambiguously from physical measurements, comparison of the reactivity with those of well-characterized copper-dioxygen complexes could shed light on their structural characteristics or, at least, help to exclude certain possibilities. We also describe reactions relevant to copper monooxygenases, particularly arene hydroxylation reactions akin to tyrosinase activity, in this section.

III.A. Reactivity Comparisons of Discrete Cu $_2$ O $_2$ Complexes

A rather detailed study has been carried out comparing the reactivity of [{{(tmpa)Cu} $_2$ (O $_2$)}] $^{2+}$ (**3**), [Cu $_2$ (XYL—O—)(O $_2$)] $^+$ (**8**) and [Cu $_2$ (N4)(O $_2$)] $^{2+}$ (**15**, $n = 4$) in reactions with a series of inorganic and organic reagents [162]. Some further comparisons can be made with other characterized complexes. The main conclusion is that **3** and **8** react as typical nucleophilic peroxo transition

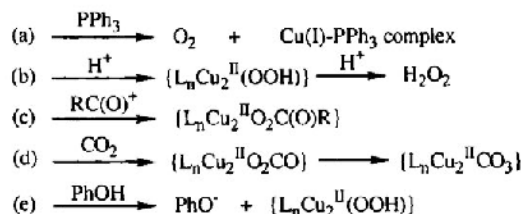
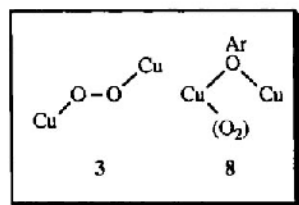


metal complexes, whereas **15** is rather different, having a nonbasic or electrophilic peroxy group (Figure 13). The possible structural basis for the differences is discussed, particularly in terms of that proposed for **15** (e.g., a side-on μ - η^2 : η^2 -peroxy dicopper(II) moiety), and this structure and its reactivity may be relevant in tyrosinase models discussed in Section III.B.

III.A.1. Reactions with PPh_3

The dioxygen in complexes **3**, **8**, and **15** can be replaced by carbon monoxide; this stabilizes copper in the 1+ oxidation state. Similar reactions occur when complexes $[\{(\text{tmpa})\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**3**) and $[\text{Cu}_2(\text{XYL}-\text{O}-)(\text{O}_2)]^+$ (**8**) are reacted with triphenylphosphane; Cu(I)— PPh_3 complexes are formed with the concomitant liberation of dioxygen, as determined quantitatively by manometry. However, $[\text{Cu}_2(\text{N4})(\text{O}_2)]^{2+}$ (**15**) does not react with triphenylphosphane under the same conditions, but triphenylphosphane oxide and $[\text{Cu}(\text{I})_2(\text{N4})(\text{PPh}_3)_2]^{2+}$ are formed when the reaction mixture is left to warm to room temperature (Figure 13).

1. Basic/Nucleophilic Peroxide



2. Non-Basic/Electrophilic Peroxide

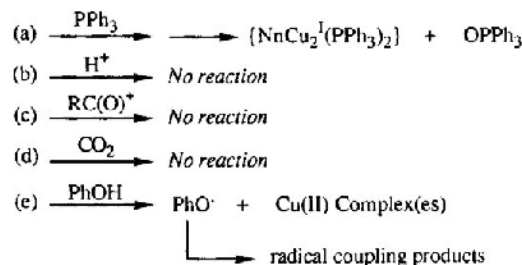
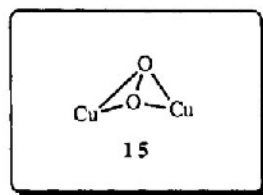
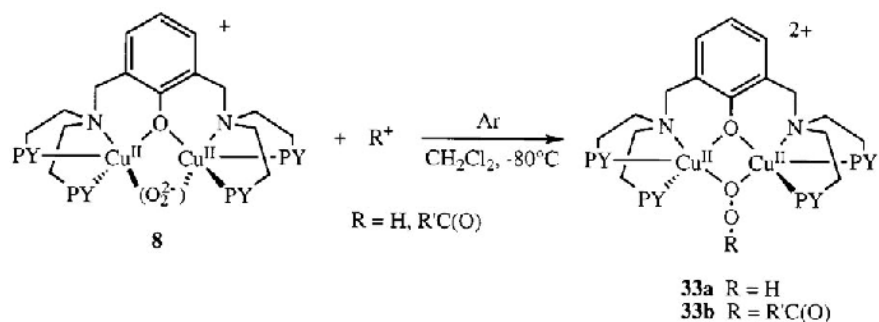


Figure 13 Reactivity comparisons of peroxo-dicopper(II) complexes **3**, **8**, and **15**.



III.A.2. Hydronation and Acylation

The dioxygen (peroxo) ligand in $[(\text{tmpa})\text{Cu}]_2(\text{O}_2)]^{2+}$ (**3**) and $[\text{Cu}_2(\text{XYL}-\text{O}-)(\text{O}_2)]^+$ (**8**) is readily hydronated or acylated. Addition of one equivalent of HBF_4 or HPF_6 to **8** at -80°C gives a hydroperoxo dicopper(II) complex, $[\text{Cu}_2(\text{XYL}-\text{O}-)(\text{OOH})]^{2+}$ (**33a**). In complex **33a**, the hydroperoxo group is probably coordinated in a μ -1,1 fashion; this postulated coordination is based on spectroscopic comparisons to structurally characterized complexes (e.g., the acylperoxo analog **33b**) and the phenoxo and hydroxo doubly bridged complex $[\text{Cu}_2(\text{XYL}-\text{O})(\text{OH})]^{2+}$ (**12**) [122]. With two or more equivalents of H^+ , dihydrogen peroxide is produced in 88% yield from **8** and 81% yield from **3** (iodometric titration). However, the addition of three to four equivalents to **15** resulted in no UV-Vis spectral change, and addition of 10-fold excess acid provides a yield of only 12% H_2O_2 , indicating that the peroxo ligand in **15** is not basic (Scheme 12).



Scheme 12

Acylation reactions of **3**, **8**, and **15** at -80°C follow the trend observed for hydronation. Reactions of $\text{R}'\text{C}(\text{O})\text{Cl}$ with $[\text{Cu}_2(\text{XYL}-\text{O})(\text{O}_2)]^+$ (**8**) are rapid and they give μ -1,1-acylperoxo dicopper(II) complexes. The use of *m*-chlorobenzoyl chloride led to the isolation of $[\text{Cu}_2(\text{XYL}-\text{O}) \times (\text{m-ClC}_6\text{H}_4\text{C}(\text{O})\text{OO}-)]^{2+}$ (**33b**, $\text{R}' = m\text{-chlorophenyl}$), which is the first structurally characterized percarboxylato-dicopper(II) complex [163]. In contrast to the dioxygen adduct $[\text{Cu}_2(\text{XYL}-\text{O})(\text{O}_2)]^+$ (**8**), complexes **33a** and **b** oxidize PPh_3 to OPPh_3 quantitatively. In other words, hydronation or acylation of the dioxygen-copper complex appears to result in activation via formation of the $\text{Cu}_n-\text{OOR}'$ species, which is capable of transferring an oxygen atom to a substrate. In the case of $[(\text{tmpa})\text{Cu}]_2(\text{O}_2)]^{2+}$ (**3**), benzoyl chloride reacts slowly and hydronation is required to cause oxygenation of added PPh_3 . In line with the lack of hydronation, there is no reaction of acyl chlorides with $[\text{Cu}_2(\text{N4})(\text{O}_2)]^{2+}$ (**15**).



III.A.3. Reactions with SO₂ and CO₂

All three dioxygen complexes (**3**, **8**, **15**) react with SO₂ to give sulfato-dicopper(II) compounds. Addition of CO₂ to [Cu₂(XYL—O—)(O₂)]⁺ (**8**) at −80°C in CH₂Cl₂ causes the loss of the characteristic 505 nm band producing a green solution (λ_{max} = 340 nm [ε = 3700] and 400 nm [ε = 3800]), presumed to contain a peroxycarbonato species, [Cu₂(XYL—O—)(CO₄)]⁺. Either by thermal decomposition or by reaction of this solution with PPh₃ (producing OPPh₃), a μ-CO₃^{2−} complex [Cu₂(XYL—O—)(CO₃)]⁺ is formed. Similar behavior is observed with [{(tmpa)Cu}₂(O₂)]²⁺ (**3**) and reaction with CO₂ causes bleaching of the characteristic bands at 525 and 590 nm. The resulting solution reacts with PPh₃ to give OPPh₃ (97% conversion) and [{(tmpa)Cu}₂(CO₃)]²⁺. By contrast, [Cu₂(N4)(O₂)]²⁺ (**15**) does not react with carbon dioxide, suggesting again that the peroxo ligand in **15** here has less nucleophilic character than in **3** or **8**.

III.A.4. Reactions with Phenols

Phenols act as acids toward [{(tmpa)Cu}₂(O₂)]²⁺ (**3**) and Cu₂(XYL—O—)(O₂)]⁺ (**8**), hydronating them to give hydrogen peroxide and phenoxo-copper(II) complexes. However, hydrogen-atom abstraction takes place when complex [Cu₂(N4)(O₂)]²⁺ (**15**) is reacted with phenols, giving phenoxy radicals that dimerize to produce biphenols or diphenoquinones, depending on the position of the substituents on the phenols. This shows that **15** is a better one-electron oxidant than **3** or **8**.

III.A.5. Reactions with ArMgBr and Ph₃C⁺ (BF₄)[−]

Dioxygen-dicopper complexes **3**, **8**, and **15** react with aromatic Grignard reagents and after hydrolytic workup phenols and biphenyls are isolated. Using 4-methylphenyl- and 4-fluorophenyl-magnesium bromides, the ratio between the yields of 4,4'-dimethylbiphenyl and 4,4'-difluorobiphenyl for complexes **3**, **8**, and **15** are 0.6, 0.8, and 1.2, respectively, showing that **15** is more electrophilic than **3** or **8**. Reactions with Ph₃C⁺ result in the formation of benzophenone in 49%, 37%, and 30% yields for complexes **3**, **8**, and **15**, respectively. This finding suggests again that **3** and **8** are more nucleophilic than **15**.

III.A.6. Summary

The results described indicate that complexes [{(tmpa)Cu}₂(O₂)]²⁺ (**3**) and [Cu₂(XYL—O—)(O₂)]⁺ (**8**) react quite similarly with the reagents employed and that they possess a peroxo ligand that is relatively basic and has a more pronounced nucleophilic character than is found for complex [Cu₂(N4)(O₂)]²⁺ (**15**). In a relative sense, complex **15** is rather electrophilic, as is more typical of early transition metal peroxo-complexes. The deviating behavior of the μ-η²:η²-peroxo



group in $[\text{Cu}_2(\text{N4})(\text{O}_2)]^{2+}$ (**15**) undoubtedly originates in the difference of the coordination environment in complexes **3**, **8**, and **15**, manifested by the $\text{XYL}-\text{O}-$, tmpa , and N4 ligands. The peroxo groups in **3** and probably **8** have end-on coordination mode. On the other hand, the binding of both oxygen atoms to both copper atoms in $[\text{Cu}_2(\text{N4})(\text{O}_2)]^{2+}$ (**15**) is feasible, since the N4 dinucleating ligand furnishes each copper ion with only three donors.

On the basis of simple valence-bond arguments, a $\mu-\eta^2:\eta^2$ -peroxo coordination may confer a partial positive charge to the oxygen atoms, since the O_2^{2-} ligand has three bonds to each O atom. Steric access of substrate to the peroxide ligand, preferably along the $\text{O}-\text{O}$ bond axis, is another important aspect discussed by Sorrell [7]; here, a substrate nucleophile can favorably attack the σ^* antibonding orbital, leading to $\text{O}-\text{O}$ bond cleavage and oxygen atom transfer to the substrate. Since the copper ion and/or ligand may substantially block access to the $\text{O}-\text{O}$ bond vector through end-on coordination in *cis*- μ -1,2- or *trans*- μ -1,2-peroxo dicopper(II) species, these latter types may be less suited for reactions where peroxide acts as an electrophile. A theoretical analysis has been offered by Ross and Solomon [22,31], which broken-symmetry SCF- $X\alpha$ -scattered wave calculations were used to detail the electronic structure of peroxide-bridged $\text{Cu}(\text{II})$ dimers; specific comparisons were made among *cis*- μ -1,2-, *trans*- μ -1,2-, and planar $\mu-\eta^2:\eta^2$ -peroxo species. An important conclusion is that there is a stronger π_σ^* donor interaction with the Cu d orbital in the $\eta^2:\eta^2$ peroxo complex as compared to the end-on species, suggesting that the negative charge on the O_2^{2-} ligand is smaller in the case of the side-on peroxo dicopper complex. These observations may account for the electrophilic behavior of a peroxide ligand bound in a $\mu-\eta^2:\eta^2$ fashion. Another finding was that the peroxide σ^* orbital acted as a π -acceptor from Cu ; this could account for the weak $\text{O}-\text{O}$ peroxide bond as manifested in an unusually low $\nu_{\text{O}-\text{O}}$ observed in $\{\text{Cu}[\text{HB}(3,5\text{-iPr}_2\text{pz})_3]\}_2(\text{O}_2)$ (**5**) and oxy-Hc ($\sim 750\text{ cm}^{-1}$).

It is useful to compare the preceding reaction characteristics with those of $\{\text{Cu}[\text{HB}(3,5\text{-iPr}_2\text{pz})_3]\}_2(\text{O}_2)$ (**5**), since the latter has a confirmed planar $\mu-\eta^2:\eta^2$ -peroxo structure. Its reactivity has not yet been studied with many of the reagents described here, but some comparisons can be made. In reactions with phenols, the dominant behavior is similar to that observed for $[\text{Cu}_2(\text{N4})(\text{O}_2)]^{2+}$ (**15**), with (presumed) one-electron oxidation to phenoxy radicals leading to oxidative coupling products; a very small yield of oxygenated products is observed [162]. The reactions of triphenylphosphane and carbon dioxide with $\{\text{Cu}[\text{HB}(3,5\text{-iPr}_2\text{pz})_3]\}_2(\text{O}_2)$ (**5**) and **15** appear to differ. PPh_3 readily displaces O_2 in **5**, but not in **15**. As described, CO_2 does not react with **15** at temperatures where this O_2 -complex is stable, contrasting with the behavior observed for **3** and **8**. Kitajima isolated a carbonato complex after allowing $\{\text{Cu}[\text{HB}(3,5\text{-iPr}_2\text{pz})_3]\}_2(\text{O}_2)$ (**5**) to warm up in the presence of CO_2 , but it appears that this is due to reaction of



carbon dioxide with an oxo- or hydroxo thermal decomposition product of **5** [164]. One can speculate that some of these variations in reactivity may be due to differences in properties of a bent (i.e., in **15**) versus planar $\mu\text{-}\eta^2\text{:}\eta^2\text{-peroxo}$ moiety (i.e., in **5**), steric effects (i.e., access to substrates), or temperature of reaction and solvent employed.

Our investigations on copper-dioxygen complexes containing simple imidazole ligands also emphasized the importance and usefulness of reactivity studies. Complex $[\{\text{L}_3\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**22**, $\text{L} = 1,2\text{-dimethylimidazole}$) proved not sufficiently stable at -90°C to determine its exact molecular structure. As mentioned, frozen solution EXAFS studies suggest either of two possible structures: a bent $\mu\text{-}\eta^2\text{:}\eta^2\text{-}$ or a *trans*- $\mu\text{-}1,2\text{-peroxo}$ coordination. The reactivity patterns observed for $[\{\text{L}_3\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**22**,) show a very close resemblance to that observed for $[\{(\text{tmpa})\text{Cu}\}_2(\text{O}_2)]^{2+}$ (**3**) and $[\text{Cu}_2(\text{XYL-O-O})(\text{O}_2)]^+$ (**8**). Tertiary phosphanes liberate the bound O_2 and excess acid hydronates the peroxo-group to form H_2O_2 in good yield. Complex **22** instantaneously reacts with CO_2 at -90°C and a carbonato-dicopper(II) complex was isolated after warming the reaction mixture to room temperature. Thus, on the basis of these reactivity comparisons, we assume that the peroxo ligand in **22** is bound in an end-on fashion, probably as a *trans*- $\mu\text{-}1,2\text{-peroxo}$ group.

III.B. Tyrosinase and Other Model Systems

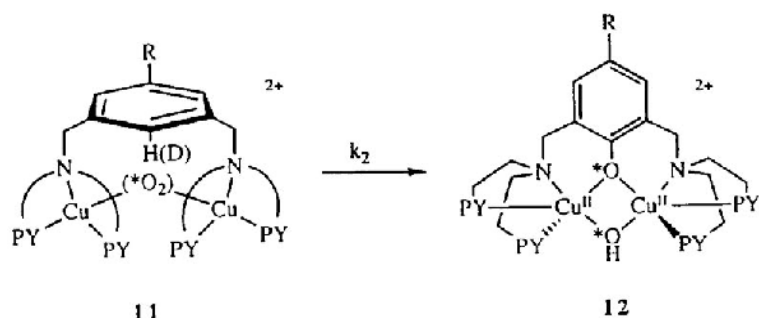
Mimicking and understanding tyrosinase activity (*o*-hydroxylation of phenols) have been of longtime interest because this was one of the earliest copper monooxygenases described and the significance of elucidating dioxygen activation mechanism(s) has widespread implications and potential applications.

III.B.1. A Xylyl-Containing Copper Monooxygenase Model System

As already mentioned in Section II.C, the complexes $[\text{Cu}_2(\text{R-XYL-H})]^{2+}$ (**10**) react with dioxygen, forming a kinetically describable intermediate dioxygen complex $[\text{Cu}_2(\text{R-XYL-H})(\text{O}_2)]^{2+}$ (**11**), and attack by the peroxo group causes ligand hydroxylation to give the phenoxo and hydroxo-bridged complex $[\text{Cu}_2(\text{R-XYL-O-O})(\text{OH})]^{2+}$ (**12**). Here, we focus on the hydroxylation event, as described by the rate constant k_2 (Scheme 13).

Stoichiometry of Reaction. One of the factors that has made this a particularly attractive system for study has been the unambiguous identification and structural characterization of the starting tricoordinate dicopper(I) complex $[\text{Cu}_2(\text{R-XYL-H})]^{2+}$ (**10**, $\text{R} = \text{H}$) and the green product $[\text{Cu}_2(\text{R-XYL-O-O})(\text{OH})]^{2+}$ (**12**, $\text{R} = \text{H}$) in a very clean process. The hydrox-





Scheme 13

ylation reaction is usually carried out in a solvent such as dimethylformamide or CH₂Cl₂ and **12** is generated in >95% yield [153].

An important point to establish in a monooxygenase reaction was that the phenolic oxygen produced in **12** was derived from dioxygen and not another source such as water. This is indeed the case. When ¹⁸O₂ was used as the source of dioxygen in a reaction with **10**, the phenoxo group in **12** contained greater than 99% labeled ¹⁸O, and direct field-desorption MS experiments even suggested that the bridging OH group in [Cu₂(H—XYL—O—)(OH)]²⁺ (**12**) also contained the label [153].

These experiments suggest that the conversion of **10** to **12** represents that stoichiometry found in enzyme monooxygenases, R—H + O₂ + 2e[−] + 2H⁺ → ROH + H₂O. Here, the two electrons are supplied by the two Cu(I) ions in **10** and the second oxygen atom (derived from O₂) is trapped as a coordinated hydroxide ion in [Cu₂(H—XYL—O—)(OH)]²⁺ (**12**). The reaction stoichiometry suggests that the hydrogen atom initially in the 2-position of Cu₂(H—XYL—H)]²⁺ (**10**) would be that found on the hydroxo group in [Cu₂(H—XYL—O—)(OH)]²⁺ (**12**). However, the oxygenation of a 2-deuterated analog Cu₂(H—XYL—D)]²⁺ (**10-D**) did not readily produce the expected product [Cu₂(H—XYL—O—)(OD)]²⁺ (**12-D**) (using IR evidence), presumably because of facile exchange with the trace amounts of water present in the solvents used [153].

Kinetic/Mechanistic Studies.

Dicopper(II) complex plus H₂O₂. In close analogy with tyrosinase, the stoichiometric hydroxylation of the xylol ligand in [Cu(I)₂(H—XYL—H)]²⁺ (**10**) to produce [Cu₂(H—XYL—O—)(OH)]²⁺ (**12**) can also be achieved by starting with a Cu(II) (“met”) form [Cu(II)₂(H—XYL—H)]⁴⁺ (**24**), in a reaction with



dihydrogen peroxide. The kinetics of this reaction were studied in 50% H₂O/dmf [165] and the rate law was determined to be

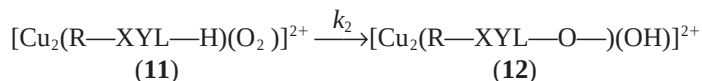
$$-\frac{d[\text{H}_2\text{O}_2]}{dt} = \frac{k[\mathbf{24}]^2[\text{H}_2\text{O}_2]}{[\text{H}^+](1 + k'[\mathbf{24}])}$$

$$k = 0.44 \text{ M}^{-1} \text{ sec}^{-1}$$

$$k' = 4500 \text{ M}^{-1}$$

The second-order dependence upon [Cu₂(XYL-II)]⁴⁺ (**34**) implies a transition state containing a total of four copper ions, which is a somewhat unexpected result considering that we know that dinuclear copper centers are sufficient in enzymes like tyrosinase and even in the reaction of **10** with dioxygen. The pH dependence and complete absence of a deuterium isotope effect for reaction of [Cu₂(H—XYL—D)]⁴⁺ (**34-D**) provide clues to the puzzle. The pH dependence suggests that peroxide binds in a μ-1,1-OOH coordinated form; therefore, we suggest that the second molecule of complex is needed as a Lewis acid to increase the nucleophilicity of the terminal OH group. The remaining oxygen atom would be a reactive electrophile, in line with suggestions for tyrosinase and consistent with the lack of an isotope effect in the hydroxylation step.

Hydroxylation of [Cu₂(R—XYL—H)]²⁺ (10**) by O₂.** As described in Section II.C.1, the complete kinetic analysis reveals an initial reversible binding of O₂ by **10** to give [Cu₂(H—XYL—H)(O₂)]²⁺ (**11**), followed by an irreversible hydroxylation reaction described by *k*₂. The kinetics preclude that a Fenton-type mechanism (production of hydroxyl radical) is involved in the reaction (i.e., that an intermediate peroxo species is further attacked by LCu(I)). We note that [Cu₂(H—XYL—H)]⁴⁺ (**34**) cleanly reacts with H₂O₂ to give product [Cu₂(H—XYL—O—)(OH)]²⁺ (**12**), whereas reaction of [Cu₂(H—XYL—H)]²⁺ (**10**) with hydrogen peroxide does not (unpublished observation). Addition of radical traps to solutions of **10** and O₂ also does not affect the hydroxylation (unpublished observation), and all the evidence points to intramolecular hydroxylation by the peroxo-dicopper species **11**.



The consistent picture being “painted” for this arene hydroxylation is an electrophilic attack of the bound peroxo ligand. The *k*₂ value observed for [Cu₂(H—XYL—D)]²⁺ (**10-D**) is within experimental error of that seen for the —H parent compound (**10**); this lack of deuterium isotope effect is consistent with electrophilic attack on the arene substrate π system, which precludes C—H bond cleavage in the rate-determining step.

Also in line with an electrophilic peroxo group is the increase of Δ*H*[‡]



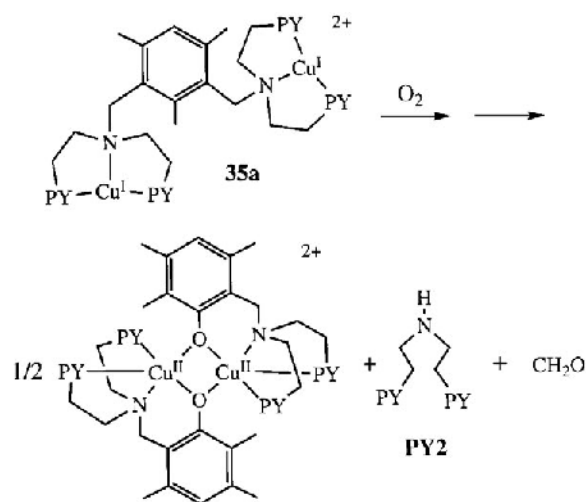
Table 3 Kinetic Parameters for Hydroxylation of $[\text{Cu}_2(\text{R}-\text{XYL}-\text{H})]^{2+}$ (**10**) at 223 K

R	k_2 (sec^{-1})	$\Delta H^\ddagger$ (kJ mol^{-1})	$\Delta S^\ddagger$ ($\text{JK}^{-1} \text{mol}^{-1}$)
NO_2	0.013	55	-32
H	0.13	50	-35
<i>t</i> -Bu	0.96	41	-59
F	0.18	39	-82

with the electron-withdrawing power of R (Table 3). The compensating effect of activation entropies $\Delta S^\ddagger$ makes a linear free energy relationship analysis based on plots of $\log k_2$ versus ρ_p^+ problematic. Nevertheless, k_2 decreases with electron withdrawing character of R in the whole temperature range studied and, for example, at -80°C a very modest ρ of ~ -2.1 is obtained. There are two main possibilities to explain the relatively weak substituent effect. The first is that k_2 is a composite rate constant, reflecting what must be multiple steps occurring between $[\text{Cu}_2(\text{R}-\text{XYL}-\text{H})(\text{O}_2)]^{2+}$ (**11**) and $[\text{Cu}_2(\text{R}-\text{XYL}-\text{O})(\text{OH})]^{2+}$ (**12**). Possible intermediate steps include attack of the aromatic ring to give a carbocation (which itself could be several steps), O—O cleavage possibly giving other important copper-oxy species (e.g., $\text{Cu}=\text{O}$), hydride migration, and hydron transfer. The second possibility is that the peroxy group in **11** may be so reactive an electrophile that it is not sensitive to arene electronic changes in its attack of the xylyl ring. Although the reactivity of the peroxy group is clearly seen to be that of an electrophile, we don't see it as being an intrinsically powerful one, and a more likely explanation for its reactivity and relative insensitivity to substituent would be the peroxy group's ideal positioning for $p-\pi$ attack along the O—O vector.

A Copper Chemistry "N.I.H. Shift" and the Suggested Mechanism of Xylyl-Copper Hydroxylation Reactions. Further supporting the notion that attack of the arene substrate in $[\text{Cu}_2(\text{R}-\text{XYL}-\text{H})]^{2+}$ (**10**) is due to an electrophilic Cu_2O_2 complex $[\text{Cu}_2(\text{R}-\text{XYL}-\text{H})(\text{O}_2)]^{2+}$ (**11**) are reactions that occur when a methyl group is placed into the 2-position of the ligand [166,167]. We have studied the oxygenation of two complexes, a trimethyl substituted derivative $[\text{Cu}_2(4,6-\text{Me}_2-\text{XYL}-\text{Me})]^{2+}$ (**35a**) and a mono-2-methyl species $[\text{Cu}_2(\text{XYL}-\text{Me})]^{2+}$ (**35b**). Instead of benzylic hydroxylation or blockage of xylyl ring attack, 2-hydroxylation and methyl migration occur, giving the organic products phenol, secondary amine PY2, and formaldehyde (Scheme 14). Yield and overall material balance were excellent, and the absolute identity and regiochemistry of the phenol were proved by isolation and x-ray structural characterization of a dinuclear copper complex of the hydroxylated product [166,167].





Scheme 14

Consistent with this monooxygenase reaction stoichiometry, using $^{18}O_2$ in the reaction resulted in labeled phenol product. Also in line with methyl group migration is the observation that placement of four deuterium atoms in the two benzylic positions of the ligand in $[Cu_2(XYL-Me)]^{2+}$ (**35b**) resulted in retention of deuterium in the phenol product, whereas formation of d_2 -formaldehyde [167] indicates that this product originated from the other benzylic carbon in **35b**.

The process occurring here is reminiscent of the "N.I.H. shift," which is well known to occur in iron hydroxylases such as cytochrome P-450 and mammalian PAH [1,167]. For example, action of PAH on $[4-^3H]$ phenylalanine produces $>90\%$ $[3-^3H]$ tyrosine. Here, a presumed electrophilic iron-oxy species produces a carbonium ion intermediate from which a 1,2-shift occurs, giving a resonance stabilized cation; rearomatization through loss of H^+ (or $^3H^+$) gives the observed product as a result of a heavy atom isotope effect. Thus, it appears that the "N.I.H. shift" mechanism for copper has been discovered for a chemical model system prior to its observation in proteins.

Concerning the mechanism, the suggestion of cationic intermediates and an oxidatively induced methyl migration reaction lead to a suggested mechanism for this unique example of a N.I.H. shift in copper chemistry, one that is also consistent with all the other chemical observations and kinetic studies carried out on these xylyl copper complexes (Figure 14).

1. Overwhelming evidence has been presented that oxygenation of xylyl derivatives $[Cu_2(R-XYL-Y)]^{2+}$ ($R = H, NO_2, CN, F, t-Bu$, and



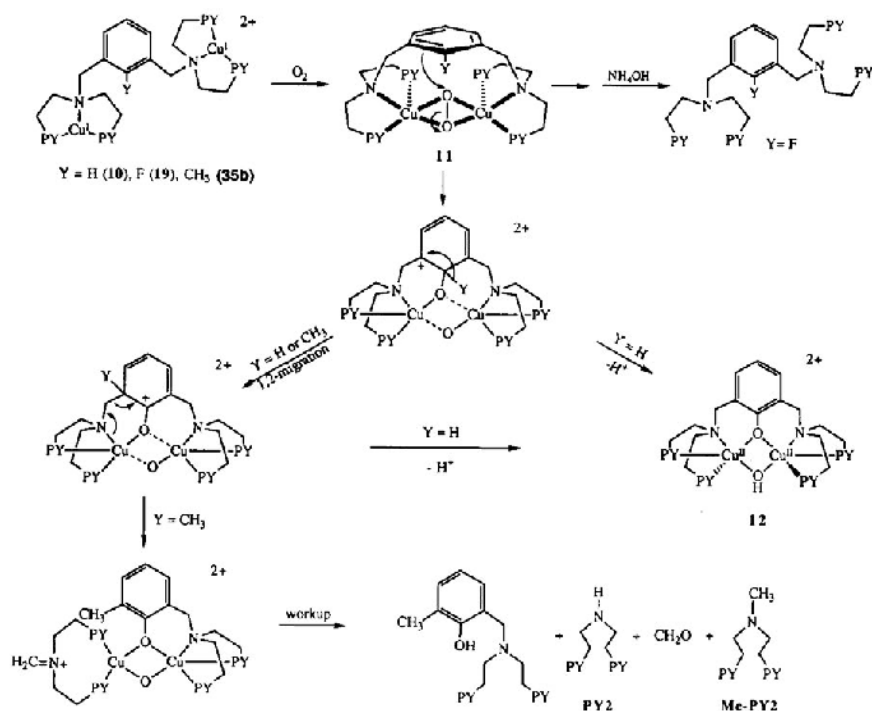


Figure 14 Unified mechanism of xylyl hydroxylation reactions, accounting for Cu₂O₂ intermediate [11] formation and copper-mediated “N.I.H. shift” (1,2-migration) reactions.

Y = H; R = H and Y = D, F, Me) results in the quasi-reversible binding of O₂ and the production of Cu₂O₂ adducts (see Sections II.B.2. and II.C.1). These species, [Cu₂(R—XYL—Y)(O₂)]²⁺ (11, R = H; Figure 14), most likely have a μ-η²:η²-O₂²⁻-dicopper(II) structure, with the peroxo group bound in close proximity to the arene “substrate.”

- When Y = F in [Cu₂(XYL—F)]²⁺ (19), neither hydroxylation nor F migration occurs and intact XYL—F ligand is recovered. The lack of reactivity may be ascribed to the presence of both a deactivated ring and a very strong C—F bond. We note that when Y = Cl, reasonable yields of copper complexes with oxidatively dehalogenated ligand are obtained [168,169]; the reaction also requires an external reductant.
- Thus, in the general case, the peroxo group in [Cu₂(R—XYL—Y)(O₂)]²⁺ (11) attacks the xylyl substrate, generating



a cationic intermediate. A variety of evidence points to an electrophilic attack by the peroxo group on the arene, consistent with the lack of a deuterium isotope effect (as discussed previously). Structural details of the intermediates are speculative, but peroxide O—O cleavage probably occurs synchronically with arene attack, which helps to drive the reaction by favorable formation of oxo or hydroxo bridged Cu—O(H)—Cu products. A stick model of $[\text{Cu}_2(\text{R—XYL—Y})(\text{O}_2)]^{2+}$ (**11**) with a $\mu\text{-}\eta^2\text{:}\eta^2\text{-O}_2^{2-}$ group suggests that the O—O vector is well aligned with and close to the $p\text{-}\pi$ orbital of the arene carbon that is attacked. For oxygen atom transfer reactions, the importance of orienting a substrate in the direction of the dicopper bound peroxide σ^* orbital (along the O—O vector) has already been discussed (Section III.A) [7,162].

4. At this point, H^+ transfer could occur directly to give the product $[\text{Cu}_2(\text{R—XYL—O—})(\text{OH})]^{2+}$ (**12**), or Y could undergo a Wagner–Meerwein rearrangement [170] (i.e., formal migration of Y^- in a N.I.H. shift) to produce another resonance stabilized carbocation intermediate.
5. Here again, loss of $\text{Y} = \text{H}$ would result in rearomatization and formation of **12**, but in the case of $\text{Y} = \text{Me}$, this cannot occur. However, assistance by the amine nitrogen lone pair can aid the rearomatization process, producing the copper-bound phenol product and an iminium salt. Hydrolysis during the workup procedure could release the 2-methylphenol product and result in a retro-Mannich reaction to give the observed secondary amine (PY2) and formaldehyde. A small amount (<10%) of N—Me—PY2 is often observed as a byproduct and its yield is at the expense of the PY2 and formaldehyde; thus, it appears to be derived from direct reduction of the intermediate iminium salt [167].

Summarizing, the discovery of the xylyl-containing copper mono-oxygenase model system [119,153] provided inspiration for a great deal of new copper bioinorganic coordination chemistry, including new LCu(I) structures and $\text{Cu(I)}_2/\text{O}_2$ reactivity studies. The latter led to the discovery of reversible O_2 binding systems and new copper-dioxygen structures. Synthetic and kinetic investigations of xylyl complex derivatives and analogs have generated fundamental insights into copper-dioxygen binding and activation.

The xylyl hydroxylating system appears to possess a number of elements seen in enzyme catalyzed reactions, and the presence of a dicopper moiety, which effects a specific aromatic ring hydroxylation, identifies it as a tyrosinase model system. Analogous to the enzyme, either dicopper(I)/ O_2 or “met” dicopper(II)/ H_2O_2 reactions result in xylyl hydroxylation. Although not yet proved absolutely, $[\text{Cu}_2(\text{R—XYL—Y})(\text{O}_2)]^{2+}$ (**11**) is suggested to possess a $\mu\text{-}\eta^2\text{:}\eta^2\text{-O}_2^{2-}$ structure, the one now favored for oxy-Hc and oxy-Tyr. Very likely, it acts as an electro-



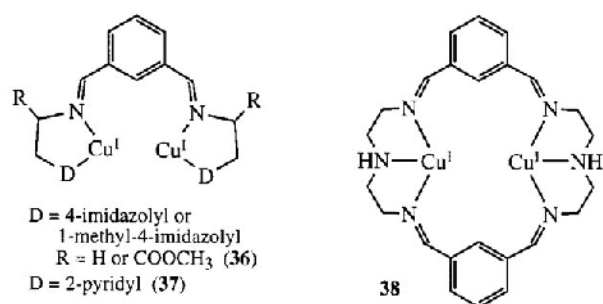
phile; coordination to dicopper(II) and placement of this species in a close and proper orientation appear to facilitate the arene attack. Indeed, these latter perspectives suggest a way for a dicopper center in either an enzyme or a chemical system to effect such a reaction. Perhaps this is the most important aspect of the system—that it mimics an enzyme active site by virtue of the fortuitous placement of a substrate (i.e., the xylyl portion of the dinucleating ligand) and reacting group (i.e., the Cu_2O_2 moiety) in appropriate juxtaposition.

III.B.2. Other Monooxygenase Models

Modified Xylyl Systems. Following our characterization of the $[\text{Cu}_2(\text{R}-\text{XYL}-\text{H})]^{2+}$ (**10**) oxygenation reaction, a number of other researchers have studied modified xylyl systems where chelating groups other than PY2 were used. Sorrell [171] has studied oxygenation of dicopper(I) complexes of some very close analogs of XYL, which, however, do not lead to hydroxylation. This is the case when 1-pyrazolyl or 2-imidazolyl donor groups fully or partially replace the 2-pyridyl ligands in $\text{XYL}-\text{H}$; all such complexes react via four-electron reduction of the O_2 molecule to give bis(μ -hydroxo)copper(II) dimers. Also, if $-\text{CH}_2\text{PY}$ (PY = 2-pyridyl) instead of $-\text{CH}_2\text{CH}_2\text{PY}$ arms are used in the xylyl dinucleating ligands, only irreversible oxidation and no ligand hydroxylation takes place (unpublished observation). Sorrell also tried to correlate the tendency for hydroxylation with either ligand basicity and/or Cu(II)/Cu(I) redox potential; however, there were no clear trends, perhaps as a result of a somewhat limited data set. Electronic considerations should be important, but copper chelation and peroxide proximity/orientation toward xylyl substrate have to be considered. The mechanism may change the course as well, since Sorrell's modified Cu(II) complexes do hydroxylate when they are reacted with hydrogen peroxide [171].

Some of these effects are also seen in the chemistry observed for xylyl-copper complexes studied by Casella [172], Feringa [173,174], and Martell [175]. Casella studied dinuclear Cu(I) complexes with Schiff-base ligands providing only two donors per copper ion (including an imidazolyl group). A solvent molecule such as acetonitrile may also coordinate. Compound **36** hydroxylates in reactions with O_2 when the imidazole N^1 is methylated (Scheme 15). Here, $\text{Cu}:\text{O}_2 = 2:1$ and a phenoxo- and hydroxo-bridged compound similar to $[\text{Cu}_2(\text{XYL}-\text{O}-)(\text{OH})]^{2+}$ (**12**) is produced. When the imidazole N^1 substituent is H, four-electron reduction of O_2 is preferred in competition with hydroxylation. Protic solvents or acids enhance the hydroxylation pathway, whereas nonprotic solvents facilitate Cu(I) oxidation to form μ -imidazolato Cu(II) compounds. The acid dependence observed suggests that hydroperoxo species may be responsible for the hydroxylation reaction. Feringa et al. examined a complex similar to that studied by Casella with 2-pyridyl donors instead of 4-imidazolyl. Thus, dicop-





Scheme 15

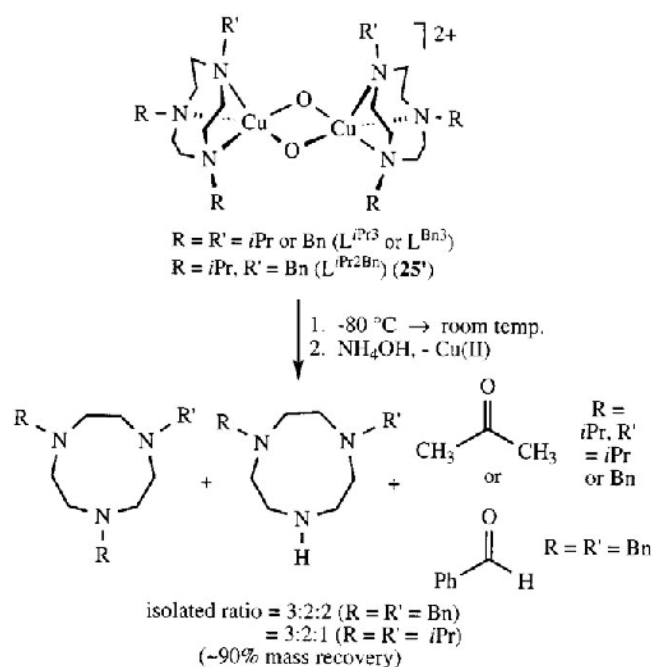
per(I) complex **37** also readily hydroxylates when exposed to O_2 and the structure of the phenoxo- and hydroxo-bridged product was verified [173]. These researchers have placed methoxy substituents in the xylyl 2- and 5-positions of this system and have observed oxidative demethylation of the 2-methoxy group; this can proceed via either aryl-oxygen or alkyl-oxygen bond cleavage pathways [174].

A further extension of *m*-xylyl dicopper complexation and xylyl hydroxylation is seen in a Schiff-base macrocycle [175]. When dicopper(I) complex **38** is reacted with O_2 , one of the two arene rings is hydroxylated, again producing a phenoxo-bridged dicopper(II) species; a peroxo-dicopper(II) intermediate is suggested as the actual oxidant.

Aliphatic Hydrocarbon Oxygenation. Monooxygenase reaction models have been observed with the substituted $\text{R}_2\text{R}'\text{-tacn}$ complexes (**23**) and their dinucleating analogs tethered by an ethylene or xylyl linker [44,135,136,157, 158,176]. For most of the complexes, oxygenation exclusively leads to cleavage of one of the substituents, producing either acetone or benzaldehyde. All these reactions exhibit a remarkable kinetic isotope effect (KIE) of up to 40, clearly establishing C—H bond cleavage as the rate-determining step. Preferential isopropyl over benzyl N-dealkylation in $\{(\text{Bn}i\text{Pr}_2\text{-tacn})\text{Cu}\}_2(\text{O})_2^{2+}$ (**25'**) may be due to equatorial disposition, and thus close contact between the reactive oxo group and the *i*Pr groups [176] (Scheme 16). Activation parameters $\Delta H^\ddagger = 49$ to 58 kJ mol^{-1} , $\Delta S^\ddagger = -36$ to $-79 \text{ JK}^{-1} \text{ mol}^{-1}$ are responsible for easily observed peroxo/bis- μ -oxo intermediates up to room temperature. Neither attack of the macrocyclic ring structure nor oxidation of the ethylene tether in dinuclear $i\text{Pr}_4$ dtne [158] has been observed to any significant extent.

An interesting observation has been made with the ligand tethering two $i\text{Pr}_2\text{-tacn}$ units by a *m*-xylyl spacer, $i\text{Pr}_4\text{-}m\text{-Xyl}$ [176]. Concentration-dependent spectra and decay rates are found for the oxygenated species, indicating preferential formation of bis- μ -oxo species at high concentrations but a $\mu\text{-}\eta^2\text{:}\eta^2\text{-}$





Scheme 16

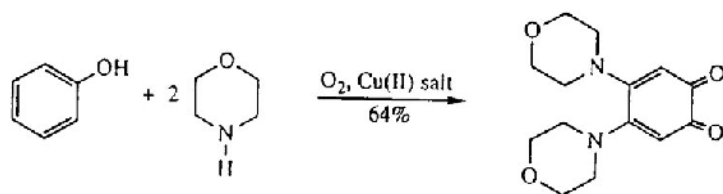
peroxo complex in dilute solution. Detailed kinetic analysis showed that the latter leads to ring hydroxylation in the 2-position, analogous to the original complex $[\text{Cu}_2(\text{R}-\text{XYL}-\text{H})]^{2+}$ (**10**) with no discernible KIE, whereas the former again leads to *i*Pr cleavage with strong KIE. The results are explained by competition of inter- and intramolecular adduct formation and point to the overall importance of proximity effects determining the relative reactivities.

In $\text{LCu(I)}_n/\text{O}_2$ reactivity studies, a number of other examples of oxidative N dealkylation [9] or O dealkylation [174,177] are known. As such, these represent possible model systems for PHM (discussed earlier). Other recent examples [134,178] of $\text{LCu(I)}_n/\text{O}_2$ -mediated hydrocarbon oxygenation reactions on coordinated ligands are also noteworthy.

External Phenol Hydroxylation. Although the xylyl systems that we have discussed are of great interest, they involve stoichiometric reactions that are more useful in mechanistic investigations than in synthesis. Tyrosinase *o*-hydroxylates phenols with turnover, and there is considerable industrial interest in catalytic phenol oxygenation.

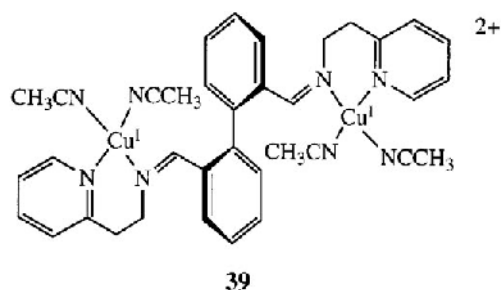


It is worthwhile to cite the pioneering work of Brackman and Havinga, carried out in the 1950s and considered as early tyrosinase models [1,2,179]. Here, conditions were found to effect the straightforward catalytic *o*-hydroxylation of phenols to give substituted *o*-quinones. A most interesting case occurs when copper salts are reacted with phenol, O₂, and morpholine (mp) in methanol, giving insoluble morpholino-substituted *o*-benzoquinone. The reaction is complicated, but a Cu(II)-peroxo-phenol-mp species is seen to be an important intermediate (Scheme 17).



Scheme 17

A number of other systems that effect catalytic phenol oxygenation reactions have been described [2,3,180]. La Monica et al. [181] reacted a hydrogen-carbonato Cu(I) complex [(phen)(PPh₃)Cu(O₂COH)] (phen = 1,10-phenanthroline) with excess phenol under O₂ and isolated a catecholates species [(phen)(Cu(OPh)OC₆H₄-2-(OH))]. In a system described by Réglier et al. [182], 2,4-*di-t*-butylphenol is converted to the corresponding *o*-quinone with up to 16 turnovers/hr when reacted with dioxygen in the presence of dicopper complex **39** and triethylamine (Scheme 18). In the absence of base, only radical coupling

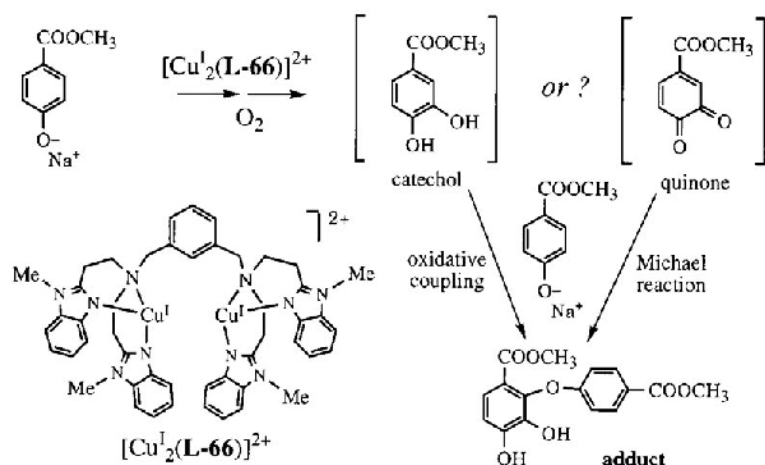


Scheme 18

product is observed, suggesting that dehydronation (loss of H⁺) and coordination of the phenol to copper are important in directing the reaction toward the oxygen-atom transfer process.



A most interesting tyrosinase model system has been described by Casella and coworkers, utilizing **L-66** [183], and/or related dinucleating ligands varying in the number of methylene groups between alkylamine and benzimidazole donors [184]. The dicopper(I) complex $[\text{Cu}_2^{\text{I}}(\text{L-66})]^{2+}$ mediates the ortho hydroxylation of exogenous phenols by reaction with O_2 . Labeling experiments indicate the expected incorporation of oxygen atoms into products, and a mononucleating analog of **L-66** shows no tyrosinase activity, consistent with the importance of a dinuclear copper complex-mediated reaction. However, the identity of the primary reaction product and course of reaction are controversial. Methyl 4-hydroxybenzoate as phenol is a convenient substrate for study; Casella has shown that appreciable quantities of catechol product are obtained, and quenching of reactions at low temperature suggest it to be the only product at early stages. Warming or longer reaction times result in the ultimate major product, which is the **adduct** shown, which is claimed to arise from a slower oxidative coupling of phenol and catechol (Scheme 19). Sayre and Nadkarni [32] also carried out experiments us-



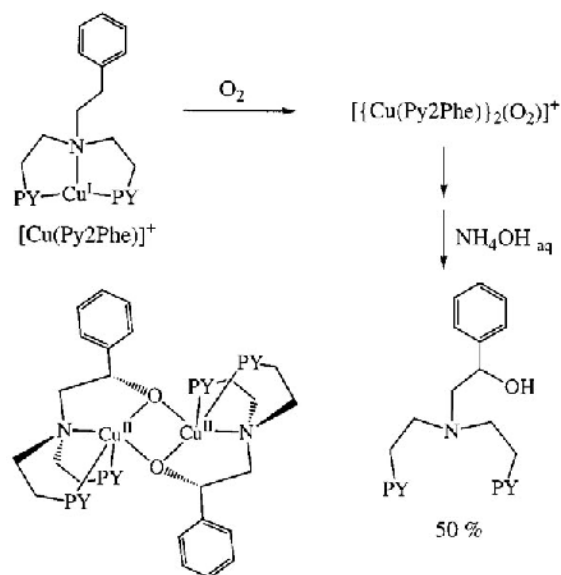
Scheme 19

ing $[\text{Cu}_2^{\text{I}}(\text{L-66})]^{2+}$. But their studies, including the observation that 4-carbomethoxy-1,2-catechol is inert to oxidation under the conditions employed, led them to conclude that the **adduct** results as a Michael addition product of phenol and 4-carbomethoxy-1,2-benzoquinone. The latter is claimed as the direct product of oxygenation in the tyrosinase-like reaction, $[\text{Cu}_2^{\text{I}}] + \text{phenol} + \text{O}_2 \rightarrow [\text{Cu}_2^{\text{I}}] + \text{quinone} + \text{H}_2\text{O}$. Notably, Kitajima [10] previously proposed a similar pathway and mechanism for tyrosinase reaction; no direct evidence for cresolase activity



in the enzyme actually exists. Using other copper oxidants, Maumy and Capdevielle [185] have also published results supporting Casella's view that catechols are directly produced by copper ion-mediated hydroxylation reactions. However, the possibility exists that quinone primary products generated in these tyrosinase mimetic reactions may be rapidly reduced to catechols by copper(I) still present, accounting for the observations by Casella [186]. Clearly, more work is required to clarify these mechanistic aspects.

A Dopamine β -Monooxygenase Model System. An important new monooxygenase model system has been described by Itoh and coworkers [187], wherein a copper–ligand complex possessing a phenylethyl pendant group undergoes specific and high-yield hydroxylation at the benzylic position, after O_2 reaction with the copper(I) complex of this mononuclear compound $[\text{Cu}(\text{Py}2\text{Phe})]^+$ (PY = 2-pyridyl). The efficiency of the reaction is very high (50% yield); oxygen atom incorporation into $\text{Py}2\text{Phe}-\text{OH}$ using labeled O_2 has been confirmed, and a $\text{Cu}^I/O_2 = 2:1$ stoichiometry is observed (Scheme 20). Spectroscopic evidence



Scheme 20

[188] indicates formation of a $\mu-\eta^2:\eta^2$ side-on peroxo intermediate $[\{\text{Cu}(\text{Py}2\text{Phe})\}_2(O_2)]^{2+}$, analogous to the RPY2 complexes we have previously studied [43] (discussed previously). It is of interest to know whether C—H ab-



straction and bis- μ -oxo formation may occur, and further mechanistic studies are in progress [188]. Another interesting feature is that the reaction can be made to produce 100% yield by using a reductant such as benzoin or hydroquinone in reaction with a copper(II) complex of the ligand, $[\text{Cu}^{\text{II}}(\text{Py2Phe})(^-\text{OCIO}_3)_2]$, followed by stirring in the presence of O_2 . The stable bis- μ -alkoxo dicopper(II) complex, $[\{\text{Cu}^{\text{II}}(\text{Py2Phe}-\text{O}^-)\}_2]^{2+}$, forms, and its structure has been determined [187].

IV. CONCLUSION

Copper-dioxygen complexes are of clear importance in chemical and industrial oxidative processes, and, within a bioinorganic context, they fit into a framework of O_2 -utilization processes, which include O_2 transport, O_2 activation for substrate oxygenation (i.e., incorporation of O atom[s]), or oxidation accompanied by O_2 reduction to dihydrogen peroxide or water. Considerable progress in synthetic modeling efforts has allowed the characterization of discrete CuO_2 (1:1) and Cu_2O_2 (2:1) Cu(I)-dioxygen adducts. This has included detailed descriptions of their (1) kinetics and thermodynamics of formation, (2) spectroscopy, and (3) structural characterization. These advances have come through the use of polydentate mono- or dinucleating ligands, which help to define and stabilize the environment of the copper-ion complex. Distinct Cu_2O_2 structures that have been elucidated through x-ray crystallographic investigations are a *trans*- μ -1,2-peroxo-coordination $[\text{Cu}(\text{II})\dots\text{Cu}(\text{II}) = 4.36 \text{ \AA}]$, a side-on planar μ - η^2 : η^2 -peroxocoordination $[\text{Cu}(\text{II})\dots\text{Cu}(\text{II}) = 3.560 \text{ \AA}]$, and an isomer of the latter with bis- μ -oxo-dicopper(III) moiety $[\text{Cu}(\text{III})\dots\text{Cu}(\text{III}) = 2.8 \text{ \AA}]$. Spectroscopic studies implicate distorted versions of these structures, and other variations clearly exist or are likely to be discovered.

The properties of Kitajima's μ - η^2 : η^2 -peroxo dicopper(II) complex lead to the conclusion that this is the likely structure in oxyhemocyanin and oxytyrosinase; this is perhaps the most important contribution from this type of model chemistry. A distorted or closely related peroxo-dicopper(II) species appears to be involved in aromatic hydroxylation proceeding in a well-characterized tyrosinase model system.

Continued research efforts in Cu_nO_2 chemistry are clearly required, as new insights concerning current systems are likely, spectroscopic/structural correlations are incomplete, and alternate structural types are likely to be found. Room-temperature stable copper-dioxygen complexes have been recently generated. However elusive, unstable Cu_nO_2 species have proved amenable to kinetic and spectroscopic characterization through the use of in situ low-temperature manipulations.

From information obtained through protein studies, it is clear that there are



other exciting new areas for future investigation. Copper ion-mediated dioxygen activation also appears to occur by other means, such as with trinuclear clusters found in multicopper “blue” oxidases, or at a mononuclear site where electron transfer from a nearby but not electronically coupled second copper ion takes place (e.g., D β H and PHM). In addition, associated novel organic cofactors such as found in phenylalanine hydroxylase, galactose oxidase, and amine oxidases are clearly critical for oxidative catalysis in these enzymes. We feel that copper-dioxygen coordination and bioinorganic chemistry are still in their infancy.

V. NOTE ADDED IN PROOF

A new oxyhemocyanin molluscan (octopus) protein x-ray structure has been reported [188a]. The active-site is very similar to that observed for the horseshoe crab structure; one of the six histidine ligands is involved in a thioether bridge, i.e., to a nearby cysteine.

For tyrosinase *o*-oxygenation of phenols, there is the question of whether formation of a catechol product is the result of direct *o*-hydroxylation, or if it arises through an indirect route, i.e., reduction of a directly produced *o*-quinone (See Sections I.B.2 and III.B.2). Recent biochemical studies [189,190] support the indirect mechanism. The x-ray structure of the monooxygenase active PHM domain of PAM (see Section I.B.2) has been published [49]. For the Cu-dependent particulate (membrane-bound) methane monooxygenase (pMMO), low-frequency EPR [191] or pulsed EPR/ENDOR [192] spectroscopic studies provide the first evidence for histidine coordination for certain of the Cu(II) centers in this enzyme. A new x-ray structure has been reported for laccase from *Coprinus cinereus* [193]; it is missing the type 2 copper ion, and the very high-potential type 1 Cu lacks the typical “blue” copper axial methionine ligand (this is replaced by a nonbonding leucine), similar to the situation found for the type 1 Cu in domain 2 of ceruloplasmin [66]. Additional reports describing synthetic models for galactose oxidase have appeared [194–197], including a system from Wang et al. [197], which catalytically oxidizes alcohols to aldehydes using O₂, and faithfully mimics the enzyme mechanism with respect to the involvement of a copper(II)-coordinated phenoxyl radical. Several more x-ray structures of amine oxidases have been published. That from *Hansenula polymorpha* provides mechanistic insights, as the TPQ cofactor is observed in a conformation poised for catalysis [198]. Structures of the holoenzyme (two forms) and apoenzyme from *Arthrobacter globiformis* have been reported; these help provide a more detailed description of proposed copper-dioxygen chemistry leading to amine oxidase cofactor biogenesis (via oxygenation of an active site tyrosine; see Section I.B.3) [199]. For both bacterial and mammalian cytochrome *c* oxidase x-ray structures, electron density of one of the Cu_B histidine ligands (at the heme-Cu dinuclear



active center) has been interpreted as involving a covalent linkage between the noncoordinated N_ε atom and the *ortho*-carbon of a close-by tyrosine; one possibility is that this Tyr-His dimer may donate an electron (with conversion to a phenoxyl radical) to an O₂-derived intermediate during catalysis [200,201]. For the bovine heart enzyme, x-ray structures of the fully reduced, reduced CO bound, oxidized (with putative peroxo ligand), and azide bound oxidized forms are now available [201]. Several important x-ray structures of copper model complexes have been recently reported. Stack et al. describe a *bis*(μ-oxo)dicopper(III) complex derived from reaction of [LCu(CH₃CN)]⁺ (L = (1*R*,2*R*)-cyclohexanediamine) with O₂ [202]. Masuda et al. [203] provide structural and spectroscopic characterization of a mononuclear Cu^{II}-OOH complex with a tripodal pyridylamine ligand; N–H hydrogen bonding from an amide ligand group to the hydroperoxo moiety provide stabilization for the compound. Additional monooxygenase model systems involving *m*-xylyl hydroxylation have been described, using copper donors with triazacyclononane [177] or methionine thioether [204] moieties. In our own *m*-xylyl systems (see Section III.B.1), a recent study revealed that the aromatic hydroxylation reactions can be followed by resonance Raman spectroscopy, which clearly implicates the intermediacy of μ-η²:η²-peroxodicopper(II) reacting species [205]. Itoh et al. [188] have further detailed their dopamine β-monooxygenase model system (see Section III.B.2), suggesting a *bis*-μ-oxodicopper(III) species as the most likely hydrogen-atom abstracting agent. In a recently advanced dopamine β-hydroxylase model system, Réglier et al. [206] generated analogs of the RPY2-copper complexes, previously investigated in our laboratories [43] and by Itoh [187, 188]; with an appended 2-aminoindane group as internal “substrate,” both regio- and stereoselective oxygen atom transfer to the benzylic C—H bond occurs.

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15

Multielectron Transfer and Catalytic Mechanisms in Oxidative Polymerization

Eishun Tsuchida, Kimihisa Yamamoto,* and Kenichi Oyaizu

Waseda University, Tokyo, Japan

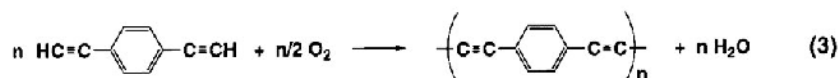
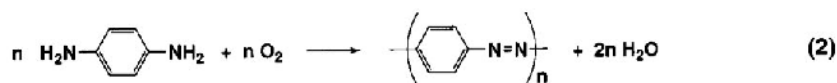
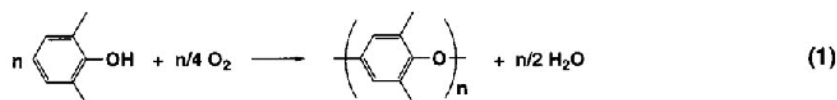
I. INTRODUCTION

Oxidative coupling polymerization provides great utility for the synthesis of high-performance polymers. Oxidative polymerization is also observed in vivo as important biosynthetic processes that, when catalyzed by metalloenzymes, proceed smoothly under an air atmosphere at room temperature. For example, lignin, which composes 30% of wood tissue, is produced by the oxidative polymerization of coniferyl alcohol catalyzed by laccase, an enzyme containing a copper complex as a reactive center. Tyrosine is an α -amino acid and is oxidatively polymerized by tyrosinase (Cu enzyme) to melanin, the black pigment in animals. These reactions proceed efficiently at room temperature in the presence of O_2 by means of catalysis by metalloenzymes. Oxidative polymerization is observed in vivo as an important biosynthetic process that proceeds efficiently by oxidases.

Organic compounds having labile hydrogen atoms, such as phenols, anilines, and acetylenes, are also oxidatively polymerized by metal-complex catalysts (Eqs. 1–3). The oxidative coupling is a dehydrogenation reaction; the polymer chain produced contains the dehydrogenated monomer structure as a repeating unit. As a remarkable example, poly(phenylene ether), one of the

* Current affiliation: Keio University, Yokohama, Japan.





widely used engineering plastics, is produced by the oxidative polymerization of 2,6-dimethylphenol with a copper–amine complex as a catalyst. The polymerization proceeds by the oxidation of the monomer, which takes place through coordination to the metal complex followed by the coupling of the monomers. The polymerization is homogeneously catalyzed by metal complexes and proceeds easily under an atmosphere of O₂ at room temperature. The catalytic efficiency is usually remarkably high as a result of the rapid reoxidation of the catalyst by O₂. The catalytic systems operate in both aqueous and organic solutions, when the complexes are stable in the media. The oxidative coupling is catalyzed by Werner-type complexes, in contrast to the polymerization of olefins, catalyzed by organometallic compounds. The latter must be carried out under strictly anaerobic conditions in inactive organic media in order to protect the catalyst from deactivation.

II. METALLOENZYMES AND OXIDATIVE COUPLING POLYMERIZATION

II.A. Role of Laccase in the Biosynthesis of Lignin

The network structure of lignin, which is made of phenol units, coagulates the cell wall in wood tissue, which is composed of cellulose and hemicellulose. Lignin is currently a waste product because of its complicated structure [1–4]. It is produced by an oxidative polymerization of coniferyl alcohol, sinapil alcohol, and cumaryl alcohol (Figure 1) catalyzed by metalloenzymes such as laccase and peroxidases. Laccase is a protein whose active center contains four coppers per one subunit [5–20].

Adler and Freudenberg reported a detailed study [21] of the polymerization of coniferyl alcohol with metalloenzymes. Coniferyl alcohol is activated by metal ions to form a radical that can be represented by four resonance states (Eq. 4) and dimerizes by coupling. In the early stage of the reaction, the formation of



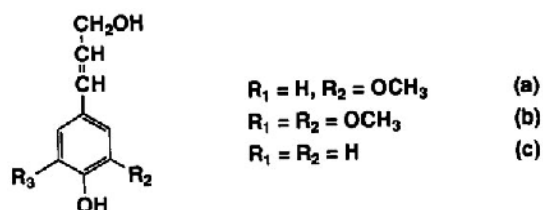
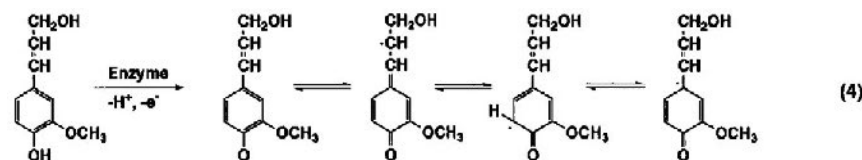


Figure 1 Monolignols; structure of coniferyl alcohol (a), sinapil alcohol (b), and cumarol alcohol (c).

the dimer preferentially proceeds by the couplings of the monomers. The dimer is reoxidized to a radical and coupled with another radical.



The formation of lignin proceeds by the uncontrolled coupling of the radical species, which leads to an irregular structure (Figure 2). The structure of lignin can be represented by the statistic ratio of each monomer, the frequency of each coupling mode, and the steric hindrance of substituents. This reaction mechanism has been successfully simulated [22].

The control of the coupling of coniferyl alcohol has been attempted. When the metalloenzyme is mixed with coniferyl alcohol, a stepwise polymerization proceeds by dimerization and tetramerization, which result in the uncontrolled couplings. When the monomer is slowly supplied to the enzyme-containing reaction mixture, the radicals attack the terminal end of the polymer chain, because the diluted radical reacts preferentially to form the dimer by the coupling; that means that comblike lignin grows on the cell wall. Some attempts have been reported to control the polymerization in the presence of cellulose as the matrix [23,24].

II.B. Formation of Melanin Catalyzed by Tyrosinase

Tyrosine is one of the important α -amino acids and is oxidatively polymerized with tyrosinase to melanin, the black pigment in animals [25–30]. Melanin plays a role in the prevention of damage to the organism that occurs through the absorption of ultraviolet light. Melanin is the only major paramagnetic organic com-



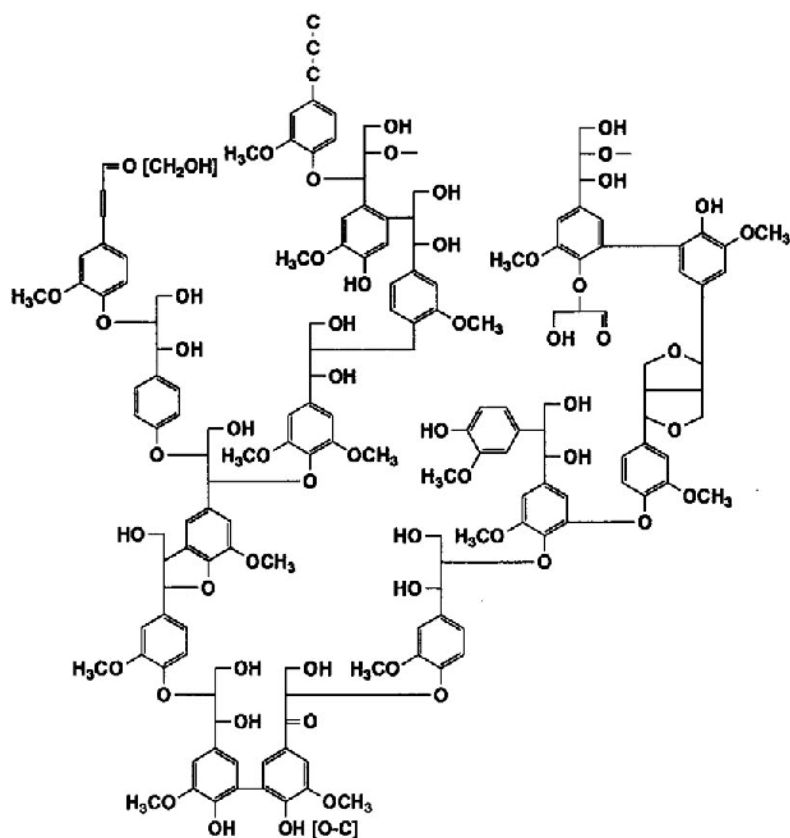


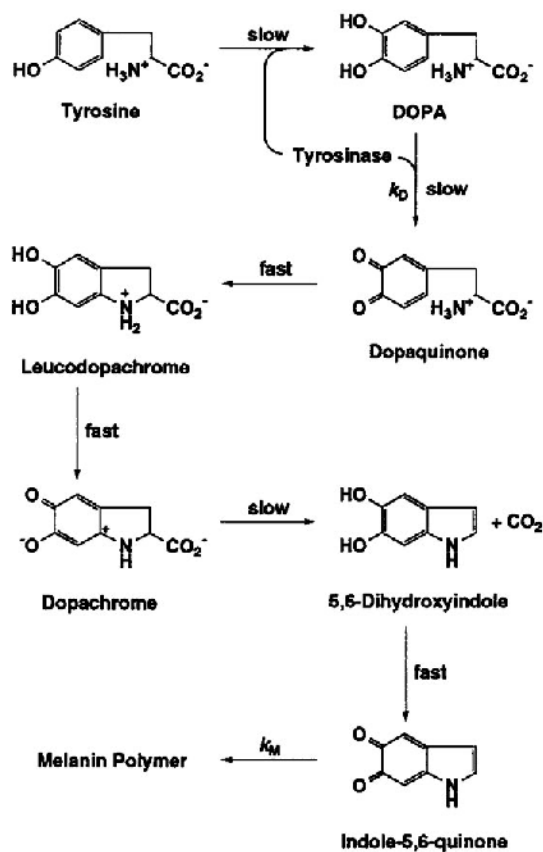
Figure 2 Proposed structure of lignin from Hondo spruce.

pound in vivo and may act as a scavenger of toxic radicals. Currently, melanin deserves much attention as a magnetic polymer containing phenoxy radicals [31,32].

Catechol melanin, a black pigment of plants, is a polymeric product formed by the oxidative polymerization of catechol. The formation route of catechol melanin (Eq. 5) is described as follows [33–37]: At first, 3-(3',4'-dihydroxyphenyl)-L-alanine (DOPA) is derived from tyrosine. It is oxidized to dopaquinone and forms dopachrome. 5,6-Dihydroxyindole is formed, accompanied by the elimination of CO_2 . The oxidative coupling polymerization produces a melanin polymer whose primary structure contains 4,7-conjugated indole units, which exist as a three-dimensional irregular polymer similar to lignin. Multistep oxidation reactions and coupling reactions in the formation of catechol melanin are catalyzed by a copper enzyme such as tyrosinase. Tyrosinase is an oxidase con-



taining four coppers with a molecular weight of 12,800. The active center exhibits diamagnetism, similar to the dinuclear site of hemocyanine, an O_2 carrier containing six histidine residues coordinated to the Cu_2 unit.



(5)

Kinetically slow steps in the formation of melanin from DOPA are the formation of dopaquinone from DOPA (step 1, k_D), the reaction of dopachrome to dihydroxyindole (step 2), and the polymerization to form melanin (step 3, k_M). Step 1 and step 2 proceed with about the same rate in the oxidative coupling polymerization catalyzed by tyrosinase. However, step 1 becomes remarkably slow when a macromolecule-metal complex is used as a catalyst. The copper complex in poly(1-vinylimidazole-co-vinylpyrrolidone) has been found [38] to act as an excellent catalyst and to exhibit the highest activity for melanin formation. The ratio of the rate constants (k_M/k_D) is approximately 3 (tyrosinase



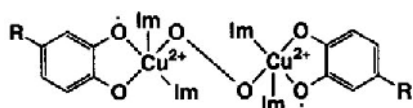


Figure 3 Proposed structure of the active center of poly(1-vinylimidazole)-Cu complex.

[$k_M/k_D = \text{ca. } 0.3$]) and is three times smaller than that for the other macromolecule-metal complex catalysts. These results suggest that the catalytic behavior of polyimidazole metal complexes is similar to that of tyrosinase. Therefore, the oxidation process to dopaquinone with a copper catalyst is promoted by using poly(vinylimidazole-co-vinylpyrrolidone) as a ligand. The effect is accelerated by the increase in vinylpyrrolidone content in the polymeric ligand. A pyrrolidone residue of only $5 \times 10^{-3}\%$ of weight in the polymeric ligand contributes to the acceleration of the polymerization. An increase in k_D is also observed by the addition of *N*-methylpyrrolidone in the reaction system catalyzed by poly(vinylimidazole)-Cu complex. The addition of ca. 5% weight of *N*-methylpyrrolidone results in similar acceleration. This indicates that the polymer ligand is effective in activating the complex.

The structure of the proposed intermediate (Figure 3) consists of substrate molecules, O_2 , and two Cu atoms as an active center and can be characterized as follows: (1) The value of k_D is maximized at a 2 : 1 [imidazole residue] to [Cu] ratio. (2) Copper(II) is not reduced by an excess of DOPA in the absence of O_2 . (3) Copper(II) is present in the steady state of the reaction. (4) The value of k_D increases with the addition of H_2O_2 and new absorption appears at 450 nm, similar to the behavior of oxyhemocyanin.

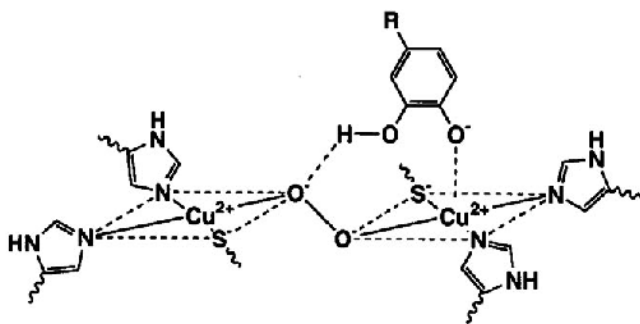


Figure 4 Proposed structure of the active center of tyrosinase.



The structure of the active center in tyrosinase has been proposed as shown in Figure 4 [39,40].

Melanin, which is prepared by polymerization with a macromolecule-metal complex, is isolated as a black powder and shows strong absorption in the ultraviolet and visible regions. The free radical species is stable against heat, acid, and base, and the EPR shows a singlet signal at $g = 2.00$. These properties are also important in the physiological characteristics of melanin.

III. OXIDATIVE POLYMERIZATION CATALYZED BY METAL COMPLEXES AS LACCASELIKE REACTIONS

III.A. Polymerization of Phenols by Metal Complexes

Organic compounds having labile hydrogens, such as phenols [41,42], phenylenediamines [43], and acetylenes [44], can be oxidatively coupled in the presence of specific metal complexes to form polymeric compounds. The oxidative polymerization of 2,6-disubstituted phenols with a copper-amine complex produces poly(2,6-disubstituted phenylene ether) [45–51]. Poly(2,6-dimethylphenylene ether) and poly(2,6-diphenylphenylene ether) are commercially produced from 2,6-dimethyl phenol and 2,6-diphenylphenol, respectively (Figure 5). These polymers exhibit excellent performance as engineering plastics.

Hay provided an improved process for the preparation of high-molecular-weight poly(phenylene ether) by oxidatively coupling a di-*ortho*-substituted phenol with O_2 under polymer-forming reaction conditions in a liquid reaction system by using a specific copper-amine complex soluble in the reaction mixture comprising copper ion, bromide ion, a specific class of hindered secondary diamines, and a specific group of tertiary monoamines [52]. The process is characterized by a reaction system that (1) results in the formation of higher-molecular-weight polymer, (2) permits the use of an extremely low copper-to-phenol ratio, and (3) allows the reaction to be carried out in a solvent system such as toluene,

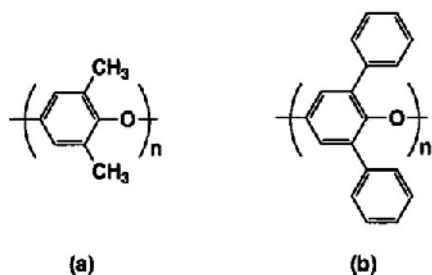


Figure 5 (a) Poly(2,6-dimethylphenylene ether); (b) poly(2,6-diphenylphenylene ether).



where H_2O produced in the reaction forms a separate phase without the necessity of removing the water phase [52]. Copper(I) bromide and copper(II) bromide form a 1:1 complex with $\text{N,N}'$ -disubstituted alkylene diamines, which have been found to have unique properties as catalysts in the oxidative coupling polymerization of phenols in solvent systems heretofore requiring removal of the H_2O formed as a by-product during the reaction [53]. Continuous polymerization of phenols has also been established [54]. 2,6-Dimethylphenol, homogeneous amine complex catalyst, solvent, and air were continuously charged to the bottom of a vertical reactor in such a way that the space velocity of air and the rate of air passing through the open part of the reactor were optimized. Poly(2,6-dimethylphenylene ether) was prepared continuously in high yield.

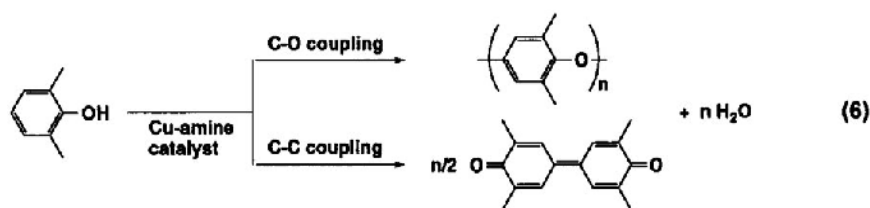
In the oxidative polymerization of phenols catalyzed by Cu complexes, the substrate coordinates to the Cu(II) complex and is then activated. The activated phenol couples in the next step. The Cu complex acts effectively as a catalyst at concentrations of 0.2–2 mol% compared to the substrate. The oxidation proceeds rapidly at room temperature under an air atmosphere to give poly(phenylene ether) in a quantitative yield. The polymerization follows Michaelis–Menten-type kinetics [55]. Enzymatic oxidation of phenols is an important pathway in the biosynthesis of lignin in plants [56] catalyzed by a metalloenzyme.

The oxidative polymerization has been proposed to proceed via a radical coupling that involves the coupling of neutral radicals or cation radicals. The former case corresponds to the oxidative polymerization of phenols and dithiols in which the neutral radical is formed by one-electron transfer after dissociation of a hydron from the monomer, or by the elimination of a hydron after the oxidation. The latter case takes place when the cation radical formed by one-electron oxidation exists as a stable species. The cation radicals then couple with each other, and the dimer is formed through solvent-catalyzed hydron elimination from the intermediate dication. Oxidative polymerization of pyrrole and thiophene uses this mechanism [57–62].

In the case of asymmetric monomers such as phenols, the coupling mode has to be controlled in order to obtain the polymer. In the polymerization of phenols, the phenolate anion is oxidized by a one-electron transfer. The C—O coupling (binding of the oxygen atom with the carbon atom in the para position) has to proceed preferentially for the formation of poly(2,6-dimethylphenylene ether), since the C—C coupling produces nonpolymerizable 3,3',5,5'-tetramethylbiphenylquinone (Eq. 6). Methyl substituents on the ortho position and orientation of phenolate anion in the coordination sphere of the metal complex prevent the side reaction (C—C coupling) from taking place [63,64]. The Cu complex catalyst takes part in the determination of the coupling mode.

Activation of the dimer in the oxidative polymerization of phenols takes place after the consumption of most of the monomer by the coupling, because the formation constant of the complex between the monomer and the copper





catalyst is much larger than that of the dimer. The mechanism indicates a stepwise reaction. The relationship between the conversion of the monomer and the molecular weight of the polymer supports the stepwise polycondensation mechanism [65–67]. The oligomer is oxidized more easily than the monomer because of its lower oxidation potential. The oligomers formed polymerize to poly(phenylene ether) through continuous repetition of the reoxidation and coupling reactions.

III.B. Catalytic Mechanism of Phenol Oxidation

2,6-Dimethylphenol is oxidatively polymerized to poly(2,6-dimethyl-1,4-phenylene ether) with a copper–amine complex by a laccaselike reaction. The activated phenols are coupled to form a dimer. The dimer is activated by a mechanism similar to that by which the polymerization proceeds. The effects of the amine ligands are to improve the solubility and the stability of the copper complex as well as the phenol-coordinated complex and to control the redox potential of the copper complex.

The catalytic cycle of Cu-catalyzed oxidation of phenol involves the coordination of the substrate to the Cu(II) complex and an electron transfer process from the substrate to Cu(II). The reduced catalyst may be reoxidized to the original Cu(II) complex by O₂. The details of the catalysis are still undetermined because of the complexity of its elementary steps.

The *d-d* absorption of the copper complex differs in each step of the catalysis because of the change in the coordination structure of the copper complex and in the oxidation state of copper. The change in the visible spectrum when phenol was added to the solution of the copper catalyst was observed by means of rapid-scanning spectroscopy [68]. The absorbance at the *d-d* transition changes; from that change the rate constants for each elementary step have been determined [69]. From the comparison of the rate constants, the electron transfer process has been determined to be the rate-determining step in the catalytic cycle.

Recently, Chen et al. [70–73] and Baesjou et al. [74] reported that the oxidative polymerization of phenol proceeds through a one-step two-electron transfer. The catalysis follows the mechanism shown in Figure 6.

In the absence of phenol, the [Cu(L)₄]²⁺ complex (compound **1** in Figure 6) is predominantly formed. The phenol itself can hardly substitute OH[−] or Cl[−]



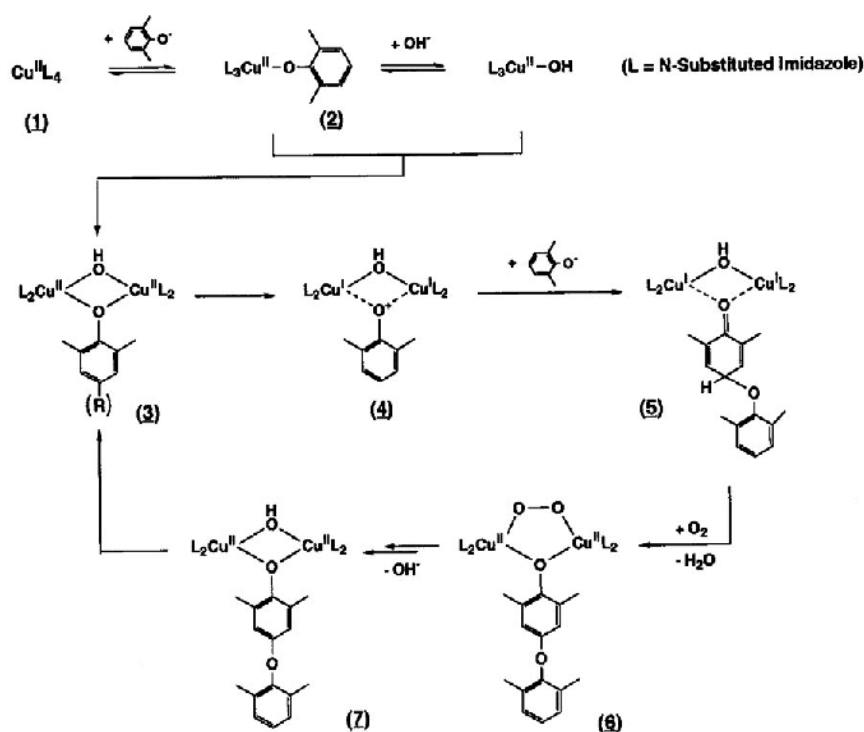


Figure 6 Catalytic mechanism for Cu-catalyzed oxidation of phenols by O_2 .

in the (bridged) copper complexes, but the phenolate anion can, as indicated by the shorter induction times and the higher initial reaction rates that are observed by the addition of a hydroxide. With a weak base, such as Et_3N , the rates are smaller. The phenolate anion (PhO^-) is coordinated to $\text{Cu}(\text{II})$ to form complexes like 2 (Figure 6).

The dimerization of the copper complex takes place to form the bis- $\text{Cu}(\text{II})$ compound 3, where the phenolate anion is the bridging ligand, just as proposed by Karlin [75], in the dinuclear complexes that act as the actual catalyst in the active state.

The electron transfer step is proposed to be rate-determining, as suggested by the significant change in the coordination structure of the complex ($\text{Cu}^{\text{II}}\text{-Cu}^{\text{II}}[\text{square planar}] = \text{Cu}^{\text{I}}\text{-Cu}^{\text{I}}[\text{tetrahedral}]$). A phenoxonium cation formed through a one-step two-electron transfer from one phenolate anion would be preferred, because the phenolate group can then keep a bridge intact in the compound



4 (Figure 6) between the two Cu(I) ions, to minimize structural changes. Such species are known for quinones [76].

The reoxidation step of the complex takes place after coordination of O₂ to the dinuclear Cu complex, forming the complex **6** (Figure 6). The μ -dioxo complex is estimated to be formed on the basis of Karlin's model [75]. In the next step the phenolate anion is oxidized by a two-electron transfer to O₂²⁻ via Cu(II). The resulting complex **7** is similarly transformed into compound **4** (Figure 6). The catalytic center is regenerated through four-electron oxidation by O₂. Such a multielectron transfer process may play a role in a metal-catalyzed enzymatic oxidation in vivo, such as photosystem II, in which metal ions often work cooperatively [77].

Electron transfer from the substrates to O₂ proceeds by a redox cycle that consists of copper(II) and copper(I). The high catalytic activity of the copper complex can be explained as follows: (1) The redox potential of Cu(I)/Cu(II) fits the redox reaction. (2) The high affinity of Cu(I) to O₂ results in rapid reoxidation of the catalyst. (3) Monomers can coordinate to, and dissociate from, the copper complex, and inner-sphere electron transfer proceeds in the intermediate complex. (4) The complex remains stable in the reaction system. It may be possible to investigate other catalysts whose redox potentials can be controlled by the selection of ligands and metal species to conform with these requisites; several other suitable catalysts for oxidative polymerization of phenols, such as manganese and iron complexes, are candidates on the basis of their redox potentials.

III.C. Metal-Catalyzed Polymerization of Aromatic Compounds Having Labile Hydrogen Atoms

Aromatic primary diamines, dithiols, and diethynyl compounds are oxidatively polymerized with metal-amine complexes as the catalyst to yield poly(azophenylene) [78], poly(disulfide) [79], and polydiyne [80], respectively (Figure 7, Eq. 7). The polymerization of compounds having active methyne groups has also been reported [81] (Eq. 8). The oxidative coupling polymerization of these monomers follows a mechanism similar to that of phenols. The catalytic cycle observed in the polymerization of *p*-phenylenediamine with Fe(edta) as the catalyst in an aqueous solution differs from that in the polymerization of phenols as follows: The activation of monomers usually involves either electron transfer from the anion or elimination of a hydrogen atom from the monomer. The oxidative polymerization of phenols uses the former mechanism of the electron transfer. In contrast, in the case of the polymerization of aromatic diamines as monomers, the neutral amines are coordinated to the catalyst, followed by the subsequent electron transfer and dehydronation. The dehydronation proceeds by the reaction with O₂. Another mechanism has also been proposed where dehydrogenation



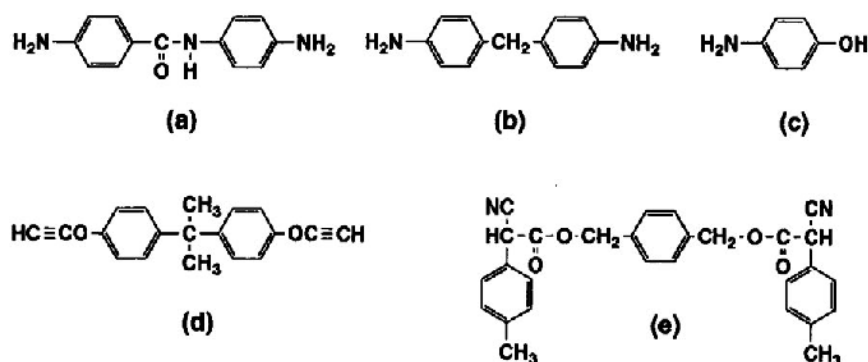
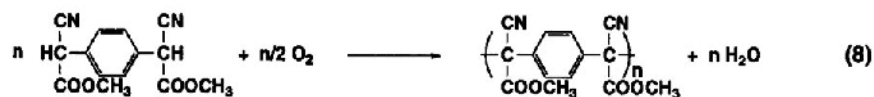
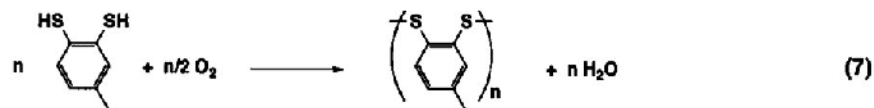


Figure 7 (a–e) Typical monomers for oxidative polymerization.

takes place by the formation of an intermediate complex that consists of O_2 , metal, and the monomer. In the oxidative polymerization of acetylenes and other compounds having active methyne groups, organometallic bonds with the copper catalyst are formed.



The orientation of the coupling is also an important issue. In the polymerization of phenols, the oxygen atom (head) is coupled with the carbon at the *para* or *ortho* position of the benzene ring (tail) through the coordination of a phenolate anion with the catalyst. On the other hand, when the monomers have two coordinating groups on both ends of the molecule, the coupling of the active radical on the coordinated atoms results in head-to-head coupling. The phenylene ring does not directly participate in the reaction but contributes to the stabilization of the formed radical. This is supported by the following results on the polymerization of aromatic diamines: (1) Azobenzene is formed by the N-N coupling from aniline. (2) Molecules having nonconjugated bonds between the coordinating groups on both sides (Figure 7b) can be treated as polymerizable monomers. (3) In asymmetric monomers, the coordinating groups with higher basicity (the amine group on the right side in Figure 7a) are preferentially coupled. The head-



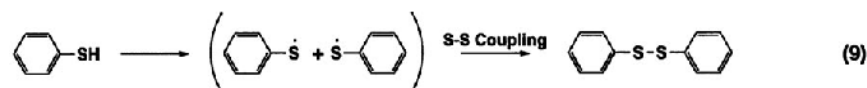
to-head coupling indicates that the reaction proceeds after the dissociation of the monomer from the coordination sphere of the metal complex.

IV. NEW CATALYTIC SYSTEMS OF METAL COMPLEXES FOR MACROMOLECULAR SYNTHESIS

IV.A. Cationic Oxidative Polymerization of Disulfides

IV.A.1. Synthesis of Poly(thio-1,4-Phenylene) by Oxidative Polymerization

The ability to polymerize readily via selective oxidation utilizing the abundant and cheap oxidant O_2 often represents a desirable low-cost method for upgrading the value of a raw material. The most successful example is the oxidative polymerization of 2,6-dimethylphenol to yield poly(2,6-dimethyl-1,4-phenylene ether) with copper-amine catalysts under an O_2 atmosphere at room temperature. Thiophenol also has a labile hydrogen but is rapidly oxidized to yield thermodynamically stable diphenyl disulfide. This formation is based on the more facilitated formation of S—S bond through radical coupling [82] in comparison with the formation of C—S—C bond through the coupling with the other molecules in the *para* position (Eq. 9).



Poly(*p*-phenylene sulfide) (PPS) deserves much attention as an engineering and a conductive plastic and in some cases as a specialty polymer with excellent performance. Lenz first reported that PPS is synthesized by the polycondensation of *p*-halothiophenolate alkali-metal salts at high temperature [83]. Commercially PPS is produced by the polycondensation of *p*-dichlorobenzene and sodium sulfide in *N*-methyl-2-pyrrolidone [84]. These polymerizations proceed only at high temperature and pressure, and it is difficult to remove the metal halides such as sodium chloride as by-products in order to obtain pure PPS; salt contamination degrades the electric performance and moldability.

A novel and convenient synthetic route for PPS via a cationic mechanism has been discovered [85–92]; diaryl disulfides and thiophenols are polymerized to PPS with high purity in high yield under an air atmosphere at room temperature by chemical oxidation and by electro-oxidation.

Thiophenol is electrochemically oxidized to diphenyl disulfide near 1.7 V (vs. Ag/AgCl), because the thiophenolate radicals undergo coupling. Although diphenyl disulfide is further oxidized under the given potential of 1.7 V, the cationic species formed are rapidly inactivated by a nucleophilic reaction with



solvents and/or supporting electrolytes and therefore do not yield PPS. It was found that the nucleophilic side reaction is effectively suppressed in the electro-oxidation of thiophenol and diphenyl disulfide in the presence of acid, which results in the formation of PPS.

Chemical oxidation can also be applied to this polymerization instead of electro-oxidation. Diphenyl disulfide reacts with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ) in dichloromethane at room temperature. The polymer has been isolated as a pure white powder (ca. 100% yield).

IV.A.2. Mechanism of Cationic Oxidative Polymerization

Methylbis(methylthio)sulfonium hexachloroantimonate ($[\text{CH}_3\text{S}(\text{SCH}_3)_2][\text{SbCl}_6]$) was isolated from the reaction mixture at -40°C by the oxidation of nonpolymerizable dimethyl disulfide [86]. This result suggests that the phenylbis(phenylthio)sulfonium cation is produced by the oxidation of diphenyl disulfide in the acidic reaction mixture [87–89]. This cation acts as the active species for the polymerization and electrophilically reacts with the *p*-position of the benzene ring to yield PPS [90].

The cationic mechanism of the polymerization is supported by the reference experiments in various solvents. The polymerization is suppressed in basic solvents such as acetonitrile and dimethylformamide. Alkyl halides and nitrobenzene are preferable for the formation of PPS in this polymerization.

IV.B. Oxidative Polymerization of Disulfides Catalyzed by Vanadyl Complexes

Aromatics for oxidative polymerization usually show high oxidation potentials. The direct oxidation of these monomers by O_2 does not proceed because of the large potential gap between the monomers and O_2 . A novel catalysis by vanadyl complexes is applicable to the synthesis of PPS by the O_2 -oxidative polymerization of diphenyl disulfide (Eq. 10) [93–103]. In the presence of $\text{VO}(\text{acac})_2$, the polymerization is accompanied by a quantitative O_2 uptake. Dioxygen is essential for the VO-catalyzed oxidative polymerization of diphenyl disulfide.



A variety of other metal complexes were utilized under the same conditions. However, most of them were not effective as catalysts (Table 1). Although an equimolar amount of DDQ and lead tetra-acetate can oxidize disulfide to yield PPS, these oxidants do not act as catalysts: the reduced species of these oxidants



Table 1 Cationic Oxidative Polymerization of Diphenyl Disulfide in the Presence of Catalytic Amounts of Metal Complexes^a

Metal complex	Conc. (mol/l)	$\frac{[\text{Catalyst}]}{[\text{Disulfide}]}$	PPS yield (wt %)	Catalytic efficiency (%) ^b
Fe(acac) ₂	0.01	0.1	0	0
Co(acac) ₂	0.01	0.1	0	0
Ni(acac) ₂	0.01	0.1	0	0
Mn(acac) ₂	0.01	0.1	0	0
Pb(CH ₃ COO) ₄	0.01	0.1	0	0
VO(acac) ₂	0.01	0.1	93	930
VO(acac) ₂	0.001	0.01	61	6,100
VOTPP	0.001	0.01	95	9,500
VOTPP	0.0001	0.001	35	35,000

^a Solvent = CH₂Cl₂, CF₃COOH = 0.1 mol/l, (CF₃CO)₂O = 0.2 mol/l.^b [PPS yield] × [disulfide]/[catalyst].

are not reoxidized by O₂. The other complexes, such as Cu(acac)₂, Fe(acac)₂, and VO(salen), cannot oxidize the disulfide.

An equimolar amount of vanadium(V) oxide (V₂O₅) reacts with diphenyl disulfide to yield PPS (>90%) even under anaerobic media. V(acac)₃ (vanadium(III) species) is oxidized to VO(acac)₂ by O₂. The activated VO(acac)₂ in the presence of acid seems to exhibit the properties of both vanadium(III) and vanadium(V) in the catalytic system.

The PPS yield depends on the acidity of the mixture. The oxidative polymerization does not proceed in the absence of acids. Strong acids, such as trifluoromethanesulfonic acid and trifluoroacetic acid, are required for the VO-catalyzed polymerization. The oxidative polymerization of diphenyl disulfide is facilitated by the activated VO(acac)₂ produced by the acid. Diphenyl disulfide is not oxidized by O₂ only or an equimolar amount of VO(acac)₂ in the absence of acid. The VO catalyst is estimated to be an excellent electron mediator, through activation by acids, to promote the electron transfer between diphenyl disulfide and molecular oxygen.

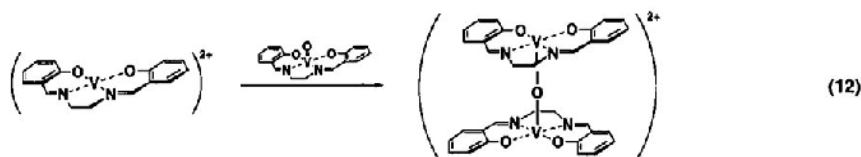
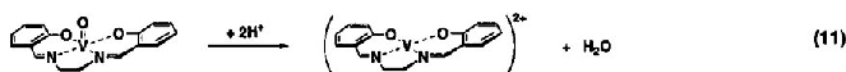
IV.C. Catalytic Cycle of Vanadium Complexes in Cationic Oxidative Polymerization

IV.C.1. Structure and Redox Properties of the Vanadium Catalyst

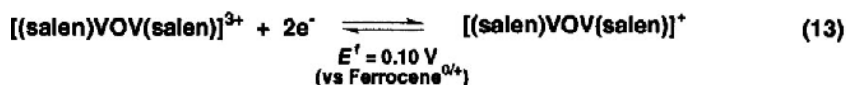
Although VO(acac)₂ acts as an excellent catalyst for the O₂-oxidative polymerization of diphenyl disulfide, mechanistic analysis of the redox process involved in



the catalytic cycle is made difficult by the lability of the β -diketone ligand [104–106]. Therefore the electron transfer chemistry of a more stable model of the acetylacetonato catalyst has been investigated. The model complex is VO(salen) [$H_2(\text{salen}) = N,N'$ -ethylenebis(salicylideneamine)]. It has been revealed that VO(salen) is deoxygenated in strongly acidic nonaqueous media (Eq. 11), and the deoxygenated complex reacts with VO(salen) itself to form a μ -oxo binuclear vanadium complex (VOV^{2+}) (Eq. 12) [107]. The oxidation of VO(salen) by O_2 to produce $VO(\text{salen})^+$ proved feasible in the presence of acid. The behavior of VO(salen) in acidic media in the presence of O_2 is of interest with regard to the mechanism by which it and its bis(acetylacetonato) analog act as auto-oxidation catalysts.



The VOV^{2+} complex can be prepared as a black powder by the acidification of VO(salen) with trifluoromethanesulfonic acid or trityl tetrafluoroborate in non-aqueous media [107]. Electro-oxidation of the VOV^{2+} complex consumed one electron per dinuclear unit and the resulting solution exhibited a single reduction wave at the electrode (Figure 8[a]). A cyclic voltammogram of the oxidized solution of VOV^{2+} contained a single reversible couple with peak currents corresponding to two electrons per dinuclear reactant (Figure 8[b]). The magnitude of the single cathodic plateau (Figure 8[a]) corresponded to the two-electron reduction. Thus, the VOV^{3+} complex is reduced directly to VOV^+ in a single step (Eq. 13).



Electrochemical and structural studies of oxovanadium complexes with Schiff-base ligands attract particular attention because of their reversible redox behavior, which allows possible applications to electrocatalysts. VO(salen) and its oxidized product $V^+O(\text{salen})ClO_4$ crystallize readily and their x-ray structures have been solved [108,109].



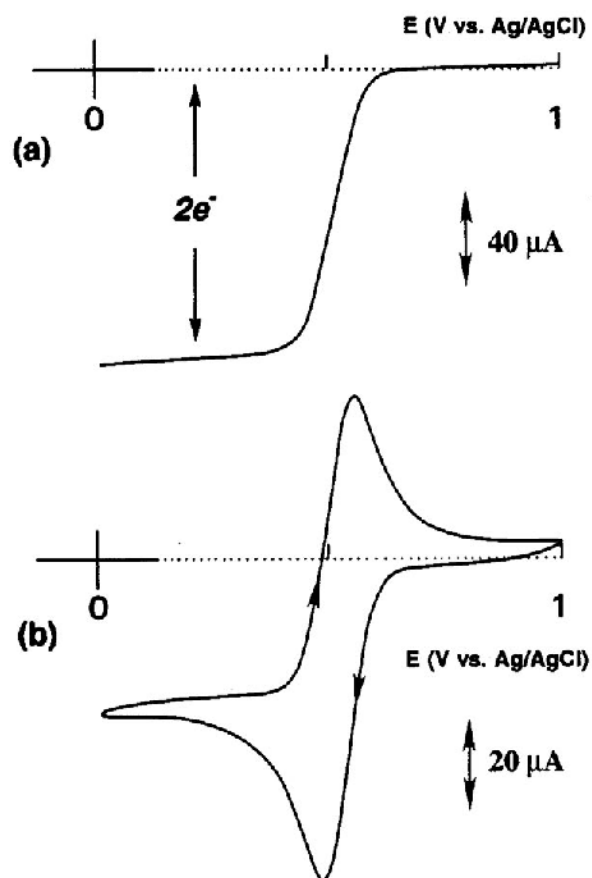


Figure 8 (a) Rotating disk current-potential curve for a 1 mmol/l solution of [(salen)-VOV(salen)][BF₄]₂ after controlled potential oxidation that consumed one electron per dinuclear molecule. Pt disk ($\phi = 4$ mm), 500 rpm, anaerobic anhydrous CH₃CN, TBABF₄ (0.1 mol/l); (b) cyclic voltammogram for the solution from (a).

The reduction of VOV²⁺ produced a mixed-valent, μ -oxo divanadium(III, IV) complex [(salen)V^{IV}OV^{III}(salen)][X] (VOV⁺). X-ray analysis revealed the molecular structure of the complex as a triiodide salt ($X = \text{I}_3^-$) as shown in Figure 9.

The phenolate oxygen in a salen ligand often serves as a bridging ligand; the typical example is provided by Cu(salen), which crystallizes in a dinuclear arrangement with non planarity of the central coordinated group [110]. Indeed,



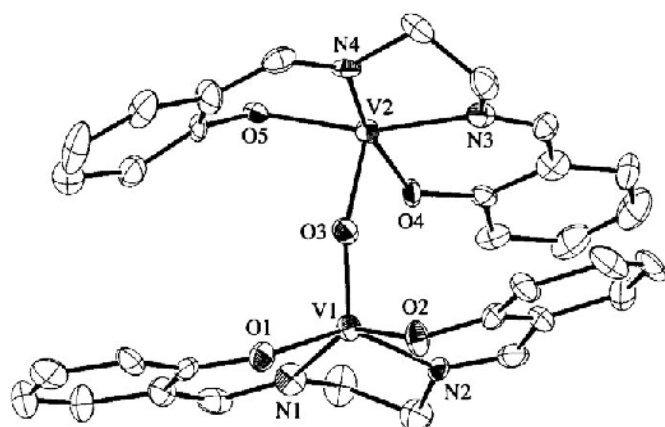


Figure 9 ORTEP view (50% probability ellipsoids) of a cation in $[(\text{salen})\text{VOV}(\text{salen})][\text{I}_3]$.

the molecular structure of VOV^+ can also be taken as tetravanadium complex with the center of symmetry located at the center of the molecule (Figure 10). It is known that vanadium(III) prefers an octahedral arrangement, whereas vanadium(IV) is more stable in square pyramidal geometry. The arrangement around vanadium(III) in the VOV^+ complex is pseudo-octahedral (Figure 9). In contrast,

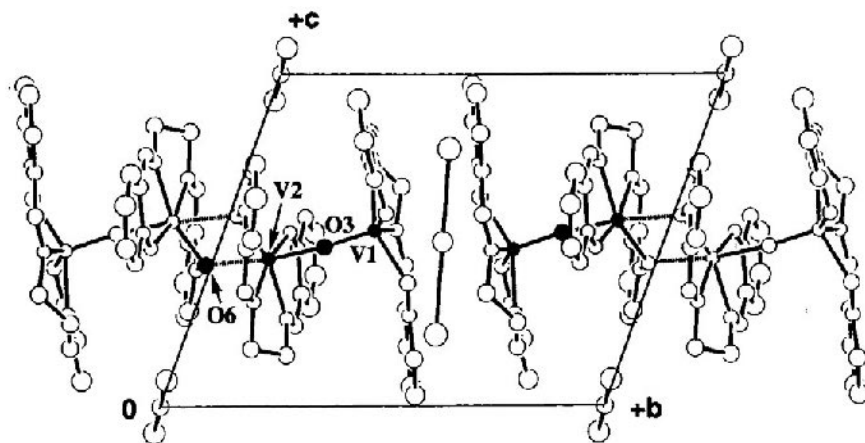


Figure 10 ORTEP drawing of the unit cell of $[(\text{salen})\text{VOV}(\text{salen})][\text{I}_3]$ viewed down the a axis.

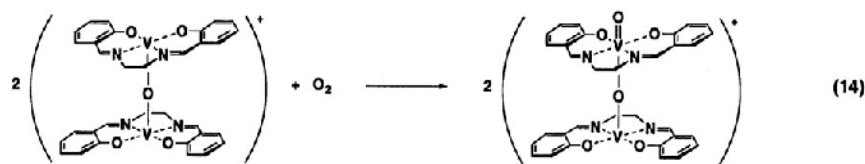


vanadium(IV) atom represent a typical square pyramidal coordination arrangement. The bond length between the vanadium atom and the equatorial phenolate oxygen of the ligand is indicative of the oxidation state.

The mixed-valent state was characterized by the near-infrared spectrum of the VOV^+ complex, which was classified into a type II mixed-valent complex ($\alpha = 3.0 \times 10^{-2}$) [111–114]; the optical and electronic properties of VOV^+ are based not only on the constituent components but also on their interaction [115].

IV.C.2. Oxidation of Low-Coordinate Vanadium(III) to Oxovanadium(V) with O_2

It is well known that the reactivity of vanadium complexes toward O_2 is responsive to the redox state and the coordination structure. A typical example is provided by acetylacetonate complexes: a 6-coordinate vanadium(III) complex $\text{V}(\text{acac})_3$ is oxidized by O_2 , whereas a 5-coordinate vanadium(IV) complex, $\text{VO}(\text{acac})_2$, is stable in air. The oxidation of VOV^+ containing the 5-coordinate vanadium(III) by O_2 in anhydrous CH_2Cl_2 proceeded almost stoichiometrically to produce a divanadium(IV,V) complex (VOVO^+) (Eq. 14). The oxidation of VOV^+ by O_2 obeyed a rate law that was pseudo-second-order in VOV^+ , which suggested a mechanism of oxidation involving a bimolecular reaction to produce an O_2 -adduct such as peroxo tetramer as the rate-limiting step. The homolytic scission of the intermediate is a likely reaction to generate two molecules of the VOVO^+ cation. The crystallization of VOVO^+ was performed in $\text{CH}_3\text{CN}/\text{Et}_2\text{O}$ to produce dark brown crystals. X-ray diffraction studies of the crystallized product revealed the formation of $[(\text{salen})\text{V}^{\text{IV}}\text{OV}^{\text{V}} = \text{O}(\text{salen})][\text{X}]$ ($\text{X} = \text{I}_3^-$, ClO_4^-) complex (Figures 11 and 12).



It is the preference of vanadium(V) to be 6-coordinated rather than 5-coordinated that drives the formation of the VOVO^+ cation. The weak axial interaction with O atoms is due to the low residual basicity of the O atoms, which are already bound to vanadium(IV).

The stoichiometry of reaction (14) is reminiscent of the electroreduction of O_2 to H_2O . It is of interest that oxygenation of vanadium(III) gives oxovanadium(V), whereas multiple one-electron redox centers have been considered to be essential for the incorporation of O_2 . An explanation for this can be found in



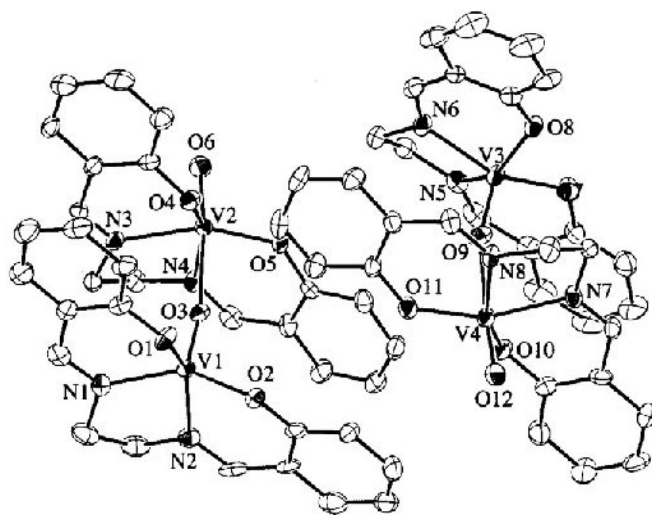


Figure 11 ORTEP view (30% probability ellipsoids) of cations in asymmetric unit of $[(\text{salen})\text{VOVO}(\text{salen})][\text{I}_3]$.

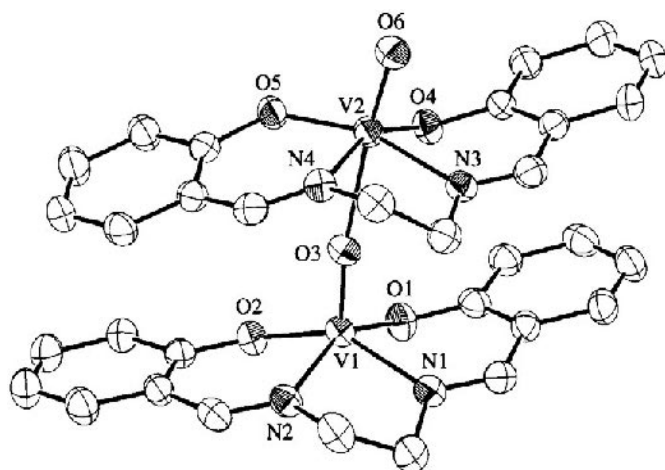
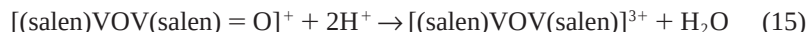


Figure 12 ORTEP view (50% probability ellipsoids) of a cation in $[(\text{salen})\text{VOVO}(\text{salen})][\text{ClO}_4]$.



the high reactivity of the five-coordinated vanadium(III) and the stability of the oxovanadium(V) species.

VO(salen) undergoes deoxygenation to give V(salen)²⁺ and H₂O in strongly acidic conditions [107]. The reactivity of VOV⁺ toward O₂ (Eq. 14) and its significant potential to lead to the selective four-electron reduction of O₂ to H₂O have prompted the study on the reaction of the VOVO⁺ cation with a strong acid to undergo deoxygenation of the VOVO⁺ cation to VOV³⁺ species (Eq. 15).



Although the lack of x-ray data prevents further determination of the electronic states, the formation of a mixed-valent VOV³⁺ cation has been evidenced by means of spectroscopic measurements [116,117].

IV.C.3. Homogeneous Catalysis of the Electroreduction of O₂

The capacity of metalloenzymes to transduce energy via synergetic electron transfer for the assimilative reductions of small molecules such as carbon dioxide and dinitrogen and O₂ oxidations in dissimilatory processes [118–121] poses a considerable challenge to modeling the biological processes. As recent topics, the x-ray structures of cytochrome *c* oxidases that provoke four-electron reduction of O₂ to H₂O have attracted much attention [122,123] though the structures of the intermediate O₂ adducts or their catalytic mechanisms have not been established yet. As to the four-electron reduction system of O₂, much effort has been spent to develop an effective electrocatalyst that operates near the thermodynamic potential [124–130]. Recent trends involve the use of multinuclear complexes having O₂ coordination sites. Anson and coworkers found that four-electron reduction of O₂ is accomplished by using a tetraruthenated cobalt tetrapyrrolylporphyrin, with continued diligence devoted to unraveling the mechanism [131–136]. On the other hand, the application of well-defined four-electron reduction systems of O₂ to molecular syntheses is also of interest. Indeed, a number of important biological and industrial processes involve metal-catalyzed oxidation of organic substrates, and for this purpose, the use of O₂ as the ultimate oxidant has obvious advantages in terms of cost and handling use [137–139].

Since the oxidative polymerization of diphenyl disulfide catalyzed by VO(acac)₂ results in selective formation of thioether bonds without any oxygenated compounds such as sulfoxides and/or sulfones, it should be noted that H₂O should be produced predominantly by the reduction of O₂ catalyzed by the vanadium complex without the formation of partially reduced side products such as H₂O₂.

In attempts to develop catalytic reduction of O₂, the vanadium complex VOV⁺ with the well-defined catalytic cycle was employed as a mediator. The electroreduction of O₂ was performed with the VOV⁺ complex in CH₂Cl₂ con-



taining trifluoroacetic acid. The VOV^+ complex has proved to be an effective electrocatalyst for the direct four-electron reduction of O_2 to H_2O .

The need for four-electron reduction of O_2 , as pointed out earlier, is the capacity to form an intercalated peroxo complex such as the $\text{Co}-\text{O}-\text{O}-\text{Co}$ complex using the difacial cobalt porphyrins and the dinuclear iridium porphyrin [124–130]. In recent studies, Anson and coworkers have demonstrated that the coordination of more than two $\text{Ru}(\text{NH}_3)_5$ groups to the meso pyridine ligands of the cobalt porphyrin ring is effective in accessing the four-electron reduction pathway [131–136]. The use of oxovanadium complex proved valuable in recent studies though it remains to be determined how hydrons take part in the oxygenation of vanadium to accelerate the overall catalytic reaction. Nevertheless, under conditions where oxygenation of vanadium(III) to oxovanadium(V) followed by acid-promoted deoxygenation proceeds, a reasonable yield of H_2O was obtained in the electroreduction of O_2 . Since the oxygenation–deoxygenation process of vanadium(III) yields two-electron-oxidized vanadium(V), it can be stated that the one-step two-electron redox couple of $\text{VOV}^{3+/+}$ that can transfer isoenergetically two electrons plays an essential role on the catalytic activity. These results also provide additional insight into the unique vanadium(III)/oxovanadium(V) redox chemistry with possible relevance to metal mono-oxygenases.

IV.C.4. Catalytic Cycle of Vanadium Complexes

The catalytic cycle containing VOV^+ and VOVO^+ , well-defined electrocatalysts for the four-electron reduction of O_2 , is described as follows: The cycle involves coordination of O_2 to VOV^+ through which oxidation to VOVO^+ takes place. As a result of metal–oxo bond formation ($\text{LM}^x + \frac{1}{2} \text{O}_2 \rightarrow \text{LM}^{x+2} = \text{O}$), the resulting complex contains two-electron oxidized vanadium(V) and a fully reduced oxo ligand. It is likely that the oxygenation process is promoted by acid. As a next step, the VOVO^+ cation is deoxygenated to produce the high-valent VOV^{3+} complex, which will act as a two-electron oxidant for substrates regenerating the low-valent starting complex VOV^+ . The unprecedented reactivity of VOV^+ toward O_2 and deoxygenation of VOVO^+ by hydrons are suggested as being key to the performance of these complexes as catalysts for reduction of O_2 to H_2O . It can be postulated that a similar catalytic cycle is also applicable to $\text{VO}(\text{acac})_2$ by which it can catalyze the O_2 -oxidative polymerization of diphenyl disulfide. Thus the overall redox cycle can be described as in Figure 13. The catalytic cycle involves one-step two-electron transfer (Eq. 13), which would also have an essential role in the catalysis. It may safely be stated that the transfer of multiple isoenergetic electrons is advantageous in the redox reaction: the two-electron oxidation of diphenyl disulfide produces a phenyl sulfenyl cation as an active species of the oxidative polymerization. It is a unique model of a catalyst operating in the four-electron reduction of O_2 to H_2O in acidic organic media.



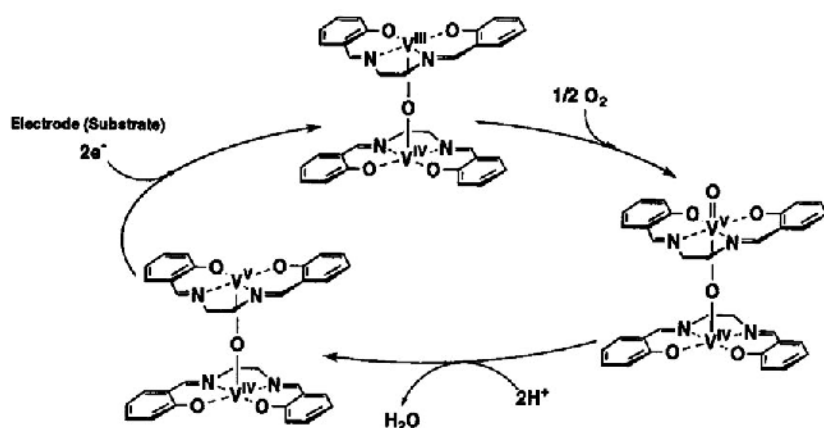


Figure 13 Catalytic cycle of VO complexes in four-electron reduction of O_2 .

V. CONCLUSION

Recent interest in the chemistry of oxometal complexes has been stimulated by their significance as reactive intermediates of oxygenase and oxidase enzymes, as new types of metallomesogens tuning intermolecular interactions, and as some of the essential structures for hydronation-triggered multielectron transfers at single electrode potentials. Of greatest importance are the synthetic applications to multielectron redox catalysts. A typical example is the epoxidation of olefins catalyzed by (tetramesitylporphyrinato)ruthenium (II), in which O_2 is reduced to epoxide by the four-electron redox process of ruthenium ($O=Ru^{VI}=O \rightarrow Ru^{II} + O_2$). As a new type of multielectron mediated system, the oxovanadium-catalyzed O_2 -oxidative polymerization of diphenyl disulfide in strongly acidic conditions has been established and provides a convenient synthesis of poly(*p*-phenylene sulfide), an important engineering plastic.

A molecular conversion system based on a four-electron transfer to O_2 was accomplished in the O_2 -oxidative polymerization of diphenyl disulfide (Figure 14) [116]. This is the first example of a multielectron mediator that is applied to molecular conversion systems. The multielectron transfer process from the reduced vanadium(III) complex (VOV^+) to O_2 not only revealed the O_2 oxidation mechanism but also provided additional insight into the unique chemistry of vanadium with possible relevance to metal mono-oxygenases.

Recent research reveals that the oxidative polymerization of phenol with O_2 also obeys the multielectron transfer with the polynuclear copper complexes [74]. The multielectron oxidation of substrates provides new active species,



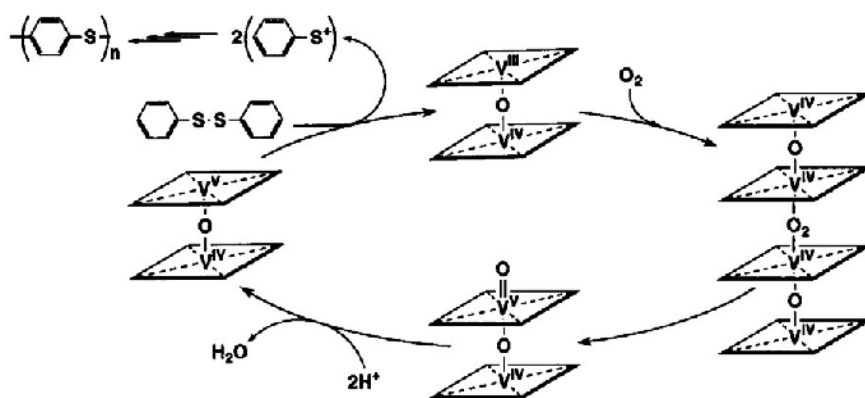


Figure 14 Proposed mechanism for the VO-catalyzed O_2 -oxidative polymerization of diphenyl disulfide.

which could be applicable to novel synthetic systems. Macromolecular metal complexes offer multielectron transfer systems where the entropy effect or the cooperative interaction is applicable as characteristic property of macromolecules.

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16

Metalloenzymes with a Quinone Cofactor

Johannis A. Duine

Delft University of Technology, Delft, The Netherlands

I. INTRODUCTION

About half of the enzymes known today require the presence of a cofactor, coenzyme, or prosthetic group, such a compound plays a direct role in catalysis by the enzyme in which it occurs (in the following, the term *cofactor* also comprises the connotation of a prosthetic group but not that of a coenzyme, as the latter is considered here to be a helper or cosubstrate in the reaction). In the past, identification of these compounds has been a matter of combined action of nutritional and biochemical research, this approach ended about 30 years ago as it was thought that all existing coenzymes, cofactors, and vitamins had been discovered. It was, therefore, a surprise when it subsequently appeared that the list should be extended because of results provided by research on the enzymology of methane dissimilation by methanotrophic bacteria and that on methane formation by methanogenic bacteria, generating several new cofactors [1]. However, most of them seemed to have a specialized role in Archaea, although it appears now that this view should not be taken too strictly (e.g., factor₄₂₀, a deazaflavin with very low redox potential that on its discovery was considered ideally suited to have a role only in the pathway of methanogenesis, has now been found to function as a cofactor in several enzymes occurring in aerobic Eubacteria [2]). Soon after its discovery in methanol dehydrogenase, pyrroloquinoline quinone, 2,7,9-tricarboxy-1*H*-pyrrolo[2,3-*f*]quinoline-4,5-dione (PQQ) (Figure 1), appeared to be exceptional in that sense that it was found in several different bacterial dehydrogenases (Table 1). Subsequently, many other enzymes were detected and were at



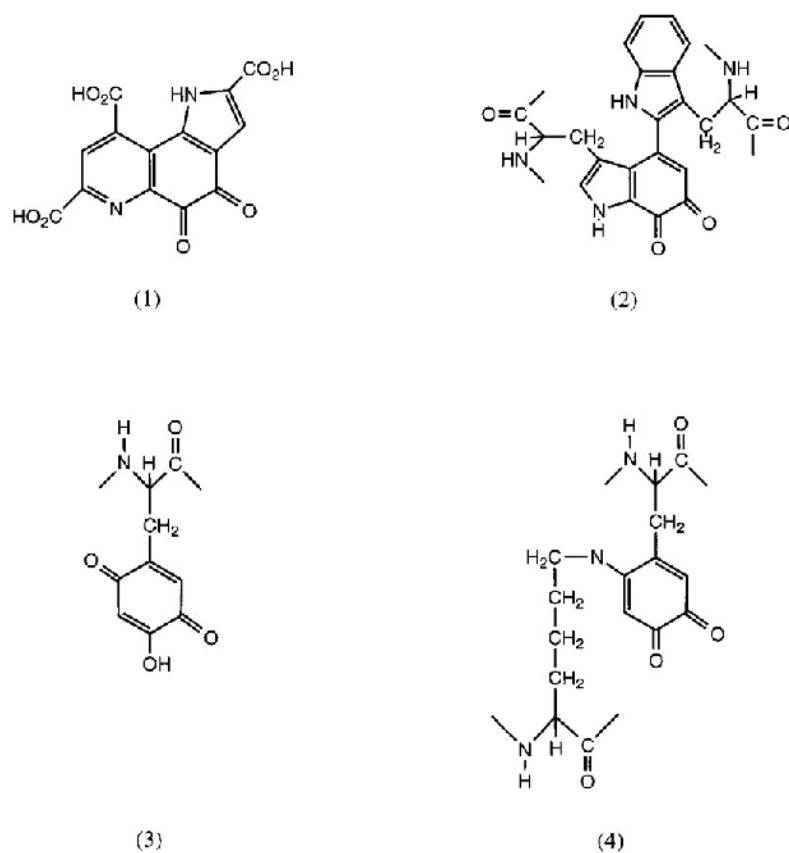


Figure 1 Structures of PQQ (1), TTQ (2), TPQ (3), and LTQ (4).

first reported to contain covalently bound PQQ. It soon became clear, however, that this was incorrect because they contained either another quinone cofactor (TTQ, TPQ, or LTQ, see Figure 1, which presents the structures for all of them) or the free radical form of a specific aromatic amino acid in their protein chain, which also functioned as a cofactor [3]. Since all these compounds have in common that they are formed by oxidation of a specific aromatic amino acid residue in the precursor protein chain (or in a peptide functioning as precursor in the biosynthesis of PQQ) and most of them require the participation of a metal ion as companion in catalysis, it could be reasoned that the following discussion will review both; however, this chapter will focus only on the quinone cofactors and



Table 1 Metal Ions and Natural Electron Acceptors for Quinoprotein Dehydrogenases

Enzyme	Cofactors	Metal ion	Electron acceptor
With PQQ			
Methanol dehydrogenase	PQQ	Ca	Cytochrome <i>c_L</i>
Alcohol dehydrogenase	PQQ	Ca	Cytochrome <i>c₅₅₁</i>
Quinohemoprotein alcohol dehydrogenases	PQQ/heme <i>c</i>	Ca	Azurin/ubiquinone/ cytochrome <i>c</i>
Polyvinylalcohol dehydrogenase	PQQ/heme <i>c</i>	Ca	?
Quinate dehydrogenase	PQQ/?	?	?
Lupanine hydroxylase	PQQ/heme <i>c</i>		
Glucose dehydrogenase (soluble, EC 1.1.99.17)	PQQ	Ca	Cytochrome <i>b₅₆₂</i>
Glucose dehydrogenase (membrane-bound)	PQQ	Mg	Ubiquinone
Fructose dehydrogenase	PQQ/heme <i>c</i>	?	
Sorbitol dehydrogenase	PQQ/heme <i>c</i>	?	
With TTQ			
Methylamine dehydrogenase	TTQ		Amicyanin
Aromatic amine dehydrogenase	TTQ		Azurin

their interplay with metal ions in “metalloquinoproteins,” since most of the combinations of free radical amino acid cofactor and metal ion are treated in other chapters (Tyr[•]/Fe in ribonucleotide reductase, in Chapter 10; tyr-cys[•]/Cu in galactose oxidase, in Chapter 14). Since knowledge of the bioinorganic aspects of Cu and lysyltyrosinequinone (LTQ) [4] (Figure 1) and the enzyme in which they occur, lysyl oxidase (protein-lysine 6-oxidase [EC 1.4.3.13]), is still lacking, this topic will also not be treated here.

II. THE QUINONE COFACTORS

II.A. General Aspects

Lipid-soluble *p*-quinones, comprising ubiquinone, menaquinone, and plastoquinone, have long been known to transfer reducing equivalents across biological membranes. Dopachrome and adrenochrome, *o*-quinones resulting from oxida-



tion of dihydroxyphenylalanine (DOPA) and adrenaline, respectively, have been inferred in melanin synthesis, in skeleton formation of insects, and in the synthesis of adhesive protein by the sea mussel. On the other hand, the detection of PQQ provided the first example of a (water-soluble) nitrogen-containing *o*-quinone involved in enzyme-catalyzed redox reactions. Similar roles are performed by the *o*-quinones TTQ and LTQ (Figure 1). Mechanistic studies on bovine plasma amine oxidase [5,6] suggest that TPQ might function as a *p*-quinone.

II.B. Pyrroloquinoline Quinone

II.B.1. General Aspects

The chemical synthesis of Pyrroloquinoline (PQQ) and its derivatives and analogs have been reviewed [7,8]. The latter have been used to detect the elements crucial for activity and binding of PQQ in quinoprotein enzymes, as well as to test their ability in curing or preventing certain diseases. From reconstitution studies on these compounds with apoquinoproteins, it is clear that the quinone moiety of PQQ should be intact and that modification of other parts always lowers activity and selectivity of the enzyme. Administration of PQQ or derivatives to mammalian organisms in some cases cures or prevents development of certain diseases. Since no PQQ-containing enzymes have been found in these organisms, this effect is most probably connected to the free radical scavenging properties of PQQ [9]. Biosynthesis of PQQ has been studied in some methanol-utilizing bacteria. All the genes involved in this are known and have been sequenced. Combined with biochemical studies, the results indicate that the biosynthesis most probably is carried out in a peptide of about 20 amino acids as precursor, by oxidation of a specific tyrosyl residue, cyclization with a specific glutamyl residue, and finally excision of the formed PQQ [10,11].

II.B.2. Redox Properties of Pyrroloquinoline Quinone

One-electron reduction of PQQ gives rise to formation of the semiquinone, PQQH[•]; whereas two-electron reduction leads to formation of the quinol form, PQQH₂ (Figure 2). Both species have been implicated as occurring in the redox reactions catalyzed by quinoprotein dehydrogenases, since electron transfer to the respiratory chain occurs in one-electron steps [12].

Pyrroloquinoline quinone, and probably also the other quinone cofactors, can be considered to be medium-potential oxidants (E_m PQQ/PQQH₂, 0.09 V (NHE) aq., pH 7), as distinguished from high-potential quinones that are of use as oxidants in organic synthesis [13]. In addition, PQQ will resist competing nucleophilic 1,4-additions of organic reductants as is observed with less heavily substituted quinones.



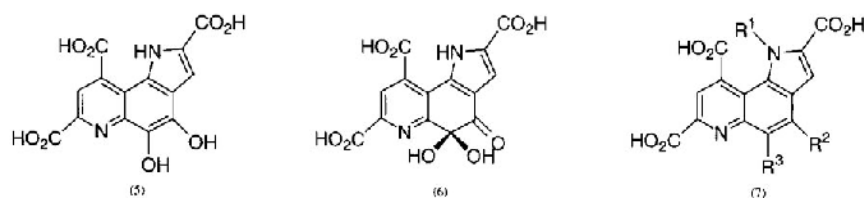


Figure 2 Structures of PQQH₂ (5), PQQ-H₂O (6), and related pyrroloquinolines (7).

The redox chemistry of PQQ has been investigated by a number of research groups. Duine et al. [14,15] performed potentiometric titrations of PQQH₂ at several pHs and measured the redox potential of PQQ/PQQH₂. Eckert et al. [16,17] compared the redox properties of PQQ with those of *o*-phenanthroline quinones. Kano et al. [18] performed cyclic voltammetry at acidic pH. Bergethon [19] investigated the amperometric detection of PQQ as a tool for HPLC. From pulse radiolysis experiments, McWhirter and Klapper [20] derived a value of -122 mV (NHE) for E_m PQQ/PQQH[•] at pH 7, as compared to the value of -218 mV calculated from mediator-linked potentiometric titrations [15].

II.B.3. Interaction of Pyrroloquinoline Quinone with Metal Ions

It has been noticed that the structural features of PQQ and its reduced forms are shared by known chelating agents [21,22]. Considering the *o*-carboxyl group of the pyridine ring, PQQ may be viewed as a derivative of the metal complexing agent α -picolinic acid [23]. In addition, PQQH₂ bears a strong resemblance to the metal complexing 8-hydroxyquinoline [24]. Initial studies by Noar et al. [21] showed equilibrium complexing of 9-decarboxy PQQ with Cd(II) to occur at pH 4.0 ($\mu = 1.0$ with NaClO₄). Formation of a 1:1 complex ($K = 1.9 \times 10^4 M^{-1}$) was deduced from plots of $\ln(A_0 - A_t)/(A_t - A_\infty)$ versus $\ln[Cd^{2+}]$ by the method of Walker, Lo, and Ree [25]. A comparison of these data with the values obtained for 7,9-didecarboxy PQQ, which showed no detectable complexing of Cd(II), suggested a strong contribution of the 9-carboxyl group to the binding of the metal ion. Since metal ion binding to the reduced species is expected to be stronger, a catalytic role for metal ions in the reduction of PQQ has been suggested [21]. Model studies with Ca revealed that the presence of these ions induced reaction of PQQ with methanol [26] and promoted the reoxidation of PQQH₂ with O₂ [27]. Ca increased the hemiketal formation of methanol with the C5-carbonyl group of PQQ as well as the base-catalyzed oxidative elimination reaction of the adduct. The absorption maxima of the complexes are presented



in Table 2 as they are useful in comparisons with several redox forms of the quinoprotein dehydrogenases (see later discussion).

X-ray crystallography of PQQ [28], (PQQ)₂(K) [28], and (PQQ)(Na)₂ [29] has been reported. Coordination of the potassium ion involves the 7-carboxyl oxygens as well as the N(6) and O(5) atoms. In the sodium complex, additional interactions with the O(4) atom and the 2-carboxyl group are observed. As yet, crystalline preparations of PQQ-heavy metal complexes have not been obtained [28]. X-ray crystallographic data of the PQQ acetone adduct [30] and the 2,4-dinitro-phenylhydrazone of PQQ triester [31] have also been presented.

II.C. Tryptophyl Tryptophan Quinone

II.C.1. General Aspects

Tryptophyl tryptophanquinone (TTQ) (Figure 1) is the cofactor of quinoprotein amine dehydrogenases. Chemical syntheses of compounds containing a 2,4'-bi-indole structure have been reported [32,33] as well as that of the model compound 3-methyl-4-(3'-methylindol-2-yl)indol-6,7-dione [34]. Spectroscopic and electrochemical properties of the model compound and its redox forms have been determined. The two indole rings are in a nonrigid conformation, although partial π conjugation exists between them. Thus, electron transfer via the tryptophyl moiety, as has been proposed to occur in the reaction of methylamine dehydrogenase (MADH) with amicyanin (discussed later), seems feasible. No indications were found that one of the carbonyl groups becomes hydrated, as is the case in PQQ [35], an indication that although the redox potentials are similar, differences in chemical reactivity exist between PQQ and TTQ. Although this could not be deduced from the model compound, the C6-carbonyl group seems to be site for nucleophilic attack, as deduced from the crystal structure of MADH inhibited with hydrazines [36]. The model compound appeared to be an efficient catalyst for the oxidative deamination of benzylamine via a transamination mechanism. The genes for biosynthesis of MADH, an enzyme in which TTQ occurs, have been determined and their sequence is known (see Ref. [37]). An interesting gene is *mauG*, of which the deduced amino acid sequence has some similarity with that of a cytochrome *c* peroxidase. Isolation and characterization of the mutant MADH produced by a mutant strain lacking *mauG* showed the presence of a phenolic precursor of TTQ, which could be converted into TTQ (detected spectroscopically as well as enzymatically) by incubating the mutant MADH with chemical agents like potassium permanganate or potassium hexacyanoferrate(III) (C. van der Palen, unpublished results, 1997). However, whether the precursor exists already in dimeric form, or whether this occurs concomitantly with the formation of the *o*-quinone moiety from the phenol group, is presently unknown. A further



intriguing question that remains to be answered is how the phenolic OH is introduced at the C₆ or C₇ position in the tryptophan moiety.

II.D. Topaquinone

II.D.1. General Aspects

Topaquinone (TPQ), the oxidized form of 2,4,5-trihydroxyphenylalanine (TOPA), is the cofactor of copper-containing amine oxidases. The following model compounds have been prepared in order to understand the catalytic function of TPQ: the *N*-pivaloyl derivative of 6-hydroxydopamine in aqueous acetonitrile [38]; topaquinone hydantoin and a series of 2-hydroxy-5-alkyl-1,4-benzoquinones in anhydrous acetonitrile (*o*- as well as *p*-quinones) [39]; 2-hydroxy-5-methyl-1,4-benzoquinone in aqueous system [40]; and 2,5-dihydroxy-1,4-benzoquinone [41]. Reaction of model compounds with 3-pyrrolines revealed why copper-quinoprotein amine oxidases cannot oxidize a secondary N [42]. The studies clearly showed that certain model compounds do not require the presence of Cu for benzylamine oxidation whereas TPQ does [38,40]; the aminotransferase mechanism proceeds via the *p*-quinone form [39]; the 470 nm band can be ascribed to a π - π^* transition of TPQ in *p*-quinonic form with the C-4 hydroxyl ionized but hydrogen bonded to some residue [40]; hydrazines attack at the C-5 carbonyl, forming an adduct in the azo form [41]. Electrochemical characterization has been carried out for free TPQ [43].

Formation of TPQ in the amine oxidases in which it occurs is initiated by binding of Cu to the precursor protein at the same site in which it functions as cofactor in the mature enzyme. Subsequently, the specific tyrosyl residue, the second amino acid in the conserved sequence Asn-Tyr-(Asp/Glu)-Tyr [44], is converted into TPQ in several steps, with the Cu catalyzing the oxidation of the precursor by O₂ [45]. Indications exist that this autocatalytic process is sometimes suboptimal; most probably the insufficient availability of Cu and/or O₂ in the posttranslational process is responsible [46].

III. QUINOPROTEIN ENZYMES

III.A. Enzymes with Pyrroloquinoline Quinone

III.A.1. General Aspects

The role of PQQ as cofactor has been established unequivocally only for a number of dehydrogenases that are located in the periplasmic space of gram-negative bacteria [3] (Table 1). These enzymes are involved in the oxidation of alcohols and sugars (to aldehydes and sugar lactones or ketosugars, respectively), especially in processes known as incomplete microbial oxidations [47] (production



of gluconic acid, vinegar), in oxidation of xenobiotics like polyethylene glycol [48] and polyvinyl alcohol [49] or natural compounds produced by secondary metabolism of plants (e.g., the alkaloid lupanine [50]). Although some gram-positive bacteria from the genus *Amycolatopsis* are known to excrete PQQ into their growth medium (M. Misset-Smits, unpublished results, 1997) no quinoprotein has been detected yet in these bacteria. It has been claimed that PQQ also occurs in eukaryotes, including mammalian organisms, but the detection method used in this is controversial and PQQ-containing enzymes have not been isolated from these organisms. Low levels of PQQ have been discovered in plants by our group, but most probably they originate from methanol-utilizing bacteria living at the surface of the leaves (see Ref. [8] for a discussion). Enzymes not listed in Table 1 are polyethyleneglycol dehydrogenase and spermidine dehydrogenase, although they have been claimed to possess PQQ [51,52]. The reason not to incorporate them is that the method used to detect PQQ has been proved not to be reliable in other cases. In the case of spermidine dehydrogenase, there is also a mechanistic reason that makes the claim suspicious. As has been established so far for TTQ-, and TPQ-containing amine oxidoreductases (see Section III.A.3), these enzymes act according to a transaminase reaction in which the amine group is transferred to the cofactor with a Schiff base as an intermediate. Since oxidation of a secondary N is principally impossible in such a mechanism, the spermidine dehydrogenase has nevertheless been claimed to oxidize the secondary N in the substrates [52], so its having a flavoprotein nature is more likely.

The group of PQQ-containing enzymes can be subdivided into quinoproteins and quinoxemoproteins, the latter containing PQQ as well as a heme *c* group. Two types of quinoxemoprotein alcohol dehydrogenases are known, QH-ADH_I, a monomeric enzyme of 73 kDa [53], and QH-ADH_{II}, a heterotrimeric enzyme with an α subunit of 72 kDa, containing PQQ as well as heme *c*; the other subunits contain heme *c* [54]. From the amino acid sequences, it appears that the major part of the sequence starting at the N terminus has significant similarity with the α subunit of the PQQ-containing alcohol dehydrogenases, whereas the minor part, ending with the C terminus, contains the heme *c* binding site [53]. Since PQQ-containing alcohol dehydrogenases have a special cytochrome *c* as natural electron acceptor [55], one would expect that a gene fusion in the evolutionary history has provided the quinoxemoproteins. However, the C-terminal part of these enzymes shows no similarity at all with the special cytochromes *c* and only very weak similarity with some unrelated cytochromes *c*. Perhaps the similar overall topology of QH-ADH_I [55a] with that of *cd*₁-nitrite reductase from *Thiosphaera pantotropha* [56] may give some clue in the future.

III.A.2. Structural Aspects

Alignment of all the amino acid sequences known for PQQ enzymes shows significant similarity for most of them, but scarcely any similarity for soluble glucose



dehydrogenase (sGDH) (EC 1.1.99.17) and polyvinylalcohol dehydrogenase (PVA-DH) (EC 1.1.99.23); (in view of this and the fact that the heme *c* is at the N-terminal side instead of the C-terminal one [49] as in QH-ADH_I, it is proposed to rubricate PVA-DH as a new type of quinohemoprotein, QH-ADH_{III}). Since sGDH is present together with membrane-bound glucose dehydrogenase (mGDH), showing sequence similarity with other PQQ enzymes in one and the same organism, whereas both contain PQQ and catalyze the same reaction, the different sequence is surprising.

A region of high sequence similarity at the C-terminal part of the chains has been found in many PQQ-containing enzymes (including sGDH), for which it was originally proposed that this could be the PQQ binding site [57]. However, the three-dimensional structure of methanol dehydrogenase (MDH; discussed later) clearly indicates that this cannot be the case. On the other hand, the conserved nature of the stretch suggests that it encodes for some, yet still unknown, common feature of this type of enzyme.

For those investigated, PQQ-containing dehydrogenases require a divalent metal ion for activity, Mg in one case and Ca in all other cases (see Table 1). For some enzymes, Ca removal leads also to detachment of PQQ, providing an apo enzyme that can be easily reconstituted by addition of PQQ and Ca. For other enzymes, removal of the metal ion plus PQQ is not so easy, but fortunately several bacteria produce apoenzyme gratuitously as they are still able to produce the protein part of the dehydrogenase, but not PQQ (at least not under the laboratory conditions used for cultivation). Also in these cases, reconstitution to holo enzyme is easy, *in vitro* as well as *in vivo*; the latter leads to active enzyme coupled to the respiratory chain. However, indications exist that a special protein is required for inserting Ca in the case of MDH [58].

A similar variation applies to reconstitution of apoenzymes with PQQ; in some cases this occurs in the absence of the metal ions, in others the presence is absolutely necessary for attachment (N.B.: It should be noted that metal ions are always required for functionalization of the enzyme). sGDH is a special case as this homodimeric enzyme contains 4 Ca ions per enzyme molecule [59,60]. It appears now that two of them are required for dimerization of the monomers (which is possible in the absence of PQQ, providing the apoenzyme) and the other two are required for functionalization of the two PQQs in the enzyme molecule. Dissociation studies [60] revealed that the metal ion and PQQ exert a synergistic action, anchoring PQQ to and locking up the metal ion in the protein so that it is unavailable for interaction with chelating agent. Since substitution by other divalent metal ions is possible for both functions, a large number of hybrid forms of this enzyme can be prepared. However, it appears from this that the metal ion specificities of the two sites involved in the two functions of the metal ion are similar. This is surprising since the functionalizing metal ion is bound to PQQ, the other metal ion involved in dimerization must have other ligands.

The three-dimensional structure of methanol dehydrogenase (MDH) has



been recently elucidated independently by two research groups [61,62], and structures based on homology modeling for mGDH [63] and for QH-ADH_I [55a] and QH-ADH_{II} [64] have been proposed. In this context, it should be stressed that MDH is always isolated in one of its reduced forms, the semiquinone or the fully reduced form [65]. As a consequence, the data obtained for the active site concern the “reduced cofactor” (either PQQH• or PQQH₂). The following striking features for the structure of MDH can be mentioned: the active site cavity consists of the reduced cofactor, sandwiched with a tryptophyl residue in π -stacking interaction underneath it and with two vicinal cysteinyl residues, forming a disulfide in a nonplanar, eight-membered ring in trans configuration above it; the wall of the cavity is lined up with hydrophobic amino acid residues; Ca is bound to the protein via the γ -carboxyl oxygens of a Glu, the amide oxygen of an Asn, and to the reduced cofactor via the C-5 oxygen, one oxygen of the C7-carboxyl group, and the pyridine nitrogen; the overall structure consists of an eight-bladed propeller, each blade containing the so-called tryptophan motif. The propeller blade structure was originally found for viral neuraminidase [66] and subsequently for the TTQ-containing methylamine dehydrogenase [67]. However, despite its frequent occurrence in quinoprotein enzymes, it is not restricted to quinoproteins since apart from viral neuraminidase it has also been found in several nonquinoprotein enzymes [68]. Moreover, even the tryptophan motifs seem not relevant since sGDH has six propeller blades with the same overall topology as MDH (A. Oubrie and B. Dijkstra, personal communication).

Although sequence homology modeling of QH-ADH_I has been carried out, the problem is that this is not possible for the heme *c*-containing C-terminal part because a homologous structure and sequence are not available for it. However, on the basis of calculations for the most efficient pathway for electron transfer between PQQH₂ and the heme *c* group, a structure has been proposed for QH-ADH_I from *Comamonas testosteroni* [55a] that explains several mechanistic features of this enzyme [69].

The sGDH form containing PQQ, but with only the two Ca ions involved in dimerization (Holo X [60]), has an absorption spectrum similar to that of PQQ, and glucose slowly transforms Holo X_{ox} into HoloX_{red}, having a spectrum similar to that of PQQH₂. Upon addition of the PQQ-functionalizing Ca ions, red shifts of the absorption maxima occur; they are especially large in the conversion of HoloX_{red} into reduced sGDH. Reduced sGDH has the same absorption maximum as (fully) reduced MDH, although MDH has only one Ca per subunit and sGDH contains neither the sulfur ring structure nor the sandwich tryptophyl residue of MDH. This suggests that the major cause of the big shift is interaction of PQQH₂ with Ca in the enzymes, a view supported by the similar shifts exhibited by the model compound (Table 2). However, in accepting this view, it is somewhat surprising to observe that on substituting the Ca required for functionalizing the PQQ in sGDH by other metal ions, only tiny red or blue shifts are induced with



Table 2 Absorption Maxima of Quinoprotein Dehydrogenase Forms and Model Complexes

Species ^a	$\lambda_{\max}$ (nm)
PQQ · TME	354
sGDH Holo X _{ox}	340
PQQ · TME-Ca	368
sGDH _{ox}	352
MDH _{ox}	348
PQQ · TME-Ca-methanol	420
MDH _{ox} -CN	402
PQQH ₂ · TME	326
sGDH Holo X _{red}	302
PQQH ₂ · TME-Ca	350
sGDH _{red}	338
MDH _{red}	338

^a Spectra of PQQ·TME, the trimethyl ester of PQQ, were measured in acetonitrile and Ca and/or methanol were added [26]. Data on the Holo X form of sGDH can be found in Refs. 59 and 60.

respect to the absorption maximum of reduced normal enzyme. Similar observations have been made for MDH (although it has been stated [70] that replacement of Ca by Sr in MDH gives a substantial shift and increases the absorbancies, comparison of the two spectra with those of MDH in semiquinone and fully reduced form suggest that the shift could also be explained by a difference in redox state). Thus the divalent metal ions that can replace Ca exert their action in the same way, without expressing their mutual differences in the spectra (substitutes are Mg, Sr, Ba, Mn, Cd).

To prepare the oxidized form of MDH, the isolated preparation has to be treated with electron acceptor in order to remove substrate and finally to oxidize the enzyme. However, the enzyme is very labile in its oxidized form (the inactivation concomitantly leading to destruction of PQQ, as deduced from the rapid disappearance of its absorption band); the only way to keep it active is to perform the oxidation in the presence of cyanide or hydroxylamine [71]. Most probably, the reason for this is adduct formation between the stabilizer and PQQ at the C-5 position, in line with the maximum of the stable oxidized form with that of the methanol adduct of the model compound (Table 2). Since sGDH is perfectly stable in its oxidized form, the origin of the instability of PQQ and the stability of PQQH[•] in MDH could be the special residues (discussed earlier) surrounding the cofactor in MDH (PQQH[•] has not been observed so far in sGDH). However, in view of the small red shift induced by Ca on PQQ in the enzymes and model



compound (Table 2) and the similarity of the EPR characteristics of MDH_{sem} and free PQQH[•], the interactions seem weak. Finally, PQQ in sGDH and MDH seems to be present in a hydrophobic environment in view of the absence of PQQ fluorescence in the oxidized enzyme forms.

III.A.3. Mechanistic Aspects

The redox potential of PQQ-containing enzymes is rather high, of the same magnitude as that of PQQ (+90 mV at pH 7). However, a substantial decrease may occur in the presence of the natural electron acceptor, as illustrated by the sGDH/cytochrome *b* couple [72]. From the evidence available, it appears that internal electron transfer takes place from PQQH₂ to the heme *c* group in QH-ADH₁ [73]. The distance between the two redox centers in the enzyme calculated by modeling indicates that this should be regarded as long-range electron transfer. Intermolecular long-range electron transfer occurs between the quinoprotein alcohol dehydrogenases and a special cytochrome *c* and between QH-ADH and the blue copper protein azurin. Strong inhibition in the first case occurs with ethylenediaminetetra-acetic acid (edta) but not when an artificial electron acceptor like *N*-methylphenaronilium methyl sulfate (PMS) is used. The inhibition appears to be due to complex formation of edta with certain lysyl residues at the cytochrome *c*_L surface, these probably being essential for contact with MDH in the electron transfer process [74]. The nature of the natural electron acceptors (relatively high redox potential) is in line with the rather high redox potential of the dehydrogenases, implying that the amount of useful energy generated via substrate oxidation by this type of dehydrogenase (the cytochromes *c* are coupled to cytochrome *c* oxidase) is rather low as compared to that generated by their nicotinamide-adenine dinucleotide- (NAD)-dependent counterparts. On the other hand, mGDH appears to be coupled to ubiquinone in the respiratory chain [75], just as QH-ADH₁ is under certain growth conditions. Thus either these enzymes have unusually low redox potentials as compared to that of free PQQ or heme *c*, respectively, or these branches of the respiratory chain are only operating at high aeration rates (high ratio of oxidized ubiquinone/reduced ubiquinone).

Although structural information is now available on the binding of Ca to a reduced form of PQQ and the protein of MDH, the mechanism of substrate oxidation and the precise role of the metal ion in this are still unknown. Some PQQ-containing, metal-ion-lacking enzymes show activity, but the tremendous increase in activity caused by addition of metal ion shows that some functionalization in the enzyme must occur [60]. Since the metal ion induces a red shift in the absorption maximum of the cofactor, it is tempting to speculate that this functionalization concerns PQQ. Several possibilities can be envisaged for the way in which this is performed. In view of the big shift induced by Ca on the



maximum of PQQH₂, an increase of the redox potential could be the cause. However, the redox potential of PQQ itself is already high enough that this explanation seems unlikely. In another one, the metal ion could act as a Lewis acid, allowing the abstraction of a hydron from the substrate (Ca indeed increases the stability of the methanol/model compound adduct [26,27] and gives a substantial red shift of the maximum). However, such a role has also been attributed to an aspartyl residue in the active site of MDH [61]. Anyhow, nucleophilic addition of the substrate at the C5-carbonyl group of PQQ, leading to the formation of a PQQ-5-hemiketal, is seen as the first step in the catalytic cycle. Evidence for the existence of this (fluorescing) adduct has been obtained in the case of MDH [76] and sGDH (in stopped-flow kinetics with deuterated glucose [unpublished results, 1997]). Whether or not the subsequent reorganization of the hemiketal, leading to PQQH₂ and product, involves a concerted or a stepwise hydride transfer is unknown. Another aspect not understood is the mechanism of ammonia as activator in the catalytic process of MDH (or of primary amines in the case of quinoprotein ADHs). Since all other PQQ-containing dehydrogenases, including the QH-ADHs, do not require such an activator, the role of the activator might be taken over by a specific amino acid residue in the active sites of the latter. In this context it is interesting to note that the structure of QH-ADH_I shows that a lysyl residue is present in the active site that is absent in that of MDH and certain esterified amino acids are even better activators than ammonia (unpublished results, 1997). Therefore, the lysyl residue might be the candidate, e.g., functioning in aldehyde release.

Kinetic investigations on MDH with PMS as electron acceptor revealed that the enzyme operates via a Ping-Pong mechanism. Using deuterated methanol, an isotope effect of 6, which could be interpreted as support for a hydride-transfer mechanism in substrate oxidation, was found. It has been proposed that O₂ competes with PMS for the reduced enzyme form, leading to the production of superoxide [77]. However, most probably this is an artifact caused by reaction of O₂ with reduced PMS [77a]. On the other hand, O₂ oxidizes reduced mGDH as it acts as a poor electron acceptor for the enzyme [77a]. Reconstitution of mGDH apoenzyme with PQQ in the way which is common for quinoproteins leads to binding of PQQ, but the holo-enzyme formed is inactive. The latter was concluded from the absence of an effect of glucose addition on the absorption spectrum of the reconstituted enzyme and on gluconolactone formation. Transformation of the inactive holo-enzyme form into an active one was achieved in the presence of sulfite. Behavior of sGDH is exceptional since it shows strong negative cooperativity kinetics in the oxidation of aldose sugars, using PMS as electron acceptor [77b]. Most probably, this is caused by binding of substrate to one of the two subunits, transforming the other into a catalytically more competent species with respect to turnover number with, however, a much lower affinity for the substrate.



Kinetic investigations on QH-ADH_I are complicated by the fact that two cofactors are present. Using hexacyanoferrate(III) as electron acceptor (most probably tapping the electrons from the reduced heme *c* group instead of PQQH₂), quantitative descriptions have been derived for steady-state kinetics as well as conversion processes of QH-ADH_I, with alcohol as a substrate and taking released product, aldehyde, also acting as a substrate, into account. With these equations the transient occurrence of aldehyde, as well as the enantioselective behavior of the enzyme, could be described [73]. Curiously, the enzyme shows an opposite preference for a certain aldehyde enantiomer from that of the corresponding alcohol enantiomer. This seems to be inherent to QH-ADH_I since QH-ADH_{II} from *Acetobacter pasteurianus* did not show it (S. Machado, unpublished results, 1997). However, with the latter enzyme it appears that in vitro and in vivo enantioselectivities are not the same since detachment of the enzyme from the membranes was found to affect the enantioselectivity.

III.B. Enzymes with Tryptophyl Tryptophanquinone

III.B.1. General Aspects

Methylamine dehydrogenase (MADH) (EC 1.4.99.3) and aromatic amine dehydrogenase (EC 1.4.99.4) are tryptophyl tryptophan quinone- (TTQ)-containing enzymes catalyzing the oxidation of primary amines to their corresponding aldehydes and ammonia. The enzymes occur in the periplasm of gram-negative bacteria (Table 1). In most organisms, the natural electron acceptor for MADH is the type I blue copper protein amicyanin [78] (in a few organisms, amicyanin is absent and its role seems to be taken over by a cytochrome *c*). Methylamine dehydrogenase (MADH) was the first quinoprotein of which the three-dimensional structure was elucidated [67]. Structural data are also available now for amicyanin [79]. Moreover, cocrystals have been obtained of a complex consisting of the dehydrogenase and amicyanin, and of a ternary complex, containing also a cytochrome *c*, presumed to be the natural electron acceptor for amicyanin [80] (however, it is questionable whether this is the correct cytochrome *c*). Kinetic studies have been performed for the binary systems MADH/amicyanin, amicyanin/cytochrome *c*₅₅₀ (the correct electron acceptor in this case), and cytochrome *c*₅₅₀/cytochrome *c* oxidase. Since the genes of these components have also been cloned, sequenced, and brought to overexpression [see Ref. [37]], the opportunities exist to study electron transfer of the process for a complete respiratory chain and to modify the individual components at will. Although the quinoprotein amine dehydrogenases do not contain a metal ion, the presence of monovalent cations (including NH₄⁺) has a dramatic effect on the rate of electron transfer rate of MADH to amicyanin; in view of the scope of this book, this aspect will be treated here.



III.B.2. Effects of Monovalent Cations

Monovalent, but not divalent, cations have large effects on the absorption spectrum of MADH_{ox} [81]: the large ones (including NH_4^+) give a red shift of the absorption maximum, and the small ones decrease the absorbances. Just as the pH, the monovalent cations have large effects on the reaction of MADH_{red} with amicyanin. The cations exert small effects on the EPR signal of MADH_{sem} [82]. Slight differences were found for enzyme reduced with substrate and with dithionite, indicating that the amino group of the substrate was still present in the substrate-reduced enzyme. Model compounds were used to determine the effect with resonance Raman spectroscopy [83]. Evidence has been put forward that the substrate binds to the enzyme in its cationic form [84]. Intermolecular electron transfer from MADH_{red} to amicyanin is linked to hydron transfer [85].

III.C. Enzymes with Topaquinone

III.C.1. General Aspects

For most of the copper-containing amine oxidases rubricated under EC 1.4.3.6, it is clear now that they contain topaquinone (TPQ) as organic cofactor. The enzymes convert primary amines with O_2 into the corresponding aldehyde, H_2O_2 , and ammonia [5]. The group of copper-containing amine oxidases includes enzymes with widely differing substrate specificities, resulting in names like benzylamine oxidase, spermidine oxidase, histaminase, etc. Some of these enzymes are distributed from bacteria to humans. A variety of roles for mammalian amine oxidases have been proposed: regulation of cell proliferation by oxidizing spermine and spermidine, lowering of the level of a biogenic amine like histamine, removal of endogenous methylamine [75], and conversion of exogenous xenobiotics like allylamine. Microbial amine oxidoreductases have a clear role as they provide the organism either with an aldehyde, used as a carbon and energy source, and/or with assimilatable nitrogen (NH_3). Insight into the role of the mammalian enzymes is hampered in several cases by the overlap in substrate specificity with flavoprotein amine oxidases. As a consequence, the nomenclature that is based on the substrate specificity of these enzymes is unsatisfactory. In order to provide another base for nomenclature, a lot of effort has been invested in the search for specific inhibitors (resulting in trivial names like semicarbazide-sensitive amine oxidases and clorgyline-resistant amine oxidase).

From the sequence of reactions found it follows that copper-quinoprotein amine oxidases catalyze an aminotransferase reaction. A different reaction sequence occurs with flavoprotein amine oxidases (EC 1.4.3.4), where formation of NH_3 is not dependent on the presence of O_2 . However, since reductive trapping of amines in the first half-reaction [86] showed attachment of substrate but not of tritium, the mechanism is also different from the aminotransferase reaction that



is commonly found for enzymes containing pyridoxal phosphate cofactors. Since most of the basic data on copper-containing amine oxidases enzymes are covered by recent reviews [87–89], only a summary and a number of uncertainties are given here.

III.C.2. The Number of Topaquinones

There is general agreement that two subunits and two Cu ions are present per enzyme molecule, but controversy exists with regard to the number of TPQs. Since a direct quantitative method is not available, the number is deduced from titrations with hydrazines (assuming that only one carbonyl of each quinone molecule reacts). Most reports mention only one hydrazine-titratable group per enzyme molecule, but other investigators find two such groups: a rapidly reacting one and a slow one that is only detectable after long standing of the preparation [90] or after subsequent addition of a different hydrazine [91], suggesting that the enzymes show half-of-the-sites reactivity. On the other hand, bovine plasma amine oxidase purified by a novel procedure showed the following characteristics: a normal Cu content, the highest specific activity ever reported, formation of two aldehydes per enzyme molecule under anaerobic titrations with substrate, and rapid reaction with 1.8 hydrazines per enzyme molecule [5]. Furthermore, indications were obtained that preparations of lower specific activity were not contaminated with other proteins. Therefore, it seems that the lower values that were obtained in the past could be due to partial inactivation or to inadequate processing of the enzyme. On the other hand, amine oxidase from *Escherichia coli* showed heterogeneity with respect to the TPQ content determined by titration and specific activity; however, the most active fraction only contained one TPQ per enzyme molecule [92]. The amount of TPQ found in the enzyme species appeared to be proportional to the specific activity and to the $A_{470\text{nm}}/A_{340\text{nm}}$ ratio. Using the same enzyme, it has been reported that long incubation with 2-hydrazinopyridine led to derivatization of both TPQs [93]. However, it cannot be excluded that during this long incubation, additional formation of TPQ took place. In conclusion, indications exist that in some enzymes or under some conditions of maturation, suboptimal amounts of TPQ are formed. On the other hand, it is also clear that in other cases, a full complement of two TPQs per enzyme molecule is present.

III.C.3. Equalities of the Two Subunits, Coppers and Topaquinones?

Electrophoresis of denatured enzyme yields only one band with half of the relative molecular mass of the native enzyme, indicating that the subunits have at least equal size. Cloning and sequencing of the gene for amine oxidase from



Hansenula polymorpha [94] revealed a gene coding for a protein with the size of the subunit. Studies on depleting copper from the enzymes show that half the amount of the copper is easily removed by dithiocarbamate whereas the other half is hardly affected; nevertheless, the enzyme is completely inhibited [95]. Moreover, adding half of the stoichiometric amount of copper to fully copper-depleted enzyme did not restore any activity [96]. The latter finding has, however, been challenged since using a slightly different procedure produced a linear relationship between copper content and activity [97]. Finally, pig plasma amine oxidase can be rendered completely inactive upon blocking with a hydrazine of the fast reacting carbonyl group [90] (this is in contradiction with the results on bovine serum amine oxidase purified according to a novel procedure in which complete inactivation requires nearly two hydrazines [5]). The asymmetry in behavior could be interpreted to result from one Cu ion's having strong ligands and the other's having weaker ligands, with both Cu ions interacting in a cooperative manner so that removal of the first copper destroys the activity of the whole system. However, this explanation is not supported by most of the EPR spectra, which suggest only one paramagnetic center (although some heterogeneity has been detected with Q-band EPR spectroscopy in plasma amine oxidase [90,98], but not for *E. coli* amine oxidase [99]) and electron spin-echo envelope modulation spectroscopy of bovine serum amine oxidase [100]). Also, the idea that only one TPQ is essential for activity, with the other modified, is not in accord with the results of resonance Raman spectroscopy [101], since the fingerprint of the enzymes is very similar to that of a peptide containing TPQ (the latter observation also excludes heterogeneity of the cofactor in the case that two TPQs are assumed to occur per enzyme molecule but could overlook TPQ still present in a precursorlike state). Most data on ENDOR, EXAFS, NMR, and the crystal structure support homogeneity of the coppers and thus their ligands. Therefore, there are convincing arguments to assume the equality of the two subunits, Cu ions, and TPQs, and also, for reasons of symmetry, to assume that each subunit contains one TPQ and one Cu. On the other hand, in view of the many reports and various techniques and research groups involved, it cannot be excluded that in some preparations the subunits and cofactors are dissimilar. This could originate from an artifact that also could be responsible for the discrepancy in the number of hydrazine-titratable groups found per enzyme molecule.

III.C.4. The Positioning of Copper and Topaquinone

The ^{19}F -NMR of bovine plasma amine oxidase [102] and *E. coli* amine oxidase [103] derivatized with fluorine-substituted hydrazines allowed estimation of the distance between the hydrazine and the active site Cu ion. The rather large distance that has been calculated precludes that the Cu ions are in the immediate



vicinity of the carbonyl group. The same conclusion has been reached from fluorescence measurements with hydrazino-acridine dyes [104]. On the other hand, electron transfer between the organic and inorganic cofactors is possible, as demonstrated [105] by the formation of the $\text{Cu(I)/PQQH}^\bullet$ couple, which is in equilibrium with Cu(II)/PQQH_2 . Moreover, the recently elucidated crystal structures of *E. coli* [93] and pea seedling [106] amine oxidases indicate that Cu and TPQ are rather close to each other, with the C2 oxygen position on the TPQ ring hydrogen bonded to the apical water ligand of the copper. It has to be concluded, therefore, that the F-NMR method discussed earlier is able to give a rough impression of the topology.

III.C.5. The Roles of Copper and Topaquinone

Reductive substrate trapping experiments, inhibition with hydrazines, and bleaching of the 480 nm band (assumed to originate from TPQ) all indicate that TPQ is involved in the first part of the catalytic cycle, as the acceptor of the amine group in the transamination reaction. Thus, in the reductive part of the catalytic cycle, amine is converted into aldehyde and TPQ into the aminophenol form ($\text{TPQH}_2 \cdot \text{NH}_2/\text{Cu(II)}$). Appreciable electron transfer occurs between the two cofactors, observed as the aminosemiquinone $\text{TPQH}^\bullet \cdot \text{NH}/\text{Cu(I)}$ by EPR spectroscopy for certain amine oxidases under anaerobic conditions [105,107]. The observation that KCN addition induces the EPR signal (the complexing of the cyanide inducing somewhat different properties, however [109]) in all amine oxidases suggests that this electron transfer step forms part of the oxidative part of the catalytic cycle. Quantitative considerations are in line with this view [108]. An additional redox form has been found for *E. coli* amine oxidase (the 400 nm species [109]). It has been proposed that this is a Zwitter ionic form of the aminosemiquinone form of TPQ. To explain that more than stoichiometric amounts of substrate are converted at high enzyme concentration under anaerobic condition, it has been proposed that the aminosemiquinone form disproportionates into $\text{TPQ}/\text{Cu(I)}$ and $\text{TPQH}_2/\text{Cu(I)}$ and that $\text{TPQ}/\text{Cu(I)}$ is enzymatically active. Evidence has been provided that H_2O_2 not only inactivates the enzyme but is also a (poor) substitute for O_2 .

IV. CONCLUDING REMARKS

Quinoprotein dehydrogenases containing PQQ or TTQ have been shown to function in various microorganisms in addition to the NAD(P)-dependent and flavo-protein dehydrogenases. The PQQ-containing dehydrogenases require Ca (or Mg) for structural as well as catalytic purposes. However, the mechanism of activation of PQQ, the substrate or the hemiketal adduct by the metal ion, is still unknown.



Cytochromes *c*, small blue-copper proteins, or an internal heme *c* group can function as natural electron acceptors for the dehydrogenases. Since these are “soluble” proteins and the genes have been cloned in most cases, they provide excellent possibilities to study electron transfer pathways in vitro and intermolecular as well as intramolecular pathways between a quinone and Cu or heme *c* in particular.

Monovalent cations affect the spectroscopic and electron transfer rates between MADH and amicyanin. The precise mechanism for this is still unknown.

Cu has a bifunctional role in copper-quinoprotein amine oxidases, catalyzing the formation of TPQ from the specific tyrosyl residue in the precursor protein and playing a role in the catalytic mechanism, most probably with O₂ reacting with Cu(I) in the oxidative part of the cycle. The recent discovery of LTQ suggests that other combinations of quinone cofactors may be found in the future.

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17

Future Developments

Elisabeth Bouwman and Jan Reedijk
Leiden University, Leiden, The Netherlands

I. INTRODUCTION

In a monograph of limited size, it is impossible to present a comprehensive picture of bioinorganic catalysis. The chosen collection of chapters does not and cannot present the totality of the bioinorganic catalysis field. However, we hope to have provided a good and balanced survey of the major interesting aspects of the catalytic role of metal ions in biological systems and low-molecular-weight analogs.

After the completion of all chapters for the present monograph, a rather extensive study in four volumes has appeared [1] that deals with biological catalysis. Readers with an interest in the general aspects, also dealing with the numerous topics in which metals do not play a key role, are referred to this work.

Although the selected chapters and authors form a consistent set and cover the most important topics in sufficient detail, a number of classic and new items of this exciting field could not be included or have at best been barely dealt with. This chapter briefly lists a few of these unmentioned items. At this stage, it should be mentioned that several major journals have added special issues on bioinorganic topics, including *Chemistry Reviews* [2] and the *Journal of the Chemical Education* [3].

II. Mg, Ca, Mn, and Zn: LEWIS-ACID CATALYSIS AND COFACTORS

Hydrolytic reactions are of crucial importance in biological systems. Since no redox chemistry is involved in such reactions, nature has selected closed-shell ions for such reactions, although in a number of cases Mn(II), and even other



redox-active metals like iron, may have such a role (see Chapters 8 and 10). A detailed treatment of these large groups of enzymes is impossible, and, therefore, the focus of this monograph has been on redox active metal ions. Nevertheless, a few exceptions are made, such as Chapter 4, which deals with zinc with a focus on biomimetic systems. Here we add a few examples to illustrate the rich chemistry of such enzymes.

Alkaline phosphatases form a well-known class of proteins that perform quite interesting and complicated reactions. As previously reported, Zn enzymes, like carboxypeptidases, thermolysin, and carbonic anhydrases, consist of only one Zn atom per active center. Most of the alkaline phosphatases consist of two 96-kDa subunits, each containing two Zn and one Mg ion. The alkaline phosphatase from *E. coli* has been crystallized and described in full detail [4], and a mechanism has been proposed. Several enzymes in this category have been mentioned in recent years, some of them also containing different metal ions, such as iron and zinc, as in the purple acid phosphatase [5]. It is likely that the detailed structure and mechanism of many more examples of enzymes that remove or add phosphate groups to proteins will become available in the next decade.

In the 3',5'-exonuclease reaction of DNA polymerase I, two of the metal ions (Mg, Zn, or Mn) are required for activity, and two slightly different metal sites are available in the enzyme, most likely in a dinuclear center [6] with phosphate and carboxylate ligands for one metal (Mg) and with carboxylates(3), phosphate, and a water ligand for the other metal (Zn) in the transition state [7].

The role of metals, and in particular Zn, in the regulation of gene expression has been a subject of recent studies in the last decade. The term "zinc finger" for this type of structure is generally accepted [8,9]. The Zn(II) ion does not act as a catalyst, but resembles more a cofactor that folds the protein in a specific conformation, as a result of which it binds to nucleic acids, such that certain genes are being activated for expression [9].

Quite recently, it has been realized that in β -lactamases, dinuclear zinc sites do frequently occur. The geometry of one zinc is tetrahedral (3xHis and a bridging OH), whereas the other is five coordinate (His, Asp, Cys, water, and a bridging hydroxide), and together they form the active site [10]. In an earlier case, only mononuclear zinc was found for the lactamase from *Bacillus cereus* [11].

Finally, the enzymes of the group xylose isomerase should be mentioned in this category. Found for the isomerization of xylose into glucose, the enzyme requires two divalent metal ions, such as magnesium or zinc. The structures of the enzymes have been reported with several metal ions and various mutants [12]. For site I, the ligands are oxygen donors (carboxylates and water); for site 2, the ligands involve one histidine and 4 carboxylates. The sugars are bound with one or two oxygens to the metal ion.

The general issue of metal-activated enzymes in nucleic acid biochemistry has been addressed in significant detail by Cowan [13], beautifully illustrating the



versatile coordination chemistry of the Lewis-acid metal ions and their flexible coordination chemistry, allowing a variety of reactions, depending on the steric possibilities of the available ligands. The phosphate ligands can be especially crucial in their reactivity, i.e., when they are coordinated to two, one, or none of the metal ions.

For general overviews of the chemistry of the elements Mg, Ca, or Mn, the reader is also referred to the published standard works on bioinorganic chemistry [14,15] and a recent popular textbook [8].

III. SELENIUM

The element Se—not really a metal—is known to play a key role in enzymes such as the well-known glutathione peroxidase, formate dehydrogenase, glycine reductase, and the previously mentioned hydrogenases (Chapter 9). The unusual amino acid selenocysteine has a unique codon on the DNA (TGA/UGA), but selenation of serine also appears to be possible [16]. A brief review on Selenium can be found in the literature [17].

IV. HOT NEWS

During the completion of several chapters, in a number of cases new important results have appeared in the literature. A selection of the most important of these is presented in the following sections.

IV.A. Nonheme Iron

Activity in the area of nonheme iron enzymes continues to flourish, with several exciting results being published in this subfield since Chapter 10 was submitted. In the area of mononuclear nonheme iron enzymes, x-ray crystal structures are now also available for the catalytic domain of human phenylalanine hydroxylase [18] and naphthalene 1,2-dioxygenase [19]. The mononuclear iron site of phenylalanine hydroxylase resembles the 2-His-1-Asp site of tyrosine hydroxylase, a result anticipated by sequence homology. More interestingly, naphthalene 1,2-dioxygenase, which catalyzes the *cis*-dihydroxylation of arene double bonds in the biodegradation of aromatics, also has a $\text{Fe}(\text{His})_2(\text{Asp})$ iron site. These two enzymes augment the increasing number of mononuclear nonheme iron enzymes with a common $\text{Fe}(\text{His})_2(\text{carboxylate})$ facial triad motif [20].

Progress continues in uncovering the mechanism of oxygen activation at nonheme diiron active sites. A diiron(III) peroxide intermediate of the mutant D84E R2 protein has been isolated and shown to have similar visible and Möss-



bauer properties to those of MMOH-P [21]. The D84E mutation does not affect the outcome of the reaction as the intermediate decays to form an equivalent of tyrosyl radical, but the lifetime of intermediate **X** appears to have been shortened significantly.

Studies on a different R2 mutant protein, F208Y, provide insight into the partitioning between one-electron versus two-electron oxidation chemistry at the R2 diiron site. In the absence of exogenous reductants, the dominant oxidation reaction is the hydroxylation of Y208 to DOPA, as previously reported by Sjöberg et al. [22]. However, when exogenous reductants are present, the principal oxidation reaction is the formation of the Y122 radical [23]. It is further demonstrated that this partitioning is dependent on the integrity of a hydrogen-bond network involving W48. Substitution of this residue for F in the double mutant W48F/F208Y results in predominant hydroxylation of Y208, regardless of external reductant concentration. This observation bolsters the proposal that the hydrogen-bond network involving W48 is the specific electron-transfer pathway between the active site and the protein surface that is key to removing one oxidizing equivalent at the active site, thus preventing an MMOH-Q like diiron(IV) species from accumulating. Control of forming such a species is likely the key to preventing alternative reactions, such as self hydroxylation, from occurring [24].

The characteristics of intermediates MMOH-Q and RNR R2-X continue to converge. The radiolytic reduction of MMOH-Q affords an Fe(III)/Fe(IV) form designated **Q_x**, which has Mössbauer properties similar to RNR R2-X [25]. This work and the recent EXAFS characterization of MMOH-Q and RNR R2-X [26–28], revealing that the two intermediates have very similar core structures, provide strong support that the O₂ reaction chemistry in these two enzymes is analogous. This notion very likely extends to the other diiron oxygen activating enzymes.

A few other cases not mentioned in the first edition deal with recently reported X-ray structure determinations of a few interesting iron proteins. These have become available during the last year and some of them could not be neglected in the present edition. First, the Rieske centers should be mentioned [29], which contain asymmetric dinuclear iron centers consisting of the chromophore (His)₂FeS₂Fe(Cys)₂. These structures were originally deduced from ENDOR using specifically labeled species, but were recently proven by XRD; such proteins are, e.g., playing a key role in phthalate deoxygenase.

The second case we want to mention deals with the X-ray structure of the non-heme iron protein lipoxxygenase. This dioxygenase enzyme occurs widely among plant and animal species, and reacts specifically with 1,4-pentadiene units, yielding hydroperoxides of conjugated dienes. The coordination geometry around the Fe(II) ion in the resting state has been found [30,31] to consist of three histidine nitrogens, a relatively distant oxygen from asparagine and the O-atom of the terminal isoleucine, leaving one coordination site open (occupied by water in the resting state).



IV.B. Peroxidases

New insights into the peroxide binding and in the catalysis, obtained through site-directed mutagenesis, have led to much-improved understanding of heme peroxidases and their possible applications. New crystal structures of peroxidases have provided much more information on the local heme environments [32].

IV.C. Nitric Oxide Enzymes

The bioinorganic chemistry of NO has only developed after the publication of the first edition of this book. Because this book deals with metal catalysis, a discussion of NO does not belong here. However, its reduction with an NO reductase [33] and its synthesis from L-arginine with an NO synthase oxygenase [34] clearly deal with metal heme systems and therefore should be mentioned here as well.

IV.D. Electron Transfer

Although electron transfer as such is not considered as catalysis, most enzymatic redox reactions require the presence of electron-transfer proteins for fast and efficiently directed electron transfer to the active sites. The ferredoxins, azurins, and cytochromes are most well known in this respect. Variations of over 15 Å in distance may occur, and as a consequence, the electron-transfer rate may vary over 10 orders of magnitude [35]. Exciting developments are ongoing in this field, and are highly relevant for the bioinorganic catalytic subject.

V. OUTLOOK

Bioinorganic chemistry will surely develop in an even wider area than it has thus far. Attention is likely to increase for studies on nonmetals, such as Se and As and their roles in, e.g., detoxification reactions. In addition, studies on elements such as aluminum (a possible causative factor in dialysis dementia and related to Alzheimer disease, senile dementia) and other abundant earth crust metals will increase. The role of bioinorganic catalysis to make and keep our environment clean has been mentioned in many of the previous chapters. It is to be expected that future catalytic processes, based on and derived from biological ones, will be as clean as possible, producing useful, harmless, and biodegradable products for the world.

As a direct consequence of an integrated approach, cross-fertilization between the various disciplines and subdisciplines is of very great importance. As a matter of fact, many interdisciplinary conferences and workshops have been



held in the bioinorganic area during the last decade, such as those in the ICBIC series, which started in 1983 with biannual meetings all over the world.

Gordon Conferences in the field of "Metals in Biology" have been held in the United States since the late 1960s; conferences of this type have also been held in Europe since 1993. Since 1985, the Federation of European Biochemical Societies has organized training courses in this area (later taken over by ESF), and as a result, many young scientists are ready for new discoveries in this exciting area and new research challenges. It is expected that the present monograph will be a useful guide in this process.

After the first edition of the present book, a new European initiative, called "Eurobic," was started, and European Conferences on Bioinorganic Chemistry were organized in 1992, 1994, 1996, and 1998.

Last but not least, we should mention that the Society of Biological Inorganic Chemistry (SBIC) was erected in 1995, and is strongly developing, not only giving attention to bioinorganic catalysis, but also to biomimetics, electron transfer, and the role of metal ions in medicine and the environment, including metal-nucleic acid interactions; for details, see the website: <http://www.sbic-home.org/>.

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