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R.H. BURDON and P.H. van KNIPPENBERG
Editors

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Edited by

R.H. BURDON — *Department of Bioscience and Biotechnology, University of Strathclyde, Glasgow*
P.H. van KNIPPENBERG — *Department of Biochemistry, University of Leiden, Leiden*

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M.H.V. Van Regenmortel

J.P. Briand

S. Muller

S. Plaué

Laboratoire d'Immunochimie
Institut de Biologie Moléculaire et Cellulaire du C.N.R.S.
15, rue Descartes
67000 Strasbourg
France

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Contents

Abbreviations

AA	amino acid
Ab	antibody
Acm	acetamidomethyl
Ag	antigen
AIDS	acquired immune deficiency syndrome
AMV	alfalfa Mosaic Virus
ANF	atrial natriuretic factor
BB Gly (Lys)	benzoylbenzoyl glycine (lysine)
BDB	bis diazobenzidine
BHA	benzhydrylamine
Boc	tert-butyloxycarbonyl
BSA	bovine serum albumin
Bzl	benzyl
cDNA	complementary DNA
CDR	complementarity determining regions
CFA	complete Freund's adjuvant
CHex	cyclohexyl
CHO	formyl
CM	carboxymethyl
DCC	dicyclohexylcarbodiimide
DCHA	dicyclohexylamine
DCM	dichloromethane
DEAE	diethylaminoethyl
DIC	diisopropylcarbodiimide
DIU	diisopropylurea
DIEA	diisopropylethylamine
DMA	dimethyl adipimidate
DMAP	4-dimethylaminopyridine
DMF	dimethylformamide
DMS	dimethyl suberimidate
DNA	deoxyribonucleic acid
DNFB	2,4-dinitrofluorobenzene

Dnp	2,4-dinitrophenol
DTT	dithiothreitol
DVB	divinylbenzene
DTPB	dimethyl 3,3′-dithiobisproprionimidate
DMP	dimethyl pimelimidate
EBIZ	*N*-ethylbenzisoxazolium fluoborate
ECDI	1-(3-dimethylamino propyl)-3-ethylcarbodiimide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPR	electron paramagnetic resonance
FMDV	foot-and-mouth disease virus
EtOH	ethanol
Fmoc	9-fluorenylmethyloxycarbonyl
FR	framework residues
HA	haemagglutinin
HBV	hepatitis B Virus
HBs Ag	hepatitis B surface antigen
Hepes	*N*-2-hydroxyethylpiperazine-*N*′-2 ethanesulfonic acid
HF	hydrogen fluoride
HOBt	1-hydroxybenzotriazole
HPLC	high-performance liquid chromatography
HTLV	human T cell leukemia virus
IBCF	isobutylchloroformate
id	intradermal
IFA	incomplete Freund's adjuvant
im	intramuscular
ip	intraperitoneal
i Pr OH	2-propanol
IPTG	isopropyl-β-D galactopyranoside
IVH	influenza virus haemagglutinin
Kd	kilodalton
KLH	keyhole limpet haemocyanin
LH-RH	luteinizing hormone-releasing hormone
mAb	monoclonal antibody
MBHA	4-methylbenzhydrylamine
MBS	*m*-Maleimidobenzoyl *N*-hydroxysuccinimide ester
MCDI	1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide methyl-*p*-toluene sulfonate
MCS	6-maleimidocaproic acyl *N*-hydroxysuccinimide ester
MDP	muramyl dipeptide
Me	methyl
Me_2S	dimethyl sulfide
MeOH	methanol
meq	milliequivalent
Met (O)	methionine sulfoxide
MHC	major histocompatibility complex

Mob	4-methoxybenzyl
Mo-MuLV	Moloney leukemia virus
Mo-MuSV	Moloney murine sarcoma virus
MPB-PE	N-(4-(*p*-maleimidophenyl) butyryl) phosphatidylethanolamine
MPPC	medium pressure preparative chromatography
M_r(K)	molecular ratio
Mtr	4-methoxy-2,3,6-trimethylbenzene sulfonyl
Mts	mesitylene-2-sulfonyl
NHS-	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance
-P	resin support
Pab	phenylacetamidobenzyl
Pam	phenylacetamidomethyl
PBS	phosphate-buffered saline
PC	phosphatidycholine
PE	phosphatidyl ethanolamine
p.f.u.	plaque-forming units
PG	phosphatidyl glycerol
Pmc	2,2,5,7,8-pentamethylchroman -6-sulfonyl
RIA	radioimmunoassay
RNA	ribonucleic acid
sc	subcutaneous
SDS	sodium dodecyl sulfate
SMCC	succinimidyl 4-(*N*-maleimidomethyl) cyclohexane 1 carboxylate
SMPB	succinimidyl 4-(*p*-maleimidophenyl) butyrate
SPDP	*N*-succinimidyl 3-(2-pyridyldithio) propionate
SPPS	solid-phase peptide synthesis
SSV	simian sarcoma virus
SV 40	simian virus 40
TBS	Tris-buffered saline
TEA	triethylamine
TFA	trifluoroacetic acid
TFMSA	trifluoromethanesulfonic acid
TLC	thin-layer chromatography
TMV	tobacco mosaic virus
Tos	*p*-Toluenesulfonyl (tosyl)
Tris	Tris(hydroxymethyl)aminomethane
Trt	triphenylmethyl
Xan	xanthenyl
Z	benzyloxycarbonyl

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CHAPTER 1

Molecular dissection of protein antigens and the prediction of epitopes

1.1. Introduction

Interest in the antigenic properties of synthetic peptides stems mainly from the fact that peptides are able to mimic the antigenic sites of proteins. Since most antigens of biological interest are proteins, one of the major goals of molecular immunology has been to elucidate the antigenic structure of proteins. The development by Merrifield (1963) of the solid-phase method of peptide synthesis made it easier to obtain short fragments of a protein by synthesis rather than by enzymatic or chemical cleavage of the protein. Natural fragments obtained by cleavage of a protein must be separated from each other, and since the level of purity required for immunological studies is extremely high, this is often no mean task. Indeed, if a fragment of a protein devoid of antigenicity is contaminated by a small amount of a highly reactive antigenic fragment derived from the same molecule, it may happen that the antigenicity is ascribed erroneously to the major, inactive peptide component in the mixture. Such misinterpretations are eliminated when synthetic fragments are used to locate antigenicity in proteins.

The molecular dissection of protein antigens has been undertaken not only in order to increase our understanding of immunological specificity, but also because such knowledge makes it possible to manipulate the immune system and leads to many useful practical applications in molecular biology, biochemistry and microbiology. For instance, available information on the location of antigenic sites in toxins, viruses and parasites has stimulated much research in the development of a new generation of synthetic peptide vaccines (Arnon et al., 1983; Shinnick et al., 1983; Lerner et al., 1985; Van Regenmortel and Neurath, 1985; Brown et al., 1986a). It was found, for instance, that protective immunity could be elicited against foot-and-mouth disease, influenza, hepatitis B and cholera by immunizing animals with synthetic peptides (see chapter 7).

Another major application of synthetic peptides relies on their ability to elicit anti-peptide antibodies that cross-react with the corresponding complete protein; such antibodies have been found to be extremely useful reagents for isolating and characterizing gene products (Lerner, 1984; Walter, 1986; Sutcliffe et al., 1983). Because of advances in gene cloning and sequencing, the information on protein sequences is nowadays increasingly derived from nucleotide sequence analysis. In many cases, the protein is not available in sufficient quantity for conventional chemical studies or its presence in the cell may even be in doubt. By synthesizing a peptide fragment of the putative protein and raising antibodies against it, it is possible to isolate and characterize the complete molecule using appropriate immunoassays (see chapter 6).

1.2. Definitions of antigenicity and the concept of epitope

The antigenic reactivity of a protein refers to its capacity to bind specifically to the functional binding sites or paratopes of certain immunoglobulin molecules. When such a binding is observed experimentally, the particular immunoglobulin becomes known as an antibody specific for the protein. Antibody molecules possess two identical paratopes made up of six highly accessible loops of hypervariable sequence known as complementarity determining regions (CDR). The CDRs are present

on the two Fab ends of the immunoglobulin molecule and interact to varying degrees with the surface of the protein antigen (Novotny et al., 1983). That portion of the antigen that comes into contact with the paratope of the antibody constitutes an antigenic determinant or epitope of the antigen. In the same way that the antibody nature of an immunoglobulin is identified only when its complementary antigen has been recognized, the epitope nature of a set of amino acids in a protein can be established only by finding an immunoglobulin able to bind to it. Clearly, an epitope is a relational entity which needs a complementary paratope for its operational definition.

According to this definition, the source and origin of the antibody used to identify a particular protein epitope are irrelevant. Usually, the antibody is obtained from an animal immunized with the protein in question, but it could also originate from an animal immunized with a related antigen possessing either the same or a cross-reacting epitope. The antibody could even be derived from a non-immunized animal (Dighiero et al., 1985) or from an animal for which the immunizing stimulus is unknown. For instance, antibodies derived from autoimmune mice have been found to recognize epitopes in DNA (Lafer et al., 1981) and in histones (Laskov et al., 1984).

Some authors are reluctant to define protein epitopes only in terms of their antigenic reactivity, i.e., their ability to bind to paratopes, for they consider that the concept of epitope involves also the property known as immunogenicity. Immunogenicity refers to the ability of a protein to induce an immune response and depends on host factors such as the immunoglobulin gene repertoire, self-tolerance, and various regulatory mechanisms (Sercarz and Berzofsky, 1988). For instance, Atassi (1984) has argued that the peptide regions in myoglobin consisting of residues 1–6 and 121–127 are not epitopes of the protein, because he could not find any antibodies recognizing these peptides in the anti-myoglobin sera he examined. The fact that peptides 1–6 and 121–127, when injected as free or conjugated peptides, were able to induce antibodies that recognized the myoglobin molecule was not taken as a sufficient criterion for considering them as myoglobin epitopes (Atassi and Young, 1985). According to Atassi (1984), therefore, an epitope refers to a region of a

protein that is recognized (by antibodies and lymphocyte receptors) during the immune response when the whole protein is used as an immunogen. Such a viewpoint, which makes the existence of epitopes in a protein depend on immunogenetic and immunoregulatory mechanisms of the immunized host, will not be followed in the present text. A different viewpoint will be adhered to, namely, that epitopes need not at all times and under all conditions possess properties both of antigenic reactivity and immunogenicity. When the two properties are dissociated, there is no difficulty in admitting, for instance, that a self antigen that is non-immunogenic in the tolerant autologous animal nevertheless possesses epitopes. It is, indeed, customary to distinguish between the antigenic reactivity and the immunogenicity of proteins, and it seems equally important to apply the same distinction to epitopes. In practice, this means, for instance, that a region of a protein that appears not to be immunogenic when the whole protein is used for immunization may be given the status of epitope when it is found to bind to antibodies induced by a peptide fragment. It should be noted that a protein epitope may appear to be non-immunogenic simply because it is not immunodominant when the complete protein molecule is injected in a particular animal. Since the phenomenon of immunodominance is poorly understood, the definition of epitope adopted here circumvents the difficulties that would arise if one were to use the relative 'strength' of immune responses as a criterion for the recognition of epitopes.

Another difficulty in defining epitopes arises from the fact that the antigenic reactivity of a protein is not the same in the native and denatured forms of the molecule. It is thus important to specify whether the epitopes being studied pertain to the native protein or not. This problem has become particularly relevant in recent years because of the popularity of solid-phase immunoassays (see chapter 5). When proteins are adsorbed to a layer of plastic in such assays, they tend to undergo some physical distortion or denaturation, and it is questionable whether the epitopes corresponding to the native state are preserved (Soderquist and Walton, 1980; Mierendorf and Dimond, 1983; Friguet et al., 1984; Al Moudallal et al., 1984; McCullough et al., 1985). In order to ascertain whether antibodies elicited by peptides of a given protein truly recognize

the 'native' form of the protein, as is often claimed (Green et al., 1982; Luka et al., 1983), it is necessary to test these antibodies in a liquid-phase type of assay that preserves the original conformation of the molecule. This is particularly important when potential peptide vaccines are being evaluated, since the neutralization epitopes may be preserved only in antigens possessing intact tertiary or quaternary structures.

1.3. Types of epitopes

It is customary to divide epitopes into a number of conceptual categories that are not easily distinguished experimentally. Sequential epitopes have been opposed to conformational epitopes on the basis that the former are defined by peptide regions in their random coil form while the latter are said to require a specific conformation in order to be recognized by their complementary paratopes (Sela et al., 1967; Sela, 1969). It is generally assumed that antibodies directed to a conformational epitope of a protein will not react with the unfolded peptide derived from the corresponding part of the native molecule. However, the distinction between conformation-dependent and -independent epitopes is somewhat artificial since it is difficult to envisage that a paratope could recognize a sequence of residues independently of its conformation.

It has been suggested (Atassi and Smith, 1978) that a more satisfactory classification consists of distinguishing between continuous and discontinuous epitopes (also called contiguous and discontiguous epitopes). Continuous epitopes are defined as a stretch of contiguous residues in direct peptide linkage endowed with distinctive conformational features, while discontinuous epitopes, also known as assembled topographic epitopes (Benjamin et al., 1984), consist of a group of residues that are not contiguous in the sequence but are brought together by the folding of the polypeptide chain or by the juxtaposition of two separate peptide chains. It is important to recognize that both types of epitopes may be sensitive to conformational changes occurring in the protein. For instance, the reactivity of a continuous epitope may depend on the ability

of a stretch of contiguous residues to assume the correct conformation. The concept of continuous epitope is thus clearly different from the earlier category of sequential epitope. The label 'continuous' seems preferable to 'contiguous' since it is the individual constitutive residues that can be said to be contiguous rather than the epitope as a whole.

In practice, the label 'continuous epitope' is attached to any linear peptide fragment of a protein that is found to react with antibodies raised against the intact molecule. It should be realized, however, that such a peptide may represent only a part of a larger discontinuous epitope, although it reacts with antibodies induced by the complex discontinuous structure. Clearly, the label 'continuous epitope' attached to an antigenically active peptide does not mean that the fragment accurately mimics the complete structure of the protein epitope. In general, antibodies induced by a protein cross-react only weakly with peptide fragments derived from it, which testifies to the limited structural resemblance between the peptides and the protein. Until recently, it was widely assumed that this low cross-reactivity was due to the fact that peptides exist in solution in thousands of different conformations, and that only a few of them are recognized by the antibody. Classical experiments performed with fragments of the staphylococcal nuclease molecule led to the suggestion that 1 in 5000 molecules of peptide fragment was correctly folded (Furie et al., 1975; Sachs et al., 1972). To-day, this interpretation has fewer adherents since there is growing evidence that peptides in solution have distinct conformational preferences (Schulze-Gahmen et al., 1985; Dyson et al., 1986). It is widely believed that the vast majority of protein epitopes are discontinuous and that short linear peptides possess a low antigenic cross-reactivity with proteins because they mimic, in a conformationally imperfect way, only part of the epitope structure (Benjamin et al., 1984; Van Regenmortel, 1984; Berzofsky, 1985). Most studies meant to unravel the nature of protein antigenicity have actually focused on the phenomenon of cross-reactive antigenicity between proteins and short peptides. As a result, our knowledge of protein epitopes concerns mainly adulterated, incomplete epitopes that have retained only part of their identity after fragmentation of the protein.

Recently, it has been argued that if the surface areas of proteins that

are recognized by paratopes are of the order of 20 × 25 Å, all protein epitopes are likely to be discontinuous (Barlow et al., 1986). Based on calculations of accessible contact areas (Lee and Richards, 1971) it was shown that in globular proteins no surface region of 20 Å diameter is likely to contain only atoms from a continuous stretch of residues. The argument of Barlow et al. (1986) rests on the premise that in all protein antigens, the epitope–paratope interaction occurs over a surface of about 16–20 Å diameter, i.e., the size found in the case of a discontinuous epitope of lysozyme (Amit et al., 1986). In the case of this epitope of lysozyme, 16 residues of the antigen were found to interact with 17 residues of the antibody belonging to all six CDRs of the paratope (see section 1.4.4). However, it cannot be excluded that in other paratope–epitope pairs, sufficient binding energy could be provided by the participation of only some of the CDRs and that in such cases the contact area between protein antigen and antibody may be smaller and closer to the size observed with small haptens (Amzel et al., 1974; Segal et al., 1974).

Two further categories of epitopes have been distinguished which are particularly relevant when discussing the antigenic structure of viruses, i.e., the so-called cryptotopes and neotopes. Cryptotopes are hidden epitopes that become expressed only after fragmentation, depolymerization or denaturation of the antigen (Jerne, 1960). In viral capsids, cryptotopes are found, for instance, on the surfaces of subunits that are turned inward and become buried after polymerization (Van Regenmortel, 1966, 1982). Cryptotopes are also responsible for the fact that many antibodies raised by immunization with denatured proteins do not react with the corresponding native molecule. Neotopes are epitopes that are specific for the quaternary structure in proteins and they are thus absent from the constituent monomeric subunits of viral capsids (Van Regenmortel, 1966; Neurath and Rubin, 1971). Neotopes arise as a result of conformational changes in the monomer induced by intersubunit bonds or by the juxtaposition of residues from neighbouring subunits.

The number of epitopes present on any antigen has been a fertile source of controversy. According to one viewpoint, each protein possesses only a small number of epitopes characterized by discrete boundaries and not influenced by the host species in which antibodies are raised

(Atassi, 1984). A more widely held view states that the entire accessible surface of a protein consists of a large number of overlapping epitopes (Benjamin et al., 1984).

Since epitopes can only be identified operationally by virtue of a relational nexus with complementary paratopes, the number of epitopes in a protein may be equated with the number of different monoclonal antibodies that can be raised against it. In the case of insulin, for instance, this number was estimated to be around 100 (Schroer et al., 1983). According to this viewpoint, the diversity of epitopes in a protein is defined by the size of the immunological repertoire in the immunized host. This means, in effect, that the analysis of the antigen is being transformed into an analysis of the antibody tool used in the study.

Clusters of overlapping epitopes have been identified at the surface of certain proteins by mapping with monoclonal antibodies. Such clusters are usually said to constitute an antigenic site. The boundaries of antigenic sites are mostly blurred and a site can be defined clearly only if it corresponds to an easily recognizable structural feature of the protein.

1.4. Methods used to delineate epitopes

Most of our knowledge concerning the location of epitopes in proteins was obtained by studying the antigenic cross-reactivity between the intact molecule and peptide fragments and could be derived because the precision of fit between paratope and epitope is not absolute. As a result, our knowledge of protein antigenicity concerns, in fact, mainly cross-reactive antigenicity (Van Regenmortel, 1987a).

Since the relationship between the two immunological partners is never of an exclusive nature, the relationship has sometimes been described as promiscuous or degenerate. Although it has been accepted for a long time that an individual epitope can be recognized by a variety of different paratopes, it is a surprising feature of the history of immunology that the reciprocal situation has become accepted much more slowly. The fact that an antibody is polyspecific, i.e., that it is able to react with a variety of more or less closely related epitopes was surmised many

years ago (Talmage, 1959; Richards and Konigsberg, 1973; Cameron and Erlanger, 1977) but this fact became generally accepted only with the advent of hybridoma technology (Lane and Koprowski, 1982). A further insight brought about by the use of monoclonal antibodies was that antibodies are not only polyspecific but also heterospecific or heteroclitic, i.e., they are often able to bind more strongly to other antigens than to the one against which they were raised (Al Moudallal et al., 1982; Van Regenmortel, 1982; Underwood, 1985). Heterospecificity appears to be a universal feature of antibodies but is seldom noticed because of lack of appropriate testing. In view of the polyspecific and heterospecific nature of antibodies, it is futile to turn the delineation of epitopes into a search for the 'true' antigenic structure to which a paratope is supposed to fit perfectly. Cross-reactive fit rather than absolute fit is the rule underlying epitope–paratope interactions (Van Regenmortel, 1986a).

1.4.1. Cross-reactivity studies between proteins and peptide fragments using anti-protein antibodies

This method measures the ability of antibodies raised against a native protein molecule to cross-react with natural or synthetic fragments of the molecule. If a fragment is able to bind to the protein antibodies and therefore able to inhibit the reaction between these antibodies and the intact protein, it is assumed that it contains a continuous epitope of the protein. Attempts are then made to locate the position of the epitope by testing peptides of decreasing size. Usually the assumption is made that the smallest peptide that still possesses some residual antigenic reactivity represents the epitope; in general this corresponds to a size of 5–7 amino acid residues. Since the degree of antigenic cross-reactivity observed with such small fragments is mostly very low, large molar excesses of peptide over intact protein are needed to demonstrate the presence of reactivity in the peptide. The major exception to this rule concerns peptides that represent chain termini in proteins. Terminal segments of proteins are less constrained and more mobile than internal sections of the polypeptide chain (Westhof et al., 1984) and in a majority of proteins

they are surface-oriented (Thornton and Sibanda, 1983). This may explain why the antigenic cross-reactivity observed with short terminal peptides is often higher than with internal peptides (Absolom and Van Regenmortel, 1977; Walter et al., 1980; Altschuh and Van Regenmortel, 1982; Bittle et al., 1982; Altschuh et al., 1983; Quesniaux et al., 1983a).

The difficulties that are encountered when one tries to determine the exact size of an epitope can be illustrated in the case of the epitope found in the region 103–112 of tobacco mosaic virus protein (TMVP). In a series of classical immunochemical studies (Young et al., 1967; Benjamini, 1977), the antigenic activity of this region was studied in direct binding assays by means of labelled synthetic peptides varying in length from dipeptide to decapeptide. Since the shortest peptide showing significant binding to TMVP antibodies was the pentapeptide 108–112, this region was identified as the epitope, although considerably more activity was present in the octapeptide 105–112 (Benjamini et al., 1968a). In subsequent work, it was shown that the entire length of the decapeptide 103–112 was needed for obtaining optimal binding to some monoclonal antibodies raised against TMVP (Morrow et al., 1984). These findings illustrate the inherent difficulty in interpreting such data: does the increase in antigenic activity observed when peptides of increased size are tested result from a direct participation of the additional residues in the epitope structure, or does it reflect the fact that longer peptides are better able to assume the conformation present in the complete molecule? It also cannot be excluded that non-specific effects, for instance hydrophobicity, may play a role, since it was found that the antigenic activity of the pentapeptide 108–112 was greatly enhanced by the addition of five alanine residues at its N-terminal end (Benjamini et al., 1968b). In the case of other peptides, it has been shown that the addition of lysine residues to a short peptide fragment can also increase its apparent reactivity in a non–specific manner (Leach, 1984; Shi et al., 1984).

Candidates for peptide synthesis are usually selected in the form of linear sequences of 6–20 residues corresponding to regions of a protein that seem likely to contain a continuous epitope. However, two other approaches have also been followed, namely surface-simulation synthe-

sis and mimotope synthesis. Atassi and collaborators developed the method of surface-simulation synthesis to simulate the structure of discontinuous epitopes of lysozyme (Atassi et al., 1976; Atassi and Lee, 1978). Attempts were made to reproduce parts of the surface topography of lysozyme by bringing together, by means of a few spacer glycine residues, certain highly accessible residues of the protein that were distant in the sequence (see section 1.4.2).

The approach known as mimotope synthesis completely ignores the sequence of the protein under study and attempts to built up short peptides with increasing binding activity for the anti-protein antibodies (Geysen et al., 1986). Starting with the 400 possible dipeptides made from the 20 common amino acids, the dipeptide possessing the highest binding activity is selected and lengthened on either side with the residues that produce the greatest increment in antigenic reactivity. In this way, hexapeptides can be constructed which possess considerable binding capacity for a particular anti-protein antibody. Such peptides, which are called mimotopes, do not necessarily reproduce the sequence of the protein epitope that induced the antibody but are believed to mimic certain features of the epitope (Geysen et al., 1986). One drawback of this approach is that it could lead to the accumulation of non-specific binding effects, caused for instance by hydrophobic and electrostatic interactions (Benjamini et al., 1968b; Leach, 1984). A number of mimotopes reacting with anti-protein antibodies have been synthesized by this method but there is no evidence yet that they are able to induce antibodies capable of recognizing the native protein antigen.

1.4.2. Cross-reactivity studies between proteins and peptide fragments using anti-peptide antibodies

Instead of testing the antigenicity of peptide fragments by means of anti-protein antibodies, this method consists in using the fragments for immunization and then ascertaining if the resulting anti-peptide antibodies react with the intact molecule. A positive cross-reaction is usually interpreted as an indication that the peptide approximates to an epitope of the protein. It has repeatedly been claimed (Green et al., 1982; Luka et

al., 1983; Niman et al., 1983; Lerner, 1982, 1984) that immunization with peptides leads to a very high frequency of induction of antibodies able to recognize the *native* protein, although the evidence for such a claim is not very convincing. In order to demonstrate that anti-peptide antibodies are able to react with a truly native protein, the test should be performed by a liquid-phase immunoassay that does not alter the conformation of the antigen. Furthermore, the antigen should preferably not be labelled since such a treatment could also induce some denaturation. It seems that these conditions were not met in the experiments that gave rise to the above mentioned claims. Since this point is of crucial importance, it will be discussed in some detail.

One series of experiments was carried out with 20 synthetic peptides representing over 75% of the haemagglutinin sequence of influenza virus. Green et al. (1982) reported that 18 of the 20 peptides elicited antibodies that reacted with purified haemagglutinin or intact virus, but that none of the peptides reacted with anti-haemagglutinin antibodies present in influenza virus antisera. No explanation was offered for this apparent contradiction. One possible explanation could have been that the immunogenic form of the peptides corresponded to a rare conformational occurrence. This idea was tested by estimating the frequency with which monoclonal antibodies directed against one of the peptides recognized the intact haemagglutinin. The results showed that 16 out of 21 monoclonal antibodies reacted with the haemagglutinin, a finding that seemed to refute the argument that a rare native-like conformation of the peptide was responsible for generating these antibodies (Niman et al., 1983). However, if the correct peptide conformation is not a rare occurrence, it is difficult to understand why none of the 20 peptides reacted with the anti-haemagglutinin antibodies. On the other hand, this apparent contradiction can be resolved if it is assumed that the anti-peptide antibodies recognized the haemagglutinin because this molecule had been denatured by the type of solid-phase immunoassay procedure used in the experiments. It is now generally recognized that proteins become at least partly denatured when they are adsorbed to a layer of plastic during a solid-phase assay (Soderquist and Walton, 1980; Kennel, 1982; Friguet et al., 1984; Altschuh et al., 1985; McCullough et al., 1985; Vai-

dya et al., 1985). As far as the extremely low level of cross-reactivity observed with influenza virus is concerned (Green et al., 1982), this could be explained by the presence of a small number of denatured haemagglutinin molecules in the preparation (Walter, 1986). In conclusion, these experiments do not substantiate the claim that when synthetic peptides are used as immunogens, antibodies cross-reacting with the *native* protein can be easily raised against almost any part of the molecule.

It should be emphasized, however, that anti-peptide antibodies tend to react extremely well with the denatured form of the corresponding protein. This may explain the high frequency with which anti-peptide antibodies react in immunoblotting with proteins that have been separated on denaturing gels. This is to be expected since a denatured protein is likely to show a greater conformational resemblance with the peptide than would the native protein. Finally, it should also be pointed out that the conformation of a peptide may be altered when it interacts with a B cell receptor during the induction of an immune response. A process of induced fit may thus influence the conformation of the immunogenic form of the peptide.

1.4.3. Cross-reactivity studies between closely related proteins

This method consists in studying the antigenic cross-reactivity between related proteins possessing known amino acid substitutions and gives unambiguous information only when monoclonal antibodies are used as probes and the tertiary structure of the protein is known (Benjamin et al., 1984; Hannum and Margoliash, 1985). The method is based on the assumption that if the substitution leads to a change in antibody binding, the mutated residue is likely to be directly involved in the structure of an epitope. Although this assumption may be valid in many cases (Hornbeck and Wilson, 1984), especially when the substitution occurs in a highly accessible surface residue, it is well known that the reactivity of epitopes can also be altered by conformational changes induced by substitutions occurring elsewhere in the protein (Hurrell et al., 1977; Ibrahimi et al., 1979; Milton et al., 1980). Since the number of available substitutions in a series of homologous proteins is always limited, and

since substitutions inside an epitope need not necessarily always affect antibody binding, this approach cannot lead to the identification of all the residues involved in epitope structures.

Immunological comparisons between the members of several families of homologous proteins have demonstrated that there is a good correlation between degree of sequence difference and degree of antigenic difference (Champion et al., 1975; Prager et al., 1978; Van Regenmortel, 1986b). From the extent of correlation, it has been inferred that about 80% of the substitutions that have accumulated during evolution of monomeric globular proteins are antigenically detectable (White et al., 1978). Such a finding is in keeping with the view that internal substitutions may influence the reactivity of epitopes located at the surface of the protein. A particularly striking example concerns a mutant of TMV in which a substitution occurs at residue 107 in the polypeptide chain, i.e., at a distance 5 nm away from the outer viral surface. This exchange was shown to alter the binding of antibody to the virus (Al Moudallal et al., 1982), presumably by long-range transmission of a conformational change through the close-packed α-helix located in residues 114–134 of the viral protein (see also Chothia and Lesk, 1985).

Instead of inferring the position of epitopes from the antigenic differences observed between naturally occurring protein variants, the same can be done by selecting antigenic mutants by means of monoclonal antibodies. A classic example of this approach is the determination of the antigenic map of influenza virus haemagglutinin (IVH) (Gerhard and Webster, 1978; Wilson et al., 1981; Caton et al., 1982). By growing the virus in the presence of neutralizing monoclonal antibodies, non-neutralizable virus mutants were selected which were found to have single amino acid substitutions in the IVH. By locating these substitutions in the known three-dimensional structure of IVH, several clusters of exchanges at the surface of the molecule were identified as likely epitopes.

1.4.4. X-ray crystallography of antigen–antibody complexes

This method is based on the study of crystals of immune complexes formed between a protein of known tertiary structure and a Fab frag-

ment of a monoclonal antibody. Although until now only two protein epitopes have been analyzed by this method (Amit et al., 1986; Colman et al., 1987), work currently in progress in several laboratories should lead to the elucidation of many more epitope structures in the near future. The information obtained in the case of a lysozyme epitope will be briefly summarized. The structure of the immune complex between hen egg white lysozyme and a specific Fab fragment from a monoclonal antibody denoted D1.3 was determined first at 6 Å resolution (Amit et al., 1985) and subsequently at 2.8 Å resolution (Amit et al., 1986). The interface between antigen and antibody was found to extend over an area of about 30 × 20 Å, the two surfaces showing extensive interpenetration. A total of 16 residues of lysozyme and of 17 residues of the antibody belonging to the six CDRs were found to interact closely (see Table 1.1). Since the residues of lysozyme belonged to two stretches of residues, i.e., 18–27 and 116–129, that were adjacent on the protein surface, the epitope is clearly discontinuous. All 6 CDRs corresponding to the hypervariable loops of the antibody as well as two framework (FR) residues (Table 1.1) interacted with the antigen. Of the antibody hypervariable regions, the region V_H CDR3 contributed the most to the binding since four of its residues interacted with 10 residues of lysozyme. This finding is in agreement with the view that V_H CDR3 plays an essential role in antigen recognition (Schilling et al., 1980).

The binding of the D1.3 Fab fragment to lysozyme measured by a solid-phase immunoassay was characterized by an affinity constant of about $10^7\ M^{-1}$ and was mediated solely through van der Waals and hydrogen bonds. Particularly noticeable was the absence of any electrostatic interactions.

The elucidation of the structure of an epitope–paratope pair achieved by Amit et al. (1986) represents a milestone in our understanding of antigen–antibody complementarity and paves the way to new experimental approaches to the study of immunological cross-reactivity. For instance, site-directed mutagenesis of both antigen and antibody leading to substitutions of residues implicated in the binding should throw light on the contribution of individual residues to the overall reaction. Another particularly fruitful approach would be to synthesize peptides such as the

TABLE 1.1
Lysozyme residues in contact with residues of antibody D1.3 in the crystallized immune complex (from Amit et al., 1986)

Lysozyme residues	Antibody residues	Position in antibody sequence
Asp 18	Tyr 50	CDR 2 L
Asn 19	Tyr 50	
	Arg 102	CDR 3 H
Arg 21	Arg 99	
Gly 22	Tyr 49	FR 2 L
	Arg 99, Asp 100, Arg 102	CDR 3 H
Tyr 23	Arg 99, Asp 100	CDR 3 H
Ser 24	Asp 100	CDR 3 H
Leu 25	Tyr 32	CDR 1 L
Asn 27	Asp 100	CDR 3 H
Lys 116	Thr 30	FR 1 H
	Gly 31, Tyr 32	CDR 1 H
Gly 117	Thr 30	FR 1 H
	Gly 31, Tyr 32	CDR 1 H
	Trp 52, Gly 53, Asp 54	CDR 2 H
Thr 118	Trp 52	CDR 2 H
	Tyr 101	CDR 3 H
Asp 119	Trp 52	CDR 2 H
	Tyr 101	CDR 3 H
Val 120	Tyr 101	CDR 3 H
Gln 121	Tyr 32	CDR 1 L
	Phe 91, Trp 92, Ser 93	CDR 3 L
	Tyr 101	CDR 3 H
Ile 124	Tyr 32	CDR 1 L
	Trp 92	CDR 3 L
Leu 129	His 30	CDR 1 L

fragments 18–27 and 116–129 of lysozyme, in order to ascertain to what extent individual linear portions of a discontinuous epitope are able to be recognized by an antibody directed to the more complex structure.

1.5. The antigenic structure of model proteins

1.5.1. Myoglobin

Sperm-whale myoglobin is a protein composed of a single polypeptide chain comprising 153 residues folded in a highly helical compact structure. Its antigenic properties have been extensively studied and several reviews of the subject are available (Crumpton, 1974; Atassi, 1977b; 1984). Following initial investigations by Crumpton and collaborators, Atassi (1975) delineated the position of five continuous epitopes by means of inhibition experiments with synthetic peptides and claimed that these five regions (residues 15–22, 56–62, 94–99, 113–119 and 145–151) accounted for the entire antigenic reactivity of the native myoglobin molecule. This claim was based on the findings that serial elution with the five peptides displaced 81% of the total elutable antibody from a myoglobin–Sepharose immunosorbent (Atassi and Koketsu, 1975) and that the five peptides achieved 89–94% inhibition of the precipitin reaction between myoglobin and its antibodies (Atassi, 1977b). However, the significance of the inhibition data is hard to assess, as no indication was given of total radioactive counts of antigen used in the experiments. If, for instance, only 2% of antigen added was precipitated, the inhibition of this precipitation would be difficult to interpret in quantitative terms.

The claim that the total antigenicity of myoglobin resides in five continuous epitopes of 6–7 residues has been challenged by several other groups (Todd et al., 1982; Benjamin et al., 1984). For instance, comparisons between several mammalian myoglobins using antibodies specific for the fragment 1–55 of myoglobin showed that the antigenicity of this fragment did not reside solely in the epitope identified by Atassi in residues 15–22 (East et al., 1980). It was also found that the synthetic peptides corresponding to residues 22–55 and 72–89 of myoglobin reacted with anti-bovine myoglobin antibodies and thus also contained epitopes (Leach, 1983). In a recent study in which several hundred synthetic peptides of myoglobin were tested for activity when attached to a plastic support, a major epitope was localized in the region 48–55 (Rodda et al.,

TABLE 1.2
Amino acid residues shown to be involved in the antigenicity of myoglobin

No.	Continuous epitopes	Reference	No.	Discontinuous epitopes	Reference
1	1– 6	a	1	34 and 113	e
2	15– 22	b	2	87 and 142	e
3	48– 55	c, d	3	4 and 79	f
4	56– 62	b	4	83, 144 and 145	f
5	72– 89	c	5	140	f
6	94– 99	b			
7	113–119	b			
8	121–127	a, d			
9	145–151	b			

a, Schmitz et al., 1983; b, Atassi, 1975; c, Leach 1983; d, Rodda et al., 1986; e, East et al., 1982; f, Berzofsky et al., 1982.

1986). Furthermore, synthetic peptides corresponding to residues 1–6 and 121–127 of myoglobin were shown to elicit antibodies that reacted with native myoglobin (Young et al., 1983; Schmitz et al., 1983a). These various studies thus bring to nine the number of continuous epitopes identified in myoglobin (Table 1.2).

A number of discontinuous epitopes were also identified in myoglobin on the basis of cross-reactivity studies between mammalian myoglobins, using monoclonal antibodies (Berzofsky et al., 1982; East et al., 1982). By identifying which surface residues are conserved among cross-reacting myoglobins and vary in non-cross-reacting myoglobins, it was possible to implicate a number of spatially adjacent residues in the formation of certain epitopes. For instance, residues 4 and 79 were implicated in one epitope and residues 34 and 113 in another (Table 1.2).

It has been argued (Young et al., 1983; Atassi, 1984) that cross-reaction studies with monoclonal antibodies may lead to erroneous assignments of discontinuous epitopes, because the investigators allegedly tend to disregard the possibility that the substitutions alter the conformation of the protein by long-range effects and are able therefore to affect epitopes elsewhere in the protein. As discussed above (section 1.4.3), this argument is less compelling when it can be shown that the substitutions affect highly accessible residues brought in close proximity by the folding of the chain. Furthermore, substitutions tend to affect antibody

binding only at one particular site and usually the reaction of monoclonal antibodies that bind to other sites remains unaffected. In this context, it is interesting to note that cleavage of myoglobin into three large fragments altered the conformation of epitopes to such a degree that reactivity with all the available monoclonal antibodies specific for myoglobin was abolished (Berzofsky et al., 1982). This finding demonstrates that any distant alterations brought about by amino acid substitutions are probably less detrimental to the conformational integrity of epitopes than fragmentation of the protein. Obviously, when an epitope is excised from a protein, conformational directives received from distal parts of the molecule before the cleavage occurred are lost. This means that both the fragmentation approach advocated by Atassi (1984) and the studies with monoclonal antibodies can produce only a rough approximation of the location of epitopes in proteins. Both approaches are complementary but neither of them is able to provide the detailed information that is obtainable, for instance, by X-ray crystallography of antigen–antibody complexes.

1.5.2. Lysozyme

Hen egg-white lysozyme consists of a single polypeptide chain of 129 amino acid residues, internally cross-linked by four disulphide bonds. Cleavage of the disulphide bonds leads to a total loss of antigenic reactivity as measured by antibodies directed to native lysozyme.

Tryptic digestion of the reduced lysozyme molecule was shown to give rise to fragments devoid of antigenic reactivity (Young and Leung, 1970). However, when the molecule was digested with trypsin without rupturing the disulphide bonds, three antigenically active peptides were obtained (Atassi et al., 1973). As these three peptides inhibited the precipitation reaction between lysozyme and its specific antiserum by 85–89%, it was claimed that they accounted for almost the entire antigenic activity of lysozyme (Atassi and Habeeb, 1977). However, as mentioned in the previous section, the significance of such inhibition data is hard to assess. There is, indeed, considerable evidence that other regions of lysozyme, such as the loop 64–80 (Teicher et al., 1973; Ibrahimi et al.,

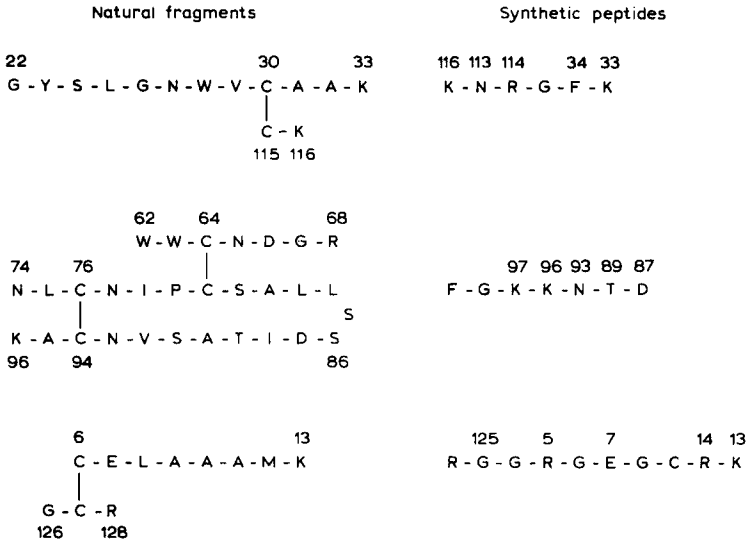

Fig. 1.1. Structure of some of the peptides implicated in the antigenic structure of lysozyme. The natural fragments were obtained by tryptic digestion and were reported to cause, together, 89% inhibition of the reaction of lysozyme with its specific antiserum (Atassi et al., 1973). The synthetic peptides are surface simulation peptides which together were reported to possess 95% inhibitory activity (Atassi and Lee. 1978).

1980) and the region 38–52 (Takagaki et al., 1980; Hirayama et al., 1985) also possess antigenic reactivity.

A more precise localization of the epitopes within the three disulphide-containing peptides isolated by Atassi et al. (1973) was attempted by a method designated 'surface-simulation synthesis' which attempts to reproduce synthetically certain features of the surface topography of the molecule (Atassi et al., 1976; Lee and Attassi, 1976). The structure of the disulphide-containing natural fragments and the synthetic counterparts that were meant to mimic their essential features is shown in Fig. 1.1. It was claimed that either set of natural or synthetic peptides accounted for 90–95% of the antigenic reactivity of native lysozyme (Atassi and Lee, 1978). However, when the two sets of peptides are compared, it is obvious that there is little structural similarity between them. When the two synthetic peptides made up of residues 5-7-13-14-

TABLE 1.3
Amino acid residues shown to be involved in the antigenicity of lysozyme

No.	Continuous epitopes	Reference	No.	Discontinuous epitopes	Reference
1	38–54	a	1	18–27, 116–121, 124, 129	c
2	64–80	b	2	5, 7, 13, 14, 125	d
			3	33, 34, 113, 114, 116	d
			4	87, 89, 93, 96, 97	d
			5	45, 47, 48, 68	e
			6	19, 21	f
			7	113, 114	f, g
			8	102, 103	f
			9	1, 41, 84	f

a, Takagaki et al., 1980; b, Teicher et al., 1973; c, Amit et al., 1986; d, Atassi and Lee, 1978; e, Smith-Gill et al., 1982; f, Smith-Gill et al., 1984; g, Metzger et al., 1984.

125 and residues 33-34-113-114-116 (interspersed with spacer glycine residues) are compared with the two corresponding natural fragments, it appears that in each case only two of the residues of the natural fragments are preserved in the synthetic peptides (see Fig. 1.1). Furthermore, three of the four retained residues are terminal lysines, residues well known to contribute to non-specific binding (Leach, 1984). Clearly, the large differences between the two sets of peptides cast doubt on the validity of the claim that both of them account quantitatively for the total antigenic activity of lysozyme.

The conclusions of Atassi and Lee (1978) are also at variance with the results of cross-reactivity studies of lysozymes of different species (Smith-Gill et al., 1982, 1984). For instance, one monoclonal antibody was shown to recognize residues 45, 47, 48 and 68, while others recognized residues 1, 41 and 84 and residues 102 and 103; none of these residues had been implicated in the three lysozyme epitopes identified by Atassi and collaborators. Furthermore, the epitope of lysozyme (section 1.4.4) that was fully delineated by X-ray crystallography (Amit et al., 1986) also does not correspond to any of the three epitopes of Atassi. All residues that have so far been implicated in the antigenicity of lysozyme are listed in Table 1.3. Although only two continuous epitopes have been experimentally identified by means of linear peptides, it seems likely that the regions 18–27 and 116–121 (Amit et al., 1986) could also be consi-

dered continuous epitopes corresponding to subsites of a large discontinuous epitope. Unfortunately, no binding experiments with fragments 18–27 and 116–121 have been reported.

1.5.3. Tobacco mosaic virus protein

TMV is a rod-shaped particle consisting of 2130 identical protein subunits arranged as a helix around an RNA molecule. The protein subunit (TMVP) which contains 158 amino acid residues, was the first viral protein whose three-dimensional structure was established by X-ray cry allography (Bloomer et al., 1978). The antigenic structure of TMVP has been extensively studied mainly by using antisera specific for dissociated viral subunits (Benjamini, 1977; Van Regenmortel, 1986b). By means of inhibition assays with fairly short cleavage and synthetic peptides, seven continuous epitopes were identified, in the vicinity of residues 1–10, 34–39, 55–61, 62–68, 80–90, 108–112 and 153–158 (Altschuh et al., 1983). When longer synthetic peptides were tested, three additional continuous epitopes were identified, in the regions 19–32, 115–134 and 134–146 (Al Moudallal et al., 1985; Table 1.4). The two antigeni-

TABLE 1.4
Continuous epitopes identified in TMVP

Position in sequence	Detected with		Reference
	Polyclonal antisera	Monoclonal antibodies	
1– 10	+	–	a, b
19– 32	+	–	b
34– 39	+	–	a
47– 61 (55–61)	+	–	c
62– 68	+	–	d
76– 88 (80–90)	+	+	a, b
103–112	+	+	e, f
115–134	+	+	b
134–146	+	+	b
149–158 (153–158)	+	+	a, g, h

a, Altschuh et al., 1983; b, Al Moudallal et al., 1985; c, Altschuh and Van Regenmortel, 1982; d, Milton and Van Regenmortel, 1979; e, Benjamini, 1977; f, Morrow et al., 1984; g, Anderer, 1963; h, Altschuh et al., 1985.

cally active peptides 115–134 and 134–146 contain within them two regions (i.e., 129–134 and 142–147) previously shown to be inactive. Furthermore, within the active peptide 19–32 three shorter synthetic peptides (18–25, 22–29, 27–33) were also found to be inactive. In the case of peptides 19–32 and 115–134, it is possible that a length of 13–20 residues was required to reveal antigenic activity, because these regions correspond to α-helices in TMVP. Although short helices in solution are known to be unstable, the environment of the carrier protein (peptides were tested as conjugates) may have stabilized the helical conformation. These peptides may also have been in a helical conformation for a long enough time to be selected by the antibody, thereby perturbing the conformational equilibrium in favour of the native structure (Crumpton, 1986). The result of such a process would not be distinguishable from an induced fit phenomenon that could also cause the peptide to adopt the native conformation. Although future X-ray crystallographic studies of antibody–peptide complexes may be able to demonstrate whether a peptide has adopted a native conformation or not, it is clear that such an analysis will be unable to establish if the process was a selection from a mixture of conformations or an induced fit subsequent to the initial interaction.

Several of the continuous epitopes of TMVP correspond to cryptotopes, i.e., to epitopes that are not expressed in the intact virus particles. Examples of such epitopes are those found in the regions 19–32, 34–39, 76–88, 90–95, 103–110 and 115–134 (Benjamini, 1977; Morrow et al., 1984; Van Regenmortel, 1986b). In a study of 30 monoclonal antibodies raised against dissociated TMVP, more that 80% of the antibodies were unable to recognize any of 18 synthetic peptides which together spanned the entire TMVP polypeptide chain (Al Moudallal et al., 1985).

A number of discontinuous epitopes were identified at the surface of TMV particles when monoclonal antibodies prepared against intact virions were used in cross-reactivity studies (Altschuh et al., 1985). By analysing the data using computer-generated images of surface residues, two discontinuous epitopes were identified which comprised residues 66, 67, 140–143, and 1, 3, 4, 9, 150, 152, 153, respectively.

1.5.4. Other proteins

There is also considerable information available regarding the antigenic structure of proteins such as cytochrome *c* (Atassi, 1984; Hannum and Margoliash, 1985; Urbanski and Margoliash, 1977; Paterson, 1985), albumin (Sakata and Atassi, 1980; Doyen et al., 1985; Benjamin et al., 1983), influenza haemagglutinin (Wiley et al., 1981; Muller et al., 1982a), scorpion toxins (El Ayeb et al., 1984; Bahraoui et al., 1986), insulin (Rathjen and Underwood, 1986), histones H2A (Muller et al., 1986) and H2B (Muller et al., 1985) and myohaemerythrin (Geysen et al., 987; Getzoff et al., 1987).

1.6. Predictions of antigenicity

For the majority of proteins identified at the present time, the only structural information available is its amino acid sequence, usually deduced from the nucleotide sequence of the corresponding gene. Attempts to raise antibody probes specific for proteins by immunization with synthetic peptides (Walter, 1986) as well as current attempts to develop synthetic peptide vaccines have underlined the usefulness of predicting the location of continuous epitopes in proteins (see Chapter 7). The accumulated knowledge of the antigenic structure of a few well-characterized proteins has led to a concerted effort to find empirical rules for predicting the position of continuous epitopes in proteins from certain features of their primary structure.

1.6.1. Correlations between antigenicity and structural features of proteins

1.6.1.1. Hydrophilicity

It has been known for many years that hydrophobic amino acids tend to be buried within the native structure of globular proteins, while hydrophilic side-chains are on the exterior where they can interact with water. By evaluating the hydrophilic and hydrophobic tendencies of stretches of residues along a polypeptide chain, it should be possible to distinguish

regions of the sequence that are buried from regions that are on the outside (Rose, 1978; Rose and Roy, 1980; Kyte and Doolittle, 1982). This approach was used by Hopp and Woods (1981, 1983) to show that the most hydrophilic segments of a protein tend to correspond to continuous epitopes. Plots of hydrophilicity along the polypeptide chain were constructed using a relative scale of hydrophilicity values for each of the 20 common amino acids and averaging this parameter over segments of 6 or 7 residues at a time. In many cases, local maxima in hydrophilicity plots were found to correspond to segments that were exposed at the surface of the molecule and at the same time were also part of epitopes. When the correlation between antigenicity and hydrophilicity was analyzed with respect to only the highest hydrophilicity peak (or for the top three peaks) in each protein, the agreement appeared to be very good (H. Hopp, 1986). However, when all the continuous epitopes of a few well-studied proteins were analyzed, the correlation between antigenicity and hydrophilicity was not particularly good (Westhof et al., 1984; Thornton et al., 1986; Geysen et al., 1987). For predicting the position of continuous epitopes, it would seem preferable, therefore, to consider only the highest peaks appearing in hydrophilicity plots.

It has been shown that methods used for predicting the secondary structure of proteins tend to generate plots in which the peaks and valleys occur in the same places as in hydrophilicity plots (Hopp, 1986). For instance, the Chou and Fasman (1978) helix prediction profile and the Garnier et al. (1978) β-strand prediction profile generate peaks in the regions of maximum hydrophobicity. This correlation arises from the fact that the centers of the largest helices and β-strands usually correspond to the tightly packed hydrophobic cores of proteins. When these secondary structure prediction scales are inverted, the resulting plots will show peaks at the same positions as in hydrophilicity plots, and such peaks can thus be used as predictors of antigenicity (Hopp, 1986). In fact, the peaks resulting from the inversion of the secondary structure scales correspond to the positions of turns and loops predicted by these methods (Garnier et al., 1978; Chou and Fasman, 1978; Rose et al., 1985b). In other words, algorithms that predict the position of loops or turns at the same time predict regions of highest hydrophilicity.

TABLE 1.5
Scales of structural parameters for the 20 common amino acids used to construct antigenicity prediction profiles[a]

Amino acid	1[b]	2	3	4	5	6	7	8	9	10
R	3.000	1.428	0.340	3.400	0.512	0.340	3.400	1.038	1.029	0.901
D	3.000	3.400	3.060	2.644	0.574	2.380	1.391	1.033	1.089	0.932
E	3.000	2.652	3.400	2.644	−0.627	0.567	1.198	1.094	1.036	0.933
K	3.000	1.938	2.380	2.947	1.819	1.587	2.125	1.093	1.082	1.057
S	0.300	2.210	1.473	0.604	−0.230	2.040	0.502	1.169	1.048	0.923
N	0.200	2.380	2.833	2.644	−0.680	2.607	1.236	1.117	1.006	0.930
Q	0.200	2.040	−0.227	2.644	−0.097	−0.227	1.333	1.165	1.028	0.885
G	0.000	1.938	2.947	0.302	−1.625	3.400	−0.309	1.142	1.042	0.923
P	0.000	0.714	1.587	1.209	−0.468	2.947	0.135	1.055	1.085	0.932
T	−0.400	1.768	−0.227	0.529	−0.397	−0.113	0.348	1.073	1.051	0.934
A	−0.500	0.714	1.813	−1.360	1.016	−0.567	−0.483	1.041	0.946	0.892
H	−0.500	0.714	1.927	2.418	2.755	−0.453	0.773	0.982	0.952	0.894
C	−1.000	0.467	−2.040	−1.889	−1.060	−2.947	0.077	0.960	0.878	0.925
M	−1.300	−1.428	−0.793	−1.436	−3.400	−2.040	−0.502	0.947	0.862	0.804
V	−1.500	−1.258	−0.340	−3.173	−0.115	−1.927	−1.043	0.982	0.927	0.913
I	−1.800	−2.720	−3.287	−3.400	−2.579	−2.833	−1.410	1.002	0.892	0.872
L	−1.800	−3.128	−0.793	−2.871	0.662	−2.833	−1.024	0.967	0.961	0.921
Y	−2.300	−0.646	−1.813	0.982	0.115	−2.267	−0.039	0.961	0.930	0.837
F	−2.500	−3.128	−1.020	−2.116	−1.245	−3.060	−1.178	0.930	0.912	0.914
W	−3.400	−3.400	1.133	0.680	−1.007	−3.400	−0.715	0.925	0.917	0.803
N-terminal		2.30								
C-terminal		3.40								

[a]Scales 2–7 are normalized wih respect to scale 1. Typical prediction profiles for myoglobin constructed with scales 1 and 8–10 are illustrated in Figs. 1.2 and 1.3.

[b] References: 1, Hopp and Woods (1981) hydrophilicity scale; 2, Parker et al. (1986) hydrophilicity scale; 3, Garnier et al. (1978) β-strand, inverted; 4, Kyte and Doolittle (1982) hydrophobicity scale, inverted; 5, Welling et al., (1985) antigenicity scale; 6, Hopp (1985) acrophilicity scale; 7, Eisenberg et al. (1984) hydrophobicity scale, inverted; 8, 9, and 10 are the original BNORM0, BNORM1 and BNORM2 segmental mobility scales of Karplus and Schulz (1985).

Different hydrophilicity and secondary structure scales of the 20 amino acids which have been used for predicting the position of epitopes along a polypeptide chain are listed in Table 1.5. In order to simplify comparisons, scales 2–7 presented in this table have been normalized with respect to the hydrophilicity scale of Hopp and Woods (1981). Such scales are based either on distribution coefficients of each amino acid between an aqueous phase and an organic phase, on peptide retention times during chromatography (Parker et al., 1986), on empirical calculations derived from the relative accessibility to solvent of residues in proteins of known structure (Rose et al., 1985a) or on secondary structure prediction algorithms (Garnier et al., 1978). The relative predictive value of the different scales is difficult to assess, since only a relatively small number of continuous epitopes have been located with sufficient precision in globular proteins. Fig. 1.2 illustrates the hydrophilicity plot obtained by using the Hopp and Woods (1981) parameters in the case of myoglobin and shows the position of continuous epitopes shorter than 18 residues (Table 1.2).

Several methods for predicting antigenicity were compared by Hopp (1986) on the basis of known epitopes in 12 proteins. The percentage of correct predictions for the top three peaks in the plots was found to be 75%, 68% and 65% using the hydrophilicity scales of Hopp and Woods (1981), Rose et al. (1985a) and Kyte and Doolittle (1982), respectively. In comparison, one of the three mobility prediction scales of Karplus and Schulz (1985) had a success rate of 70% (see section 1.6.1.3).

The distinguishing feature of the Hopp and Woods (1981) scale is that the four charged residues (K, R, D and E) were given the maximum value of 3.0 because this seemed to improve epitope selection within the set of 12 proteins studied. On the other hand, the scale of Kyte and Doolittle (1982) somewhat arbitrarily emphasized the hydrophilic nature of R and K, which results in predictions that tend to overlook negatively charged epitopes. The scale of Parker et al. (1986) is the only one that takes into account the increased hydrophilicity brought about by the presence of charged groups at the N- and C-termini.

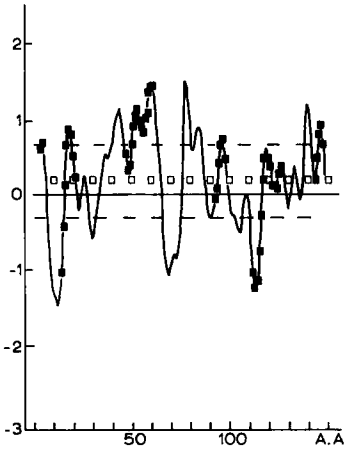

Fig. 1.2. Hydrophilicity profile of myoglobin calculated with the scale of Hopp and Woods (1981) listed in Table 1.5, column 1. The smoothing procedure of Van Regenmortel and Daney de Marcillac (1988) was used. The black squares correspond to amino acids that are part of the known continuous epitopes of myoglobin (see Table 1.2). The white squares represent the average value of the hydrophilicity parameter over the entire sequence and the two horizontal broken lines represent ±0.7 SD from the mean. Such an interval corresponds to approximately 50% of the population. Note that plots are traced from the 4th residue onwards and until the $(n-3)^{rd}$residue.

1.6.1.2. Amphipathicity

A segment of protein secondary structure is said to be amphipathic (or amphiphilic) when one side is appreciably more apolar than the other, the segment being viewed down its axis. Amphipathicity is a contributory factor in the folding of the protein and arises from a periodicity of hydrophobic residues in the amino acid sequence. The degree of amphipathicity can be measured by the hydrophobic moment μ which is the sum of the components of the hydrophobicity vector in the periodic structure (Eisenberg et al., 1984). If the periodic segment is specified by m, the number of residues per turn, $\delta = 2\pi/m$, in which δ is the angle in radians at which successive side-chains emerge from the backbone when the segment is viewed down its axis. For an α-helix, $\delta = 100°$ ($m = 3.6$) and for a strand of β structure, δ is in the range $160° - 180°$ ($m = 2.3$ to 2.0). When the hydrophobic moment μ of an amphiphilic helix is plotted

as a function of δ, a curve is obtained which shows a large maximum at δ = 100° (Eisenberg et al., 1984).

DeLisi and Berzofsky (1985) described a method to determine the presence of periodic hydrophobicity along a peptide chain and showed that the T cell antigenic sites of proteins tend to be located in amphiphilic segments.

A computer program for analyzing the hydrophilicity and amphipathicity of proteins has been described (Sette et al., 1986). This analysis uses blocks of 7 residues, and calculates amphipathic peaks and the corresponding δ angles, on the basis of one of the classical hydrophilicity scales described in section 1.6.1.1.

Epitopes located in α-helices present at the surface of a protein have also been identified by using the helix wheel representation. This method provides a projection of all side chains along the helix axis. In the case of an ideal surface helix, this simplified amphipathic analysis splits the helix into two clearly hydrophobic and hydrophilic faces. The majority of large helices in proteins have a substantial hydrophobic face which allows stable packing of the helix against the core of the molecule. On the basis of such an analysis, Pfaff et al. (1982) synthesized a peptide corresponding to the region 144–159 of protein VP1 of foot-and-mouth disease virus, predicted to be a surface helix, and showed that it was able to induce antibodies that neutralized the virus.

1.6.1.3. Segmental mobility

It is increasingly appreciated that protein molecules are not static structures and that their functional activity is often linked to dynamic conformational variations (Gurd and Rothgeb, 1979; Karplus and McCammon, 1983; Ringe and Petsko, 1985). Information about local mobility in proteins comes from NMR studies, which depend on motions such as the rapid flipping of side chains, and from the crystallographic refinement of protein structures. These refinement methods provide the atomic temperature factors (also known as B values or Debye-Waller factors) given by the equation $B = (8/3)^2\pi\bar{u}^2$, where $\bar{u}$ is the mean square atomic displacement from the crystal equilibrium position. When plotted against residue number, temperature factors provide a graphic

image of the degree of mobility existing along the polypeptide chain. Regions of highest mobility usually coincide with highly accessible segments at the surface of the molecule.

When plots of segmental mobility for TMV protein, myoglobin and lysozyme (taking into account only the mobility of main-chain atoms) were compared with the known location of continuous epitopes in these antigens, a striking correlation was observed between the peaks in the mobility plot and the position of epitopes of a length of 6–10 residues (Westhof et al., 1984). Similar correlations between segmental mobility and antigenicity were also reported in the case of other proteins such as insulin and cytochrome *c* (Tainer et al., 1985).

When antibodies were raised against peptide fragments of the protein myohaemerythrin, it was found that anti-peptide antibodies against highly mobile regions reacted strongly with the protein whereas antibodies raised against the less mobile regions did not (Tainer et al., 1984). In other words, these anti-peptide antibodies recognized the corresponding intact protein best when the target site in the protein was mobile.

In subsequent studies, the epitopes of myohaemerythrin were mapped by measuring the ability of synthetic peptides, 6 to 14 residues long, to bind antibodies raised against myohaemerythrin (Geysen et al., 1987). The 113 hexapeptides encompassing all 118 residues of the myohaemerythrin sequence were synthesized and tested by ELISA against rabbit antisera using rod-coupled peptides (Geysen et al., 1984, 1985a). Four peptides were recognized by 6 of the 7 antisera, 31 peptides reacted with only a single antiserum and 35 did not react with any antiserum. The entire surface of the protein was found to be antigenic using either the hexapeptides tested by ELISA or the longer 10–14-residue peptides tested by immunoprecipitation or conjugated to carriers. A good correlation was observed between the location of the most antigenic peptides (based on mean titres and number of responding rabbits) and structural parameters such as segmental mobility and surface accessibility (Geysen et al., 1987). The 5 peaks in the mobility curve corresponded to regions of higher that average antigenicity, whereas the mobility minima were located in regions of low antigenicity. In comparison,

the surface accessibility plot did not correlate as clearly with the peaks of antigenicity. These results show that although segmental mobility is not an absolute requirement for antigenicity, this property is associated with higher than average antigenic reactivity as assayed with short peptides. There is of course no evidence that antibodies recognize only mobile regions, but only that they preferentially tend to recognize short peptides when these correspond to mobile segments of a native protein. As discussed elsewhere (Van Regenmortel, 1985, 1986c) all studies of protein antigenicity are operationally biased and the methods and probes used in the study largely determine the type and location of the epitopes that are revealed. When longer peptides that are likely to have a more defined conformation are used as probes, regions of the protein that are structured and possess low mobility are also found to be antigenic (Al Moudallal et al., 1985).

It has been suggested that the ability of short peptides to cross-react preferentially with antibodies directed to mobile regions of the protein is a reflection of the fact that the possibilities for steric complementarity are maximized when both the immunogen and the test antigen possess some mobility enabling them to adapt to a pre-existing paratope (Westhof et al., 1984). It should be emphasized that the magnitude of the motions found in segmental mobility in small (1–2 Å root mean square) and that, contrary to some claims (Novotny and Haber, 1986), the energetic cost for binding is thus not necessarily prohibitive. Movements of a few Å within the epitope could have beneficial effects on the critical positioning of residues (allowing for instance highly directional hydrogen bonding) and this could result in an induced fit and increased binding affinity. (Edmundson and Ely, 1986; Getzoff et al., 1987). In a recent X-ray crystallographic study of an immune complex between influenza neuraminidase and antibody, shifts of about 1 Å in epitope residues and of 3 Å in antibody residues have been reported (Colman et al., 1987). In the case of the epitope of lysozyme delineated by Amit et al. (1986), it is possible to reconcile the observed equilibrium constant of $K_0 = 4.5 \times 10^7$ M^{-1} (equivalent to 10.4 kcal/mole) with an induced fit component of 4.5 kcal/mole, assuming that each $Å^2$ of the interacting surface contributes 20 cal/mole to the binding energy (i.e., 748 Å × 20 cal/mole = 14.9 kcal/

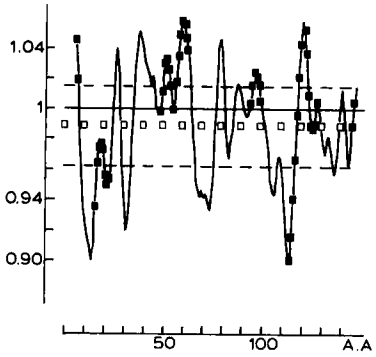

Fig. 1.3. Segmental mobility profile of myglobin calculated with the scale of Karplus and Schulz (1985) listed in Table 1.5. The black squares correspond to amino acids that are part of the known continuous epitopes of myoglobin (see also Fig. 1.2). Other symbols as in Fig. 1.2.

mole) (Getzoff et al., 1987).

A method for predicting segmental mobility from the primary structure of proteins has been described by Karplus and Schultz (1985). These authors developed normalized scales of mobility for each of the 20 amino acids which include a nearest neighbour analysis and are based on the *B* values (for α carbons) available for 31 proteins in the Brookhaven Protein Data bank (see Table 1.5). Using these scales, it is possible to construct plots of predicted average segmental mobility along the polypeptide chain. Such a plot corresponding to the predicted mobility in myoglobin is shown in Fig. 1.3.

1.6.1.4. Static surface accessibility

Since antibodies bind to the surface of proteins it is to be expected that epitopes will tend to consist of residues exposed at the protein surface. Recently, several groups have suggested that the primary reason why certain segments of a polypeptide chain are antigenic is their exceptional surface exposure. Thornton et al. (1986) analyzed the location of epitopes in three proteins of known tertiary structure and calculated a protrusion index for each amino acid which reflects the degree to which it protrudes from the protein's globular surface. Regions that protruded

were found to correlate with the position of continuous epitopes. The same regions were shown to correlate equally well with segmental mobility but less well with hydrophilicity.

Fanning et al. (1986), Novotny and Haber (1986) and Novotny et al. (1986) used contour maps of protein surfaces and profiles of contact areas of several proteins of known tertiary structure to show that the most exposed portions of the surface correlated with known epitopes of these molecules. The accessible molecular surface was evaluated by the algorithms of Lee and Richards (1971) and Connolly (1983) which calculate the surface accessible to contacts with spherical probes ranging from 1.4 to 10 Å in radius.

Proponents of the idea that static surface accessibility is sufficient to explain the location of antigenic regions in proteins view accessibility as a *primary* correlate and tend to equate surface exposure with antigenicity. Novotny and Haber (1986), for instance, claimed to have found additional, previously undetected *antigenic* regions in a scorpion neurotoxin by the large probe accessibility method of analysis and concluded that protein antigenicity is *synonymous* with exceptional surface exposure. It should always be remembered that antigenicity is not a structural property of molecules per se but is a functional category that can be measured only by using antibody combining sites as a detecting device. The existence of correlations between structural parameters and a functional property such as antigenicity need not imply that any particular structural feature such as accessibility or segmental mobility is an essential and sufficient condition for antigenicity.

That static accessibility is not a sufficient condition for antigenicity has been shown by the finding that highly accessible stretches of 5–7 residues in a protein are not necessarily scored as antigenic when the corresponding short peptides are used as probes (Van Regenmortel et al., 1986). Another instance concerns a region of myohaemerythrin (residues 53–60) that was predicted by Novotny et al. (1986), on the basis of poor accessibility, to possess little or no antigenicity, but was found experimentally to be highly antigenic (Geysen et al., 1987). The partially buried epitope of influenza haemagglutinin studied by Wilson et al. (1984) is another case illustrating the fact that dynamic factors may also contrib-

ute to antigenicity. To oppose static and dynamic explanations of antigenicity as mutually exclusive (Novotny and Haber, 1986) is to overlook the fact that both notions may need to be invoked, at times, to account for the stereo-complementarity and functional interactions between proteins.

In view of the correlation between surface accessibility and antigenicity, attempts have been made to predict which regions in a protein of unknown tertiary structure are likely to possess a surface orientation. Hopp (1985) developed an acrophilicity scale for the 20 amino acids, based on the frequency with which they are found in exposed locations in 49 proteins of known tertiary structure (see Table 1.5). However, the rate of success in predicting antigenic regions by this method was found to be relatively low (Hopp, 1986).

1.6.1.5. Chain termination

In a majority of proteins, the N- and C-termini are located at the surface of the molecule and in close proximity to each other (Thornton and Sibanda, 1983). Being at the end of the chain, they are less constrained than internal segments of the peptide and have a high relative flexibility. These features are probably responsible for the finding that the termini of many proteins correspond to continuous epitopes (Anderer, 1963; Absolom and Van Regenmortel, 1977; Walter et al., 1980; Altschuh et al., 1983; Quesniaux et al., 1983a, b). Synthetic peptides corresponding to the 10–15 terminal residues of any protein are thus likely to be suitable immunogens for raising antibodies that cross-react with the intact protein.

Another reason that has been advanced for the exceptionally high rate of antigenic cross-reactivity between terminal peptides and the whole molecule is the presence of free NH_2 and COOH terminal groups in both the peptides and the end regions of the protein. In contrast, in the case of peptides corresponding to inner regions of the protein, these free end groups in the peptide are not found as such in the protein, since they are involved in the formation of a peptide bond. In fact, in order to increase the similarity between an inner peptide and the corresponding region of the protein, some workers introduce an acetyl group at the N-terminal

end and a carboxamide group at the C-terminal end of the peptide (Audibert et al., 1981; Hodges et al., 1984). However, such a manoeuvre tends to decrease the solubility of the peptide, especially if it is very short; furthermore when these modified peptides are used as immunogens, a significant proportion of the antibodies that are obtained are specific for the amide or acetyl groups and do not recognize the unmodified peptide or the parent protein (Gras-Masse et al., 1985).

1.6.1.6. Sequence variability

In families of homologous proteins, it is commonly observed that the regions that present high sequence variability tend to correspond to the location of epitopes (Crumpton, 1974; Reichlin, 1975). As pointed out by Jemmerson and Paterson (1985) regions of the protein in which changes in local conformation arising from point mutation can be tolerated are most likely to be on the surface, where they are not involved in the long-range interactions that stabilize the internal folding of the molecule. Natural selection clearly favours those amino acid substitutions that do not perturb the overall tertiary structure of the protein because they occur in highly accessible, mobile regions. It is not surprising, therefore, that evolutionary variability, surface accessibility and segmental mobility are related and can be used to predict antigenicity.

This approach has often been used to localize epitopes in viral proteins. In a highly variable virus such as FMDV, regions of high sequence variability in different serotypes, for instance, residues 138–160 of VP1, have been shown to correspond to major epitopes (Bittle et al., 1982; Brown, 1986).

1.6.2. The selection of peptides for synthesis

Many structural parameters (see preceding sections) have been shown to be correlated with the location of continuous epitopes in proteins, and there are conflicting claims in the literature as to which prediction method is best able to locate antigenic regions in a protein of unknown tertiary structure. Hopp (1986), for instance, concluded that his hydrophilicity prediction method was superior to the other prediction methods

that he tested. In contrast, Thornton et al. (1986) reported that the correlation between antigenicity and predicted hydrophilicity was not as good as that found between antigenicity and the accessibility and segmental mobility parameters measured in molecules of known structure. Parker et al. (1986) investigated a combination of various parameters for improving the prediction of antigenicity. These authors concluded that a composite profile based on the maximum values for individual residue positions obtained from a HPLC hydrophilicity scale, an accessibility scale (Janin, 1979) and the mobility scale of Karplus and Schultz (1985) provided the best prediction results.

The usefulness of methods for predicting antigenicity lies in the fact that if stretches of 6–15 residues along a protein sequence can be identified as corresponding to continuous epitopes, these regions can be synthesized and used to raise antibodies that will react specifically with the whole protein molecule. The applicability of prediction methods is therefore limited to a search for continuous epitopes and the validity of different procedures should therefore be tested with respect to the known location of continuous epitopes in model proteins that have been systematically analyzed for antigenicity. Epitopes that have been located only with respect to a few individual residues, as is the case for most discontinuous epitopes, are clearly not suitable for determining the comparative value of different prediction algorithms. The same is true for so-called continuous epitopes of 20–40 residues in which the precise location of the antigenic residues is unknown. If such long stretches of sequence were included in the testing of prediction methods, the structural parameters suitable for identifying epitopes of 6–8 residues would no longer be useful for distinguishing antigenic from non-antigenic peptides.

Recently, 8 of the commonly used prediction methods were compared with respect to their ability to correctly identify 29 continuous epitopes (of up to 17 residues in length) in 4 well-studied proteins, myoglobin, lysozyme, myohaemerythrin and TMVP (Van Regenmortel and Daney de Marcillac, 1988). The structural parameters used in these comparisons correspond to the 8 scales for the amino acids in Table 1.5. When assessed by their ability to predict the greatest possible number of

TABLE 1.6

Prediction of antigenic residues in myoglobin based on eight different scales of structural parameters for the 20 amino acids (see Table 1.5). The cut-off value was set on the mean of each parameter over the entire sequence (see Fig. 1.2)

Scales	A	B	C	A/B	A/C
Hopp and Woods, 1981	42	79	56	0.53	0.75
Parker et al., 1986	46	90	56	0.51	0.82
Garnier et al., 1978	48	90	56	0.53	0.86
Kyte and Doolittle, 1982	41	85	56	0.48	0.73
Welling et al., 1985	32	73	56	0.43	0.57
Hopp, 1985	46	91	56	0.50	0.82
Eisenberg et al., 1984	34	76	56	0.61	0.44
Karplus and Schulz, 1985	43	87	56	0.49	0.77

A, Number of residues correctly predicted to be antigenic.

B, Total number of residues predicted to be antigenic.

C, Total number of residues known to be antigenic, i.e., belonging to continuous epitopes of a length of 6–8 residues (see Table 1.2).

epitopes in the different proteins, none of the 8 prediction methods proved consistently superior to all the others. A particular method which gave the best prediction score with one protein could be the least successful with another protein.

In order to make a rigorous comparison of the predictive value of each method, tables were constructed from the prediction profiles (see Fig. 1.2) indicating the number of residues known to be antigenic in each protein, the total number of residues predicted to be antigenic by each method and the number of residues correctly predicted to be antigenic. Each residue was scored 1 or 0 for its predicted antigenicity, according to whether its parameter value in the profile was greater or less than a certain threshold value. Two different thresholds were considered, i.e., the overall mean of the parameter over the entire plot and the mean +0.7 standard deviation. An example of such a calculation in which the cut-off value was set at the mean of each structural parameter is shown in Table 1.6. It can be seen that in the case of myoglobin, the hydrophilicity scale of Hopp and Woods (1981) predicted that 79 residues were antigenic, but that only 42 of these residues actually belonged to known epitopes of the protein. In other words, only 53% of the predictions turned out to be correct. Compared to the known location of the 56 antigenic residues in

TABLE 1.7

χ^2 values from 2 × 2 contingency tables for the observed location of antigenic residues in four proteins against eight different structural parameters used to construct antigenicity prediction profiles. [a] (From Van Regenmortel and Daney de Marcillac, 1988.)

Scales	Myoglobin[c]		Tobacco mosaic virus protein		Myohaemerythrin		Lysozyme	
	1[d]	2[e]	1	2	1	2	1	2
Hopp and Woods, 1981[b]	19.31	7.90	1.43	–[f]	0.64	0.79	2.12	4.13
Parker et al., 1986	19.83	18.41	4.09	0.71	1.31	3.69	15.25	28.35
Garnier et al., 1978	26.37	25.19	1.12	0.10	0.47	–	5.01	2.84
Kyte and Doolittle, 1982	11.15	2.53	0.13	1.59	–	0.05	21.32	16.80
Welling et al., 1985	3.14	2.06	0.61	1.43	0.08	–	1.39	14.86
Hopp, 1985	18.82	5.15	5.95	3.65	1.80	3.69	30.13	19.98
Eisenberg et al., 1984	4.54	2.64	1.76	5.02	0.43	1.31	8.81	13.03
Karplus and Schulz, 1985	14.29	9.87	14.22	9.80	0.95	0.83	9.14	15.14

[a] The larger the value of χ^2, the more the method differentiates between antigenic and non-antigenic residues and leads to correct predictions. Values of χ^2 below 3.84 are not statistically significant.

[b] The 8 normalized scales for the 20 amino acids are listed in Table 1.5.

[c] Examples of prediction profiles for myoglobin are shown in Figs. 1.2 and 1.3.

[d] Cut-off level was the mean of each parameter over the entire sequence.

[e] Cut-off level was the mean +0.7 standard deviation of each parameter.

[f] Indicates that the χ^2 value was below 0.01.

myoglobin, the method correctly identified 75% of them. When the cut-off value was set at the mean +0.7 standard deviation of the hydrophilicity parameter (Fig. 1.2), the method correctly identified only 39% of the antigenic residues.

In their analysis, Van Regenmortel and Daney de Marcillac (1988) also calculated χ^2 values from 2 × 2 contingency tables of each parameter against the observed location of antigenic residues. The results of this statistical analysis are shown in Table 1.7. The larger the value of χ^2, the more the method differentiates between antigenic and non-antigenic residues and leads to correct predictions; values of χ^2 below 3.84 are not statistically significant. The data in Table 1.7 show that the segmental mobility scale of Karplus and Schulz (1985) gave significant χ^2 values in the largest number of cases, followed by the hydrophilicity scale of Parker et al. (1986) and the acrophilicity scale of Hopp (1985). The worst level of prediction was obtained with the antigenicity scale of Welling et al. (1985). It is noteworthy that not a single significant χ^2 value was obtained when the different prediction methods were applied to myohaemerythrin. However, in this case all the known continuous epitopes of the protein (see Getzoff et al., 1987) had been identified by the solid phase method of Geysen et al. (1984) and this may have introduced a bias.

There clearly is a need for analyzing the comparative value of different prediction methods by an objective statistical method such as the one illustrated in Table 1.7. Conflicting claims as to which are the best prediction algorithms to use with different classes of proteins can only be resolved by this type of analysis, and it is to be hoped that such procedures will become more widely used.

In the author's laboratory, the scales of Karplus and Schulz (1985) and Parker et al. (1986) are commonly used with considerable success. In addition, 15 residue-long peptides corresponding to the termini of the polypeptide chain are usually also synthesized. In view of the relative abundance of continuous antigenic regions in most globular proteins, it is unlikely that after synthesizing three peptides chosen in this manner, a suitable one able to induce cross-reactive antibodies will not have been found.

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CHAPTER 2

Solid-phase peptide synthesis

2.0. Introduction

The prodigious range of applications of synthetic peptides which is observed today is the consequence of the development of the chemistry of peptides synthesis over the last thirty years. It was, in fact, in 1954 that Du Vigneaud and his colleagues (Du Vigneaud et al., 1954) accomplished the synthesis of oxytocin, the first naturally occurring peptide hormone to be prepared in the laboratory, and in 1963 that the first chemical synthesis of human insulin was achieved by Meienhoffer et al. These two hormones were obtained by application of the classical methods of synthesis in solution, which require the purification and characterization of the intermediate peptide at every step. This approach, both time-consuming and complicated and the preserve of chemical specialists, is used in the pharmaceutical industry to prepare large quantities (of the order of kg) of certain peptides.

The introduction of the concept of solid-phase peptide synthesis (SPPS) by Merrifield in 1963 considerably modified the existing state of the art. In this approach, the growing peptide chain is bound covalently through its C-terminus to an insoluble solid support. Synthesis is then carried out by the successive addition of amino acids in the desired se-

quence. All of the intermediate steps of purification, which are necessary for synthesis in solution, are reduced in this case to simple washings, since most of the side-products of reaction and degradation are dissolved in the reaction mixture. The advantages of SPPS are to be found in its speed, the relative ease of its implementation and the fact that it is a method which can be partially or completely automated. At present, the maximal amounts of peptide synthesized on solid phase are limited to several tens of grams, but quite often several tens of milligrams, or even fractions of milligrams, are amply sufficient to meet the present need in the various areas of investigation cited in Chapter 1. Since the synthesis of the first peptide Leu-Ala-Gly-Val by Merrifield in 1963, solid phase peptide synthesis has been developed extensively by Merrifield's group and in many other laboratories. The literature in this field is abundant and we shall mention only a few general reviews for reference (Barany and Merrifield, 1980; Kent, 1980, Kent and Clark-Lewis, 1985; Sheppard, 1986).

Figure 2.1 presents the standard scheme for SPPS as developed by Merrifield in 1963.

(A) The first protected amino acid is linked to the solid support (P) through a covalent bond which must remain stable throughout the synthesis.

(B) The free amino group of the bound residue is regenerated under conditions to which the protecting groups of the side chain functions are stable: this is the deprotection step.

(C) The next protected amino acid is condensed with the first to give rise to the formation of an amide bond: this is the coupling step. The steps B and C are repeated until the synthesis is complete.

(D) At the end of the synthesis, the peptide is released from the solid support and freed from the amino acid side chain protecting groups.

Figure 2.2 presents the manual apparatus for synthesis which we have used for several years in our laboratory for the preparation of peptides and on which our students are still given their initial training. The various solvents are introduced into the glass reaction vessel (Fig. 2.2A) by means of a wash bottle and the amino acids by means of a pipette. We use nitrogen both for agitation of the resin in the solvents used in synthe-

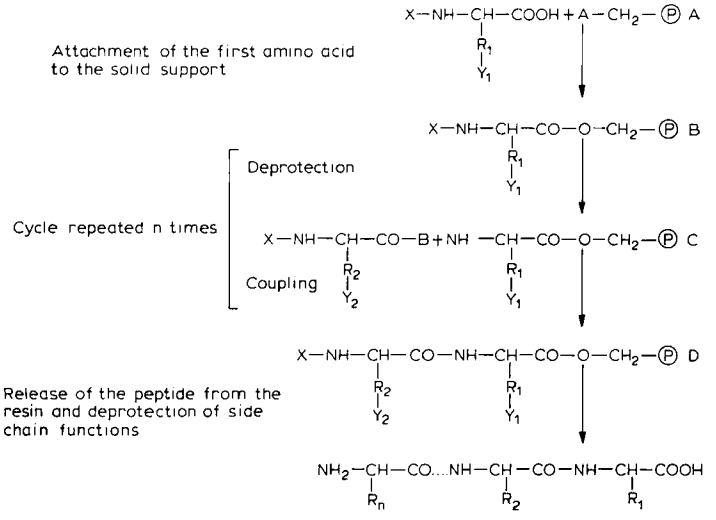


Fig. 2.1. Basic scheme for solid-phase peptide synthesis according to Merrifield. X : protecting group for the α-NH_2 function; Y_1, Y_2: protecting groups for side chain functions; A: functional group on the resin; B: activating group for the carboxylic acid function.

sis (the flow rate is regulated by a two-way valve) (Fig. 2.2B) and to remove waste solvents (Fig. 2.2C). It is in comparison with such a simple and inexpensive system, which we have taken as reference, that we have judged the performance of the automatic synthesizers we wished to test.

A considerable number of combinations of amino acid protecting groups have been described in the literature. However, at present, only two of these combinations are commonly used in most laboratories. The first is the *tert*-butoxycarbonyl (Boc)/benzyl combination which has been used by Merrifield since 1963 and which still remains the most widely used. More recently, the fluorenylmethoxycarbonyl (Fmoc)/*tert*-butyl system, developed by Sheppard and his group, has found increasingly wide application.

In this chapter we shall describe these two approaches to synthesis,

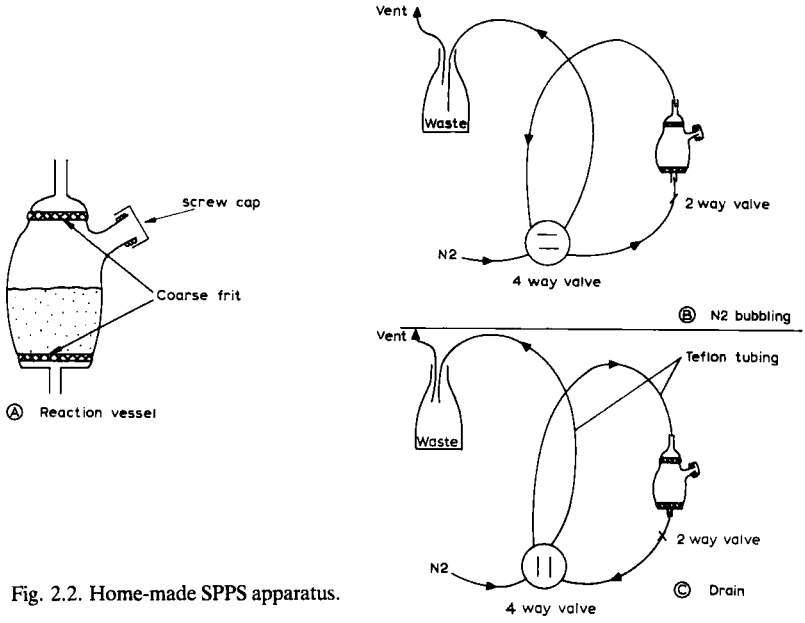


Fig. 2.2. Home-made SPPS apparatus.

paying particular attention to the resins and the protecting groups employed, and to the various methods for amino acid coupling and the types of procedure for cleaving the peptide from the resin which are the most widely used. We shall also discuss the advantages and disadvantages of both the Boc and Fmoc approaches as well as their automation. The last section will be devoted to methods for the purification and analysis of peptides. We shall try to give as many practical details as possible and shall restrict ourselves to essentials as far as theory is concerned. This review is to be regarded as providing practical orientation to the reader who is seeking an introduction to peptide synthesis and not as an exhaustive review of all of the possibilities which exist in the field. In addition, we have included at the end of this book appendices listing sources of chemicals that we routinely use as well as the main suppliers of apparatus in the field of peptide synthesis. In the same perspective, the reader will find it profitable to consult the practical manual by J. Stewart and J. Young entitled 'Solid Phase Peptide Synthesis', published in 1984.

2.1. The 'Boc' synthesis

2.1.1. The polymeric support

In order to be suitable for use in peptide synthesis, the solid support must meet a certain number of well-defined criteria. Not only must it show physical and chemical stability to the various operations of synthesis but it must also allow solvents and reagents to diffuse readily to the peptide chain, both during synthesis itself and during cleavage of the peptide from the resin.

The first successful support used by Merrifield (1963) was a copolymer of polystyrene cross-linked with 2% divinylbenzene (DVB). In 1971 Gutte and Merrifield recommended polystyrene cross-linked with 1% DVB as the support of choice for the large majority of applications. Competition experiments between amine components attached to this polymer and free in solution have shown that the rates of reaction of both species with a resin-bound reactive entity are practically identical (Kent, 1980). Moreover, measurements of rotational correlation times by nuclear magnetic resonance (NMR) and electron paramagnetic resonance (EPR) of entities attached to a support and those same entities free in solution have given similar results which indicate that they possess very similar degrees of freedom of rotation. In fact, molecular events take place in the interior of a resin bead in the same manner as they do in homogeneous solution and so one may speak of insolubility at the macroscopic level and of solubility at the microscopic level (Kent, 1980). Furthermore, the polymer has a solvating effect on the peptide which may be very considerable: a given peptide may be perfectly solvated when it is attached to the resin whereas the same peptide in the same solvent may precipitate when it is cleaved from the polymer. For all of these reasons the polystyrene copolymer (1% DVB) is the one most commonly used at present.

Arshady et al. (1981) proposed the use of a support of the polyamide type (see Section 2.2.2.2). This support, which is more hydrophilic than polystyrene, would be expected to confer better solvation properties on the growing peptide chain and hence facilitate synthesis. Nonetheless,

conclusive proof that this support is superior to polystyrene has still not been provided.

2.1.2. Functionalization of the polymer. Introduction of the first amino acid

2.1.2.1. General

Functional groups are introduced into the polymer in order to make it possible to link the first amino acid to the insoluble support in a covalent manner. Of the very large number of groups which have been described in the literature (for a review, see Barany and Merrifield, 1980), we shall limit ourselves here to those which are the most commonly used and we shall discuss their advantages and disadvantages.

The number of functional groups introduced into the polymer determines its degree of functionality and, consequently, the number of growing peptide chains which can be incorporated into it. Opinions are divided as to the optimal degree of functionality of a resin (in milliequivalent (meq)/g). Our own experience has led us to use three different types of substitution depending on the size of the peptide to be synthesized: (a) for peptides containing less than 15 residues, a functional group content of up to 1.8 meq/g may be used; (b) for peptides containing 15–30 residues, a functional group content of 0.6–0.9 meq/g gives the best results; (c) for peptides containing more than 30 residues, a functional group content of less than 0.5 meq/g seems to be the most appropriate.

There exists a large number of resin-bound functional groups which lead to derivatization of the C-terminal function of the peptide (see Fig. 2.3). We shall limit ourselves here to those leading ultimately to the formation of peptide free acids and peptide amides.

2.1.2.2. Resins for synthesis of peptide free acid

2.1.2.2.1. Chloromethyl resins The standard type of derivatization carried out on polystyrene containing 1% DVB is chloromethylation. The chloromethyl groups are introduced into the support by a Friedel Crafts alkylation in the presence of a Lewis acid (Fig. 2.4). This is achieved by using chloromethyl methyl ether in the presence of $ZnCl_2$ (Feinberg and

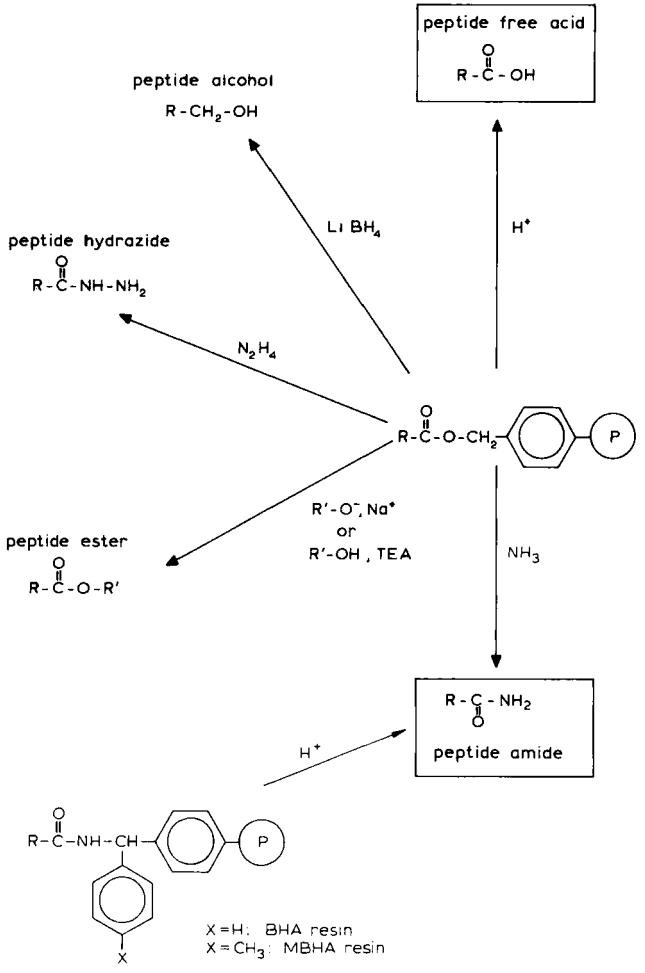


Fig. 2.3. Cleavage of peptides from resins.

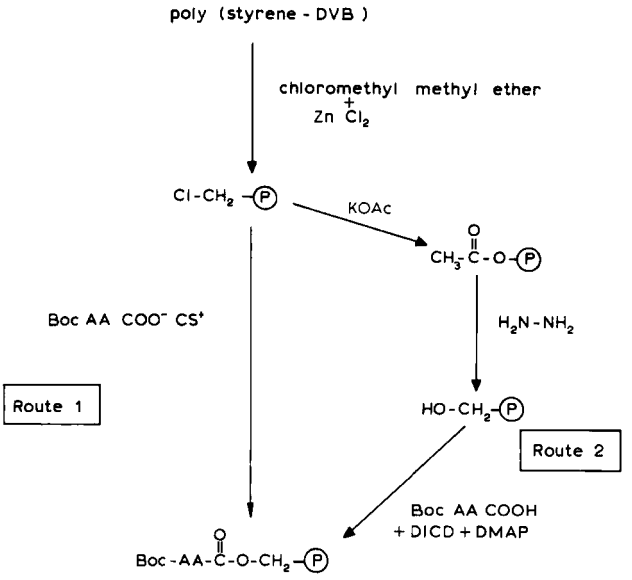


Fig. 2.4. Functionalization of the polystyrene and esterification of the first amino acid to the resin.

Merrifield, 1974). In view of the fact that chloromethyl methyl ether is a highly carcinogenic substance, it is preferable to buy the chloromethyl resin rather than prepare it oneself if adequate facilities are not available. However, it must be borne in mind that the quality of the commercially available resins may vary very much, depending on the supplier and even on the batch obtained from a given supplier. One reason for this is that the chloromethylation of polystyrene may lead to the formation of interchain bridges within the support and, as a result, may cause a quite considerable increase in the degree of cross-linking. This side reaction may occur when the amounts of the Lewis acid used are too high or when the reaction time is too long.

The surest but also the most tedious way of testing a commercial resin is to carry out on this resin a synthesis which is well-known in the laboratory and to compare the crude product obtained with the best crude sample of the reference product. In a more general manner, this approach

enables us to test each of the parameters of the synthesis, including a new batch of solvent or a new cylinder of hydrogen fluoride (HF), for example.

The method most commonly used to attach the first amino acid to the chloromethyl resin is that of Gisin (1973). It consists of forming the cesium salt of the protected amino acid to be coupled and of then esterifying it directly to the resin. This method is the simplest and leads to a minimum of side reactions.

General procedure

Preparation of the cesium salt

2 g of Boc amino acid are dissolved in a mixture of 10–15 ml of ethanol and 3.5 ml of H_2O. The pH of the solution is adjusted to 7.0 by means of a 1.5 M solution of cesium hydrogen carbonate; the mixture is then evaporated to dryness and taken up several times in either benzene or dichloromethane (DCM) in order to remove the last traces of H_2O; the product is then dried for 5 h over P_2O_5. The salt may then be used without further purification.

Esterification of the resin (Fig. 2.4, Route 1)

The cesium salt is used at a 1.5–2 excess with respect to the functionalized resin. 1 g of resin is suspended in 8 to 10 ml of dimethylformamide (DMF) at 50°C, and the suspension is agitated overnight. The resin is then filtered off, washed successively with DMF, a DMF/H_2O mixture (8 v/2v), DMF, DCM, methanol (MeOH), and finally dried. The yield of the esterification reaction may be determined by total hydrolysis of a sample of the amino acid resin, followed by quantitative analysis on an amino acid analyzer. Most of the amino acids may be esterified to a chloromethyl resin by use of the method we have just described; there are, however, certain difficulties with some of them.

Glycine gives relatively low esterification yields (60–80%), Boc Arg (Tos) also gives low yields on account of steric hindrance. Application to methionine must be avoided owing to the risk of S-alkylation; this problem is less acute with the different derivatives of Boc cysteine. However, these two amino acids may be esterified to a hydroxymethyl resin. Nonetheless, other difficulties are associated with the use of this type of

resin (see Section 2.1.2.2.2). Asparagine should not be esterified in the form of a salt owing to the risk of intramolecular cyclization. In order to avoid this side reaction, use is made of Boc Asp (α-benzyl ester) which is bound to a benzhydrylamine resin through the intermediary of an amide bond. On completion of the synthesis, the asparagine residue is generated by acidolysis of the peptide. In the case of glutamine, similar precautions are recommended. In the case of histidine, acylation catalyzed by the imidazole nucleus may occur; this should be taken into account in the case of Boc His (Tos) and it is advisable to use Boc His (DNP) in order to eliminate the risk of this reaction.

2.1.2.2.2. Hydroxymethyl resins A hydroxymethyl resin may be obtained by treatment of the corresponding chloromethyl resin with potassium acetate, followed by hydrazinolysis (Wang, 1975) (Fig. 2.4, route 2). These resins are also commercially available. A protected amino acid may be esterified to this resin by the use of dicyclohexylcarbodiimide (DCC) or diisopropylcarbodiimide (DIC) in the presence of 4-dimethylaminopyridine (DMAP).

General procedure

The Boc amino acid (3 eq) and DMAP (0.6 meq) are dissolved in a minimum of DMF, then added to the hydroxymethyl resin suspended in DCM (10 ml/g of resin); finally, 3 eq of DCC are added. After the mixture has been agitated for 4–8 h, the resin is washed twice with DCM, then three times with DMF. The resin is then treated with benzoyl chloride in order to block the remaining free OH groups: the reaction is carried out for 2 h in DMF in the presence of 10 eq of benzoyl chloride and 10 eq of pyridine.

A few comments are necessary at this point with regard to the use of the hydroxymethyl resin and the method of esterification described above. It has, in fact, been shown that DMAP can induce the racemization of the amino acid residue introduced onto the support (Atherton et al., 1981). The risk of this happening can be minimized by using catalytic quantities of DMAP but under such conditions esterification proceeds much more slowly and the possibility of racemization is not totally

excluded. This results in it being difficult to predict the yields of the esterification and benzoylation reactions, and OH groups remaining free can pose quite serious problems in the course of synthesis as we shall see later (Section 2.1.2.2.3). Consequently, we prefer the chloromethyl resins which are widely used in the laboratories of peptide chemistry and which are commercially available in the form of the Boc–amino acid–resin. However, for the assembly of 20–30 residues and more it is generally accepted that the phenylacetamidomethyl resins are the ideal supports in solid phase peptide synthesis (following Section).

2.1.2.2.3. The phenylacetamidomethyl (Pam) resins It was observed at a very early stage that the benzyl ester bond linking the first amino acid to the resin is not completely stable to the various steps involved in the removal of the Boc group, especially the trifluoroacetic acid (TFA) step (usually 50% TFA in DCM for 30 min or 65% TFA in DCM for 15 min). On the one hand, a loss of up to 1% of the peptide is observed per cycle and, on the other, the cleavage of this ester linkage leads to the regeneration of an hydroxymethyl function on the resin. This hydroxyl function is easily triofluoroacetylated and this acetate can then lead to the formation of a trifluoroacetamide as the result of a nucleophilic attack of the α-NH_2 function of the growing peptide chain during the neutralization step (Kent et al., 1979; Fig. 2.5). In order to avoid this type of side reaction, which may represent a contamination of up to 30% in the synthesis of a peptide of 20 residues, two essential precautions must be taken. On the one hand, it is essential to eliminate the hydroxyl functions still existing on the resin (and this may be difficult in the case of an hydroxymethyl resin); on the other hand, the generation of new hydroxyl groups during the various cycles of deprotection must be prevented. These problems have been resolved, in large measure, by the introduction of Pam resins (Mitchell et al., 1978). In this resin, a 4-(carboxamidomethyl) benzyl alcohol grouping is introduced between the first amino acid and the resin. Under these conditions, the ester bond linking the first amino acid to the resin is one hundred times more stable to the conditions of TFA deprotection than the classical benzyl ester bond, but remains, nonetheless, completely susceptible to HF cleavage.

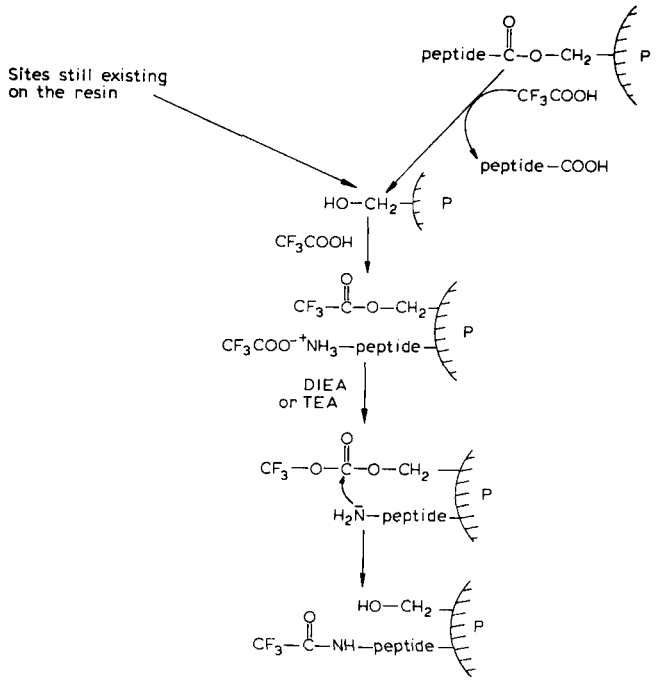


Fig. 2.5. Mechanism of trifluoroacetylation.

The best way to prepare this resin is to synthesize the Boc amino acid handle in solution and then to couple it to an aminomethyl resin (Tam et al., 1979; Plaué and Heissler, 1987; Fig. 2.6). The aminomethyl resin is commercially available but it can be synthesized quite easily (Mitchell et al., 1978). Boc–amino acids–Pam resins have recently been marketed by various companies but they are very expensive (see appendices).

Consequently, it may be said that, at present, the Pam resins are the best suited to solid phase peptide synthesis but that they are relatively little used owing to the fact that the route to their synthesis is long and tedious.

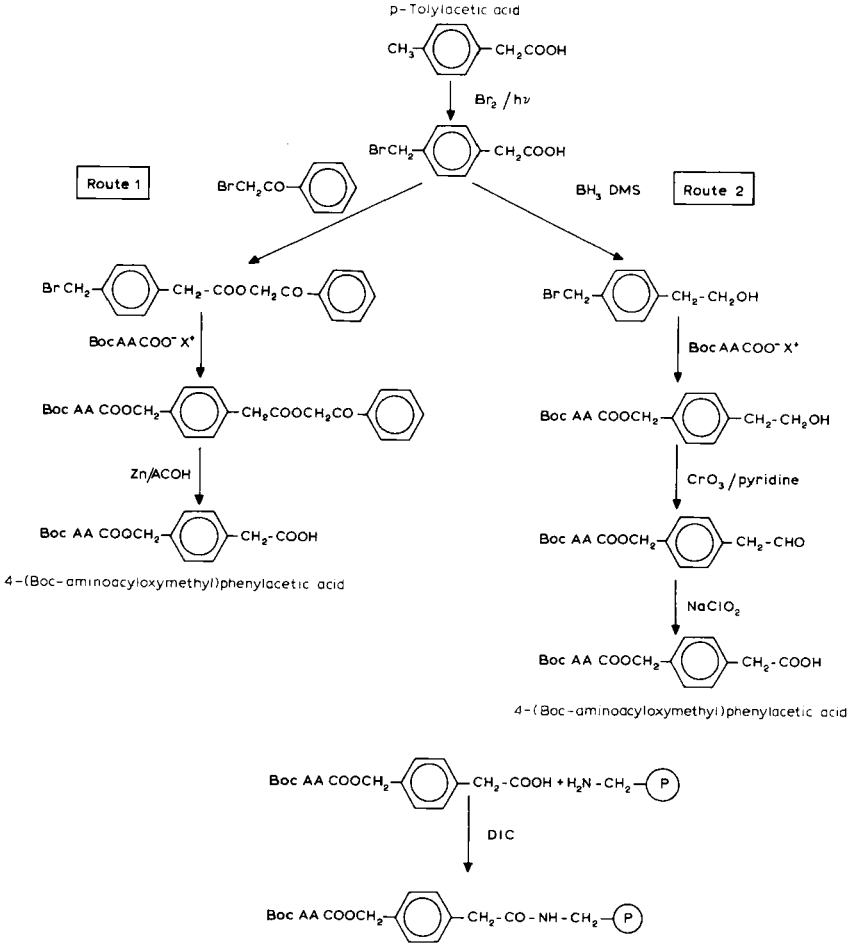


Fig. 2.6. Preparation of the Pam resin according to Tam et al. (1979) (route 1) and Plaué and Heissler (1987) (route 2).

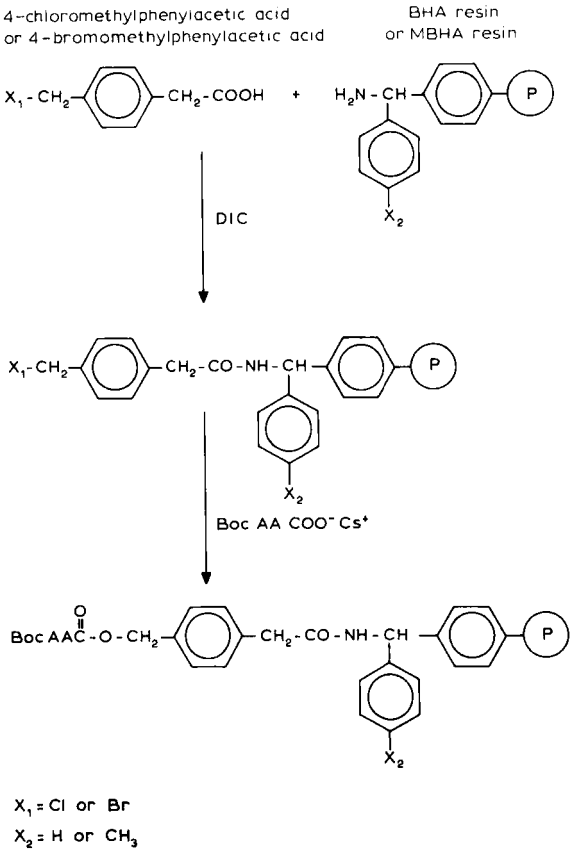


Fig. 2.7. Preparation of the Pab resin and esterification of the first amino acid to the resin.

2.1.2.2.4. The phenylacetamidobenzyl (Pab) resins An interesting alternative to the use of Pam resins was introduced in 1981 by Giralt et al. (1981), namely the Pab resins. They are prepared by condensing 4-bromomethylphenylacetic acid or 4-chloromethylphenylacetic acid with benzhydrylamine (BHA) resin. An α-halogenated resin is thus obtained to which a Boc amino acid in the form of its cesium salt can be esterified under the conditions described in Section 1.2.2.1. (Fig. 2.7). The advantage of this modification is that the stability of the ester bond linking the

first amino acid to the resin is quite comparable to that of a Boc–amino acid–Pam resin and that its synthesis is considerably simpler. The disadvantage is that, as in the case of the standard Merrifield resins, certain amino acids such as Boc Met, Boc Asn, Boc Gln and Boc His (Tos) cannot be esterified.

General procedure

BHA or 4-methylbenzhydrylamine (MBHA) resins are commercially available, as is 4-bromomethylphenylacetic acid (see Addenda). However, the latter compound may be easily prepared according to the method described by Mitchell et al. (1978), and 4-chloromethylphenylacetic acid can be prepared according to Bogdanov (1958). 1.3–2 eq of handle are dissolved in a minimum of DMF and added to 1 g of resin suspended in 10–15 ml of DCM. 1.3–2 eq of DCC or DIC are then added and the mixture is agitated overnight. The progress of the reaction can be monitored by the classical ninhydrin test (see Section 2.1.4.5).

2.1.2.3. Resins for synthesis of peptide amides

There are a number of biologically active peptides possessing a C-terminal amide function. The simplest method for the preparation of peptide amide is to use the MBHA resin (Matsueda and Stewart, 1981).

Use of this type of resin leads to the $CONH_2$ function being obtained directly after HF cleavage. The first amino acid is attached to the resin by a standard coupling reaction (Fig. 2.8). The MBHA resins are commercially available but here again their quality may vary very much from one supplier to another. In fact, it needs to be borne in mind that there are many methods for the synthesis of the MBHA resin and that most of them lead to the incorporation in the polymer of undesirable chemical functionalities. Recently, Bryan (1986) has published a route to the synthesis of MBHA which entails a minimum of side reactions. We recommend the use of this method to those who wish to prepare their own MBHA resin. The standard analytical techniques are of limited usefulness in verifying the quality of the commercially available resins. Nonetheless, the use of infra-red spectroscopy and the recently introduced gel-phase NMR (Giralt et al., 1984) may be extremely useful.

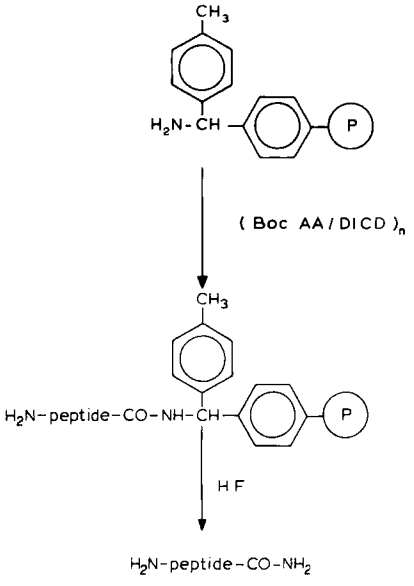


Fig. 2.8. The use of 4-methylbenzhydrylamine resin.

2.1.3. Side chain protecting groups for Boc amino acids

Within the scope of this review, we shall limit ourselves to the Boc amino acids which are most commonly used and the side chain functions of most of which can be deprotected by HF cleavage (see also Table 2.1). In the case of a final cleavage with TFA-TFMSA (Section 2.1.5.4), it is necessary to introduce into some Boc amino acids protecting groups which are still not widely used but which will be discussed in the section dealing with this type of cleavage.

(a) *Arginine:* Boc Arg (Tos) is the most suitable derivative although it sometimes poses problems at the coupling step (see Section 2.1.4.4.2).

(b) *Asparagine and glutamine:* they are used preferably in the non-protected form although their coupling does pose problems (Section 2.1.4.4.2). Their amide function can be protected as the xanthenyl derivative. However, Boc Asn (Xan) and Boc Gln (Xan) are only soluble in DMF.

(c) *Aspartic acid and glutamic acid:* Boc Asp (OcHex) and Boc Glu (OcHex) are preferred to the benzyl ester derivatives since they lead to a minimum of side reactions during the cleavage of the peptide from the resin (especially γ-acylation reactions and the formation of aspartimides).

(d) *Cysteine:* there are many commercially available cysteine derivatives but there are only two which are really essential to synthesis. When the final peptide is required to contain free SH functions, Boc Cys (Me Bzl) is the protecting group which gives the best results. When the synthetic target is a peptide containing disulfide bridges, different strategies are possible but the most useful in our opinion consists of using Boc Cys (Acm). This protecting group is very stable in acid solution and is practically resistant to cleavage by HF. The peptide may then be purified with the cysteine residues protected and removal of the Acm protecting groups and the formation of the disulfide bridge can then be carried out in one step using iodine (Kamber, 1971).

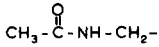
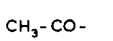
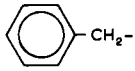
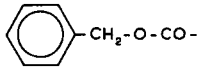
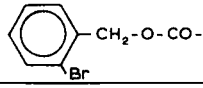
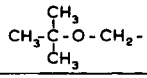
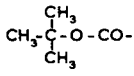
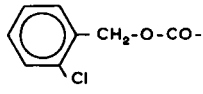
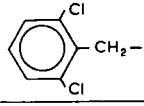
(e) *Histidine:* this is commercially available in the form of three different protected derivatives.

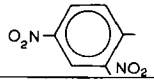
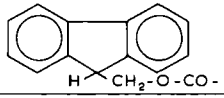
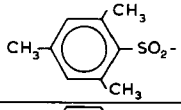
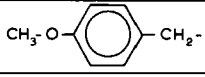
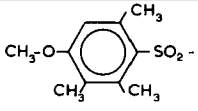
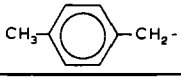
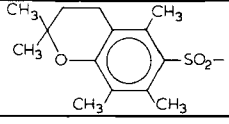
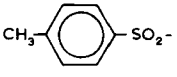
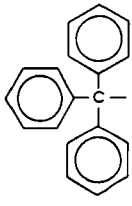
– Boc His (Tos): the tosyl group is very stable towards acids but is easily removed by HF cleavage. However, it has been shown that hydroxybenzotriazole (HOBt), which is frequently used in the activation of Boc amino acids (Section 2.1.4.4.3), can also cleave the tosyl group. Thus, this type of activation must be avoided when a His (Tos) derivative is being introduced into the peptide chain. It must also be kept in mind that Boc His (Tos) is relatively unstable at ambient temperature: it should thus be purchased as its salt, dicyclohexylammonium (DCHA) and converted to the free acid just before use.

– Boc His (Dnp): the dinitrophenyl group is even more stable to acid than the tosyl group with the result that it is not cleaved in the presence of HF. However, in this case no particular problems are encountered upon activation in the presence of HOBt. The 2,4-dinitrophenol (Dnp) group can be removed either before or after, but preferably before the final cleavage of the peptide from the resin. In both cases, deprotection is carried out by treatment with a 0.1 M solution of thiophenol in dimethylformamide (DMF) at ambient temperature for 30 min. Nonethe-

TABLE 2.1

LIST OF THE MOST COMMON PROTECTING GROUPS FOR AMINO ACIDS

NAME	FORMULA	ABBREVIATION
Acetamidomethyl		Acm
Acetyl		Ac
Benzyl		Bzl
Benzyloxycarbonyl		Z
2-Bromobenzyloxycarbonyl		2-BrZ
tert-Butylthio		s-tBu
tert-Butyloxymethyl		Bum
tert-Butyl		tBu
tert-Butyloxycarbonyl		tBoc
2-Chlorobenzyloxycarbonyl		2-Clz
Cyclohexyl		cHex
2,6-Dichlorobenzyl		2,6-ClBzl

2,4-Dinitrophenyl		Dnp
9-Fluorenylmethoxycarbonyl		Fmoc
Formyl	-CHO	For
Mesitylene-2-sulfonyl		Mts
4-Methoxybenzyl		Mob
4-Methoxy-2,3,6-trimethylbenzenesulfonyl		Mtr
4-Methylbenzyl		MeBzl
2,2,5,7,8-Pentamethylchroman 6-sulfonyl		Pmc
p-Toluenesulfonyl		Tos
Trityl		Trt

less, the sensitivity of the dinitrophenyl group towards nucleophiles must be mentioned and, as a consequence, the use of thiols must be avoided during the deprotection step. Finally, commercially available Boc His (Dnp) usually needs to be recrystallized before use (ethanol (EtOH) or 2-propanol (iPrOH)/hexane).

– Boc His (Z): this derivative has recently been introduced in solid-phase peptide synthesis (SPPS). It can be introduced as a symmetrical anhydride or an HOBt active ester. It does not require a desalting step and does not necessitate a prior deprotection step as it is cleavable by HF or trifluoromethanesulfonic acid (TFMSA). We recently compared the quality of an HF crude peptide synthesized with Boc His (Tos) or Boc His (Z); the Boc His (Tos) produced a highly impure product whereas the Boc His (Z) yielded a product of a good quality. These results suggest that the use of Boc His (Z) for routine synthesis of peptides containing histidine may be advantageous.

(f) *Lysine:* Boc Lys (2-ClZ) is the most commonly used. This derivative is available as a crystalline compound; its stability is higher than that of Boc Lys (Z) and equivalent to that of Boc Lys (2-BrZ) which is much more expensive and is not crystalline.

(g) *Methionine:* Boc Met is most often incorporated into peptides in the unprotected form. Nonetheless, the thioether function is susceptible to alkylation or to oxidation to the sulfoxide or sulfone. In order to prevent S-alkylation, the use of cation trapping agents such as ethanedithiol is recommended during the TFA deprotection steps. When peptide synthesis is carried out under nitrogen, oxidation is a side reaction which can be neglected.

The other possibility is to introduce methionine in the form of Boc Met sulfoxide (O) which can easily be reduced to methionine at the final cleavage of the peptide from the resin by the Low-High HF procedure (Section 2.1.5.3). A particular reason for recommending this second procedure is that Boc Met (O) is commercially available (but is relatively expensive). It must, however, be borne in mind that methionine can be easily oxidized during the different purification steps to which the peptide is subject and even when the lyophilized peptide is not stored in an inert atmosphere (nitrogen or argon).

(h) *Boc Ser (Bzl) and Boc Thr (Bzl):* their use is standard and poses no particular problems.

(i) *Tryptophan:* tryptophan always poses problems in peptide synthesis. The indole nucleus of tryptophan is very sensitive to oxidation and alkylation reactions, especially in acid solution. In order to partially prevent side reactions of this type, two solutions may be envisaged:

– Boc Trp may be used without a protecting group for the indole nucleus and scavengers must be present throughout the synthesis. For example, ethanedithiol may be included in the deprotection solvent in such a case. However, the use of these trapping agents is only a partial solution, especially when the tryptophan residue is introduced early in the synthesis of a peptide of 15–20 residues, the protection conferred by ethanedithiol is not always sufficient.

– A tryptophan derivative is used in which the indole nucleus is protected and in this case formyl is the group most commonly used. Its use is nonetheless exacting since it is not cleaved by the standard HF treatment and special procedures are made necessary: Low-High HF or basic treatment of the peptide after cleavage from the resin. The problem is that none of these solutions is totally satisfactory. Either the tryptophan residue is not completely deprotected or the peptide is partially destroyed under the reaction conditions used.

– Recently, the mesitylene-2-sulfonyl (Mts) group was proposed for the protection of the indole nucleus. It would appear that this protecting group is stable during synthesis and easily removed at the final cleavage. The usefulness of Boc Trp (Mts) is presently being studied in various laboratories, and it is commercially available.

(j) *Tyrosine:* Boc Tyr (2-BrZ) and Boc Tyr (2,6-ClBzl) may be used interchangeably. Boc Tyr (Bzl) gives rise to more side reactions during HF cleavage.

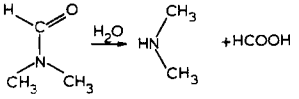
2.1.4. The steps of the synthesis

2.1.4.1. Purification and control of solvents

The use of solvents of high purity is essential to the success of peptide synthesis.

2.1.4.1.1. Dichloromethane (DCM) Technical grade DCM may be used after being dried over $MgSO_4$ or K_2CO_3 and carefully distilled. However, the DCM must be used within 10 days of being distilled. Analysis by gas chromatography can be used to check the purity of this solvent.

2.1.4.1.2. Dimethylformamide (DMF) DMF is a solvent which is not very stable for long periods; in the presence of water, it decomposes to form formic acid and dimethylamine.



It is important to exclude dimethylamine and especially formic acid during the coupling step. Formic acid can be easily detected by gas chromatography and a colorimetric test using dinitrofluorobenzene (DNFB) makes possible the detection of dimethylamine according to the following procedure:

Prepare a 1 mg/ml solution of DNFB in 95% EtOH. Mix 1 ml of this solution with 1 ml of DMF and leave to stand in the dark for 30 min. Then read the OD of the sample at 381 nm against a solution of DNFB in ethanol. The purity of the DMF is considered to be satisfactory if the OD measured is less than 0.15. Should it be required, a simple method for the purification of DMF consists in placing it over molecular sieves (4 Å) and bubbling nitrogen or argon through the solvent. The 'aeration' of the solvent is continued until the DNFB test is negative (several days if necessary). The best way to store DMF is to keep it in bottles of 1 or 2.5 litres under nitrogen.

2.1.4.1.3. Trifluoroacetic acid (TFA) The TFA must not contain anhydrides, aldehydes or water. In general, the commercially available material must be distilled. Two techniques are available for this operation.

(a) The TFA is stored over $CaSO_4$ overnight and then distilled in the absence of drying agent. Since the anhydride has a lower boiling point than the acid, the first fractions collected must be discarded.
(b) The TFA is refluxed overnight over chromium trioxide (Cr_2O_6). Any precipitate which forms is filtered off at room temperature and then the TFA is distilled from phenylalanine at normal pressure, the first fractions being again discarded.

2.1.4.1.4. Diisopropylethylamine (DIEA) The commercial grade material must be distilled over ninhydrin. The mixtures DIEA/DCM and DMF must be made up freshly each day.

2.1.4.2. Preparation of the resin
The commercially available Boc amino acid resins can usually be used directly without prior treatment. After the resin has been allowed to swell for several minutes in DCM, three one-minute washes with DCM are performed. These are followed by the deprotection step.

The aminomethyl resins and the MBHA resins are available as their hydrochloride salts; a neutralization step is thus required before the addition of the first amino acid. The following procedure is used:
(a) Suspend the resin in DMF for 2–3 min
(b) DMF washes: 3 × 1 min
(c) 10% DIEA/DMF washes: 2 × 1 min
(d) DMF washes: 3 × 1 min
(e) Addition of the first amino acid (see Section 2.1.4.4).

2.1.4.3. The deprotection and neutralization steps
Deprotection is usually performed with 65% TFA in DCM containing 0.25% ethanedithiol for 15 min. At this point, a number of comments are called for.

A large number of scavengers have been incorporated into the TFA/DCM deprotection solvent but it would appear that ethanedithiol is the most effective for trapping *tert*-butyl carbocations. Basically these scavengers are only really of importance after the deprotection of Met or Trp if the latter are used without side chain protection. The use of thiols

must be avoided after the incorporation of His (Dnp) (see Sections 2.1–2.3); in this case, ethanedithiol may be advantageously replaced by indole. However, indole is to be used with caution; it may give rise to the formation of indole dimers with Trp and when used in automatic or semi-automatic synthesizers, indole may crystallize in the valves and the solvent flow network.

The important point, as far as the neutralization step is concerned, is the speed of the reaction; times of 1 min, then 2 min are amply sufficient. Neutralization can be carried out with 10% DIEA–DCM; in this case, the two DMF washes preceding neutralization are replaced by DCM washes and neutralization is followed by three DCM washes.

General procedure
(a) DCM washes: 3 × 1 min
(b) 65% TFA–DCM: 1 min
(c) 65% TFA–DCM: 15 min
(d) DCM washes: 5 × 1 min
(e) DMF washes: 2 × 1 min
(f) 10% DIEA–DMF: 1 min
(g) 10% DIEA–DMF: 2 min
(h) DMF washes: 3 × 1 min

2.1.4.4. The coupling reaction
Irrespective of the coupling procedure used, the first step consists of activating the COOH function of the amino acid to the *O*-acyl-isourea by means of a carbodiimide (Fig. 2.9, route A). Subsequently, three different possibilities exist for the formation of the peptide bond: route (B), which is the standard carbodiimide coupling, and routes (C) or (D) which involve the intermediate formation of a symmetrical anhydride or an active ester.

Until recently, dicyclohexylcarbodiimide (DCC) was the most widely used coupling agent. However, the acylureas which form during the reaction are insoluble in DCM and only very slightly soluble in DMF. Moreover, the melting point of DCC is about 35°C, which means that at ambient temperature it exists in the form of a solid mass which is difficult and dangerous to handle. Diisopropylcarbodiimide (DIC) is a liquid at

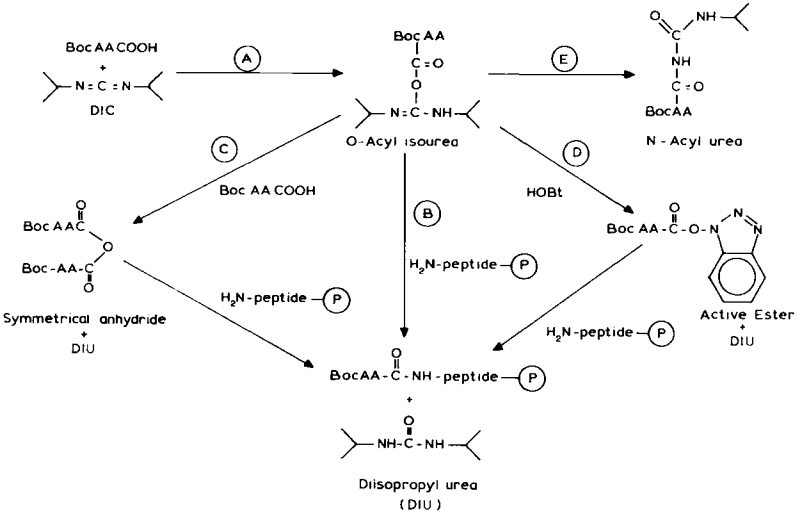


Fig. 2.9. Scheme of the different coupling procedures.

ambient temperature and the acylureas to which it can give rise are soluble in DMF; it is also commercially available. Since the reactivity of these two carbodiimides is similar, we recommend the use of DIC for practical reasons. If, however, DCC is preferred for the preparation of the symmetrical anhydrides and active esters, the acylureas which form in DCM can be eliminated by filtration, although it must not be forgotten that some anhydrides and active esters may co-precipitate with the acylureas.

The solvents used in the coupling step are DMF, DCM or a mixture of the two. We recommend that DMF be used systematically when the amino acid has been activated beforehand. When a standard coupling with carbodiimide is carried out, however, it is important to use DCM. In both cases, the reaction volume should be kept as small as possible. Finally, we have observed very often that when a coupling proceeds with difficulty in DMF, for example, reaction can be brought rapidly to completion by the addition of about 50% of DCM, and vice versa.

2.1.4.4.1. Standard carbodiimide coupling (Fig. 2.9, route B) This is the oldest coupling procedure and one which is very widely used.

Procedure: 3 eq of Boc amino acid are dissolved in a minimum of DMF and the solution is added to the resin suspended in DCM (10–15 ml/g of resin). 3 eq of DIC are then added and the mixture is stirred for 30 to 90 min.

Advantages: this method can be used for all of the amino acids even though it is necessary to use an amide protecting group in the case of Asn and Gln. It is certainly the easiest method to use.

Disadvantages: the reaction must be carried out in DCM. DMF, which provides better solvation of the peptide resin, may not be used since it promotes *N*-acylurea formation (Fig. 2.9, route E). The reaction times are longer than those needed for other methods of activation. During the coupling reaction, a not inconsiderable amount of symmetrical anhydride is formed which leads to other difficulties as we shall see in the following paragraph. Finally, the side chain protecting groups of Asn and Gln give rise to problems of solubility and steric hindrance.

2.1.4.4.2. Symmetrical anhydride coupling (Fig. 9, route C) The symmetrical anhydride is the most reactive entity to be used in peptide coupling reactions.

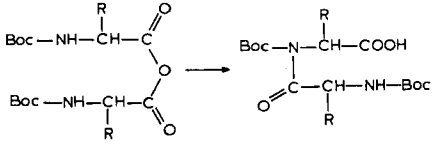
Procedure: 4 eq of Boc amino acid are dissolved in a minimal volume of DCM (containing a minimum of DMF, if necessary). 2 eq of DIC are then added and the mixture is stirred at room temperature for 10–20 min. The DCM is subsequently removed by evaporation at 40°C and the symmetrical anhydride is dissolved in a minimum volume of DMF and the solution is added to the resin.

Advantages: symmetrical anhydrides are very reactive; the mean coupling time is about 15–20 min and very often couplings which are difficult to carry out under standard conditions in DCM can be easily performed using symmetrical anhydrides. When the symmetrical anhydride is prepared immediately before use, the coupling may be done in DMF, which is an undoubted advantage compared with the preceding procedure in which DCM is used as the reaction medium.

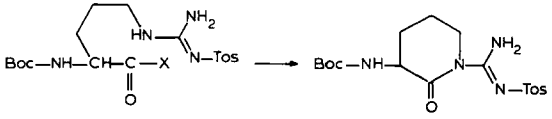
Disadvantages: the use of symmetrical anhydrides is incompatible

with the employment of Boc Asn and Boc Gln in which the amide group is unprotected since the amide function of Asn and Gln can be dehydrated to give the corresponding nitrile in the presence of DIC. Moreover, the Boc Asn (Xan) and Boc Gln (Xan) derivatives are not ideal as they are only soluble in DMF.

Another problem is that posed by the possibility of an intramolecular rearrangement, giving rise to the formation of a protected dipeptide. This protected dipeptide may then be incorporated into the growing peptide chain.



The amino acids more likely to undergo this reaction are those not possessing bulky side chains, specially glycine, although an intramolecular re-arrangement is also possible with Boc Arg (Tos):



This reaction occurs all the more easily if X is a good leaving group (the best being Boc COO^-). The delta lactam is also an acylating entity but it reacts much more slowly than the anhydride. Nonetheless, it has been found that, in a solution of the symmetrical anhydride of Boc Arg(Tos), the major acylating agent was the δ-lactam. Therefore, the coupling of Boc Arg(Tos) by means of a symmetrical anhydride must be avoided.

In conclusion, the use of symmetrical anhydrides certainly provides an attractive alternative to the standard carbodiimide coupling. However, their use cannot be general owing to the difficulties which remain.

2.1.4.4.3. Active ester coupling (Fig. 9, route D) A great variety of reagents has been used to prepare active esters, particularly for synthesis in solution. The only one which has found significant application in solid-phase peptide synthesis is hydroxybenzotriazole (HOBt).

Procedure: 3 eq of Boc amino acid and 3 eq of HOBt are dissolved in a minimum of DMF and 10–15 ml of DCM at 0°C with stirring. 3 eq of DIC are then added and stirring is continued for 15–20 min. The DCM is evaporated, the active ester is dissolved in DMF and the solution obtained is added to the resin.

Advantages: all of the Boc amino acids can be coupled in the form of their benzotriazole ester, except Boc His (Tos). The mean reaction time (20–30 min) is usually longer than that taken in the case of a symmetrical anhydride coupling but it remains acceptably short.

Disadvantages: the major disadvantage of this method is that it cannot be used for the introduction of Boc His (Tos) (Section 2.1.3). However, subsequent amino acid couplings using HOBt active esters appear not to lead to serious problems. On the other hand, Boc His (Dnp) does not pose problems of this kind. However, if it is used, an additional deprotection step is required since the Dnp group is stable to HF (Section 2.1.3).

2.1.4.4.4. Conclusions and comments The different possibilities for coupling which we have just discussed are by no means exhaustive. They simply represent general methods which give satisfactory results in most cases. It is recommended that the relevant section in the review of Barany and Merrifield (1980) be read for a general discussion. As far as coupling times are concerned, values given are only mean values and have no absolute significance since they may show considerable variations from one peptide sequence to another for the same amino acid. At the end of each coupling step, it is thus preferable to carry out a chemical test to assess the level of residual free amino groups on the resin. In point of fact, such considerations call into question the principle of the complete automation of solid phase peptide synthesis. This matter will be discussed in more detail later.

2.1.4.5. Monitoring of coupling reactions

The basic principle of SPPS requires complete reaction at each step in order to limit the number of by-products formed which may be difficult to separate from the desired product in the final purification steps.

It is standard practice to monitor the progress of the coupling reaction by colorimetric detection of the α-NH_2 which remain free. One of the most widely used tests is the ninhydrin reaction (Kaiser et al., 1970). It is quite sensitive (0.5% of primary amines are normally detected) and is very useful for routine qualitative analysis. A quantitative ninhydrin assay also exists (Sarin et al., 1981) but it is more time-consuming and, in our opinion, the results obtained do not justify its systematic use.

Procedure for the qualitative ninhydrin assay

The following reagents are prepared:

(1) 5% ninhydrin in absolute ethanol (1 g in 20 ml)

(2) 80 g phenol in 20 ml of absolute ethanol

(3) pyridine.

A 1 to 2 mg sample of peptide resin is taken from the reactor and washed with methanol in a hemolysis tube. Once the resin beads have settled, the methanol is removed by aspiration and the washing procedure is repeated twice. 2 drops of each of the reagents 1, 2 and 3 are added to the resin beads and the mixture is heated to 110°C for 4–5 min. If primary amino groups are absent, the reaction solution is pale yellow and the beads perfectly translucent; if primary amino groups are present a blue color develops on the beads and/or in the solution.

However, the ninhydrin test may present difficulties of interpretation after the coupling on to the residues Pro, Asp, Ser, Cys and Asn. In this case, the test with 2,4,6-trinitrobenzene sulfonic acid (Hancock and Battersby, 1976) is very useful and quite easy to use.

2.1.4.6. Double coupling and acetylation

Generally speaking, it is inadvisable to prolong the reaction time beyond 1 h even if the coupling is incomplete. In such a case, the coupling should be repeated and be carried out in the following manner:

(a) DCM washes: 3 × 1 min

(b) 10% DIEA/DCM washes: 2 × 1 min
(c) DCM washes: 3 × 1 min.

The double coupling is carried out with 1, 2 or 3 eq of the amino acid, depending on the intensity of the ninhydrin color. If the solvent used for the first coupling was, for example, DMF, we advise using DCM (or a DCM/DMF mixture) for the second coupling. This change of solvent may, in fact, modify the conformation of the peptide on the resin and render the still unreacted amino groups more accessible. In certain cases a triple coupling may prove to be necessary and the third coupling maintained for an extended period of time (overnight, for example). The procedure is identical with that described for the double coupling. It is recommended that a minimum of DMF be used in the reaction solvent if the triple coupling is to last several hours in order to keep the risks of racemization to a minimum.

If, after these various attempts, the coupling is still not complete, acetylation is carried out in order to block irreversibly the peptide chains which have not reacted. After three DCM washes, 10 eq of acetic anhydride and 10 eq of 10% DIEA/DCM are added to the resin and the mixture is left for 10 min. The ninhydrin test ought then to be negative.

2.1.5. The cleavage of the peptide from the resin

When the Boc synthesis is complete, a strong acid is required to deprotect the side chain functions of the amino acids and to cleave the peptide from the resin support. The acid most commonly used is anhydrous hydrogen fluoride, HF. At ambient temperature, this acid exists as a gas; it is very corrosive and requires special equipment or a vacuum HF line. These HF lines, made of Teflon-Kel-F, are commercially available (see appendix) and easy to use; a particularly detailed description of the handling of a HF line is provided by Stewart and Young (1984). We shall pay particular attention to the discussion of this type of cleavage which we have practised several hundred times in our laboratory, without any major difficulty. However, before one does an HF cleavage on one's own, it is strongly advisable to have done a cleavage under the guidance of an experienced person. We shall also mention cleavage by tri-

fluoromethanesulfonic acid (TFMSA) which is becoming increasingly used and which may be substituted for HF cleavage in certain instances.

2.1.5.1. Preparation of the peptide resin

Whatever method of cleavage is used, the preliminary treatment of the peptide resin is the same. When the last Boc amino acid has been added, a complete deprotection cycle is carried out and the resin is washed with DCM (5 × 1 min), then with ethyl ether (3 × 1 min). Subsequently, the peptide resin is dried in a stream of nitrogen, and then under vacuum in a desiccator.

2.1.5.2. Standard HF cleavage

Of the protecting groups which we have described above, it will be recalled that the only ones not to be cleaved are the dinitrophenyl group of histidine, the formyl group of tryptophan, and the acetamidomethyl group of cysteine. The most frequently used scavengers for trapping carbocations during the reaction are anisole and *p*-cresol. Generally speaking, the proportions of reagents used are the following : 90% HF/10% *p*-cresol (v/v). If the peptide contains Trp, Met or Cys, the mixture is the following: 90% HF/9% *p*-cresol/1% 1,2-ethanedithiol.

General procedure for 0.3 mmol of peptide resin

After the resin has been placed in the Teflon reaction vessel, the addition of 1 ml of *p*-cresol or of 0.9 ml of *p*-cresol and 0.1 ml of ethanedithiol is made. The reaction vessel is then cooled for 10 min in a dry ice/ethanol mixture, then a magnetic stirring bar is slid down the walls of the vessel by the external application of another magnet. The reaction vessel is then screwed onto the HF line before being cooled again in the dry ice/ethanol mixture for 20 min. In our laboratory, a vacuum is created in the line by means of a rotary vacuum pump (4 m^3/h) protected by a metallic trap containing calcium oxide mounted in series with a glass trap cooled to −70°C. The reaction vessel is evacuated for 1 min, then the HF container is opened and 10 ml of HF are condensed onto the peptide resin.

Reaction then proceeds with magnetic stirring during 45 min at −2°C in an ice/salt mixture. After the HF has been evaporated, a viscous mix-

ture of resin, peptide and scavenger is left in the reaction vessel. This mixture is then washed on sintered glass with 3 × 10 ml of anhydrous ether or ethyl acetate in order to precipitate the peptide and remove the scavengers. The peptide is finally eluted with a solution of 10% acetic acid in water; if the peptide is particularly hydrophobic, glacial acetic acid or acetonitrile may be used for the elution. The eluate is then lyophilized and the crude synthetic peptide is ready for purification.

Comments: The reagent used in this final step must be of the same high quality as those used during the synthesis. Traces of impurities or moisture in the HF can cause considerable damage to the peptide. Even when the maximum number of precautions have been taken, there remain a large number of side reactions which may occur in the presence of HF and they are discussed in detail in the review by Barany and Merrifield (1980). Basically, most of these reactions are due to benzylic carbocations which are formed in the strong acid medium and which are very powerful alkylating species. In order to prevent the formation of these cations Tam et al. (1983) have suggested the use of a mixture of strong acid–weak base during the HF cleavage, the so-called Low–High HF cleavage procedure.

2.1.5.3. The 'Low–High' HF system

Principle: In the first step for 'Low HF' cleavage, a mixture of strong acid (HF) and weak base (dimethyl sulfide) is used which leads to the deprotection of the side chain functions of the trifunctional amino acids by a mechanism of the SN_2 type, i.e. without the formation of carbocationic intermediates. In this way, all of the side reactions due to this type of chemical species are suppressed. All of the side chain protecting groups are cleaved except Arg (Tos), Asp (OcHex), Glu (OcHex), and Cys (4-Me Bzl), Met (O) is reduced to Met and the formyl group of Trp (CHO) is cleaved to a large extent. The bond linking the peptide to the support may be cleaved in the case where a chloromethyl resin has been used, but when the synthesis has been performed on resins of the Pam or MBHA type, the low HF must be followed by a standard high HF cleavage which eliminates the remaining protecting groups: Tos, cHex and 4-Me Bzl.

General procedure for 0.3 mmol of peptide resin

After the resin has been placed in the reaction vessel, 1 ml of *p*-cresol and 6.5 ml of dimethyl sulfide (Me_2S) are added successively. In the case in which the peptide contains Trp (CHO) or Met (O), the mixture used is 0.5 ml *p*-cresol, 0.5 ml thiocresol and 6.5 ml of Me_2S. After the magnetic stirring bar has been introduced, the reaction vessel is screwed onto the HF line and cooled in a mixture of ethanol/dry ice for 30 min. The reaction vessel is then evacuated for 1 min, then 2.5 ml of HF are condensed and the mixture is then stirred for 2 h at 0°C. HF and Me_2S are removed by evaporation to dryness and the resin is washed on a sintered glass frit with 3 × 10 ml of DCM, then with 3 × 10 ml of ethyl acetate, dried in a vacuum for 30 min and returned to the reaction vessel. The standard High HF cleavage is then carried out as described in Section 2.1.5.2.

Comments: Although this procedure has been criticized by some workers, we ourselves have experienced no particular difficulty except the incomplete deprotection of Trp (CHO). Thus the HPLC analysis after Low–High HF cleavage of peptides containing Trp (CHO) has often shown a peak representing 5%–10% of the material and corresponding to the protected peptide. However, we have systematically observed in particularly critical cases a gain in the purity of the final peptide product of 15–30%.

2.1.5.4. TFMSA cleavage

The cleavage of certain types of protecting group by TFMSA was first described by Yajima et al. in 1974. More recently, Tam et al. (1986) have studied the mechanisms of acidolytic deprotection of benzyl groups in the system TFMSA–TFA–Me_2S (10 : 60 : 30, v/v) and they have developed a 'Low–High acidity TFMSA' deprotection procedure. At the moment the Applied Biosystems Company suggests TFMSA for routine use in the final cleavage of the peptide from the resin as a means of avoiding the use of HF (Bergot et al., 1986).

Advantages: This method does not require special equipment, it can be adapted to any quantity of resin, large or small, and it can be used to cleave several peptide resins simultaneously.

Disadvantages: Peptide synthesis must be carried out using Boc amino acids which are compatible with this type of cleavage: Asp (OBzl), Glu(OBzl), His(Z) or His(Tos), Lys(Cl-Z), Met(O), Ser(Bzl), Thr(Bzl), Arg(Mts), Tyr(Br-Z), Trp(Mts) or Trp(CHO), Cys(Mob). The times required for cleavage of the peptide–resin link and the yields of peptide obtained are reasonable when chloromethyl or Pam resins are used, but reaction times are longer when the synthesis is conducted on a MBHA resin. Each type of peptide resin would seem to require a preliminary investigation of the TFMSA type of cleavage and it also appears that the results obtained are scarcely superior to those afforded by the conventional HF cleavage. Finally, it must be borne in mind that TFMSA is a very corrosive liquid which must be handled with great care and in a hood. Nonetheless, we recommend that the reader follow the development of this technique attentively.

2.2. *The 'Fmoc' synthesis*

2.2.1. *Introduction*

The various problems which are encountered in the course of the Boc type of synthesis are due essentially to the acid conditions used both for the deprotection steps and for the final cleavage of the peptide from the resin. These considerations have led to the development of a method of synthesis based on the use of the fluorenylmethoxycarbonyl (Fmoc) group for the protection of the α-NH_2 function in combination with the *tert*-butyl group for the protection of the side chain functionalities of the amino acids (Carpino and Han, 1970). This combination avoids both repeated acid treatments and the use of HF at the end of the synthesis. The synthetic procedure is orthogonal since Fmoc is cleaved by a basic treatment (usually piperidine) and the peptide is cleaved from the resin by an acidic treatment (TFA). Although this approach is also not an ideal one, it has been developed particularly intensively in recent years. We shall describe this approach briefly: the abundant literature relating to it may be found in a recent review by Sheppard (1986).

2.2.2. The solid support

At present, there exist 3 types of solid support which may be used for the Fmoc type of solid-phase peptide synthesis.

2.2.2.1. Polystyrene resin

This polymer is identical with that used for the Boc approach to synthesis. The only difference relates to the introduction of the functional groups (the details will be given in the relevant Section below).

2.2.2.2. Polyacrylamide resin

This is a copolymer of dimethylacrylamide and ethylenebisacrylamide. The functional sites are introduced during polymerization by using defined quantities of acryloylsarcosine methyl ester (Arshady et al., 1981). This polymer possesses superior swelling properties to those of polystyrene in most organic solvents and its polyamide nature is more compatible with the structure of the peptide than is that of the highly hydrophobic polystyrene. In theory, this polymer would be expected to exhibit solvation properties similar to those of the growing peptide chain, leading to improved permeation by solvents and reagents and resulting in higher yields in synthesis. In practice, the efficacy of this polymer seems to be quite comparable to that of polystyrene.

2.2.2.3. Composite resin

This consists of a polyacrylamide resin identical with that just described but co-polymerized with silica gel. This gives rise to a very rigid support which can be used as packing material for columns of the HPLC type. All solvents and reagents flow continuously through the system and the progress of the reactions is monitored by spectrophotometry (Dryland and Sheppard, 1986). The resins and the equipment necessary for this type of continuous flow synthesis are already commercially available.

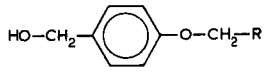
Wade et al. (1986) have recently compared the synthesis of ANF using the Fmoc approach on this type of resin with the same synthesis conducted by the Boc approach on the standard resin. The results of the analyses performed on the crude products obtained by HPLC are quite comparable.

2.2.3. Introduction of functional groups and attachment of the first amino acid to the resin

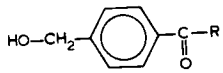
The employment of TFA for the final cleavage of the peptide from the resin requires an ester linkage which is relatively labile to acid. This requirement has led to the introduction of handles between the first amino acid and the resin. These handles are the same, irrespective of the resin used. In order to prepare 'free acid' peptides, handles derived from 4-hydroxymethylphenoxyacetic acid are usually used (Sheppard and Williams, 1982), and to prepare peptide amides, the handles used are derived from 4-hydroxymethylbenzoic acid (Atherton at al., 1981b).

2.2.3.1. Functionalization of the polystyrene resins

The appropriately functionalized polystyrene resins are commercially available. These are the 4-hydroxymethylphenoxymethyl resins:



and the 4-hydroxymethylbenzoyl resins:



However, these resins can be prepared in the laboratory according to a method which is relatively straightforward (Fig. 2.10). The active esters of 4-hydroxymethylphenoxyacetic acid or 4-hydroxymethylbenzoic acid, those formed with trichlorophenol for example, can be used to directly acylate an aminomethyl resin in the presence of HOBt. This reaction leads to a resin functionalized with hydroxyl groups to which the C-terminal amino acid of the peptide to be synthesized may be esterified according to the method described above (Section 2.1.2.2.2). The OH functions which have not reacted are then acetylated according to the procedure described in the same section.

Another method, described in Fig. 2.11, consists of first attaching an unusual protein Fmoc amino acid, NLeu for example, to the

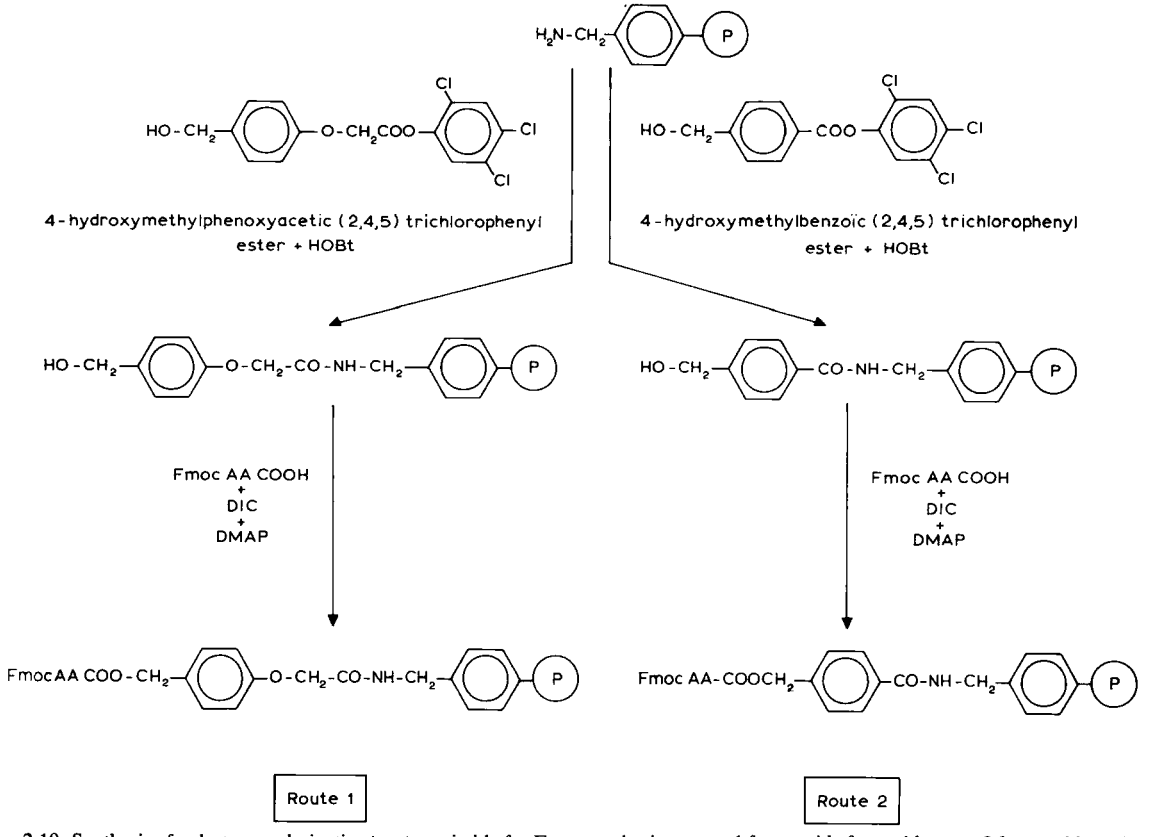


Fig. 2.10. Synthesis of polystyrene derivatized resins suitable for Fmoc synthesis – route 1 for peptide free acid, route 2 for peptide amide.

aminomethyl resin. After removal of the Fmoc group the free α-NH_2 function is allowed to react with the active ester of 4-hydroxymethylphenoxyacetic acid or 4-hydroxymethylbenzoic acid in the presence of HOBt. The first protected amino acid of the sequence to be synthesized is then esterified as described above. After acetylation, the amino acid/NLeu ratio, determined by amino acid analysis of the resin, enables the esterification yield to be calculated very precisely.

A third method exists in which the 4-hydroxymethylbenzyloxymethyl

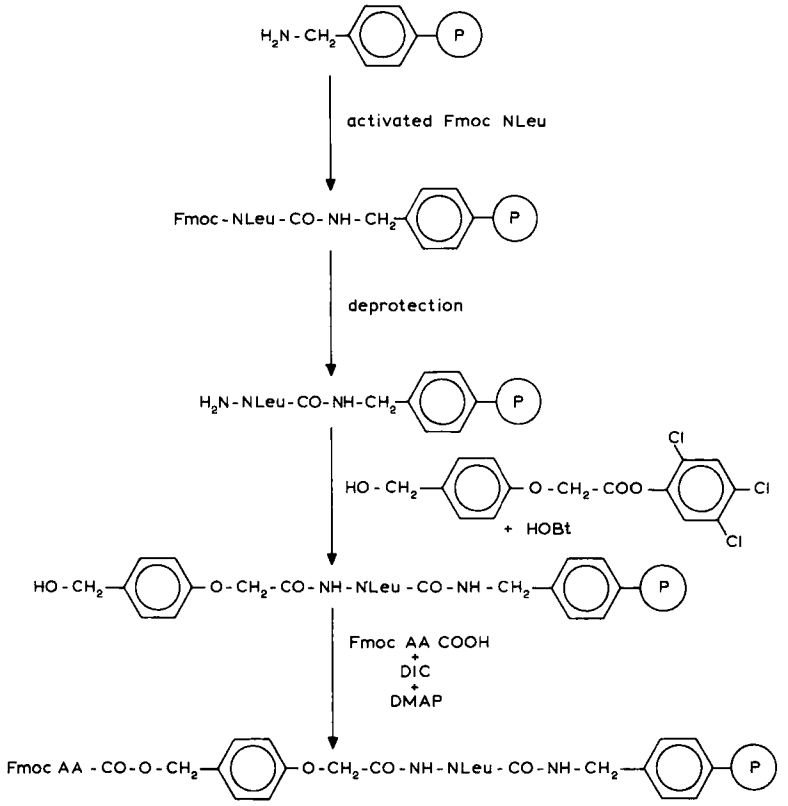
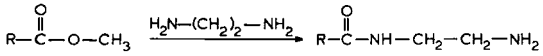


Fig. 2.11. Derivatization of polystyrene support for Fmoc peptide synthesis through intercalation of NLeu.

resin is prepared from a chloromethyl polystyrene (Lu et al., 1981). This type of resin is commercially available and gives results identical with those obtained in the systems described above. However, it is not possible to intercalate an amino acid reference in this case.

2.2.3.2. Functionalization of the polyamide resins

The functional groups of the polyamide resin are methyl esters which are converted into amines by treatment with ethylenediamine (40 ml/g of resin for 10 h).



This step results in the production of an aminomethyl type of resin and the various methods described in the previous section may be used to esterify the first amino acid to the resin.

2.2.3.3. Conclusions and comments

It is difficult to recommend one type of resin rather than another since their efficacy in peptide synthesis is identical in most cases. The user will thus have to make his choice in the light of his own assessment. As far as the esterification of the first amino acid to the residue is concerned, the possibility of racemization in the presence of 4-dimethylaminopyridine (DMAP) must not be ignored. In addition, 4-DMAP may lead to premature deprotection of the Fmoc group which then causes a double incorporation of the first amino acid. This approach must thus be used with the necessary caution. The interference from OH groups which may have survived acetylation is very limited in the Fmoc type of synthesis since deprotection is carried out under basic conditions and, as a result, the side reactions of the type which are encountered in the Boc type of synthesis do not occur.

2.2.4. The protecting groups (see also Table 2.1)

All of the Fmoc amino acids commonly used in synthesis are commercially available. Generally speaking, *tert*-butyl derivatives are used for

the protection of side chain functions: *tert*-butyl esters for Asp and Glu, *tert*-butyl ethers for Ser, Thr and Tyr, *tert*-butoxycarbonyl for Lys and His. The *tert*-butyloxymethyl group may also be used in the case of histidine. Fmoc Cys (Trt) is used if a free SH group is desired at the end of the synthesis and Fmoc Cys (S-tButhio) is used if the SH group must remain protected, subsequent deprotection being carried out by treatment with tributylphosphine. Fmoc Asn and Fmoc Gln may be used without special protection provided certain precautions are taken at the coupling step (see Section 2.2.5.2). As for the 4-methoxy-2,3,6-trimethylbenzene sulfonyl (Mtr) group, it is the least unsatisfactory protecting group presently available for the guanidine function of arginine.

2.2.5. The steps of deprotection and coupling

2.2.5.1. The deprotection step

Deprotection is usually carried out by treatment with piperidine according to the following procedurec (Fig. 2.12):

(a) DMF washes: 10 × 1 min
(b) 20% piperidine/DMF: 1 × 2 min or 50% piperidine/DMF 1 × 2 min
(c) 20% piperidine/DMF: 1 × 8 min or 50% piperidine/DMF 1 × 2 min
(d) DMF washes: 10 × 1 min.

Comments: Since the Fmoc group is very sensitive to primary and secondary amines, many DMF washes are necessary in order to eliminate

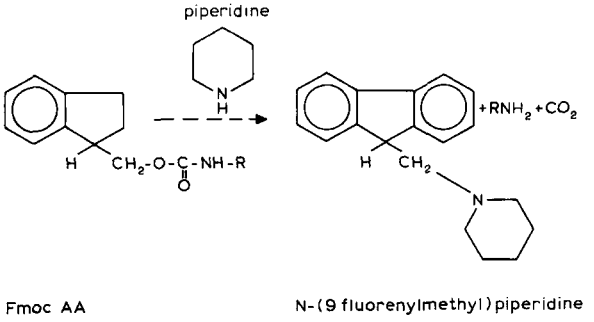


Fig. 2.12. Cleavage of Fmoc protecting group with piperidine.

all trace of piperidine. If such precautions are not taken this may lead to the double incorporation of the amino acid added at the next coupling step. Analytical grade piperidine is usually sufficiently pure for use but all traces of dimethylamine must be removed from the DMF used in the coupling step. In this case, the purification procedure and the tests for amines with DNFB described above (Section 2.1.4.3.2) may be used.

2.2.5.2. The coupling step

Generally speaking, the methods described for the coupling of the Boc amino acids are valid for the coupling of the Fmoc amino acids. When a standard coupling with DIC is carried out or when the method of symmetrical anhydrides is used, the usual precautions must be taken for Gly, Asn, Gln and Arg. Fmoc Asn and Fmoc Gln, in which the primary amide function is protected, are commercially available and this enables them to be coupled in the form of the anhydride. It nonetheless remains a fact that these Fmoc amino acids are very expensive.

The active ester method poses no difficulties with the Fmoc amino acids, all of the derivatives may be activated with HOBt. Recently, the pentafluorophenyl esters of Fmoc amino acids have been successfully used by Atherton and Sheppard (1985). These active esters can be crystallized and are easy to store, thus making manual pre-activation superfluous. In addition, these pentafluorophenyl esters are commercially available but they may be easily synthesized following the procedure described by Kisfaludy and Schön (1983). They are used in excess (2–3 eq) in the presence of the same excess of HOBt.

Generally speaking, the speeds of reaction of the different types of active ester are more or less equivalent (5 to 45 min).

2.2.5.3. Monitoring of the coupling reaction

The ninhydrin and trinitrobenzene sulfonic acid tests may be used quite satisfactorily to assess the progress of the coupling reaction.

It is interesting to note that the *N*-(9-fluorenylmethyl)piperidine which is formed during deprotection (see Fig. 2.12) absorbs strongly at 301 nm (Σ = 7,800). Thus, by collecting the washes after the piperidine

treatment, the chromophores formed can be determined spectrophotometrically in a very precise manner and hence the deprotection yields can be assessed.

2.2.6. Cleavage of the peptide from the resin

2.2.6.1. Preparation of the peptide free acid

The synthesis of the peptide must be carried out on a 4-hydroxymethylphenoxymethyl resin. At the end of the synthesis, the last amino acid is deprotected and the resin is washed in the following manner:

(a) Washes with *tert*-amyl alcohol: 5 × 1 min

(b) DCM washes: 5 × 1 min

(c) Washes with ethyl ether: 5 × 1 min.

The peptide resin is dried in a stream of nitrogen, then in a desiccator over P_2O_5. The reaction volumes used for the cleavage itself are of the order of 100 ml/g of peptide resin. The composition of the reaction mixture and the times of reaction are modified as a function of the sequence of the peptide.

General procedure

(a) If the peptide does not contain Arg, Cys, Met and Trp, the following volumes of reagents are used : 90–98% TFA/1–5% anisole/1–5% H_2O. The reaction is performed in a round-bottomed flask at room temperature for 1 h. The proportion of anisole may be increased and that of H_2O decreased if the peptide contains a number of trifunctional amino acids.

(b) If the peptide contains arginine, the reaction mixture is identical with that just mentioned but the time of reaction is extended to an average time of 6 h.

(c) If the peptide contains Cys, Met and Trp, the following mixture is used: 98% TFA/1% anisole/1% ethanedithiol. The time of reaction is about 1 h if the peptide does not contain arginine.

At the end of the reaction, the reaction mixture is filtered on to a glass frit and the resin is washed 2–3 times with TFA; all of the washes are then collected and evaporated to dryness. The peptide is then taken up 2 to 3

times in hexane and re-evaporated in order to remove the last traces of TFA; it is finally taken up in water and lyophilized. At this last step, the aqueous phase may be extracted again 2 to 3 times with the same volume of ether in order to remove traces of anisole and other organic products resulting from cleavage.

2.2.6.2. Preparation of the peptide amide

It will be recalled that the peptide must be synthesized on a 4-hydroxymethylbenzoyl resin. At the end of the synthesis, the last amino acid is left in the protected form. Cleavage is performed in two stages:

1st stage: Deprotection of the peptide on the resin. The procedure is the following :

(a) DMF washes : 3 × 1 min

(b) Washes with *tert*-amyl alcohol: 5 × 1 min

(c) TFA treatment 1 × 5 min, then 1 × 30 min.

For this treatment, 50 ml of 90% TFA/10% H_2O (v/v) per gram of resin are usually used. If the peptide contains Cys, Met, Trp or Arg, see the previous section.

(d) Washes with acetic acid: 5 × 1 min

(e) Washes with *tert*-amyl alcohol: 5 × 1 min

(f) DMF washes: 5 × 1 min

(g) 20% piperidine/DMF 1 × 3 min, then 1 × 5 min

(h) DMF washes: 10 × 1 min.

2nd stage: Cleavage of the peptide from the resin. As soon as step (h) is finished and without special drying, the resin is treated with a solution of methanol saturated with anhydrous ammonia (20 ml/0.1 mmol of peptide). The mixture is prepared at 0°C and added to the resin contained in the flask in an inert atmosphere (nitrogen or argon). The reaction proceeds at ambient temperature for 10 to 20 h with stirring. The resin is then filtered off onto a glass frit and the work-up is the same as that described in the previous section.

2.2.6.3. Conclusions

It is quite clear that the final treatment with TFA to deprotect the peptide and to cleave it from the resin will cause less damage to the peptide than the more drastic HF treatment. The deprotection of arginine remains the major problem; in fact, the cleavage of the Mtr[1] group requires 4 to 6 hours of reaction in TFA and, under these conditions, the so-called 'sensitive' peptides, namely those containing Trp, Met or Cys residues, may be subject to serious degradation.

2.3. Semi-automated, automated and multiple synthesis

In the introduction to this chapter we described the manual apparatus for the synthesis of peptides which we have used for several years in our laboratory and on which our students are still given their initial training in peptide synthesis. Using this manual apparatus, which is simple and inexpensive, it is possible to incorporate five to six amino acids a day when the synthesis itself poses no major problems.

Starting with this basic system, we have developed our own semi-automated multi-synthesizer, that is, a synthesizer which enables four different peptides to be synthesized in parallel in four different reaction vessels (see appendices). When used routinely in the laboratory, this synthesizer allows us to prepare four peptides of 15–20 residues per week and thus easily meet the requirements of our Institute. This system can be adapted to either the Boc approach or Fmoc approach to peptide synthesis on standard polystyrene resins. We have adopted the semi-automated procedure in order to be able to intervene at critical points of the synthesis, namely, the coupling step (choice of the form of activation and the solvent to be used) and the control of the progress of the reaction, our strategy having always consisted of paying careful attention to the steps of the actual synthesis in order to save precious time on subsequent purification. Undoubtedly a molecular biologist without experience in peptide synthesis will be tempted to buy a completely automated machine. Several such very sophisticated pieces of equipment are now available on the market. They are relatively expensive but the publicity

which surrounds them suggests that they give excellent results. The most critical manner in which the capacities of such a machine can be tested is to have it at one's disposal for several weeks, and to prepare a number of critical peptides presenting particular difficulties for synthesis and which have been synthesized in a manual apparatus for comparison. The crude cleavage products obtained after lyophilisation should then be analysed under the conditions described in Section 2.5.3.

It should, however, be borne in mind that the synthesis itself is only one step in the preparation of a peptide since it is subsequently necessary to cleave the peptide from the resin, purify it and analyse it before it can be used.

Recently, Geysen et al. (1984) have presented an interesting method which makes possible the simultaneous and rapid testing of large numbers of short peptides still attached to the support used for their synthesis. The peptide synthesis is developed on polyethylene rods which have been previously immersed in a solution of acrylic acid and γ-irradiated. Rods so prepared are assembled into a polyethylene holder with the format and spacing of a microtitre plate. Subsequent reactions at the tip of the rods are carried out in a Teflon tray with a matrix of wells to match the rod spacing. A Boc Lys methyl ester is first coupled to the polyethylene/polyacrylic acid via the NΣ-amino groups and the following residues are coupled in DMF in the presence of HOBt. At the end, the side chain protecting groups are removed by treatment with borontris (trifluor-acetate) in trifluoroacetic acid for 90 min at room temperature. Before testing by ELISA, support-coupled peptides are washed several times with phosphate-buffered saline. Since peptides adsorbed or linked to a solid phase may have different antigenic properties compared to when they are free in solution, this method of peptide synthesis appears to have some limitations.

Houghten (1985) has published another procedure allowing the rapid synthesis of large numbers of peptides. In this system the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods. This procedure allows the carrying-out of between 4 and 8 couplings per day

on at least 100 different peptides simultaneously. At the end, the protected peptide resins are cleaved by using the conventional HF/anisole procedure in a modified vessel which allows cleavage of 20 peptide resins at once.

These new approaches which are now commercialized (see appendices) make it possible to carry out a rapid screening of the ability of anti-protein antibodies to recognize peptide fragments. When an antigenic determinant has been localized in this manner, the peptide can then be synthesized and further purified according to the conventional methods.

2.4. Boc synthesis or Fmoc synthesis

As stated at the beginning of this chapter, there is a growing tendency for the Fmoc approach to peptide synthesis to be used at the expense of the Boc approach and some laboratories and some synthesizers are oriented exclusively toward this approach. It should be pointed out that one point in its favour is the avoidance of the use of hydrogen fluoride for the final cleavage of the peptide from the resin: the use and handling of HF may pose problems to the uninitiated and may even be forbidden in certain laboratories.

We are not concerned to take sides in this debate; rather, we believe that both approaches should be available in the laboratory so that one or other may be chosen depending on the particular difficulties presented by the peptide to be synthesized. At present, two different kinds of argument can be presented in support of maintaining the Boc approach to synthesis:

– The Boc approach has been under study and development for 25 years. Most of the side reactions likely to occur are perfectly well known and, in most cases, this approach has led to the resolution of the problems posed by synthesis. The Fmoc approach is more recent and thus less well studied and progress remains to be made in various respects, particularly with regard to the choice of side chain protection groups for certain amino acids.

– The cost of the Fmoc type of synthesis is still two to four times higher

than that based on Boc, and the Fmoc derivatives of the amino acids remain expensive in spite of the increasingly wide use of the method.

2.5. *Methods for the purification and analysis of peptides*

Purification of the crude product obtained immediately after synthesis is necessary in all cases when the peptide is intended for use in immunochemical experiments. Although it is certain that a crude synthetic peptide of five to fifteen residues presenting no particular difficulty has often been found to exhibit a degree of purity varying from 70 to 90%, gel filtration, on Sephadex G-15 for example, proves to be essential for the removal of solvent residues and salts resulting from the final cleavage of the peptide and leads to a product which is easier to store (Section 2.5.1). In general, and irrespective of their size, we have used peptides exhibiting a degree of purity of more than 80–85% in our immunochemical experiments. In certain, well-defined cases, it was necessary to prepare a final product with a degree of purity higher than 95%. To achieve this we employed a series of quite standard purification methods such as ion exchange chromatography (Section 2.5.2) and preparative HPLC (Section 2.5.4).

The analytical method based on HPLC which we used to analyse the crude synthetic peptide or the purified final product is described in Section 2.5.3.

2.5.1. Gel filtration

The type of gel used depends essentially on the size of the peptide. This purification step is intended to remove salts and solvent residues remaining after cleavage. It should be carried out as soon as possible after the lyophilization of the crude peptide. It is often illusory to expect any purification of the major product during this operation, since, for the most part, the contaminants are peptide fragments resulting from dele-

tions or chain terminations and which thus have a similar molecular mass. However, in certain cases gels of the type Fractogel® TSK HW-40 particularly, when used under low pressure (1–3 bars), have proved to be extremely powerful tools.

The elution solvent is usually a 5 to 10% solution of acetic acid in water. In the case of hydrophobic peptides it is sometimes preferable to use either a 30% acetic acid solution or a 5 to 10% formic acid solution. If the peptide is acidic it is advisable to use 0.1 to 1 M ammonium acetate solution, depending on the overall negative charge, or, if necessary, a 0.05 to 0.1% solution of ammonia.

I the case of peptides containing methionine it is particularly advisable ι work at acid pH to minimize the risk of oxidation and it is also necessary to de-gas the solvents by bubbling nitrogen or helium through them. In certain cases, depending on the nature of the peptide, the risk of oxidation of methionine residues is particularly acute. The peptide containing methionine sulfoxide is easily detected by analytical HPLC as it emerges just ahead of the methionine peptide.

The same precautions must be taken in the case of peptides containing cysteine residues with unprotected thiol groups and it is for this reason that it is often advisable to introduce cysteine residues into the peptide chain in the form of Boc Cys (Acm) during synthesis. In this way, the side chain reaction of the cysteine residue is maintained throughout all of the purification steps to which the peptide is subjected. The final deprotection step is then carried out with mercuric acetate (Veber et al., 1972). When it is desired to prepare a peptide which requires cyclization through the formation of disulfide bridges, cleavage of the Acm protecting group and oxidation are carried out in one step by treatment with iodine (Kamber, 1971).

2.5.2. Ion exchange chromatography

This technique is widely used and gives excellent results for the purification of peptides. It is often an essential step, carried out just prior to the final purification step consisting of preparative HPLC. We have very frequently used resins of the carboxymethyl (CM) and diethylaminoethyl

(DEAE) type in conjunction with systems of volatile solvents such as ammonium acetate or ammonium bicarbonate. We have used ionic strength or pH elution gradients, or both. Other supports and other solvents are described in the book by Stewart and Young (1984) and general information relating to ion exchange chromatography is available in standard text books.

2.5.3. Analysis of peptides by HPLC

2.5.3.1. General

High performance liquid chromatography has been developed since the early 1970s and has led to an enormous reduction in the time required for analysis while simultaneously improving the resolution obtained. Currently, pressures of the order of 200 to 300 bars, corresponding to 3000 to 4500 PSI, are used.

The increase in the pressures which may be used in the columns is not the only explanation of the present explosive development of this technique. Better understanding of the chromatographic parameters and, in particular, the possibility of testing them played a decisive role.

It will serve to draw attention to the chief of these parameters, the size of the particles used as supports: spherical supports of very low particle size (3 to 5 μm) are gradually replacing supports consisting of spheroidal or irregularly shaped particles of 20 to 200 μm. This development has been accompanied by an improvement in the control of the size distribution of the particles and by their improved resistance to pressure.

A description and a discussion of the parameters influencing the resolution obtained on HPLC may be found in the monographs by Rosset et al. (1982) and Fallon et al. (1987). We can only give them brief mention in the section which follows.

2.5.3.2. Factors influencing separation

– *The diameter of the particles.*

The number of theoretical plates in a column varies inversely with the size of the particles.

– *The length of the column.*

A column 5 cm in length possesses 75% of the efficacy of a column which is 25 cm long.

– *The diameter of the column.*

Although the present tendency is to reduce the internal diameter of the columns (saving of time and solvents), it will be recalled that a column of large diameter makes it possible to approach the ideal conditions of separation (interaction with the walls is minimized).

– *Loss of pressure.*

The smaller the diameter of the particles of the support, the higher the pressure at the head of the column (inversely proportional to the square of the diameter). It is therefore essential to find a compromise.

– *Rate of flow of solvents.*

The pressure at the head of the column is also determined by the viscosity of the eluant chosen. A linear flow rate compatible with the resistance of the support varies, in general, between 0.1 and 2 or 3 cm/sec.

– *The porosity of the support.*

Pores of 60 or 80 Å permit the separation of small peptides but not of small proteins. A porosity of 300 Å enables small peptides and proteins of molecular weight of 50,000 to be separated equally well.

– *Choice of solvents.*

In general, it is difficult to predict exactly what the ideal composition of the elution solvent should be. A progressive mixing of two solvents (A and B) is thus used in order to resolve as many of the products to be separated as possible. In the case where the solvent A is water, solvent B (with high elution power) is acetonitrile in the case of peptides (used in preference to methanol or acetone).

– *Choice of support.*

Almost all of the supports used in HPLC consist of porous silica beads onto the surface of which may be grafted a stationary phase consisting of various types of organic residues.

2.5.3.3. Detection

The form of detection most commonly used is based on the absorption of UV (210 nm) or visible light by the molecule eluted. Detection by differential refractometry, fluorimetry or by electrochemical means are

also common. HPLC coupled to mass spectrometry is being developed.

2.5.3.4. The future

The pumps presently used in chromatography can generate constant pressures in the range 400 to 600 bars. Under these conditions and using particles of diameters varying from 0.5 to 1 μm (and these are not yet commercially available) the time of analysis could be reduced to a few seconds.

2.5.3.5. HPLC analysis of peptides

The first step in the analysis of synthetic peptides consists in carrying out a gradient elution in order to assess the state of purity of the crude material. This gradient provides information concerning impurities such as deletion fragments, components of the cleavage mixture, etc. At the last step of purification or analysis, the gradient elution must be followed by an isocratic elution in order to obtain a more precise estimate of the impurities which are very similar to the desired peptide: diastereoisomers, deletions which have only a slight effect on the hydrophobic character of the peptide, etc.

The slope of the elution gradient is chosen as a function of the state of purity of the peptide; in the final step of the purification it is advisable to use a shallow elution gradient of the order of 1% of solvent B per minute.

The solvent A (non-eluting) is chosen from among aqueous solutions of triethylammonium phosphate, TFA (reverse phase) or acetate, phosphate, etc., buffers (ion exchange HPLC). The pH and the molarity vary between 2 and 6.5 and from 0.05 to 1 M, respectively.

2.5.3.5.1. Reverse phase HPLC – (a) Peptides of low molecular weight. Moderately hydrophobic peptides containing fewer than 12–16 amino acids can easily be separated on columns of silica beads of 80 Å porosity onto which C18 chains have been grafted. For very hydrophobic peptides a C1 graft is preferable.

The case of extremely hydrophilic or basic peptides must be considered separately: it is usual for them to be eluted in the dead volume of

the commercially available HPLC columns. Their purification requires the use of a different type of support: normal phase or a completely synthetic resin (capable of withstanding high pH values) must then be used and this leads us to the subject of ion exchange HPLC.
– (b) Peptides of high molecular weight. It is preferable to use column material of higher porosity, of the order of 300 Å, for peptides containing 15 to 100 amino acids. The graft employed may be C18, C10 or C4, the last-mentioned being preferred.

2.5.3.5.2. Ion exchange HPLC This type of chromatography has been developed only in recent years. The choice obviously lies between using weakly or strongly charged cationic or anionic resins. Grafted silica beads may also be used. The usual pitfall with this method lies in choosing a phase which retains the peptides too strongly: the peptide (or its impurities) is not eluted from the column even after numerous washings; subsequent chromatographies then become contaminated or the peptide remains on the column and is lost.

These synthetic resins are still so expensive that they are not very widely used.

2.5.4. Preparative HPLC

A choice has to be made between the purity required, the extent of recovery of the peptides, the time taken and the volume of solvent needed for the purification. The purification may be carried out in one passage through a column of large diameter or in several passages through a column of smaller diameter. This latter approach is by far the most widely used since it does not require large investments in equipment.

In preparative HPLC, the most important parameter is the selectivity (followed by the capacity of the column). The differences between the sizes of the particles used in the analytical columns and the preparative columns are such that the search for ideal conditions in analytical HPLC cannot justifiably be pushed to the limit before switching to preparative HPLC. The need to obtain high yields may lead to the employment of non-linear chromatography. In this case, the choice of support will be

different: in linear chromatography the form and (small) diameter of the particles are important; in non-linear chromatography, homogeneous distribution of the particles is of no importance, a condition which makes it possible to use columns of large diameter and less expensive supports.

The capacity of the grafted silica beads increases as the hydrocarbon surface (e.g., C18 increases the capacity by 20%).

The choice of solvent determines the way in which the purified peptide is subsequently treated: if the solvent is difficult to lyophilize, then the solution of purified peptide must first be desalted.

In cases where separation is particularly difficult, the sample must be chromatographed with recycling.

The duplication of peaks, often observed in preparative HPLC, is often the result of different rates of flow of the elution buffer at the centre and at the periphery of the column.

Future of preparative HPLC

This technique is gradually replacing Medium Pressure Preparative Chromatography (MPPC). However, the capacity of the columns remains far short of 1 meq/g of support.

2.6. Conclusions

Peptide synthesis = assembly of amino acids + cleavage from the resin + purification + HPLC analysis + amino acid analysis + possible mass spectrometry. This equation is once again intended to emphasize the fact that the assembly of the amino acids represents only a part of the total process of preparing a peptide. In this respect, peptide synthesis is fundamentally different from oligonucleotide synthesis, a fact of which molecular biologists are not always aware. On the other hand, peptide synthesis is relatively expensive, since it is generally admitted that each coupling step costs an average of US$ 20 for a synthesis of 0.3–0.5 mmole of peptide by the Boc procedure. Even though the use of an automated or semi-automated synthesizer can certainly facilitate considerably routine synthetic work, we believe that peptide synthesis requires the presence in the laboratory of a chemist highly qualified in this area.

2.6.1. Addendum

New protecting group for the guanidine function of arginine (see Table 2.1).

The 4-methoxy-2, 3, 6-trimethylbenzene sulfonyl (Mtr) group has hitherto proved to be the least unsatisfactory of the groups presently available for the protection of the guanidine function of arginine. Very recently, Ramage and Green (Tetrahedron Letters *28*, 2287, 1987) have suggested the 2,2,5,7,8-pentamethylchroman-6-sulfonyl group for this purpose; it seems to offer considerable promise since it is much more labile to acid than the Mtr group.

CHAPTER 3

Peptide–carrier conjugation

3.0. Introduction

Most authors agree that in order to produce antibodies against small peptides of M_r 700 to 1500, it is necessary to enhance their immunogenicity by coupling them to protein carriers (see Butler and Beiser, 1973). Furthermore, when short peptides are used as the immobilized antigen in solid-phase immunoassays it is also necessary to use peptide–carrier conjugates since peptides of 6 to 15 residues generally do not bind satisfactorily to plastic surfaces.

The different coupling procedures that have been used to prepare peptide–carrier conjugates are listed in Table 3.1. In many instances, the chemistry of these conjugation reactions has not yet been elucidated. The aim of this chapter is to describe the different techniques in such a way that the investigator is able to choose the most appropriate procedure for his particular peptide. The choice of conjugation procedure is important since the antigenic activity of a peptide may be drastically affected by different coupling procedures (Talamo et al., 1968; Pique et al., 1978; Briand et al., 1985).

The coupling of small molecules to carriers has many applications outside immunology (Widder and Green, 1985). Several comprehensive re-

views dealing with chemical modifications of proteins can be consulted to obtain information on the types of chemical intramolecular bridges that can be introduced into proteins (Glazer et al., 1975; Atassi, 1977a; Peters and Richards, 1977; Thorell and Larson, 1978; Kiefer, 1979; Erlanger, 1980; Feeney et al., 1982; Han et al., 1984).

Most procedures for preparing peptide–protein conjugates are based on the use of symmetrical or asymmetrical bifunctional reagents which

TABLE 3.1.
Principal reagents used for peptide–protein conjugation*

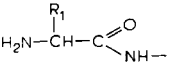
Coupling agents	Modified amino acid		References
	Primary reactions	Secondary reactions	
Glutaraldehyde	ε-NH_2, α-NH_2, SH-Cys	Tyr, His	Habeeb and Hiramoto (1968)
Bis imido esters	α-NH_2, ε-NH_2 Lys	negligible	Means and Feeney (1971)
BDB	Tyr, SH-Cys, His, ε-NH_2 Lys	Trp, Arg	Glazer et al. (1975)
Carbodiimides (ECDI, MCDI)	α-NH_2, ε-NH_2 Lys, α-COOH, Glu, Asp	Tyr, Cys	Goodfriend et al. (1964)
MBS	Cys-SH, NH_2 bridges	n.o.	Kitagawa and Aikawa (1976)
SPDP, MCS	Cys-SH, NH_2 bridges	n.o.	Carlsson et al. (1978); Lee et al. (1980)
Imido esters (2-iminothiolane)	Cys-SH, NH_2 bridges	n.o.	King et al. (1978)
IBCF	-COOH, NH_2 bridges	n.o.	Thorell and Larson (1978)
Toluene diisocyanate	α-NH_2, ε-NH_2 Lys	n.o.	Talamo et al. (1968)
p-nitrobenzoyl chloride	Tyr, His, SH-Cys, ε-NH_2 Lys	Trp, Arg	Anderer and Schlumberger (1965a)
p-amino phenyl acetic acid	α-NH_2, ε-NH_2 Lys	His, Tyr	Spirer et al. (1977)
cystamine dihydrochloride	SH-Cys	n.o.	Gilliland and Collier (1980)
EBIZ	α-NH_2, ε-NH_2 Lys, -COOH	n.o.	Likhite and Sehon (1967)

n.o. = Not observed.
* For abbreviations, see text, and list, p. vii.

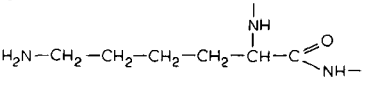
either become incorporated into the final conjugate or activate certain reactive sites of the carrier protein molecule for subsequent linkage with the peptide. During the coupling reaction inter- but also intramolecular bridging can take place. Several factors such as protein concentration, ratio of coupling agent to protein, ionic strength and pH govern the kind of linkage obtained in the final product. For example, at low concentration of reactants, unwanted intramolecular linking reactions often predominate.

TABLE 3.2.
Functional groups in proteins used in peptide-carrier conjugation

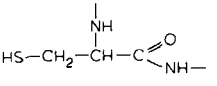
α-amino groups



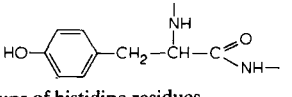
ε-amino groups of lysine residues



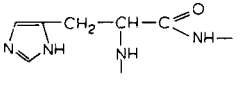
sulfhydryl groups of cysteine residues



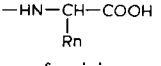
phenolic hydroxyl groups of tyrosine residues



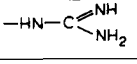
imidazole groups of histidine residues



α-carboxyl groups



guanido groups of arginine



Although a wide range of coupling reagents has been reported in the literature, nearly all of them react primarily with the ε-amino group of lysine and the nucleophilic thiol of cysteine and only secondarily with the imidazole group of histidine and the phenolic hydroxyl group of tyrosine (Tables 3.1 and 3.2). Only a few compounds such as bis-imido esters are group-specific protein reagents.

3.1. Choice of carrier

Carrier molecules are chosen on the basis of criteria such as availability of reactive sites, size, solubility, immunogenicity, commercial availability and cost. The most commonly used carriers are listed in Table 3.3. One of the most important parameters that determines the suitability of a particular carrier molecule is the solubility of the final conjugate, since this may influence the accessibility of antigenic sites. When using the same coupling agent and peptide, some carriers may give insoluble conjugates while others give rise to soluble conjugates. Certain carriers are

TABLE 3.3.
Principal carriers used for coupling peptides

Carriers [a]	Usual abbreviation	M_r, K	Number of groups				References [d]
			ε-NH_2	-SH	phenol	imidazole	
bovine serum albumin	BSA	67	59[b]	35	19	17	1
ovalbumin	–	43	20	4	10	7	2
myoglobin	–	17	19	0	3	12	1
tetanus toxoid	TT	150	106	10	81	14	3
keyhole limpet haemocyanin[c]	KLH	>2000	6.9	1.7	7.0	8.7	4

[a] Other carriers have also been used such as thyroglobulin (669 K), diphtheria toxoid, rabbit serum albumin, bovine or mouse gamma globulin (150 K), poly (L-lysine) (15–300 K), poly (L-glutamic acid) (15–100 K), dipalmityl lysine, Ficoll (40 K) (Talamo et al., 1968; Boyle et al., 1983; Hopp, 1984; Fok et al., 1982; Wheat et al., 1985; Lee et al., 1980).

[b] Only 30–35 of the 59 lysine residues of BSA are accessible.

[c] For KLH, the amino acid composition is expressed in g amino acid / 100 g.

[d] References: (1) Dayhoff, 1976; (2) Nisbet et al., 1981; (3) Bizzini et al., 1970; (4) Malley et al., 1965.

preferable when the peptide–carrier ratio is to be determined by amino acid analysis. Keyhole limpet haemocyanin (KLH), for instance, is not appropriate in this case since it has a high molecular weight and commercial preparations usually contain many impurities. In our laboratory we currently use bovine serum albumin (BSA) for preparing peptide conjugates intended for use as antigens, and ovalbumin for preparing conjugates to be used for immunization. When this combination of carriers is used, BSA can be included as blocking agent in immunoassays without interference by the anti-ovalbumin antibodies present in the antipeptide-carrier antiserum.

3.2. Optimal peptide density on carrier protein

Although it is possible to cover the surface of large carriers such as haemocyanin with as many as 200–500 peptide molecules, this is seldom necessary and may even be detrimental for certain studies. It was found, for instance, that in the case of DNP–BSA conjugates, a small number (in the range 5–20) of DNP ligands per mole of carrier was sufficient for obtaining a good anti-DNP immune response whereas higher levels of substitution induced a tolerance effect (Klause and Cross, 1974; Desaymard and Howard, 1975). A ratio of 5 to 20 mol of peptide per mole of carrier was found to be suitable for conjugates used either as immunogen or as antigen (Muller et al., 1986).

3.3. Point of attachment on peptide chain

For evident reasons, the coupling reaction should not affect the configuration of the amino acids that constitute the epitope. It is important therefore, to select a coupling reagent that will crosslink through specific groups situated preferably at either end of the peptide and away from the presumed location of the epitope. It is often advantageous during peptide synthesis to add at either end of the relevant sequence amino acids such as cysteine or tyrosine (see 3.4.4 to 3.4.7) that will serve as specific

anchoring points for coupling. The same type of residue should, of course, not already be present in the antigenic sequence.

Some authors prefer joining the peptide to the carrier via a spacer in order to enhance the accessibility of the peptide moiety and avoid harmful effects due to carrier proximity. A small number of glycine or alanine residues are commonly introduced as spacers (Emini et al., 1983; McMillan et al., 1983; Mäkelä and Seppällä, 1986).

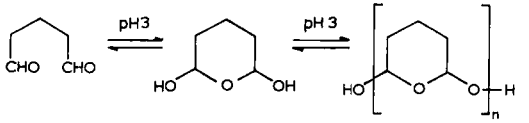
3.4. Coupling procedures

In the following section, the properties of the most commonly used coupling agents will be described.

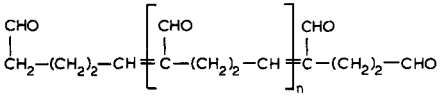
3.4.1. Glutaraldehyde

3.4.1.1. Reaction mechanism

Although glutaraldehyde is the most extensively used coupling reagent, the mechanism of reaction of glutaraldehyde with proteins is still not definitively established. Commercial preparations of aqueous solution of 25% glutaraldehyde contain unstable derivatives of the aldehyde in the form of compounds of variable molecular weight which easily revert to glutaraldehyde (Monsan et al., 1975). At acidic pH (the pH of commercial solution is 3.1) glutaraldehyde is in equilibrium with its cyclic hemiacetal and polymers of the cyclic hemiacetal :

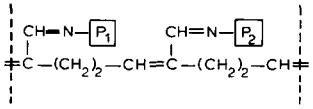


For the coupling reaction, the pH is raised to neutrality or to a slightly basic value (pH 7–8) and the dialdehyde is transformed in α, β-unsaturated aldehyde polymers :



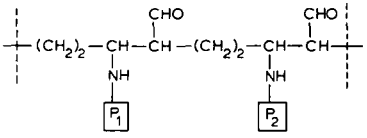
As the pH is raised, *n* increases until the polymer precipitates from solution (Peters and Richards, 1977).

At pH 7–8, the major reactive species (α, β-unsaturated aldehyde) appears to form a labile intermediate Schiff base with amino groups of the protein :



The resonance interaction of the Schiff base with the double bond is believed to lead to a stable final product. Another possible stabilization mechanism is a Michael-type addition which may occur at various sites when the local amine concentration is particularly high (Peters and Richards, 1977) :

e.g.:



The reaction of glutaraldehyde with proteins involves mainly lysine residues (4 mol glutaraldehyde react per mole of lysine; Korn et al., 1972) as well as the α amino group and sulfhydryl group of cysteine. Secondary reactions with phenolic and imidazole groups have also been described (see Table 3.1).

3.4.1.2. Coupling procedure (one-step method)

(a) Add peptide to a 1 mg/ml solution of carrier protein in phosphate buffered saline (PBS) pH 7.4 at a molar ratio of protein:peptide varying from 1:20 to 1:40. Cool at 4°C.

(b) Just before use prepare a 2% (v/v) glutaraldehyde solution in water (e.g. Serva, FRG, 23114 or Fluka, Switzerland, 49626).

(c) Add dropwise an equal volume (e.g. 1 ml) of 2% glutaraldehyde solution to the protein mixture with constant stirring.

(d) After 1 h stop the reaction by addition of sodium borohydride ($NaBH_4$) to a final concentration of 10 mg/ml. Keep 1 h at 4°C.

(e) Dialyse against PBS (3 changes) and store the conjugate at 4°C in presence of 0.02% sodium azide or in aliquots at −20°C.

Note

This method is particularly adapted to peptides which do not contain internal lysine or cysteine residues. By using 0.05% glutaraldehyde instead to 1%, active conjugates have also been prepared with certain basic peptides. However, the coupling yield then drops from 70–80% to about 20% and the conjugate stability is decreased (Briand et al., 1985).

Applications

Different coupling methods using glutaraldehyde have been successfully employed, for example with hormones (Reichlin, 1980), peptides of the large tumour antigen of simian virus 40 (Walter et al., 1980) and a $(Gly)_4$ peptide (Escribano, 1974).

3.4.2. Bisimido esters

3.4.2.1. Reaction mechanism

Bisimido esters are very soluble in water and react under mild conditions (pH 7–10) with a high degree of specificity with α- and ε-amino groups. The side reactions with thiol, phenolic, carboxyl, imidazolyl and guanidyl groups are negligible (Peters and Richards, 1977) (Table 3.1). The resulting amidine is stable and resists total acid hydrolysis. Most of the bisimido esters are commercially available in highly purified form (Pierce, USA or Merck, FRG) (Table 3.4). The reaction of imido esters can be summarized as follows (Hartman and Wold, 1966):

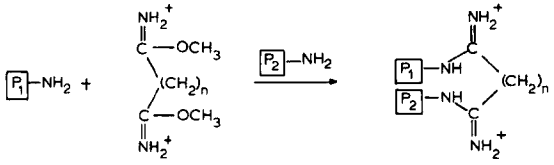
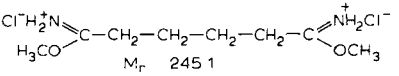
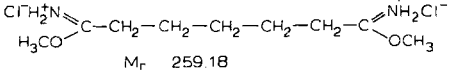
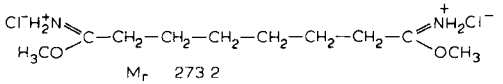
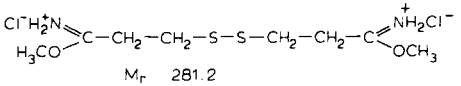


TABLE 3.4.
Principal bisimido esters used in coupling methods

Dimethyl adipimidate (DMA)	 M_r 245.1
Dimethyl pimelimidate (DMP)	 M_r 259.18
Dimethyl suberimidate (DMS)	 M_r 273.2

Dimethyl 3,3'-dithiobispropionimidate (DTPB)


M_r 281.2

3.4.2.2. *Procedure used in the case of dimethyl suberimidate (one-step method)*

(a) Add peptide to a 1 mg/ml solution of BSA in 0.2 M triethanolamine hydrochloride pH 8.5 (molar ratio protein:peptide = 1:20 to 1:30).

(b) Just before use, dissolve dimethyl suberimidate (DMS) in the same buffer at 0°C and within 30 sec add it to the peptide–protein mixture (molar ratio DMS:peptide = 3:1).

(c) After 3 h incubation at 22°C, the mixture is dialyzed against PBS (3 changes). The reaction need not be stopped as the reagent is largely consumed after 3 h.

(d) Store the conjugate at 4°C in presence of 0.02% sodium azide or in aliquots at −20°C. This procedure gives a coupling efficiency around 40–70% (6 to 13 mol peptide/mole of carrier).

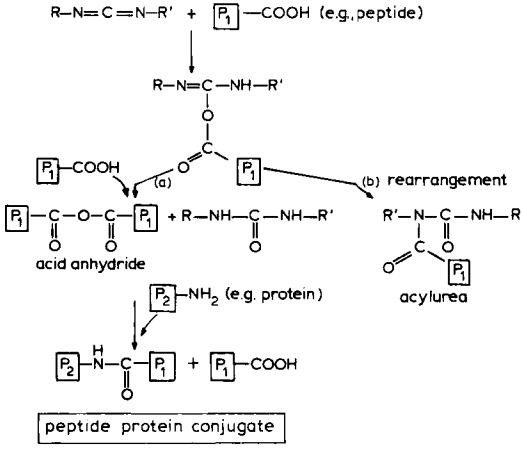
Applications

- Cross-linking of pancreatic ribonuclease to DNA (Hartman and Wold, 1966).
- Binding of multivalent immune complexes to Fc receptors (Dower et al., 1981).
- Several other applications are listed by Han et al. (1984).

3.4.3. Carbodiimides

3.4.3.1. Reaction mechanism

Carbodiimides comprise a group of compounds whose general formula is R − N = C = N − R′ (Table 3.5). R and R′ are aliphatic or aromatic groups. The mechanism of the reaction is not yet fully understood. It is postulated that an intermediate product is formed that can react through two possible pathways, the desired one being obtained at lower temperature (see pathway (a) below) by reaction between the intermediate product and amino groups (Goodfriend et al., 1964; Bauminger and Wilche , 1980).

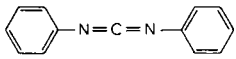
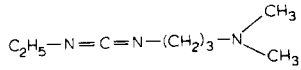
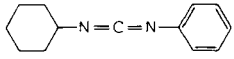
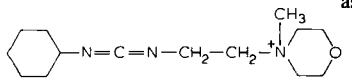


In addition to reacting with α and ε amino groups and carboxyl groups, carbodiimides also react with sulfhydryl and phenolic hydroxyl groups (Table 3.1). The tyrosine can be regenerated by treatment of the derivative with 0.5 M hydroxylamine at pH 7, 25°C for 5 h (Glazer et al., 1975).

3.4.3.2. Procedure (Goodfriend et al., 1964)

(a) Dissolve peptide in 1 ml of distilled water and add water-soluble 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (ECDI) (Pierce,

TABLE 3.5.
Principal carbodiimides employed for coupling methods

diethylcarbodiimide	$C_2H_5-N=C=N-C_2H_5$	symmetrical, aliphatic
diphenylcarbodiimide		symmetrical, aromatic
ethylpropylcarbodiimide	$C_2H_5-N=C=N-C_3H_7$	asymmetrical, aliphatic
1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (ECDI)		asymmetrical, aliphatic
cyclohexylphenyl-carbodiimide		asymmetrical, aromatic
1-cyclohexyl-3-(2-morpholinyl-4-ethyl)-carbodiimide methyl-*p*-toluenesulfonate (MCDI)		asymmetrical, aliphatic

USA, 22980) or 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide methyl- *p*-toluenesulfonate (MCDI) (Aldrich, FRG, C10640-2) to a final concentration of 10 mg/ml with constant mixing. Allow to stand 5–10 min at room temperature with gentle agitation.

(b) Add carrier protein to the mixture at a final protein:peptide molar ratio of 1:20 to 1:40. Stir for 2 h at room temperature.

(c) Dialyze for 24 h at 4°C against 2–3 changes of PBS (1 litre). Store at 4°C in presence of sodium azide (0.02%) or in aliquots at −20°C.

Notes

(i) The carbodiimide should be as fresh as possible.

(ii) Using the above method a coupling yield of 40–70% is generally obtained. According to Staros et al. (1986) the efficiency may be further enhanced by the addition of 5 mM *N*-hydroxysulfosuccinimide (e.g. Pierce, USA, 24510 or Fluka, Switzerland, 56485).

(iii) In order to avoid modifications of amino groups in the peptide, the latter can be pretreated with citraconic anhydride as described in Section 3.5. In this case, the coupling is performed at pH 8. Follow-

ing the reaction with carbodiimide (step b), the citraconylated amino groups are deprotected by dialysis against 5% acetic acid. Finally the product is dialysed against PBS as in step c.

Applications

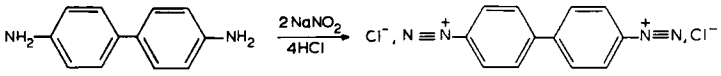
The method has been used successfully with hormones and peptides of cytochrome *c* (Likhite and Sehon, 1967), peptides of luteinizing hormone-releasing hormone (LH–RH) (Pique et al., 1978), hydroxyl–steroids and prostaglandins (Clausen, 1981), $pp60^{scr}$ peptides of Rous sarcoma virus (Tamura et al., 1983) and peptides of influenza haemagglutinin (Shapira et al., 1984).

3.4.4. Bis diazobenzidine

3.4.4.1. Reaction mechanism

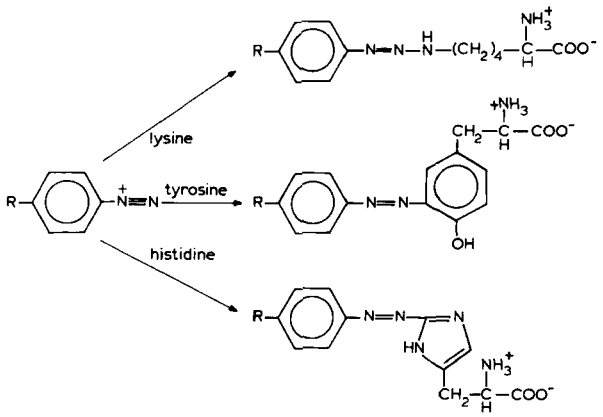
Bis diazobenzidine (BDB) reacts through its diazonium functional groups with the phenolic hydroxyl group of tyrosine, the imidazole group of histidine, the sulfhydryl group of cysteine and primary α and ε amino groups (Table 3.1). Secondary reactions involving guanido groups of arginine and indole groups of tryptophan have also been observed.

Activated BDB is obtained by treatment of benzidine with nitrous acid ($NaNO_2$ and HCl) :



Activated BDB then reacts with different groups in protein molecules (Glazer et al., 1975). In the case of tyrosine, lysine and histidine, the products shown on page107 are formed.

Diazo derivatives are unstable at low pH and can be stored at 0–5°C only for a short period.



3.4.4.2. Procedure (Tamura and Bauer, 1982; Briand et al., 1985)

(a) Preparation of BDB: dissolve 3.5 mg $NaNO_2$ in 1 ml of benzidine (5 mg/ml; Sigma B 3383) in 0.2 M HCl at 4°C with constant agitation. Stir at 4°C for 2 h. Use precautionary measures because of the carcinogenic properties of benzidine. Aliquots can be stored at −60°C for more than one year.

(b) Dissolve 2.5 mg of carrier protein in 10 ml borate buffer pH 9.0 ($Na_2B_4O_7$ 0.16 M–NaCl 0.13 M) and add the peptide in a molar ratio protein:peptide of 1:30 to 1:40. Cool on ice.

(c) Add dropwise 0.1 ml of activated BDB to the mixture. The solution turns dark brown. Stir continuously for 2 h at 4°C. During this time adjust the pH to 9.0 with 0.5 M NaOH. The mixture will turn yellow with time.

(d) Dialyse against PBS and store at −20°C.

Notes

(i) As for carbodiimide coupling, amino groups of peptide can be blocked first by citraconylation (Section 3.5). In this case, coupling will occur mainly through tyrosine.

(ii) Sometimes a precipitate forms during the dialysis step at 4°C (step d). This can be counteracted by decreasing the time BDB is allowed to react with the peptide protein mixture (e.g. 1 h instead of 2 h);

however, the coupling yield will then be decreased.

(iii) A coupling yield of 45–60% is usually obtained. Since the conjugate is very unstable at 4°C, it is important to check the peptide:carrier ratio after storage (see Section 3.8). In some cases, it may be preferable to prepare fresh conjugate each time an animal has to be immunized.

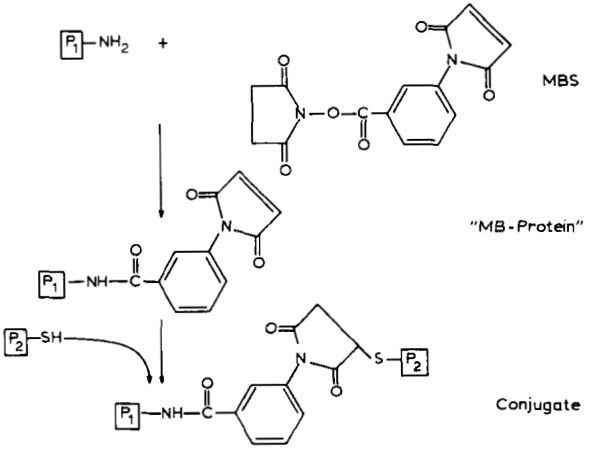
Applications

The method was used successfully with peptides of LH-RH (Pique et al., 1978), simian virus 40 (Harvey et al., 1982), $pp60^{scr}$ protein (Tamura and Bauer, 1982), poliovirus (Emini et al., 1983) and interferon (Russell et al., 1986).

3.4.5. m-Maleimido benzoyl-N-hydroxysuccinimide ester (MBS)

3.4.5.1. Reaction mechanism

This is the best known hetero-bifunctional reagent (Table 3.1). In neutral aqueous solution, MBS reacts first by acylation of amino groups via the active *N*-hydroxysuccinimide ester followed by formation of a thioether bond through addition of a thiol group to the double bond of the maleimide (Kitagawa and Aikawa, 1976) :



The linkage proceeds via two separate reactions, thus avoiding formation of bonds between identical molecules. The method can be applied for instance to peptides that contain a cysteine residue or in which an –SH group has been introduced by thiolation. Numerous procedures for introducing thiol groups in proteins have been described (White, 1972; Kitagawa and Aikawa, 1976; Carlsson et al., 1978; Imagawa et al., 1982; Duncan et al., 1983).

Alternatively, an additional cysteine can be incorporated during peptide synthesis or a thiol group can be introduced at the N-terminal end of the peptide during solid-phase synthesis, using the reagent 5-acetylthioglycolic acid (Presentini et al., 1986). The *S*-acetyl protecting group was found to be stable to HF during deprotection and cleavage of the peptide from the resin. Conjugates could be prepared with maleimidated protein carriers by deprotecting the thiolic group with aqueous hydroxylamine at the time of reaction with the carrier.

3.4.5.2. Procedure (two-step method) (from Liu et al., 1979, modified by Green et al., 1982)

(a) Dissolve carrier protein in 250 µl PBS pH 7.4 and add 100 µl MBS (Pierce, USA, 22310) solution (10 mg/ml) in dimethylformamide (DMF) (final molar ratio of protein to MBS of 1:40). Stir for 30 min at room temperature.

(b) Remove the excess of MBS by filtration through a Sephadex G-25 column (14 × 0.9 cm) equilibrated with 50 mM phosphate buffer pH 6.0. Wash the gel with the same buffer. Monitor by recording absorbance at 280 nm.

(c) Pool the fractions containing activated carrier protein ('MB-protein') and immediately allow to react at pH 7–7.5 with peptide in a molar ratio peptide:protein of 30–40:1.

(d) After 3 h reaction at room temperature with constant stirring, dialyse the mixture against PBS. Store aliquots at −20°C or at 4°C in presence of sodium azide (0.02%).

Notes

(i) A coupling yield of 30 to 50% is usually obtained. The efficiency is strongly dependent on availability of thiol groups. Preliminary trials under different pH conditions (such as PBS pH 7.5, borate buffer pH 9.0, acetate buffer pH 4.0) may be necessary to determine the optimal conditions linked to the particular environment of -SH groups in a peptide (Green et al., 1982).

(ii) Although MBS is the best known *N*-hydroxysuccinimido ester used for peptide–protein coupling, other compounds of this type have also been successfully applied in immunochemical studies (Table 3.6). For instance 6-maleimidocaproic acyl *N*-hydroxysuccinimide ester (MCS) has frequently been used in a one-step procedure (Lee et al., 1980; Clark et al., 1985; Wheat et al., 1985; Stevens et al., 1986a). Under appropriate conditions, MCS can react specifically with amino groups on the carrier and with thiol groups on peptides.

Applications

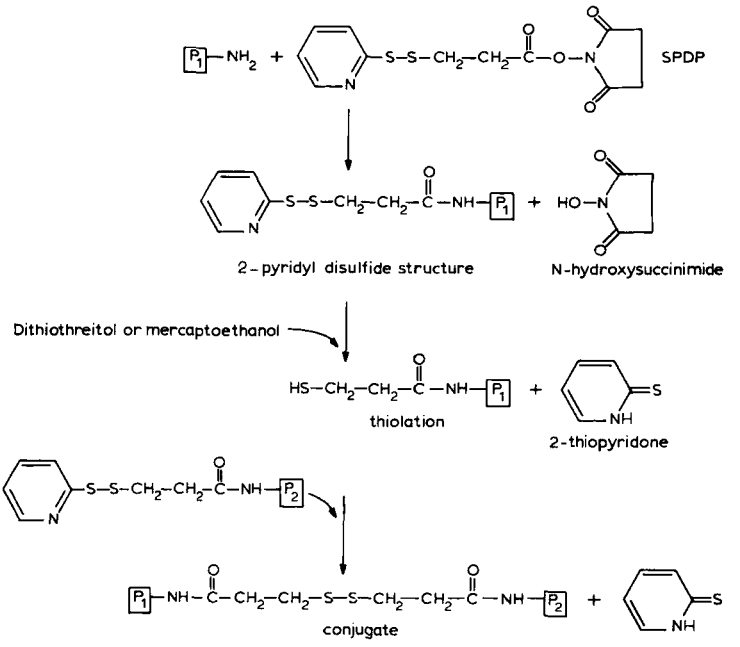
Peptides of hepatitis B (Lerner et al., 1981), influenza haemagglutinin (Green et al., 1982), murine cμ chain (Ghose and Karush, 1985), hormone fragments (Antoni et al., 1985), HTLV-III virus (Kennedy et al., 1986), SV40 large T antigen (Goldfarb et al., 1986) and a *ras* oncogene product (Wong et al., 1986).

3.4.6. N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP)

3.4.6.1. Reaction mechanism

SPDP is a hetero-bifunctional reagent (Table 3.6) which, under mild conditions, reacts by its NHS-ester group with amino groups of the protein. The 2-pyridyl disulfide structure then reacts with aliphatic thiols (e.g. -SH of peptide) via a thiol-disulfide exchange reaction (shown on page 111).

SPDP was first synthesized by Carlsson et al. (1978) and is now commercially available. The coupling reaction is efficient and proceeds rapidly at pH 5–9. The degree of substitution can be monitored spectrophotometrically. No side reactions have been described.



3.4.6.2. Procedure (two-step method) (Granier, Bahraoui and Van Rietschoten, 1988)

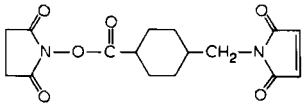
(a) Dissolve 66 mg (1 μmol) BSA in 3 ml 100 mM phosphate buffer, 100 mM NaCl, pH 7.5. Add 6.5 mg SPDP (Pharmacia, Sweden) suspended in 1 ml 2-propanol and allow to incubate during 30 min at room temperature.

(b) Collect activated BSA by filtration through a 2.5 × 100 cm Biogel P2 column (Bio-Rad, USA) equilibrated and washed in 50 mM ammonium acetate buffer, pH 8.5. Pool fractions (identified by monitoring at 280 nm).

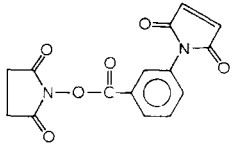
(c) The number of thiopyridyl groups introduced in BSA is estimated spectrophotometrically (Grassetti and Murray, 1967): transfer 1 ml of fractions containing modified BSA in a spectrophotometer cuvette

TABLE 3.6.
Principal *N*-hydroxysuccinimide esters used for coupling

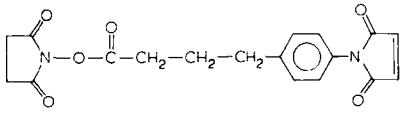
Succinimidyl 4-(*N*-maleimidomethyl)cyclohexane 1-carboxylate (SMCC), M_r 334.3



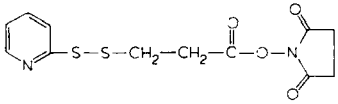
m-maleimidobenzoyl *N*-hydroxysuccinimide ester (MBS), M_r 314.2



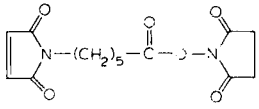
Succinimidyl 4-(*p*-maleimidophenyl) butyrate (SMPB), M_r 356.3



N-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), M_r 312.4



6-maleimidocaproic acyl *N*-hydroxysuccinimide ester (MCS), M_r 308



and add 200 µl of 50 mM mercaptoethanol in 100 mM phosphate buffer, pH 7. Measure absorbance at 343 nm before and after addition of mercaptoethanol. Evaluate the quantity of thiopyridone liberated (this is directly proportional to introduced thiopyridyl groups) using $A_{343nm} = 8000\ M^{-1}cm^{-1}$. In standard conditions described above, 7–10 µmol thiopyridyl groups are generally introduced per µmole of BSA. If more thiopyridyl groups are introduced, BSA tends to precipitate.

(d) A synthetic peptide containing a cysteine residue is allowed to react with 8-fold excess of 2,2′-dipyridyl disulfide. The peptide is then purified by gel filtration as *S*-thiopyridyl derivative.
(e) Before conjugating the peptide to BSA, the cysteine thiol group is activated with tributyl phosphine (Merck, FRG; Rüegg and Rudinger, 1977): in a glass tube, introduce 10 μmol of peptide dissolved in 1 ml 0.1 M Tris, pH 8 and 10 μmol of tributyl phosphine in 1 ml 2-propanol. Keep the mixture during 20 h under stirring in the dark. Tris buffer and propanol must be saturated with nitrogen and the reaction must be carried out under nitrogen atmosphere.
(f) Add deprotected peptide to 10 ml activated BSA solution (7–10 μmol thiopyridyl groups) in 0.1 M Tris, pH 8. Stir during 1 h. The efficiency of coupling can be controlled by measuring the amount of thiopyridone liberated during this incubation.
(g) Stop the reaction by freezing. Lyophilize and redissolve in 2 ml of 50 mM ammonium acetate buffer, pH 8.5. Apply to a Bio-Gel P2 column for filtration and wash in the same buffer. Collect fractions that absorb at 280 nm. Pool and store at −20°C until use. Under these conditions the coupling efficiency is usually 5–6 mol of peptide per mole of BSA.

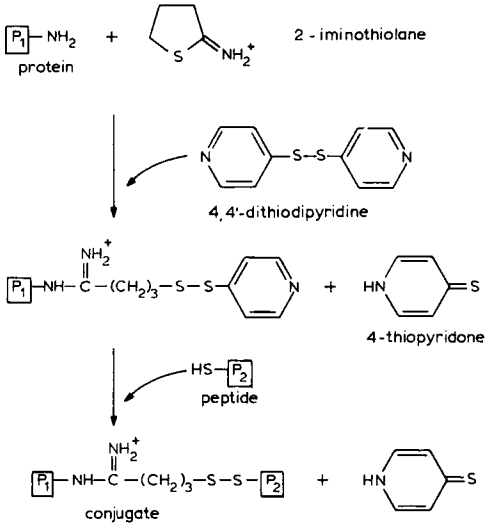
Applications
- Peptides of calmodulin (Van Eldick et al., 1983), scorpion toxin (Bahraoui et al., 1986), thymosin α1 (Incefy et al., 1986) and of foot and mouth disease virus protein (Di Marchi et al., 1986).
- Further information is available in the booklet 'SPDP, hetero-bifunctional reagent', published by Pharmacia, Sweden.

3.4.7. Imidoesters: 2-iminothiolane or 2-iminotetrahydrothiophene

3.4.7.1. Reaction mechanism

Unlike most imidoesters, 2-iminothiolane is very stable in solution at acidic and neutral pH. This compound, also known as Traut's reagent, reacts first with primary amino groups in the presence of 4,4′-dithiodipyridine leading to an average of 1 to 8 thiols or 4-dithiopyridyl

groups per mole of protein. The final conjugate contains an intramolecular disulfide bond. The reaction scheme can be simplified as follows (from King et al., 1978):



The required thiol group in P_2 can also be introduced as described in 3.4.5.1.

3.4.7.2. Procedure (one-step method)

The following method is a modification from that of King et al. (1978).

(a) Dissolve 1 mg BSA in 0.45 ml borate buffer 25 mM, pH 9, and add successively 0.1 ml 4,4'-dithiodipyridine (Sigma D 8136) at 2.2 mg/ml in acetonitrile (CH_3CN) and 0.45 ml 20 mM 2-iminothiolane (Sigma I-6256) in borate buffer, pH 9. Stir for 2 h at room temperature. Note that precipitation is often observed.

(b) For introducing sulfhydryl groups in the peptide, dissolve peptide (in a molar ratio carrier:peptide of 40) in 0.5 ml 25 mM borate buffer, pH 9, and add 2-iminothiolane in a 200 molar excess compared to α-NH_2 groups contained in the peptide. Stir for 2 h at room temperature.

(c) Mix activated BSA and thiolated peptide and add 0.5 ml phosphate buffer 4 mM EDTA, pH 8; leave to react overnight at room temperature keeping the reaction vessel under nitrogen.
(d) Apply to a Sephadex G100 column (60 × 1.2 cm) equilibrated in PBS, pH 7.4, and wash with the same buffer. Collect fractions that absorb at 280 nm. The first peak corresponds to conjugated (and unconjugated) BSA. Pool and store at 4°C or −20°C. The coupling efficiency is usually 6 to 10 mol of peptide per mole BSA.

Notes

(i) Prepare stock solution of 2-iminothiolane just before use.
(ii) The extent of BSA substitution may be estimated before peptide coupling by the spectrophotometric method described by Van Eldik and Lukas (1986).

Applications

Calmodulin peptides (Van Eldick et al., 1983).

3.4.8. Other coupling agents

In addition to the procedures described above, the following reagents have also been used for preparing peptide–carrier conjugates (see Table 3.1).

Toluene diisocyanate which only affects primary amino groups of the protein (Talamo et al., 1968; Wold, 1972).

p-nitrobenzoyl chloride which converts aliphatic amino groups of the peptide to a *p*-nitrobenzoylamide; the latter is then reduced to a *p*-aminobenzoyl derivative and coupled to proteins by diazotization (see 3.4.4.1 for description of the primary and secondary reactions observed with azo coupling). This method was used by Anderer and Schlumberger (1965a) with a series of C-terminal peptides of TMVP and by Deodhar (1960) with angiotensin.

p-aminophenylacetic acid derivatives (Jacob et al., 1985) which link the peptide to carrier protein via an azo bond (see also Rojo et al., 1986).

Cystamine dihydrochloride (Gilliland and Collier, 1980; Johnson et al., 1982) which acts by the formation of disulfide bonds.

N-ethylbenzisoxazolium fluoroborate (EBIZ) which links peptide to carrier protein via an amido bond. This procedure was successfully used by Likhite and Sehon (1967) for angiotensin.

Isobutylchloroformate (IBCF) which has been extensively used for coupling a variety of carboxyl group-containing peptides to proteins. The reaction proceeds in two steps: first, the peptide is conjugated to IBCF in the presence of a tri-*N*-alkylamine resulting in the formation of a mixed anhydride. The mixed anhydride is then added to the protein solution where the peptide reacts with free amino groups, usually lysine side chains, linking the peptide to protein via an amido bond (Thorell and Larson, 1978; Samokhin and Filimonov, 1985; Gendloff et al., 1986).

3.4.9. Photochemical coupling

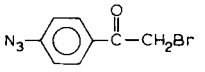
3.4.9.1. Mechanism

Since the introduction of aryl azides as a photoreactive cross-linking functional group by Fleet et al. (1969), several of these reagents have become commercially available (Pierce, USA). The main photochemical reagents have been listed by Han et al. (1984) (see also Table 3.7). Photoreactive cross-linking reagents generally involve alkyl or aryl azides ($R\text{-}N_3$) which can be photolysed to a highly reactive nitrene intermediate at 290 nm and 350 nm respectively. The latter then reacts with various bonds such as C-H or N-H (Peters and Richards, 1977). Photoprobes which have the advantage of being inert until photolysis are able to link peptides to any accessible residue of the carrier. Using aryl azides no direct photochemical damage is observed in the protein when an intense light pulse is applied at 350 nm. In this respect aryl azides ap-

TABLE 3.7.
Photochemical reagents used for coupling

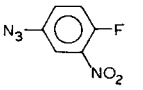
Photoreactive aryl nitrenes:

p-azidophenacyl bromide



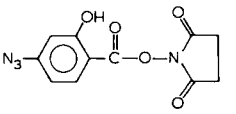
M_r 240.0

4-fluoro-3-nitrophenyl azide



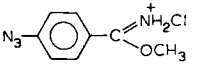
M_r 182.1

N-hydroxysuccinimidyl-4-azidosalicylic acid



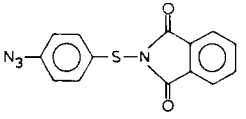
M_r 272.2

methyl-4-azidobenzoimidate·HCl



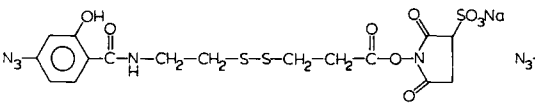
M_r 212.6

N-(4-Azidophenylthio)phthalimide



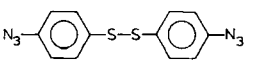
M_r 296.3

sulfosuccinimidyl 2-(p-azidosalicylamido)ethyl 1,3'-dithiopropionate



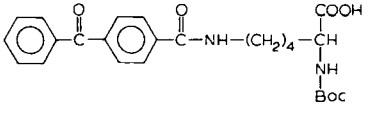
M_r 541.51

4,4'-Dithiobisphenylazide



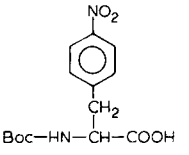
M_r 300 3

Benzophenone derivative



Boc BB Lys

p-nitrophenylalanine derivative



Boc-p-NO_2-Phe

pear to be more appropriate than alkyl azides since modifications can be generated in proteins at 290 nm. The half-life of the nitrene is of the order of a millisecond.

Many applications and experimental details concerning covalent binding by photochemical reagents have been discussed by Han et al. (1984). The method has been found useful for cross-linking macromolecular assemblies such as hormone–receptor complexes, ribosomes etc.

A drawback in using reagents such as *p*-azidobenzoate as photoprobes is that active nitrene intermediates are generated which react strongly with the aqueous solvent. In 1973, Galardy et al. introduced aromatic ketones as a new photochemical probe. Reagents such as benzophenone are characterized by a triplet state which apparently does not react with water, does not rearrange or react intramolecularly, and is easily generated at long wavelengths (>320 nm). Recently Parker and Hodges (1985) used such benzophenone photoprobes for linking peptides to a protein carrier. The photoprobes are directly coupled to the protected peptide attached to the solid-phase resin, using the conditions employed for amino acid coupling (see chapter 2). The benzophenone species (BB

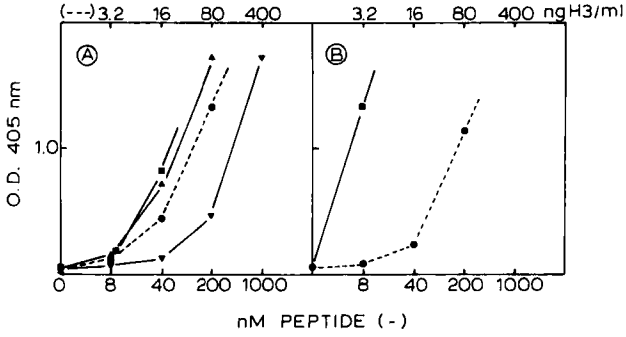


Fig. 3.1. Binding in ELISA of antibodies to H3 (A) and to peptide 130–135 H3 (B) with various concentrations of H3 (B) and various conjugates of peptide IRGERA– carrier protein. Sera were diluted 1:2000 and allowed to react with histone H3 (●), conjugate IRGERA–BSA (19:1) prepared with glutaraldehyde (▲), conjugated IRGERA–BSA (■) and IRGERA–ovalbumin (▼) (with a molar ratio peptide: protein of 4:1 and 2:1, respectively) prepared by photochemical coupling. Antiserum to peptide 130–135 H3 (B) was raised by injecting peptide–ovalbumin conjugate obtained with glutaraldehyde. Background values detected with normal rabbit serum were less than 0.1. Substrate hydrolysis time was 45 min.

Gly or BB Lys, see Table 3.7) and *p*-nitrophenylalanine (*p*-NO_2-Phe) are not affected by the hydrofluoric acid used for deprotection and cleavage of the peptide from the resin support. The peptide probes are then photolytically coupled to the carrier at 350 nm as described below. Photolysis can be performed in 8 M urea solution in a pH range of 2.5 to 8.5. The coupling efficiency obtained by Parker and Hodges (1985) was around 50%, and conjugates containing up to 72 mol of peptide per mole of BSA have been described (Worobec et al., 1983). This procedure avoids potential modifications of functional groups in the peptide. Furthermore, since the photoprobe is inert until photolysis, the synthetic peptide probe can be purified by HPLC before cross-linking to the carrier molecule.

The conditions used in our laboratory for coupling BB Gly-IRGERA (C-terminal hexapeptide of histone H3) to BSA and ovalbumin are described below. The reactivity of this conjugate was tested in ELISA using anti-peptide antibodies and anti-H3 antisera (Fig. 3.1) while the IRGERA–ovalbumin conjugate was used to raise specific antisera in rabbits (see chapter 4, Fig. 4.5).

3.4.9.2. Procedure

(a) BB Gly IRGERA was synthesized according to Parker and Hodges (1985) and purified by filtration on a G15 Sephadex column.
(b) Dissolve 10.75 mg ovalbumin (0.25 μmol) in 450 μl distilled water and add 50 μl 1 M KH_2PO_4 buffer pH 6.8.
(c) Dissolve 2.41 mg BB Gly peptide (2.5 μmol) in 500 μl 0.1 M KH_2PO_4 buffer, pH 6.8. In the case of very hydrophilic BB peptides it is possible to use as much as 10 μmol of the peptide without causing precipitation.
(d) Mix the two solutions in a 1 ml quartz cuvette equilibrated at 10°C and containing a small magnetic stirrer.
(e) Photolysis is carried out at 350 nm for 1 1/2 h (at 10°C) under stirring using a Xenon lamp (Photolysis system, Müller Optic, FRG) with a Karatos monochromator condensor. Light intensity is checked by a Kipp and Zohnen thermopile.
(f) Dialyse the mixture for 24 h at 4°C against 3 changes of 1 litre PBS.

Using these conditions 2 mol of BB IRGERA were coupled to 1 mol of ovalbumin. The coupling efficiency was 20%.

Notes

(i) Conjugates were stable for at least 2 months at 4°C.
(ii) The method is particularly adapted to hydrophilic peptides which are often difficult to couple, without chemical damage, by means of classical reagents.
(iii) The main limitation of the method is that the hydrophobic character of benzophenone decreases the solubility of neutral and hydrophobic peptides with the result that it may be difficult to handle some peptide probes.

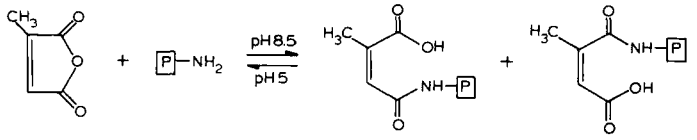
Applications

The method was used successfully with a large number of macromolecular ligand-receptor systems (see for example Ji and Ji, 1982) as well as with apamin, a 2000 M_r peptide purified from bee venom (Seager et al., 1986). Photochemical linking of primary aromatic amines to carrier proteins has also been described (Pandey et al., 1986).

3.5. Reversible protection of amino groups with citraconic anhydride

Several coupling methods lead to the random, indiscriminate linking of any amino or carboxyl groups of the peptide to the carrier molecule, with the result that the antigenic and immunogenic properties of the peptide are often extensively modified.

It may be useful, therefore, to protect certain residues of the epitope during the coupling reaction. Treatment with citraconic anhydride is a particularly suitable method for protecting amino groups in a reversible fashion, thereby preserving the antigenic properties of peptides (Atassi and Habeeb, 1972; Briand et al., 1985).



Citraconic anhydride reacts with α and ε amino groups to give two equally stable reaction products as well as with sulfhydryl groups and hydroxyl amino groups. Phenolic hydroxyl groups seem to be unaffected (Atassi and Habeeb, 1972).

3.5.1. Procedure

(a) Dissolve the peptide in Hepes buffer 50 mM, pH 8.5 (0.2 mg/ml).
(b) Add citraconic anhydride (Merck, FRG, 8013 63 or Sigma, USA, C 2395) in a molar ratio of peptide amino groups:citraconic anhydride of 1:10. Adjust pH continuously to 8.5–9 with 1 M NaOH. Repeat the addition until pH remains constant to 8.5–9. When pH remains stable, add 100 µl citraconic anhydride. If the pH does not change, the reaction has gone to completion. Otherwise readjust with NaOH and repeat the addition of citraconic anhydride.
(c) Incubate the mixture for 1 h at room temperature under slow stirring.
(d) Proceed with actual coupling experiment (see Section 3.4.3 or 3.4.4).
(e) Deprotect masked residues by dialysis against 1 litre 5% acetic acid for 3 h at 4°C.
(f) Dialyse for 24 h at 4°C against 3 changes of 1 litre PBS.

3.6. Coupling of peptides to liposomes

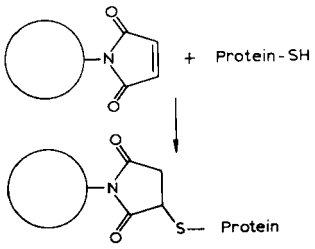
3.6.1. Reaction mechanism

In recent years, liposomes have gained recognition as potential carriers for various antigens, e.g. haptens, proteins and peptides, in eliciting antibody formation. Liposomes are vesicles prepared from phospholipids; they may differ in size (0.1 to 10 nm), their composition (one or several phospholipids, with or without cholesterol), their charge and their struc-

ture (uni- or multilamellar). All these variables control liposomal adjuvanticity and their importance in the immune response will be addressed later (chapter 4). The present section is concerned only with the preparation of liposomes.

Numerous techniques of liposome preparation, including freeze-dried forms, are described in the literature (for reviews see for example Szoka and Papahadjopoulos, 1980; Alving and Richards, 1983). Different methods have been described for attaching the antigen to the liposome. The antigens can be entrapped in the aqueous compartment of the vesicles (i.e., encapsulated antigen) or can be covalently bound to the outer surface of the liposomes. Although available data are not extensive, it seems that in some cases the association of the antigens to the liposome surface resulted in a marked increase of the immunoadjuvant effect (Alving and Richards, 1983; Van Rooijen and Van Nieuwmegen, 1983; Allison and Byars, 1986).

Encapsulation of the antigens is achieved by preparing the vesicles in the presence of antigen, for example by using the 'reverse phase evaporation' technique (Szoka and Papahadjopoulos, 1978). Methods used for the covalent coupling of proteins or peptides to liposomes have recently been reviewed (Heath and Martin, 1986). Several techniques are available which involve azo-coupling (Snyder and Vannier, 1984), reagents such as glutaraldehyde (Torchilin et al., 1979), and carbodiimides (Endoh et al., 1981; Neurath et al., 1984a). The conjugation occurs between the amino-group of phosphatidylethanolamine (PE), incorporated in the preformed liposomes, and an appropriate functional group of the antigen. Coupling can also be obtained by reacting a protein with an aldehyde, generated by IO_4-oxidation of a glycolipid present in the liposome, followed by the Schiff base reduction with a borohydride (Heath et al., 1981). Derivatized proteins or peptides with fatty acids can also be incorporated into the bilayers of the liposomes during their formation (Huang et al., 1980). A general scheme for ligand coupling to preformed liposomes can be summarized as follows:



It involves the incorporation of MPB–PE, obtained by reaction of PE and the bifunctional reagent succinimidyl 4-(*p*-maleimidophenyl) butyrate (SMPB) into liposomes (Martin and Papahadjopoulos, 1982). The maleimide group reacts, under very mild conditions, with a thiol residue present or introduced in the antigen. A similar technique has been developed (Leserman et al., 1980) in which PE was derivatized with another bifunctional reagent, SPDP; in this case a thiol function of the ligand performs a thiol exchange reaction and becomes linked to the liposomes through an -S-S- bond (Huang et al., 1980; Goldmacher, 1983). Recently, the use of succinimidyl-*S*-acetylthioacetate was found convenient for the thiolation of proteins in view of their coupling to liposomes (Derksen and Scherphof, 1985).

3.6.2. Procedure (Frisch et al., 1988)

In this section we describe the encapsulation of a synthetic model peptide (CGIRGERA) into large unilamellar liposomes and its coupling to preformed vesicles. The coupling involves the reaction between the thiol function of its cysteine residue with the maleimido group of a derivatized phosphatidylethanolamine (MPB-PE).

3.6.2.1. Synthesis of N-(4-(p-maleimidophenyl)butyryl)phosphatidylethanolamine (MPB-PE)

MPB-PE was synthesized according to the method of Martin and Papahadjopoulos (1982).

100 μmol of PE (transesterified egg phosphatidylcholine, Sigma) were

dissolved in 5 ml of anhydrous methanol containing 100 μmoles of triethylamine and 50 mg of SMPB (Pierce). The mixture was kept under argon at room temperature and the reaction progress was monitored by thin-layer chromatography (TLC) on silica gel 60-F_{254} (Merck) eluted in a chloroform/methanol/water (65:25:4, v/v/v) system. The reaction was essentially complete after about 2 h. Methanol was evaporated under vacuum and phospholipids were redissolved in 5 ml of chloroform. A double extraction with a saline (1% w/v NaCl in water) was then required to eliminate water-soluble byproducts and unreacted SMPB. MPB-PE was then purified by chromatography on a 1 × 15 cm Bio-Sil HA (BioRad) column activated overnight at 150°C and equilibrated with 100 ml of chloroform. The column was eluted with successively 20 ml of chloroform, 20 ml of chloroform/methanol 40:1, 20 ml of chloroform/methanol 30:1, 20 ml of chloroform/methanol 25:1, 20 ml of chloroform/methanol 15:1 and 100 ml of chloroform/methanol 10:1. Each fraction was tested on silica gel as described and fractions indicating a single phosphate-positive (molybdophosphoric acid revelation) and ninhyndrin-negative spot were collected (fractions 15:1 and 10:1). Solvent was evaporated under reduced pressure and MPB-PE was redissolved in chloroform. At all steps the solutions were flushed and kept under argon. After phosphate determination (Rouser et al., 1970), concentration of MPB-PE was adjusted at 1 or 1.5 μmol/ml of chloroform and stored at −20°C in 2-ml sealed (under argon) ampules. The yield of the synthesis was about 95%.

3.6.2.2. Preparation of large unilamellar vesicles and peptide coupling

Large unilamellar vesicles were prepared by the reverse-phase evaporation method of Szoka and Papahadjopoulos (1978).

6.37 μmol of egg phosphatidylcholine (PC), 2.12 μmol of phosphatidylglycerol (PG) (Sigma), 4.25 μmol of cholesterol (Sigma) and 1.5 μmol of MPB-PE were mixed in a 1 × 10 cm glass tube. Chloroform was evaporated under vacuum and phospholipids were dissolved in 1 ml of diethylether. A 10 mM Hepes buffer, pH 6.5, containing 145 mM NaCl (330 μl) was then added and the mixture was emulsified by sonication for 3 min in a bath-type sonicator (Laboratory Supply Co., Hicksville, NY).

Ether was removed under partial vacuum (550 mm Hg) until a gel-phase was obtained. The gel was broken up by vortexing and ether evaporation was resumed. Finally the homogeneous suspension of liposomes was placed under full vacuum water-pump in order to eliminate residual ether (about 15 min).

The liposomal suspension was then mixed with 660 µl of Hepes 10 mM, pH 6.5, 145 mM NaCl containing 7.5 µmol of the thiol-derivatized peptide in order to obtain a 5:1 thiol/maleimide ratio. The coupling reaction was performed under argon at room temperature overnight. Vesicles were then further treated with 10 µmol of mercaptoethanol in order to derivatize unreacted maleimido residues. Grafted liposomes were separated from free peptide and mercaptoethanol by filtration on a 1 × 15 cm Sephadex G-75 (Pharmacia) column equilibrated and eluted with a 0.9% NaCl solution. After 4 ml of void volume, liposomes were collected in 2 ml of saline. Vesicles were tested for their phosphate content according to Rouser et al. (1970). Usually, the yield of coupling estimated from available maleimido groups at the vesicles surface is quantitative.

Vesicles are kept under argon at 4°C in the dark until use.

3.6.2.3. Preparation of encapsulated peptide

7.5 µmol of egg PC, 2.5 µmol of PG and 5 µmol of cholesterol were treated as mentioned above. After redissolving the lipid film in diethylether, 330 µl of Hepes 10 mM, pH 6.5, 145 mM NaCl containing 7.15 µmol of the peptide were added and the two phases were emulsified as mentioned above. The liposomes were formed as described above.

Encapsulated peptide was then separated from free material by filtration on a Sephadex G-75 column. The collected vesicles contained about 25% of the initial amount of peptide. They are kept under argon at 4°C in the dark until use.

3.7. Coupling of peptides to solid supports

Solid supports have been used in immunology mainly for preparing immunoadsorbents intended for the purification of antibodies, antigens or cells, although other applications such as microencapsulation of monoclonal antibodies or drugs have also been described (Widder and Green, 1985).

A variety of materials have been used, e.g., nylon fibres, polystyrene beads and tubes, polysaccharide beads, agarose, polyacrylamide, polyvinyl chloride and glass. When the support has been activated, the same methods can be applied as for peptide–protein coupling (see for example 6.3.2.2). Numerous activation procedures have been developed and used mainly for the preparation of affinity absorbants (Jakoby and Wilchek, 1974; Kiefer, 1979; Kohn and Wilchek, 1983; Dean et al., 1985; Mäkelä and Seppällä, 1986; Kricka, 1985).

Activated beads are commercially available, e.g., ECDI-activated agarose (Pierce, USA), CNBr-activated Sepharose and epoxy-activated Sepharose (Pharmacia, Sweden), glutaraldehyde-activated Ultrogel (IBF, France) etc. Spacers have also been introduced in order to preserve the peptide conformation and enhance its accessibility.

In the case of polystyrene tubes, dishes or beads (latex), one method used for introducing functional groups is nitration followed by reduction of the nitro group to yield active aromatic amines. In the case of nylon (a copolymer of hexamethylenediamine and adipic acid) the terminal amino and carboxyl groups can be modified. The number of these functional groups at the surface can be increased by mild acid hydrolysis (3N HCl) or *O*-alkylation (Kricka, 1985).

Controlled pore glass is the most commonly employed inorganic matrix for immobilizing biological molecules. Activation can be performed by several methods (Robinson et al., 1971; Weetall and Filbert, 1974; Polson et al., 1985). Until now, there have been only few reports describing the use of such immobilized haptens and peptides for immunochemical studies (Kiefer, 1979 with DNP; Gazin et al., 1986; Hui et al., 1986; Berzins et al., 1986).

3.8. Determination of peptide:carrier ratio of conjugates

The molar ratio peptide:protein can be easily obtained from the amino acid composition of the conjugate determined after complete hydrolysis. Obviously, the conjugate preparation should be dialyzed before analysis to remove any uncoupled peptide. The method of calculation is illustrated in Table 3.8.

The most accurate method of determining the number of moles of

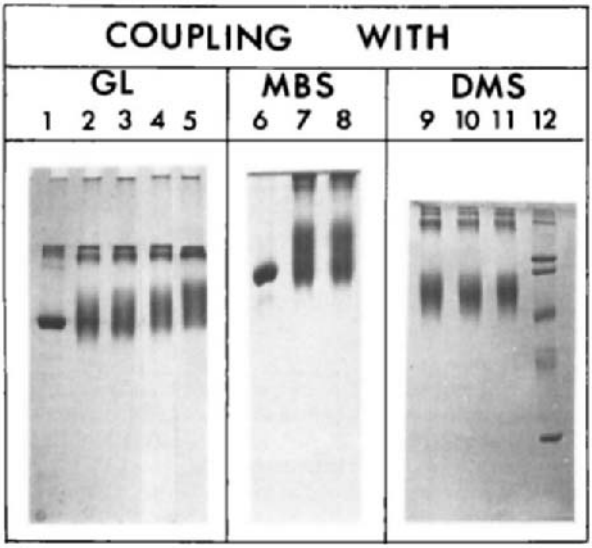


Fig. 3.2. Electrophoretic analysis of peptide-carrier conjugates. BSA–peptide conjugates (10 μg expressed as BSA) were separated by conventional SDS-electrophoresis on 10% polyacrylamide gel (Tijssen, 1985b; Laemmli, 1970). 1, control BSA; 2, BSA treated with 1% glutaraldehyde (without peptide); 3, BSA treated with 0.1% glutaraldehyde (without peptide); 4, conjugate peptide 44–61 H2A–BSA (coupling with 1% glutaraldehyde), molar ratio 24:1; 5, conjugate peptide 85–100 H2A–BSA (using 0.1% glutaraldehyde), molar ratio 13:1; 6, control BSA; 7, BSA treated by MBS; 8, conjugate peptide Arg·14·Ala–BSA (using MBS), molar ratio 20:1; 9, conjugate peptide 1–11 H2B–BSA (using DMS), molar ratio 13:1; 10, conjugate peptide 40–55 H3–BSA (using DMS) molar ratio 5:1; 11, BSA treated with DMS alone (without peptide); 12, low molecular weight markers.

GL: glutaraldehyde; MBS: *m*-maleimidobenzoyl -*N*-hydroxysuccinimide ester; DMS: dimethyl suberimidate.

TABLE 3.8.
Determination of the peptide–carrier ratio of conjugates by amino acid analysis

The method is illustrated in the case of the hexapeptide IRGERA of histone H3 coupled to ovalbumin by glutaraldehyde.

Residues	1[a]	2[b]		3[c]	4[d]		5[e]	6[f]	7[g]
		A	B		A	B			
Asp	31	37			1269				
Thr	15	18			689				
Ser	38	35			1238				
Glu	44		44	1		2325	65	21	21
Pro	14	12			500				
Gly	21		21	1		1650	46	25	25
Ala	31		31	1		1962	55	24	24
Val	31	25			1007				
Ile	17		17	1		1007	28	11	11
Leu	32	27			1144				
Tyr	10	11			376				
Phe	20	19			584				
His	7	7			283				
Lys	20	19			449				
Arg	37		37	2		2964	83	46	23
		$\Sigma = 218$			$\Sigma = 7752$				
								average value: 23	

[a] Theoretical amino acid composition of ovalbumin (moles of residues/mole ovalbumin).
[b] Experimental amino acid composition of ovalbumin used for the coupling. A: residues not present in IRGERA peptide (total = 218). B: residues present in IRGERA peptide.
[c] Theoretical amino acid composition of IRGERA peptide.
[d] Experimental amino acid composition of ovalbumin-IRGERA conjugate. A: residues not present in IRGERA peptide (total = 7752). B: residues present in IRGERA peptide. The values in column 2A are added: the result found is 218. The values in column 4A are added: the result found is 7752. A factor f is obtained from the ratio 4A/2A (in this example $f = 0.028$).
[e] Number of moles of each amino acid residue common to peptide and carrier per mole of conjugate. This value is obtained by multiplying the figures in column 4B by a factor $f = 0.028$ corresponding to the ratio of the summations of columns 2A and 4A (218/7752).
[f] Number of moles of each amino acid residue of the peptide per mole of conjugate. This is obtained by subtracting the values of 2B from those of column 5.
[g] Number of moles of peptide per mole of carrier calculated for each amino acid residue (values of column 6 divided by those of column 3). As in the case of glutaraldehyde coupling the N-terminal residue (Ile) of the peptide is involved in the binding to the carrier, a lower value is obtained which is not used for calculating the average molar ratio. An average value of 23 is obtained.
(From Briand et al., 1985.)

coupled peptide consists in utilizing a radioactive peptide and determining the total radioactivity of the purified conjugate.

Methods based on ultraviolet or visible absorption or fluorescence of the peptide have limitations, since, if the peptide reacts with more than one kind of residue in the protein, each derivative may have different spectral characteristics. Also, the molar absorption or emission quantum may differ after conjugation depending on the microenvironment in the protein.

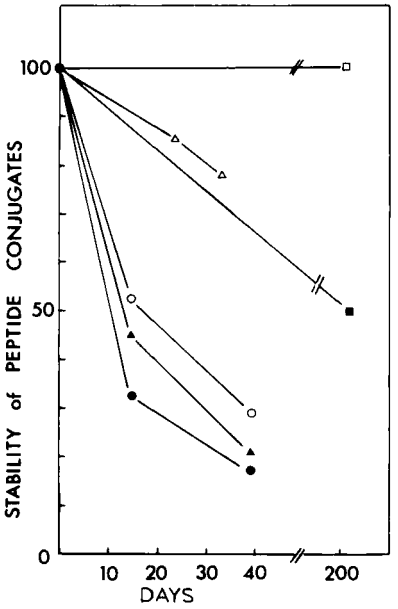


Fig. 3.3. Determination of the stability of peptide conjugates. The peptide/carrier ratio of different conjugates was determined by amino acid analysis at different periods after conjugation. The conjugates were kept in solution at 4°C and dialyzed against PBS before each amino acid analysis. (□——□) peptide 43–50 H_2B coupled to BSA with 1% glutaraldehyde (100% = initial molar ratio peptide/carrier of 14); (■——■) peptide 61–74 TMV-P coupled to BSA with 0.05% glutaraldehyde (100%≙14 molar ratio); (○——○) peptide(Y)1-8 H_4 coupled to BSA with 0.05% glutaraldehyde (100% = 8 molar ratio); (▲——▲) peptide 1-9(Y)H_2A coupled to BSA with BDB (100%= 13 molar ratio); (●——●) peptide(Y)1-8 H_4 coupled to BSA with BDB (100% = 19 molar ratio); (△—△) peptide(C)39–47 H_2B coupled to BSA with MBS (100% = 14 molar ratio). (From Briand et al., 1985.).

It is not possible to verify that the peptide is covalently linked to the carrier protein by observing the mobility of the conjugate in SDS gel electrophoresis. As shown in Fig. 3.2, bands corresponding to a conjugate may present a migration pattern different from the one shown by the carrier protein alone but not necessarily distinguishable from bands formed by carrier molecules modified by the coupling agent in absence of peptide.

A knowledge of the peptide–carrier ratio is important for controlling the stability of conjugates during storage. As shown in Fig. 3.3, peptide conjugates obtained with bis-diazotized benzidine or low concentration of glutaraldehyde are relatively unstable (Briand et al., 1985). When conjugates are to be used over any length of time, it is advisable to verify that the peptide:carrier ratio remains unchanged.

CHAPTER 4

Immunization with peptides

4.1. General remarks

As most synthetic peptides are good immunogens, there is usually no difficulty in raising antibodies against them by classical immunization procedures (Niman et al., 1983). In many cases, the titre of antipeptide sera measured with respect to the peptide used for immunization is very high. However, it should be remembered that the most common purpose for raising antibodies against a peptide fragment is to obtain a reagent that will also react strongly and specifically with the complete protein. Those antipeptide antibodies in the antiserum that cross-react with the complete protein have been designated antibodies of predetermined specificity since they react with a single antigenic region of the protein (Lerner, 1982, 1984). Such polyclonal antibodies share with monoclonal antibodies raised against the protein the property of binding to a discrete region of the protein antigen; however, they differ from the monoclonal antibodies in being less specific for the native conformation of the protein and they usually bind to the protein with a lower affinity constant.

In this chapter, methods for raising antipeptide sera will be discussed mainly in terms of the ability of the resulting antibodies to cross-react with the whole protein.

Practical details concerning the inoculation and bleeding of animals have been described in several reviews (Mayer and Walker, 1980; Vaitukaitis, 1981; Tijssen, 1985; Dresser, 1986; Herbert and Kristensen, 1986). As far as dosage of immunogen is concerned, it should be stressed that there is no direct relation between the size of an animal and the quantity of antigenic material required for efficient immunization: a rabbit of 5 kg requires approximately the same dose of immunogen as a mature goat of 50 kg or a mouse of 25 g (Ritchie, 1986; Van Regenmortel, 1987b).

The adjuvants most commonly used to enhance the humoral response are the complete or incomplete Freund's adjuvants (for details on the composition and the preparation of these adjuvants, see Clausen, 1981; Goding, 1983; Tijssen, 1985b; Dresser, 1986). Good results have also been obtained with alum (Green et al., 1982) which is an aqueous 10% solution of $AlK(SO_4)_2 \cdot 12\ H_2O$ (Goding, 1983; Tijssen, 1985) and with muramyl dipeptide (MDP) or derivatives and analogues of MDP. This compound (*N*-acetyl-muramyl-L-alanyl-D-isoglutamine) is the minimal structure that can substitute for the mycobacteria in complete Freund's adjuvant (CFA) (Ellouz et al., 1974; Jolivet et al., 1983; Arnon et al., 1983; Neurath et al., 1984a; Clough et al., 1985; Stevens et al., 1986a). Several MDP derivatives have been chemically synthesized and biologically tested. When mixed with a conjugate made up of peptide P2 of MS2 coliphage and poly(DL-alanyl)-poly(L-lysine) synthetic carrier, MDP was shown to cause a slight increase in antibody production. When it was covalently linked, the resulting MDP–P2–carrier conjugate administered in PBS elicited in rabbits almost as good an anti-MS2 response as did P2–carrier conjugate in CFA (Arnon et al., 1980). MDP has been shown to be an effective adjuvant in various systems (Audibert, 1987; Jacob et al., 1986/1987) although some undesirable effects have been reported (Allison and Byars, 1986). Further research is required to develop better adjuvant formulations that will enable the formation of protective antibodies without unacceptable side effects (Ribi, 1986).

4.2. Specific immunization protocols

There have been no systematic studies of the most efficient immunization procedures for obtaining antipeptide antibodies. Since the immune response of individual outbred animals submitted to the same immunization protocol can vary greatly, it would be necessary to collect extensive comparative data to demonstrate the superiority of any particular procedure (Dresser, 1986; Van Regenmortel, 1987b). Published information on methods used for raising antipeptide antibodies concerns mostly data obtained from a small number of animals; furthermore, the relative merits of different immunization schedules are rarely compared and unsuccessful attempts to obtain antipeptide antibodies are seldom reported (Palfreyman et al., 1984; Murdin and Doel, 1987a, b).

In the case of biologically active peptides that are administered to humans for therapeutic purposes, the aim is mostly to avoid eliciting an immune response as far as possible (Lee, 1986). Studies in this area are likely to increase our knowledge of the fate of peptides after they have been injected, and as a spin-off useful clues may be obtained on how to increase their immunogenicity.

In view of animal variability, it is essential to immunize several animals at a time. It is commonly observed that animals which receive the same immunization regimen produce antisera with completely different characteristics. Furthermore, the kinetics of appearance and disappearance of various antibody types during the course of immunization can also vary considerably in different animals. This is illustrated in Fig. 4.1 (A and B) which shows the variation in antigenic response observed with two rabbits immunized with peptide 28–42 of histone H2A (Muller et al., 1986). Although the titre of the antisera measured in ELISA using peptide 28–42 of H2A as antigen increased in successive bleedings, this was not the case for the cross-reactive titre measured in rabbit 2 when histone H2A was used as the antigen.

Another example showing that homologous titres and cross-reactive titres can evolve differently during the course of immunization is given in Fig. 4.1 (C). The rabbit was immunized with an unconjugated peptide corresponding to residues 85–100 of histone H2A. The homologous titre

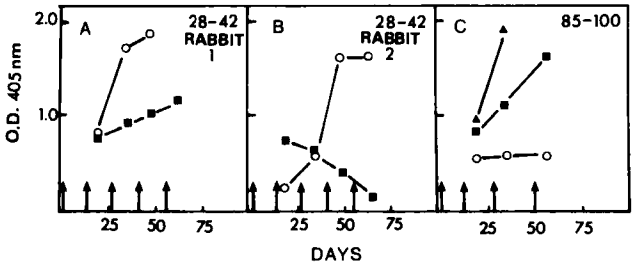


Fig. 4.1. Production of antipeptide antibodies by immunization of rabbits with free peptides of H2A (from Muller et al., 1986). Antibody levels measured by ELISA using plates coated with: 400 ng H2A/ml (■); 2 μM homologous peptide–BSA conjugates (▲); 2 μM homologous free peptide (○). Arrows indicate the immunization schedule (100 μg unconjugated peptide/injection).

in ELISA measured with the unconjugated peptide as antigen did not increase after the second injection, whereas the titre measured with the peptide–BSA conjugate or with histone H2A increased sharply.

A similar lack of correlation between the antipeptide and cross-reactive anti-protein responses has been observed in other systems (Neurath et al., 1984b) and it does not seem to depend on whether the peptide is injected as a conjugate or in free form. These findings illustrate the need to bleed immunized animals repeatedly and to test each bleeding with all relevant antigens. If the complete protein is not available for selecting the best bleeding, as is often the case when the antipeptide serum is made in an attempt to identify a putative gene product, experiments should be done with as many different bleedings as possible.

It seems that very few systematic studies have been made regarding the relative merits of different strains of mice, rats, guinea pigs and rabbits as experimental animals for raising antipeptide sera (Atassi and Young, 1985; Murdin and Doel, 1987a, b). As a general rule, it is clearly advantageous to use as many different types and strains of animals for immunization as possible as this will minimize difficulties linked to the presence of a limited immunological repertoire in certain animals. When antisera of low titre are obtained using a particular breed or strain of animal, it is preferable to switch to another type of animal rather than re-

peating the immunization protocol with another series of individuals of the same breed.

Immunological tolerance, i.e., a specific non-reactivity of lymphoid cells to a given antigen, seems to pose fewer problems when peptide fragments are used for immunization compared to when the complete protein molecule is the immunogen. Tolerance phenomena arise when the injected protein is closely related to a homologous protein present in the animal host. However, when peptides corresponding to conserved regions of homologous proteins are used for immunization, antibodies can be readily obtained, as shown, for instance, in the case of the N-terminal tetrapeptide of cytochrome *c* (Jemmerson et al., 1985) or the C-terminal hexapeptide of histone H3 (Muller et al., 1982). It is possible that these peptides were immunogenic because they were injected in the form of conjugates, in which the conserved peptide region was presented in the unnatural environment of the carrier. Immunization with peptides corresponding to conserved regions of autologous or closely related antigens may in fact be a useful method for overcoming tolerance to self antigens.

In view of the wide variety of successful immunization procedures reported in the literature, only a few examples will be presented here, mainly to illustrate the range of conditions that can be used.

4.2.1. Method of Walter et al. (1980); Patschinsky et al. (1984)

Animals: rabbits, male, 5–6 months old.
Peptides: 8–10 residues long, conjugated to BSA with glutaraldehyde or BDB (see 3.4.1 and 3.4.4).
1st injection: intradermal route (id) at 20 different sites; 1 mg conjugate emulsified in 2 ml CFA.
Subsequent injections: weeks 4 and 8, intramuscular (im) and subcutaneous injections (sc).
Bleedings: from week 10 onwards.

This procedure is similar to that described by Müller et al. (1982) and Shapira et al. (1984) for immunizing rabbits with synthetic peptides of in-

fluenza virus haemagglutinin; these peptides were conjugated to tetanus toxoid by ECDI according to procedure 3.4.3.

4.2.2. Method of Green et al. (1982)

Animals: rabbits.
Peptides: 8–10 residues long, conjugated to KLH by MBS (see 3.4.5).
1st injection: 200 μg conjugated peptide emulsified in CFA (1:1), sc route.
Subsequent injections: day 14, same injection but with incomplete Freund's adjuvant (IFA); day 21, intraperitoneal (ip) inoculation of 200 μg conjugated peptide with alum (4 mg); several booster injections given in the same conditions every 5 weeks.
Bleedings: from week 4.

This procedure was also used by Niman (1984) with peptides corresponding to segments of protein sequences predicted from the nucleotide sequences of viral oncogenes and by Tainer et al. (1984) with myohaemerythrin peptides.

4.2.3. Method of Tanaka et al. (1985)

Animals: New Zealand white rabbits.
Peptides: 7–20 residues long, conjugated to KLH with glutaraldehyde.
1st injection: 300 μg conjugate emulsified in CFA; sc inoculation.
Subsequent injections: sc inoculation of 175 μg conjugate in IFA (week 4); at intervals of 2 weeks to 4 months, sc inoculations of 175 μg/injection.
Bleedings: ten days after each injection.

4.2.4. Method of Muller et al. (1986)

Animals: rabbits, female, 10–12 weeks old (2–4 rabbits/peptide).
Peptides: 14–20 residues long peptides were used unconjugated; peptides shorter than 14 residues were mostly conjugated to ovalbumin,

using a variety of procedures.
1st injection: 200 μg peptide emulsified in CFA (1:1, v/v); multiple sc inoculations at 5–10 injection sites for unconjugated peptides; im inoculation for conjugated peptides.
Subsequent injections: 200 μg peptide (weeks 2, 4, 6 etc.) emulsified in CFA for unconjugated peptides (sc injections), in IFA for conjugated peptides (im injections).
Bleedings: 8 days after each injection starting from week 5.

4.2.5. Method of Nussberger et al. (1985)

Animals: guinea pigs, female, Hartley, 6 weeks, 250–350 g, 10 animals/peptide.
Peptides: octapeptide, conjugated by means of carbodiimide.
injection: a single id injection at several points, emulsion in CFA, 500 μg/animal.
Bleedings: 8 days after the injection and then every 2 weeks.

Other procedures for immunizing guinea pigs are described by Audibert et al. (1981) and Fujio et al. (1985).

4.2.6. Method of Choppin et al. (1986)

The following method has been described for obtaining antipeptide antibodies from mice ascites.

Animals: BALB/K female mice, 12–16 weeks old, 5 mice/peptide.
Peptides: 18–24 residues long, conjugated or free.
Schedule:
day 0: ip injection of a mixture 1:9 (v/v) $9^0/_{00}$ NaCl–CFA (200 μl final)
day 14: ip injection of 10 μg peptide in 200 μl emulsified in CFA (1:9, v/v)
days 23, 32, 43, 53, 83: idem
Generally ascites form around days 28–32, sometimes only after 6–7

injections. Ascites fluids are collected by ip puncture and the cells are removed by centrifugation (5000×*g* for 5–10 min). A yield of 40–60 ml/mouse is common. A boost can be given intraperitoneally when the production of ascites decreases.

Generally ascites does not clot. Sodium azide at a final concentration of 10 mM can be added to discourage microbial growth. Small aliquots are best stored at −70°C.

This method, first described by Tung et al. (1976), has a number of advantages over the usual procedures for immunizing mice. Large volumes of ascites can be collected compared to the small quantities of serum obtained by bleeding. An example of ascites antibody activity directed against two peptides of the HLA-B7 heavy chain is shown in Fig. 4.2 (Choppin, 1988). This technique has been reviewed by Tung (1983) and various applications have been described by Thèze and Sommé (1979).

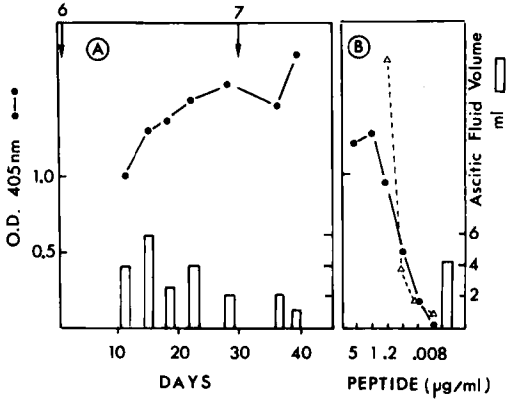


Fig. 4.2. Antipeptide antibody level in ascitic fluid of a BALB/K mouse immunized with peptide 138–157 of HLA-B7 heavy chain (see 4.2.6). In (A) the arrows 6 and 7 refer to the 6th and 7th ip injections of peptide conjugated to ovalbumin (10 μg peptide/mouse). A total volume of 22.1 ml of ascitic fluid was obtained from this mouse. For ELISA, ascitic fluids were diluted 1:20 and allowed to react with peptide-coated plates (200 ng/ml). In (B), antibody levels to the peptide 99–118 of HLA-B7 heavy chain in ascitic fluid (obtained 11 days after the 6th ip injection) and in a rabbit antiserum are compared. The ascitic fluid was diluted 1:20 (•—•), the rabbit antipeptide antiserum was diluted 1:100 (△---△). (From Choppin, 1988.)

4.2.7. Young et al. (1983)

Animals: BALB/c By J (H-2^d) mice; 5 animals/peptide.
Peptide: 6–8 residues long; unconjugated.
1st injection: 100 μg peptide emulsified in CFA; id injection (foot-pad).
Subsequent injections: weeks 2 and 4, 100 μg peptide in CFA by ip inoculation.
Bleedings: from the tail vein, weeks 2, 4 and 8.
This technique was used to obtain monoclonal antibodies (mAb) able to recognize native sperm-whale myoglobin (Schmitz et al., 1983a, b; Atassi and Young, 1985).

Antipeptide mAb has also been obtained by immunizing mice with conjugated peptides. Fok et al. (1982) immunized mice with the nonapeptide corresponding to the thymic hormone serum thymic factor conjugated to IgG by glutaraldehyde. Shapira et al. (1984) have obtained antibodies directed against influenza virus haemagglutinin peptides by immunizing (SJL/J × BALB/c)Fl mice by ip route with peptides coupled to tetanus toxoid by ECDI (50 μg peptide/animal). Other protocols have been described by Niman et al. (1983), Bellet et al. (1984), Darsley and Rees (1985), Scheefers-Borchel et al. (1985), Caraux et al. (1985), Antoni et al. (1985) and Price et al. (1986). De Boer et al. (1987) have prepared monoclonal antibodies to thyroglobulin by in vitro immunization with a free 19-residue synthetic peptide.

Generally authors have immunized BALB/c mice or mice derived from this strain, mainly for ensuring compatibility during the fusion with available murine myeloma cells. For some immunogens, other mouse strains showing differences in the haplotype of the I-region of H-2 (MHC genes in the mouse) were used to obtain an immune response, e.g., SWR strain (Darsley and Rees, 1985), 129 GIX^+ strain (Niman et al., 1983), B10 and C57 Bl strains (Atassi and Young, 1985).

4.3. Free versus conjugated peptides as immunogens

In view of the absence of comparative studies specifically devoted to the

immunogenic potential of peptides (Palfreyman et al., 1984), the general procedures described for raising antibodies against proteins should be followed (Butler and Beiser, 1973; Crumpton, 1974; Mayer and Walker, 1978; Dresser, 1986; Ritchie, 1986).

It is commonly assumed that antigens with a molecular weight smaller than $2–5 \times 10^3$ behave like haptens and are not immunogenic. This belief accounts for the widespread practice of immunizing animals with peptides conjugated to a carrier molecule. There are indeed many reports in the literature indicating that immunization with conjugated peptides leads to antisera of higher titre than immunization with the corresponding free peptides (Delmas et al., 1985; Choppin et al., 1986; Schulze-Gahmen et al., 1986; Alving et al., 1986).

Studies performed in our own laboratory have shown that immunization with free peptides of a length of 14–25 residues (using protocol 4.2.4) could lead to a moderate antibody response after 2–3 injections (Fig. 4.1); however, in order to obtain antisera of adequate titre, 5–6 injections were usually necessary. Some authors have reported that satisfactory antibody levels could be obtained by immunizing rabbits and mice with free peptides of a length of 6–8 residues (Young et al., 1983; Atassi and Young, 1985; Antoni et al., 1985; Young and Atassi, 1985). In our own laboratory, attempts to raise antibodies by immunization with free hexapeptides corresponding to fragments of histone molecules failed.

Since the main purpose of raising antipeptide antibodies is usually to obtain a reagent capable of reacting with the parent protein, the success of an immunization protocol depends on the cross-reactive potential of the antiserum and not solely on the titre measured with respect to the homologous peptide immunogen. When a conjugated peptide is used for immunization, the proximity of the carrier molecule may induce a conformation in the peptide which is close to that present in the parent protein. In such a case, the resulting antipeptide antibodies would be expected to show considerable cross-reactivity with the complete protein molecule. Cyclization of peptides is another method used for making peptides resemble the conformation of the corresponding segment of the parent protein. Immunization with cyclized peptides has been found to

lead to antisera possessing a high degree of cross-reactivity with the intact protein (Dreesman et al., 1982; Dorow et al., 1985; Kanda et al., 1986).

As the length of a peptide fragment increases, the likelihood that its conformation will resemble that found in the corresponding part of the complete protein also increases. This may explain why longer peptides react better with antiprotein antibodies than do shorter peptides, and why they also induce antibodies that cross-react more strongly with the parent protein (Van Eldick et al., 1983; Dorow et al., 1985; Welling and Fries, 1985; Tanaka et al., 1985; Al Moudallal et al., 1985; Van Regenmortel et al., 1986). However, it may be impossible to predict for any particular peptide whether conjugation to a carrier or cyclization is likely to lead to a structure that better mimics the conformation found in the native protein. For many years, it was commonly assumed that free peptides could adopt a very large number of different conformations in aqueous solution (see chapter 1). However, recent experimental observations show that this is not always the case and that peptides may in fact adopt a few preferred conformations (Dyson et al., 1985). The extent to which any peptide is able to mimic a conformation present in the intact protein depends, in the last analysis, on its individual sequence. Since we cannot yet predict, in a reliable manner, the conformation which a peptide is likely to adopt, it is not possible to state which immunization approach is most likely to succeed in any particular instance. It seems best to follow an empirical approach and to test a variety of immunization procedures using free, conjugated and cyclized peptides in succession until the desired antibodies are obtained.

4.4. Immunization with conjugated peptides

Several protein carriers are commonly used to obtain peptide conjugates suitable for immunization (see Table 3.3). The use of highly immunogenic substances such as KLH can be detrimental in certain cases, probably because of antigenic competition phenomena (Taussig, 1971; Sarnesto et al., 1983). In the case of peptides that are only poorly im-

munogenic, it may be preferable to use a less immunogenic protein such as BSA as carrier.

In chapter 3 the important of choosing the correct coupling agent for conjugation has been emphasized. As far as the optimal number of moles of peptide per mole of carrier is concerned, it seems that conjugates comprising 6 to 30 peptide molecules per 100 kDa of carrier molecule give the best results. Many studies have been performed with small haptens such as dinitrophenol, azobenzenarsonate, and fluorescein to determine the optimal degree of carrier substitution (Ivanyi and Cerny, 1969; Klaus and Cross, 1974; de Weck, 1974; Desaymard and Howard, 1975; Turk and Parker, 1978). As few as two molecules of hapten per carrier have been shown to lead to a specific antibody response. At high coupling densities, immunogenicity may be reduced, a phenomenon known as immunological paralysis.

The immunogenicity of peptides can be enhanced by coupling them to MDP (see Section 4.1), to long fatty acid chains such as dipalmityl lysine (Hopp, 1984), to preform iscoms (Lovgren et al., 1987) or to liposomes. It seems that liposomes may act both as a vector for the peptide and as an adjuvant compatible with human use (Gregoriadis, 1985). In addition to being biodegradable, liposomes interact strongly with macrophages; they also possess considerable configurational fluidity which can be modified according to need (Allison and Gregoriadis, 1974; Lifshitz et al., 1981; Alving and Richards, 1983; Neurath et al., 1984a; Alving et al., 1986; Zigterman et al., 1987). Different types of liposomes as well as the methods used for anchoring peptides to them have been described in section 3.6. Considerable success has been achieved by coupling peptides of biological interest to liposomes, such as peptides of diphtheria and cholera toxins, surface antigen of hepatitis B virus, and haemagglutinin and neuraminidase of influenza virus. A variety of cross-linking agents and carriers were compared by Neurath et al. (1984a) to assess their efficacy in increasing the immunogenicity of a synthetic peptide corresponding to residues 135–155 of hepatitis B surface (HBs) antigen. Two rabbits were immunized with each of the 26 different conjugates tested and the antisera were assessed with respect to antipeptide and anti-HBs response. Animals immunized with the peptide in free form did not respond.

Polymers of the peptide prepared by linking to diaminoalkanes as well as conjugates prepared by binding the peptide to liposomes or to polylysine were found to be immunogenic. A poor correlation was observed between antipeptide and anti-HBs response elicited by these conjugates. Glutaraldehyde-fixed liposomes were the best carriers for inducing an anti-HBs response (Neurath et al., 1984a) and gave better results than MDP-peptides.

The potential value of liposomes for eliciting antibodies to peptides is increasingly recognized. Methods have been devised to increase their stability and rigidity, and this has been shown to render the linked peptides more immunogenic (Yasuda et al., 1977). Furthermore, liposomes are completely non-toxic and induce no local or systemic reaction in the immunized animals. There is also no antibody induction against the lipid component of liposomes, at least when they contain lecithin (Alving et al., 1980). In view of the current interest in the development of new synthetic peptide vaccines (chapter 7), the use of liposomes as a substitute for protein carriers is likely to increase in future.

Antipeptide antibodies have also been raised in mice by intraperitoneal implantation of paper disks derivatized with a synthetic peptide (Viamontes et al., 1986). A peptide corresponding to cysthymopoietin 28–39 was coupled by diazo linkages to aminophenyl thioether-derivatized paper disks. After four implantations 6/6 mice developed antibodies reactive with native thymopoietin. In contrast, mice conventionally immunized with peptide alone or with peptide conjugated to thyroglobulin by means of SPDP all failed to produce antibodies. Another successful method was described by Smith et al. (1986) who implanted subcutaneously in mice pieces of nitrocellulose containing absorbed antigens. Only a single implantation was necessary and spleen cells of the inoculated mouse could be used for producing monoclonal antibodies.

Peptide conjugates can also be studied using an approach in which the synthetic peptide is still bound to the resin used during solid-phase synthesis. As additional residues are added to the growing peptide chain, it is possible to study by a suitable immunoassay the influence of parameters such as composition, length and conformation of the chain on the

antigenic activity of the peptide. Spacers have been introduced to render the peptide more accessible to antibodies. This approach has been used to localize continuous epitopes in leghaemoglobin (Hurrell et al., 1978), myoglobin (Smith et al., 1977; Shi et al., 1984; Rodda et al., 1986), apoliprotein AII (Bhatnagar et al., 1983), parathyroid hormone (Delmas et al., 1985), cytochrome *c* (Paterson, 1985) and foot-and-mouth disease viral protein (Geysen et al., 1984, 1985b). Recently Kanda et al. (1986) immunized rabbits with an HBs peptide still attached to a polydimethylacrylamide resin and cyclized by reconstitution of a disulfide bridge. The resulting antibodies recognized the HBs antigen whereas antibodies raised against the same peptide attached to the resin but not cyclized, did not.

CHAPTER 5

Solid-phase immunoassays

5.1. Introduction

It is beyond the scope of this volume to describe all the immunoassays that can be used with synthetic peptides. Numerous textbooks and reviews are available which describe the immunological testing of small and medium-sized molecules especially by classical solution phase immunoassays (see Crumpton, 1974; Parker, 1976; Weir, 1986).

When synthetic peptides corresponding to protein fragments are studied for the purpose of localizing the epitopes of a protein, the most common approach consists of measuring the capacity of the peptides to inhibit the reaction of the protein with its homologous antibodies. The following serological methods are widely used for this purpose: inhibition of the precipitation reaction (Atassi, 1977b), inhibition of radioimmunoassay (Milton et al., 1980), inhibition of complement fixation (Benjamini, 1977; Milton and Van Regenmortel, 1979) and inhibition of enzyme-linked immunosorbent assay (ELISA) (Altschuh and Van Regenmortel, 1982). It should be stressed that in all these inhibition assays, the peptide is used free in solution.

It is important to realize that the type of immunoassay format used can determine whether a peptide shows antigenic activity or not. Many of the

current controversies in the field (see Atassi, 1984; Benjamin et al., 1984) can be ascribed to the fact that investigators are not always sufficiently aware of the operational nature of any definition of antigenicity. In the classical studies of Atassi (1977b) on the antigenic structure of myoglobin, five continuous epitopes of 6–7 residues were identified by inhibition of the precipitation reaction between myoglobin and its specific antibodies (see chapter 1). These results could not be confirmed in a subsequent study in which all the possible overlapping hexa-, hepta- and octapeptides of myoglobin were synthesized on a polymer of polyacrylic acid and were tested for antigenicity while still bound to the solid-phase. By this method, only one major epitope was identified in residues 48–55 (Rodda et al., 1986), a region which in the earlier work had been found to contain no antigenic activity. Furthermore, the epitopes identified by Atassi were not picked up by method of Rodda et al. (1986) in which the peptides are attached to a solid phase. It seems that the origin of such conflicting results lies in the fact that peptides adopt different conformations in different types of assays and that covalent or non-covalent attachment of peptides to a carrier or a solid phase may cause certain epitopes to be masked. The interpretation of such discrepant results would be made easier if the investigators were prepared to exchange their reagents. In this way, it would be possible, for instance, to demonstrate whether the differences are caused by the unique properties of particular antibody populations or by the use of different assays. Studies in which synthetic peptides were tested in the same laboratory by different immunoassays using the same antisera demonstrated that the antigenic activity of peptides may differ when they are tested as free peptides in solution, conjugated to a carrier or adsorbed to a solid-phase (Muller et al., 1986; Quesniaux et al., 1986).

In recent years, solid-phase immunoassays such as ELISA and solid-phase radioimmunoassay (RIA) have become increasingly popular (Clark and Engvall, 1980; Oellerich, 1984; Tijssen, 1985; Yolken, 1985; Chard, 1987) and these assays are now commonly used for measuring the antigenic activity of synthetic peptides. This chapter will describe some of the solid-phase assay formats that have been used with peptides and will review some of the difficulties likely to be encountered in such tests.

In spite of the numerous advantages of solid-phase assays, it should always be remembered that (a) any polypeptide immobilized on a solid-phase will have part of its surface unavailable for binding to antibody; (b) adsorption of a polypeptide on a polymer surface will alter its conformation; and (c) the kinetics and equilibrium characteristics observed in liquid-phase antigen–antibody interaction are altered when one of the reactants is immobilized (Stevens et al., 1986b).

5.2. Types of solid-phase immunoassays

The simplest type of solid-phase immunoassay that can be used with peptides is one in which the synthetic peptide is allowed to react with antibodies while still attached to the resin support used for its synthesis (Smith et al., 1977; Modrow et al., 1988). By immunological testing at each step of the synthesis, it is possible to assess the contribution of each successive amino acid to the antigenic reactivity of the growing peptide.

Geysen et al. (1984) have described a method which allows the rapid concurrent synthesis of hundreds of peptides on polyethylene rods, and in which the synthesized peptides are tested for antigenic activity without removing them from the support (see chapter 2). The rods are assembled into a polyethylene holder with the format and spacing of a microtitre plate, which allows the peptides to be tested easily by ELISA. Another approach consists in coupling peptides synthesized by standard solid-phase methods directly to radiation-grafted polyethylene using reagents such as MBS or glutaraldehyde (Geysen et al., 1985a,b).

Instead of being covalently attached to the solid support, the free peptide is, more commonly, made to adsorb to a plastic solid-phase, for instance to the wells of a microtitre plate. In general, peptides that are at least 15 residues long will become adsorbed to plastic after overnight incubation of the peptide solution in the microtitre wells. However, this is not an absolute rule, as longer peptides sometimes do not become adsorbed (Muller et al., 1987) while shorter ones do (Houghten, 1985). This procedure (Procedure 1, Table 5.1) is the simplest method for measuring the activity of antipeptide sera. Serial dilutions of the antiserum are incubated in the peptide-coated wells and the bound anti-

TABLE 5.1
Solid-phase immunoassays used with peptides

Procedure	Successive steps of assay[a]			
1	Peptide	$\underline{Ab}^R$	$antiR^G$–E	
2	Peptide-conjugate	$\underline{Ab}^R$	$antiR^G$–E	
3	Ab^R	Protein	$\underline{Ab}^R$–E	
4	$\underline{Ab}^C$	Protein	$\underline{Ab}^R$	$antiR^G$–E

[a] Ab = antibody; R = rabbit; C = chicken; G = goat; E = enzyme label; $antiR^G$ = goat anti-rabbit globulin reagent. Underlined antibodies refer to antibodies raised against the peptide.

bodies are revealed by an enzyme-labelled anti-immunoglobulin reagent or by radiolabelled protein A (see Sections 5.3.1 and 5.3.3).

Some authors have advocated the use of substances such as glutaraldehyde, poly-L-lysine or cyanogen bromide to improve the attachment of polypeptides to the solid phase (Lehtonen and Viljanen, 1980; Corthier and Franz, 1981; Suter, 1982; Brennand et al., 1986). When this approach is used with short peptides, it should be established that the immobilizing reagent does not modify the antigenicity and that the pretreatment of plates with such substances does not lead to undesirable high background readings.

When the peptide is shorter than 15 residues, it is preferable to use it in the immunoassay in the form of a peptide conjugate (Procedure 2, Table 5.2). Methods used for coupling peptides to carrier proteins are described in chapter 3. It is of course essential in this instance to couple

TABLE 5.2
Competitive solid-phase immunoassays used with peptides

Procedure	Successive steps of assay[a]			
1	Peptide	$\underline{Ab}^R$	$antiR^G$–E	
2	$\underline{Ab}^C$	Protein	$\underline{Ab}^R$ + peptide	$antiR^G$–E
3	Peptide-conjugate	$\underline{Ab}^R$ + peptide	$antiR^G$–E	
4	Ab^R	Peptide-L + peptide		
5	$antiM^G$	$\underline{Ab}^M$	Peptide-L + peptide	
6	Biotin-conjugate	Avidin	Ab^M-biotin	Peptide-L + peptide

[a] For meaning of abbreviations, see Table 5.1. L = label, e.g., radio-label, enzyme or biotin, ^{M} = mass.

the peptide to another carrier than the one used to prepare the peptide conjugate intended for immunization. It is not always realized that the peptide antiserum, in addition to containing antibodies directed to the peptide moiety and to the carrier protein, is likely also to contain a considerable proportion of antibodies directed to the coupling agent used for conjugation (Goodfriend et al., 1964; Briand et al., 1985). It is thus necessary to use in the immunoassay a peptide conjugate prepared with both a different carrier protein and a different coupling agent. If this precaution is not taken, antibodies directed against glutaraldehyde- or carbodiimide-modified residues, for instance, will contribute to the overall reaction. This will lead to an overestimation of the level of peptide antibodies present in the antiserum (see Fig. 5.1).

In order to measure the subpopulation of antibodies in a peptide antiserum that is able to cross-react with the cognate complete protein, the microtitre plates should be coated with protein instead of peptide (Procedure 1, Table 5.1). The plastic wells can be coated, for instance, by incubation with a 0.1–1.0 μg/ml protein preparation diluted in carbo-

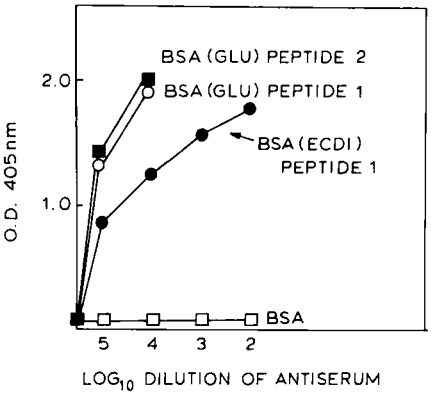


Fig. 5.1. Binding in ELISA of antiserum raised against peptide 1 (conjugated to KLH with glutaraldehyde) with homologous and heterologous peptides. The antigens were used at a concentration of 2.5 μmol. Peptide 1 corresponds to sequence 80–89 of histone H4 (TVTAMDVVYA) conjugated to BSA with glutaraldehyde (o) or with e.c.d.i. (carbodiimide) (•). Peptide 2 corresponds to sequence 130–135 of histone H3 (IRGERA) conjugated to BSA with glutaraldehyde (■). Note that the reaction of antibodies specific for peptide 1 can only be demonstrated with a peptide 1 conjugate prepared with a different coupling agent from the one used to prepare the immunogen (adapted from Briand et al., 1985).

nate buffer, pH 9.6, or phosphate buffer, pH 7.0. Optimal incubation conditions (reagent concentrations, pH, time of incubation) must be determined empirically.

It should be emphasized that, contrary to some claims (Green et al., 1982), this type of assay does not necessarily measure antibodies able to cross-react with the native protein antigen. There is now a considerable body of evidence indicating that proteins become at least partly denatured when they are adsorbed to a solid-phase (Soderquist and Walton, 1980; Kennel, 1982; Mierendorf and Dimond, 1983; Al Moudallal et al., 1984; Altschuh et al., 1985; Dierks et al., 1986; Butler et al., 1986). The widespread use of monoclonal antibodies, which are often specific for a particular conformation of the antigen, has contributed to the growing realization that proteins adsorbed to surfaces undergo conformational changes (Friguet et al., 1984; Al Moudallal et al., 1985; Vaidya et al., 1985).

If it is important to measure only those antibodies in the peptide antiserum that recognize the native protein molecule, it is possible to use either a double antibody sandwich immunoassay in which the protein is bound to a first layer of antiprotein antibodies (Procedures 3 and 4, Table 5.1) or a competition type of assay (Procedure 2, Table 5.2). It should be remembered that attachment of the antigen to a first layer of immobilized antibody may also lead to a local change in protein conformation by an allosteric effect, although this is likely to be of minor importance compared to the drastic changes induced by direct adsorption of the protein to the solid-phase.

When peptide antisera are screened in an immunoassay in order to determine the titre of antibodies cross-reacting with the whole protein and to evaluate whether the antisera are likely to contain neutralizing antibodies (for instance in experiments designed to develop a synthetic peptide vaccine), it is best to use an assay in which the native conformation of the antigen is preserved. Antibodies that recognize, for instance, a denatured form of the viral antigen but not the native conformation are unlikely to possess neutralizing or protective potential.

The methods discussed so far (Table 5.1) are particularly suited for analyzing the properties of antisera raised against peptides. On the other

hand, when the antigenic reactivity of synthetic peptides is tested for the purpose of delineating the epitopes of a protein, a competition format of solid-phase immunoassay is more appropriate. The simplest form of assay consists of incubating the peptide with antibodies directed to the protein and measuring thereafter the amount of unreacted antibodies still able to bind to the protein immobilized on the solid-phase (Procedure 1, Table 5.2). The degree of antigenic reactivity of the peptide is inversely proportional to the level of protein antibody that can still be detected by the labelled anti-immunoglobulin reagent. However, the concentration of the different reagents has to be adjusted with care to ensure maximum sensitivity; it was found, for instance, that the extent of inhibitory activity exhibited by different peptides was dependent on the concentration of protein used for coating the wells (Altschuh and Van Regenmortel, 1982).

To ensure that the peptides compete with antibodies specific for the native protein, the protein must be immobilized on the solid-phase via a first layer of protein antibodies (Procedure 2, Table 5.2). In this type of double antibody sandwich assay, the trapping antibody should be obtained from a different animal species than the antibody that is made to interact with the peptide. Avian antibodies which are serologically unrelated to mammalian immunoglobulins (Leslie and Clem, 1969) are a particularly good choice for use as trapping antibodies since they will not be detected by an enzyme conjugate directed against mammalian globulins (Van Regenmortel and Burckard, 1980; Al Moudallal et al., 1984). Another approach consists of using as trapping antibody Fab_2 fragments devoid of the Fc portion of immunoglobulin molecules; in the subsequent step of the assay, the same intact antibody molecule can then be used and its binding revealed by an anti-Fc enzyme conjugate.

Solid-phase immunoassays can also be used to compare the antigenicity of a series of peptide analogues, for instance in experiments designed to assess the contribution of individual amino acids to the antigenic reactivity of the peptide. This can be done, for instance, by the methods developed by Geysen et al. (1984) and by Houghten (1985), i.e., by synthesizing a replacement set of peptide analogues in which each residue of the peptide is, in turn, replaced by the other 19 amino acids (Rodda et

al., 1986; Getzoff et al., 1987). In view of the disadvantages linked to the testing of short peptides that are still attached to the support used for synthesis or that are adsorbed non-covalently to the plastic, it may be preferable to assess the comparative antigenicity of a series of peptide analogues by a competitive immunoassay (Quesniaux et al., 1986). A simple assay consists of assessing the capacity of peptide analogues to inhibit the binding of antibodies to a peptide conjugate immobilized on the solid-phase (Procedure 3, Table 5.2). Another approach consists of letting a series of peptide analogues compete with a labelled peptide (Procedure 4, Table 5.2). In this case, it is important to verify that the binding roperties of the antibody are not affected by adsorption to the solid-phas . Since this effect can be particularly troublesome with monoclonal antibodies (Butler et al., 1986), it may be preferable to trap them on the plastic by a first layer of anti-mouse immunoglobulins (Procedures 5 and 6, respectively, Table 5.2) or by a biotin – avidin bridge (Suter and Butler, 1986).

5.3. Specific procedures

A very large number of solid-phase immunoassay formats have been developed and it would be impracticable to describe them all in detail. Only a few procedures that were found to work satisfactorily with peptides will be described. Depending on the properties of molecules under study, each investigator should determine the optimal parameters of the test in terms of incubation time, reagent concentration, buffer composition, etc.

5.3.1. Indirect ELISA using immobilized peptide (Procedures 1 and 2, Table 5.1)

This test can be used to quantitate the amount of antibodies present in antisera raised against a peptide or the corresponding protein which are capable of reacting with an immobilized peptide. It consists of the following steps.

(a) Microtitre wells (e.g., polyvinyl plates; Falcon, USA, Ref 3912) are coated by incubation overnight at 4°C or for 2–6 h at 20 or 37°C with 100–300 μl of 0.2–2.5 μM peptide solution diluted in carbonate buffer, pH 9.6 (15 mM Na_2CO_3; 35 mM $NaHCO_3$; 0.2 g/l NaN_3). If the peptide does not become adsorbed when this buffer is used, other buffers in the pH range 4–8 should be tried (Geerligs et al., 1988). When peptide conjugated to a carrier protein is used for coating the wells, the concentration of conjugate expressed as peptide should also be in the range 0.2–2.5 μM (usually 1 μM).

(b) Repeated washings (at least 3) with phosphate-buffered saline, pH 7.4, containing 0.05% Tween-20 (PBS-T).

(c) Blocking of remaining sites on the plastic by incubation with 10 mg/ml bovine serum albumin in PBS-T, for 1 h at 37°C.

(d) Repeated washings with PBS-T.

(e) Incubation with the antiserum to be tested, diluted in PBS-T, for 2 h at 37°C. The range of two-fold dilutions appropriate for a peptide antiserum is usually from 1/100 to 1/12 800 although dilution end points of 10^{-5}–10^{-6} may sometimes be observed.

(f) Repeated washings with PBS-T.

(g) Incubation with a suitable anti-immunoglobulin enzyme conjugate, diluted in PBS-T in the range 1/500 to 1/8000, for 1–3 h at 37°C. If the peptide antiserum was raised in a rabbit, the enzyme conjugate could be goat anti-rabbit globulin conjugated to alkaline phosphatase (Procedure 1, Table 5.1).

(h) Repeated washings with PBS-T.

(i) Incubation with an appropriate enzyme substrate for 1–3 h at 37°C. If the enzyme conjugate was prepared with alkaline phosphatase, the substrate *p*-nitrophenyl phosphate at 1 mg/ml in 0.1 M diethanolamine buffer, pH 9.8, is used. This substrate is available as tablets (Sigma, St Louis, Ref 104105) or in powder form (Boehringer, FRG, Ref 107905). Several other enzyme-substrate systems are commercially available (Tijssen, 1985).

(j) Reading of the optical density (at 405 nm for the above mentioned system) in a suitable ELISA reader such as the Titertek Multiskan MC (Flow Laboratories) or the Autoreader Micro ELISA (Dynatech).

Typical titration curves of peptide antisera obtained by this method are presented in Figs. 3.1 and 5.1. The reactivity of antisera raised against the C-terminal hexapeptide of histone H3 with the corresponding peptide and with the whole histone is illustrated in Fig. 5.2.

Instead of using the indirect immunoassay approach, it is also feasible to use antipeptide labelled antibodies. This method was used for instance to detect the cyclic undecapeptide, cyclosporin, in an inhibition format of enzyme immunoassay and was found to be more sensitive than liquid-phase RIA or other solid-phase immunoassays (Quesniaux et al., 1987). In this competitive assay using enzyme-labelled monoclonal antibodies, 0.2 ng of cyclosporine caused 50% inhibition of binding (Fig. 5.3). Under the conditions used, the within-assay coefficient of variation was in the range 1.5 to 10.5%.

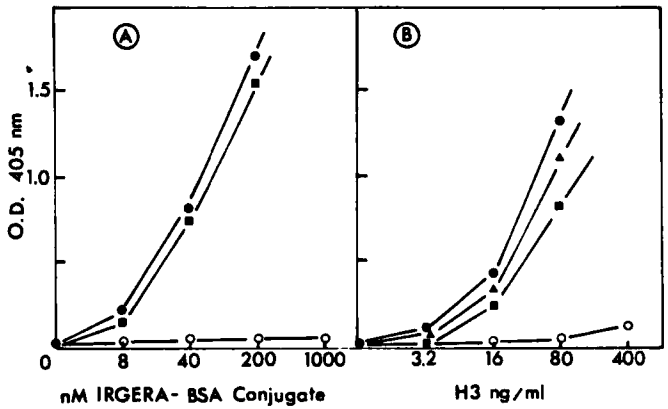


Fig. 5.2. Reactivity in indirect ELISA of two antisera raised against the C-terminal hexapeptide of histone H3 (residues 130–135 of sequence IRGERA). Antisera diluted 1:1000 were tested against an IRGERA–BSA conjugate (coupling ratio 21:1) prepared with glutaraldehyde (in A) and against the complete H3 histone molecule (in B). A concentration of 16 ng/ml of H3 corresponds to about 1 nM. ●: Antiserum to IRGERA–ovalbumin conjugate was prepared by photochemical coupling (coupling ratio 2:1); ▲: antiserum to IRGERA–ovalbumin conjugate prepared with glutaraldehyde (coupling ratio 13:1); ■: antiserum to H3; ○: antiserum to an unrelated peptide conjugated to ovalbumin by photochemical coupling (see section 3.4.9). The ELISA procedure described in section 5.3.1 was used. Substrate hydrolysis time was 45 min (P. Claudon and S. Muller, unpublished results).

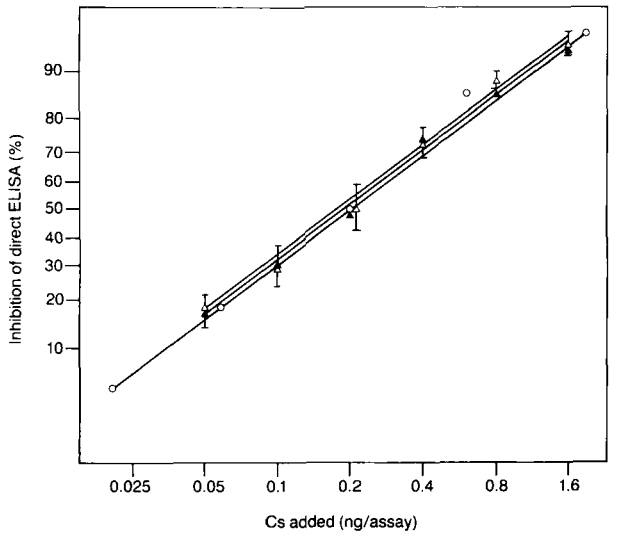


Fig. 5.3. Detection of the cyclic peptide cyclosporine by inhibition of direct ELISA. Microtitre plates coated with 0.25 μg/ml of BSA–cyclosporine conjugate were incubated with enzyme-labelled monoclonal antibody (1 μg/ml of antibody) for 2 h at 4°C in the presence of increasing quantities of cyclosporine inhibitor. Inhibition standard curves correspond to three experiments performed on different days (△, ○, ▲). Bars indicate 2 standard deviations (from Quesniaux et al., 1987).

5.3.2. Double antibody sandwich assay

In this type of assay, the antigen will become attached to two different layers of antibodies and it must therefore be multivalent. This means that either a protein or at least a fairly long peptide must be used as antigen.

As enzyme conjugate, it is possible to utilize the antipeptide serum labelled with an enzyme (Procedure 3, Table 5.2). However, this means that each antiserum to be tested has to be labelled. It is therefore more practical to use an indirect procedure with antisera obtained from two animal species such as chickens and rabbits (Procedure 4, Table 5.2). An example of such an assay is presented in Fig. 5.4. The assay consists of the following steps.

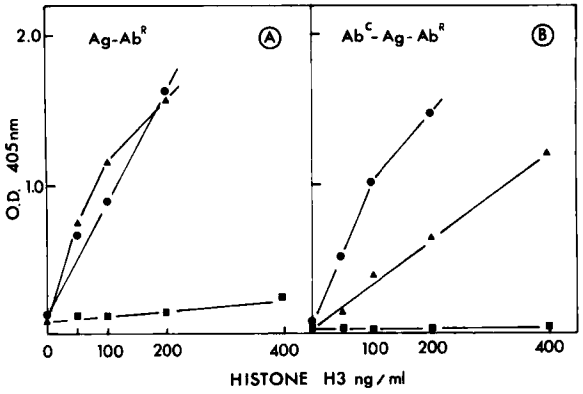


Fig. 5.4. Comparative reactivity in indirect ELISA (A) and in double antibody sandwich ELISA (B) of two antisera raised against the hexapeptide IRGERA of histone H3. The same antisera as in Fig. 5.2. were used, i.e., prepared against a conjugate prepared with glutaraldehyde (●) and against a conjugate prepared by photochemical coupling (▲). Normal rabbit serum was used as control (■). (A) H3-coated plates were incubated with antipeptide rabbit antisera (diluted 1:1000) for 2 h at 37°C. After blocking and washing an anti-rabbit globulin enzyme conjugate was incubated for 1 h. Substrate incubation time was 1 h (see section 5.3.1). (B) Plates were coated with 10 μg/ml chicken anti-H3 globulins for 2 h at 37°C. Subsequent incubations were with H3 (2 h) and with antipeptide rabbit antisera (2 h). Enzyme conjugate and substrate were as in (A) (P. Claudon and S. Muller, unpublished results).

(a) Wells are coated by incubation (2 h at 37°C) with 1–10 μg/ml chicken immunoglobulins diluted in PBS-T. These immunoglobulins can be obtained from laying hens immunized with the protein and are easily extracted from the egg yolks (Polson et al., 1980; Van Regenmortel, 1982).

(b) Repeated washings with PBS-T (see section 5.3.1).

(c) Blocking with 1% BSA in PBS-T, followed by washing.

(d) Incubation with the protein diluted in the range 20–500 ng/ml in PBS-T, for 2 h at 37°C.

(e) Repeated washings with PBS-T.

(f) Incubation with the antipeptide rabbit serum diluted in the range 1/100 to 1/10 000 in PBS-T containing 1% BSA, for 2 h at 37°C.

(g) Repeated washings with PBS-T.

(h) Incubation with goat anti-rabbit globulin enzyme conjugate, and further steps as outlined in section 5.3.1.

This assay can also be used in the inhibition format, in which case the antipeptide serum is incubated for 1–2 h with the peptide or a series of peptide analogues (Procedure 2, Table 5.2). To prevent the peptides from binding to the antibody-coated plates, this incubation must be carried out outside of the microtitre wells. In common with all competitive assays, this method is particularly suited for comparing the antigenic reactivity of a large number of different peptides.

5.3.3. Solid-phase radioimmunoassays (RIA)

The various assays listed in Tables 5.1 and 5.2 can also be used in the RIA format by replacing the enzyme conjugate by a radiolabelled antibody or alternatively by detecting the antigen–antibody complex by means of ^{125}I-labelled protein A. Following the incubation step with radiolabelled reagent (for instance ^{125}I-protein A from Amersham, UK, 60–100 μCi/μg, using 4×10^4 cpm/ml incubated for 1 h at 37°C) and repeated washings to remove excess radiolabelled reagent, the wells are cut from the plate and read in a gamma counter (Muller et al., 1982b).

Practical instructions concerning the performance of different types of radioimmunoassays are available in several textbooks (Parratt et al., 1982; Newby et al., 1986). When a competitive assay is used (Procedure 4, Table 5.2), peptides containing a tyrosyl residue can be radiolabelled with [^{125}I] sodium iodide using the chloramine-T method (Greenwood et al., 1963). Peptides containing cysteine and methionine residues can be labelled with Iodogen (Pierce Chemicals, Rockford, Illinois) using the method of Fraker and Speck (1978).

5.4. Monitoring of the immune response to peptides

Solid-phase immunoassays are widely used to follow the development of the immune response in animals immunized with synthetic peptides.

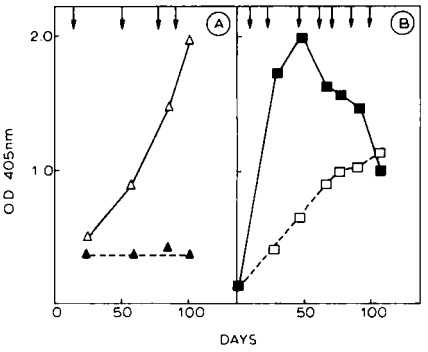


Fig. 5.5. Variability of the antibody response to peptides in rabbits subjected to the same immunization protocol. (A) Antibody levels measured by indirect ELISA in two rabbits (△ and ▲) immunized by a series of subcutaneous injections (indicated by arrows) with 100 μg of a 15-residue-long peptide of histone H2A. Antisera were diluted 1:500. Plates were coated with 2 μM unconjugated peptide. (B) Development of antibody response to an 18-residue-long peptide conjugated to ovalbumin in two rabbits (□ and ■) immunized by one subcutaneous and several subsequent intramuscular injections (arrows). Antibody levels were measured by indirect ELISA using plates coated with 2 μM unconjugated peptide and antisera diluted 1:1000.

Since the outcome of an immunization protocol is unpredictable, it is essential to bleed the animals frequently and to test each serum for the presence of antibodies reacting with different forms of the peptide as well as with the cognate protein. The variability of the immune response to peptides in rabbits receiving the same immunization schedule is illustrated in Fig. 5.5.

For instance, a 15-residue-long peptide (residues 12–26 of histone H2A) when used as a free, unconjugated peptide was found to elicit an immune response in only one of two rabbits (Fig. 5.5.A). In recent years, a few reports have appeared indicating that small synthetic peptides of 5–10 residues could be immunogenic when administered in free form (Young and Atassi, 1982); however, the affinity of the antibodies obtained in this way was about 10^3-fold lower than the values obtained with carrier-conjugated peptide (Mariani et al., 1987).

The data presented in Fig. 5.5.B show that antiserum titres do not necessarily continue to rise during the course of immunization, indicating the need to collect serum at frequent intervals.

CHAPTER 6

Detection of gene products with antipeptide antibodies

6.1. Introduction

A powerful method for identifying previously undetected proteins consists in revealing their presence by means of antibodies raised against synthetic peptides, the sequence to be synthesized being deduced from the nucleotide sequence of the protein gene. The peptide sequences selected for synthesis usually correspond to regions of the protein that are believed to be particularly antigenic on the basis of prediction algorithms (see chapter 1). These algorithms are derived from established correlations between antigenically active regions and structural features of the protein such as chain termination, hydrophilicity and segmental mobility. Antisera raised against these peptides are expected to recognize epitopes on the protein synthesized *in vivo*. The feasibility of this approach has been demonstrated in many studies, especially for the detection of viral proteins (for reviews, see Lerner, 1982, 1984; Sutcliffe et al., 1983; Walter, 1986).

When the proteins are synthesized in living organisms, other immunological techniques have been developed for identifying them.

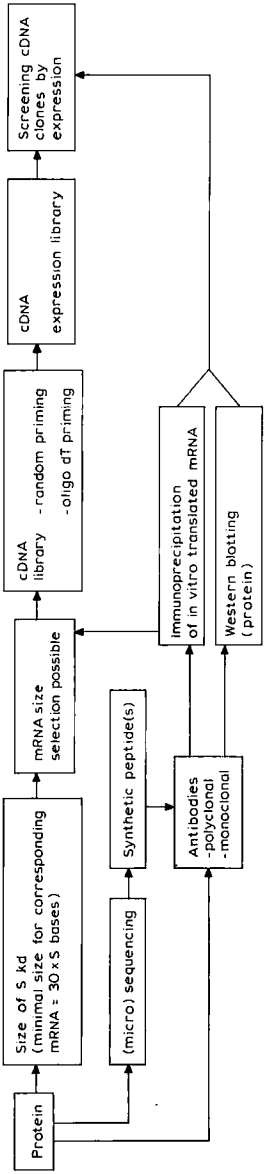


Fig. 6.1. Detection of gene products with antipeptide antibodies (from Jeltsch, 1988).

Some investigators, for example, used fusion proteins of which a part was encoded by an inserted DNA sequence and the remainder was encoded by a vector gene. These fusion proteins were used either to stimulate antibody production (Kleid et al., 1981; Coppel et al., 1983; Schlomai and Zadok, 1984) or to select by affinity chromatography those antibodies present in a polyspecific antiserum that recognize the protein encoded by the cloned sequence (Hall et al., 1984; Crewther et al., 1986).

The aim of this chapter is to describe some of the methods involving antipeptide antibodies that can be used for finding gene products in a cellular extract and for detecting *in vitro* translation products of viral RNA in cell-free extracts (Fig. 6.1).

Specific experimental details concerning DNA cloning as well as transcription and translation techniques have been described extensively in numerous texts and reviews (Maniatis et al., 1983; Hames and Higgins, 1984; Glover, 1985; Hirama, 1985). A number of suitable vectors have been developed for cDNA bacterial expression such as pBR322, λgt11, pEX. The detection of expressed cloned sequences by antibody screening methods is simplified by using expression vectors that produce insoluble hybrid protein products (Stanley and Luzio, 1984) and by working with bacterial hosts that are defective in protein degradation pathways (Young and Davis, 1983a).

As pointed out by Lerner (1984), it is important, in order to correctly identify a particular gene product, to use antibodies raised against peptides corresponding to several different regions of the putative protein. First, a single antipeptide antiserum may not recognize certain forms of the unprocessed gene product. Second, in order to ensure that the detected product pertains to the studied protein, it is important to show that antibodies directed to more than one peptide recognize the protein and that non-related antibodies do not bind to it. By using a double antibody sandwich immunoassay with antipeptide antibodies specific for two different epitopes of the protein, the possibility of erroneously identifying a protein owing to nonspecific reactions is virtually eliminated (Walter, 1986). The specificity of the observed reaction can also be demonstrated by inhibition experiments with the free peptide. As the

total amount of protein used in screening assays is often very high, problems caused by coprecipitation and nonspecific absorption of antibody are often encountered and it is therefore essential to use suitable controls to establish binding specificity.

In the following two sections, the main applications of antipeptide antibodies for identifying gene products will be briefly summarized and a few selected techniques will be described.

6.2. Use of antipeptide antibodies for detecting gene products

6.2.1. Detection of putative proteins on the basis of nucleic acid sequences

Antipeptide antibodies have been found particularly useful for identifying DNA and RNA tumour virus proteins implicated in cell transformation, and it is in this field that putative proteins were first successfully characterized by means of antipeptide antibodies. Sutcliffe et al. (1980) succeeded in identifying in infected cells a new retroviral gene product, the protein R from Moloney leukaemia virus (Mo-MuLV). They used an antiserum to a pentadecapeptide corresponding to the C-terminal region of the R protein predicted from the nucleotide sequence. Using a similar approach, Papkoff et al. (1981) identified a product from the '*mos*' gene of Moloney murine sarcoma virus (Mo-MuSV).

Antipeptide antibodies have been used to demonstrate the existence of relationships between different proteins. For instance, Robbins et al. (1983) and Devare et al. (1983), using this approach, observed a relationship between the '*sis*' oncogene protein of simian sarcoma virus (SSV) and a normal human protein of known function, the human platelet derived growth factor. These studies were carried out by means of antipeptide antisera raised against the N- and C-terminal regions of the predicted oncogene protein of SSV. Niman (1984) confirmed this result using antibodies to a peptide of 17 residues which corresponded to an internal segment of the SSV transforming protein.

Proteins encoded by viral v-*myc* and cellular c-*myc* oncogenes were also identified by means of antipeptide antibodies (Hann et al., 1983;

Giallongo et al., 1983; Persson et al., 1984; Patschinsky et al., 1984; Gazin et al., 1986). There is considerable interest in the c-*myc* gene since Burkitt lymphoma cells present translocations that bring the c-*myc* gene in close proximity to immunoglobulin heavy and light chain loci (Taub et al., 1982; Dalla-Favera et al., 1982). Some *myc* oncogene products have been shown by means of antipeptide antibodies to be evolutionarily highly conserved (Persson et al., 1984). The oncogene c-*myc* has also been identified in normal cells (Hann et al., 1983). The proteins that were detected by means of antipeptide antibodies were phosphorylated and localized in the cell nucleus of infected cells. In non-infected cells, the *myc*-related protein did not appear to be phosphorylated (Hann et al., 1983). The *myc* gene consists of three exons and two introns, exons 2 and 3 coding for the cellular homologue of the v-*myc* protein. Gazin et al. (1986) recently showed that a protein with an apparent size of 58 kDa was a dimer of a 32 kDa protein and represented a product derived from the *myc* exon 1. Both 58 kDa and 32 kDa proteins were recognized using five different antipeptide antisera elicited by peptides, ranging from 21 to 28 residues, which corresponded to non-overlapping regions of the putative product of this exon. The role played by the *myc* gene in cellular metabolism and in ontogeny still remains unclear.

Antipeptide antibodies have also been used for identifying gene products in the case of the large T antigen of simian virus 40 (SV40) and polyoma virus (Walter et al., 1980, 1981; Dietrich, 1985). In these experiments, Walter and his colleagues used antibodies to the C-terminal hexapeptide of polyoma virus medium T antigen as well as antibodies to a hexapeptide from the C-terminus of SV40 large T antigen (Walter, 1986).

Antibodies to peptides have also been used to track viral or cellular protein precursors during their processing. For example, Semler et al. (1982), and Baron and Baltimore (1982a, b) have demonstrated the existence of a common precursor for the poliovirus replicase and the polio RNA-linked protein, VPg. Other examples of this approach are the analysis of precursor proteins encoded by the '*gag*' gene of human T cell leukemia virus (HTLV) (Hattori et al., 1984) and of proteins found in cells infected with Mo-MuLV (Green et al., 1981) and influenza virus

(Sutcliffe et al., 1983; Lerner, 1984).

In their study of the human T cell leukemia viruses, HTLV-I and HTLV-II, Slamon et al. (1984) used antipeptide antibodies for characterizing products encoded by a region previously termed 'X' which is located between the gene *'env'* and the 3' long terminal repeat of these viruses. These workers prepared three synthetic peptides corresponding to the sequence of the 'X' region of the HTLV-I virus. Antisera were prepared and tested in immunoprecipitation experiments with lymphoid cells transformed or infected by HTLV-I or HTLV-II. Two proteins of 40 kDa and 37 kDa were characterized in HTLV-I and HTLV-II infected cells, respectively. It has been suggested that the 'X' region proteins may be involved in the regulation of viral RNA expression (Sodroski et al., 1984).

Immunodetection by means of antipeptide antibodies was also used successfully for identifying several unknown mitochondrial proteins whose existence had been predicted on the basis of unidentified open reading frames in mitochondrial DNA (Chomyn et al., 1983; Mariottini et al., 1983). This study also showed that antipeptide antibodies are valuable probes both for assigning reading frames and for detecting the products of overlapping genes.

Many other applications have been reported, for instance the identification of several non-structural proteins in virus-infected plants (Xiong et al., 1984; Ziegler et al., 1985; Laquel et al., 1986; Berna et al., 1986), the identification of proteins postulated to be present in malignant tumors, e.g., the protease transin (Matrisian et al., 1986), the pS2 protein found in the human breast cancer cells MCF-7 (Nunez et al., 1987), and the p21 proteins, encoded by the *'ras'* family of cellular genes, which are modified at a single amino acid residue (at position 12 in the sequence) in their oncogenic forms (Clark et al., 1985; Wong et al., 1986).

Many studies have also been carried out on the coding sequences of hepatitis B viral genes (Neurath et al., 1986 a, b; Neurath and Kent, 1987). One of the open reading frames on hepatitis B virus (HBV) DNA comprises the coding region for the viral envelope proteins, known as the *'env'* gene. This gene has the capacity to code for three related

proteins: the S protein (226 amino acids) (Tiollais et al., 1981), the M protein, containing an additional 55 amino acids at the N-terminal end of S protein (coded by a portion of the *'env'* gene upstream of the S gene known as pre-S2), and the L protein, corresponding to the sequence of M protein with an additional 108 N-terminal amino acids (encoded by the pre-S1-region). Using antibodies raised to synthetic peptides corresponding to the pre-S gene, Neurath et al. (1985a) showed that all three *'env'* encoded proteins were present in HBV particles and that pre-S epitopes played an important role both in hepatitis B virus infection and in the human immune response to HBV. The presence of pre-S sequences has been shown to enhance the immune response to S protein (Milich et al., 1985; Coursaget et al., 1985). A new immunoenzymatic assay was developed (Neurath et al., 1985b, 1986a) for detecting the presence of pre-S sequences in HBV preparations and this test also made it possible to evaluate hepatitis B vaccines for the presence of sequences corresponding to the pre-S regions. In this assay, antibodies to the synthetic pre-S peptide (residues 120–145) were used to coat polystyrene beads. Recently Itoh et al. (1986) showed that chimpanzees could be protected against HBV infection by a synthetic peptide vaccine that contained the sequence 14–32 of the pre-S2 region (see chapter 7).

6.2.2. Immunodetection of in vitro translation products using antipeptide antibodies

In vitro translation products have been characterized by many different methods. Most of them require that the proteins be labelled during their synthesis by incorporation of radioactive amino acids. However, if antibodies specific for the translation products are available, they represent an alternative powerful method for identifying and quantifying these products. The specificity of these antibodies makes it possible to avoid the problems caused by multiple translation that occur when many mRNA species are present. The strategy used for identifying translation products involves either immunoprecipitation experiments or a separation of translated products on SDS-gel, followed by transfer to nitrocellulose sheets and immunodetection by specific antibodies (Towbin et

al., 1979; Clemens, 1984). This approach was used, for instance, by Berna et al. (1984, 1985, 1986) in their characterization of the non-structural proteins of alfalfa mosaic virus (AMV) found in infected tobacco plants. Antisera to synthetic peptides corresponding to the C-termini of the three predicted non-structural proteins, P1, P2, P3, were used to detect these proteins in crude membrane fractions of AMV-infected leaves. In a wheat germ translation system, the three antisera also recognized the respective translation products derived from the viral RNA species 1, 2 and 3 (see Section 6.3.3).

6.3. Selected techniques used for the immunological detection of gene products

Detailed accounts of the relevant techniques have been published in numerous publications (Kemp and Cowman, 1981; Clemens, 1984; Huynh et al., 1985; Wang and Esen, 1985; Ridley et al., 1986; Snyder et al., 1987). In the present section, the basic methodology used in all immunochemical studies of gene products will be described. Three examples from different fields will be used to illustrate the wide range of possible applications.

6.3.1. Gene isolation with antibody probes using λgt11 expression vector

Young and Davis (1983 a, b) have developed a very efficient technique for isolating gene sequences by screening *E. coli* expression libraries with antibody probes, using the bacteriophage expression vector λgt11. This vector has become increasingly popular for the construction of cDNA or genomic libraries, mainly because of its ability to express high levels of the desired proteins. Cloning into the unique *Eco*RI site located in the 3′ end of the λgt11's Lac Z gene can result in the expression of the foreign DNA as a part of a β-galactosidase fusion protein in a lawn protease deficient *E. coli* strain. These fusion proteins are generally stable enough to be detected with antibody probes.

The procedure for isolating genes by immunoscreening developed by

Snyder et al. (1987), and slightly modified by Gradwohl and de Murcia (1988), is described below.

6.3.1.1. Construction of a recombinant DNA library in λgt11

Genomic libraries are used for organisms such as Drosophila or yeast that have a small genomic size and few introns in their coding sequences. In this case the number of genomic recombinants that must be screened in order to isolate the gene of interest is not too large. For organisms such as mammals which have a large genomic size, it is necessary to use cDNA libraries. The construction of cDNA and genomic libraries has been described in detail elsewhere (Huynh et al., 1985; Snyder et al., 1987).

6.3.1.2. Preparation of antibody probes

Both polyclonal and monoclonal antibodies have been used successfully for isolating genes by means of λgt11 libraries. When polyclonal antibodies are used, problems are sometimes encountered owing to the presence of anti-*E. coli* antibodies in the serum. These contaminating antibodies can be removed by incubating the antiserum with an *E. coli* lysate bound to Sepharose 4B (Young and Davis, 1983a) or nitrocellulose filters (Schleicher and Schüll BA 85, 0.45 μm) (Johnson et al., 1985). The sensitivity of the assay can be enhanced by using affinity-purified antibodies (O'Brien et al., 1986).

6.3.1.3. Screening λgt11 libraries with antibody probes

The method developed by Young and Davis (1983 a, b) and modified by Huynh et al. (1985) consists of the following steps.

– *E. coli* Y1090 cells are infected with phages of the λgt11 library and plated on LB soft agar, at approximately 10^5 plaques per 150 mm plate.

– The plates are incubated for 4 h at 42°C.

– A dry nitrocellulose filter previously saturated with 10 mM isopropyl-β-D-galactopyranoside (IPTG) and air dried is placed on the agar and incubated for 8 h at 37°C. The filter position is marked with a needle dipped in waterproof ink.

– The filters are removed from plates and are washed for 5 min in TBS (10 mM Tris, pH 8, 150 mM NaCl).

For duplicate screening a second IPTG-treated filter is overlaid on plates and incubated for an additional 3 h at 37°C.

– Filters are incubated in the antibody solution at a suitable dilution in TBS, 0.05% Tween 20 (TBS-T) and 5% powdered milk (12.5 ml/large filter) for 2 h at room temperature (Johnson et al., 1984).

– The filters are washed 3 times in TBS-T for 10 min.

– Detection of the antibody probes :

(a) ^{125}I *protein A*

Treat filters with ^{125}I-labelled protein A (> 30 mCi/mg) (Amersham IM 144) at a concentration of 1 μCi in 10 ml TBS-T per large filter for 5 h.

Wash filters 3 times in TBS-T for 10 min.

Dry the filters and expose at −70°C to Kodak X-O-mat AR film and a Cronex Lightning Plus intensifying screen.

(b) Horse-radish peroxidase conjugated second antibody

Incubate filters for 2 h with horse-radish peroxidase-conjugated second antibody at a 1:1000 dilution in TBS-T, 5% powdered milk at room temperature.

Wash the filters 3 times in TBS-T for 10 min.

Incubate the filters 30 min in the following solution: 10 ml of 10 mM Tris, pH 7.4, 200 μl orthodianizidine, 4 μl H_2O_2 30%.

– Remove an agar plug at the position of each positive signal and incubate it in 10 mM Tris, pH 7.5, 10 mM $MgSO_4$.

– Replate the phages at a lower density (use serial dilutions, for example 10^{-3}, 10^{-4}, 10^{-5}) and rescreen until all plaques on the plate produce a positive signal.

6.3.1.4. Preparation of E. coli lysates

The most common method for examining translation products derived from λgt11 recombinants consists of lysogenizing positive phages in *E. coli* Y1089 (Glover, 1985). After induction of the lytic cycle, IPTG is added, and the proteins of the lysates are examined on SDS–polyacrylamide gel and by Western blot analysis. If difficulties are experienced with this method, the procedure developed by Gradwohl and de

Murcia (1988) is recommended :

5×10^3 p.f.u. are plated on a lawn of *E. coli* Y1090 ; one 90 mm diameter plate is prepared for each lysate. After 4 h incubation at 42°C, 1 ml of 10 mM IPTG is added to the plates and the plates are incubated for an additional 5 h at 37°C. Lysates are recovered with 0.5 ml 25% β-mercaptoethanol/10% SDS/6 M urea/300 mM Tris, pH 6.8. Aliquots (25 µl) are subjected to SDS–polyacrylamide gel and Western blot analysis in order to identify the fusion protein.

6.3.1.5. Comments

– The antibody solution, when kept at −80°C between successive uses, can be reused up to 15 times.

– To avoid false positives it is better, especially for the first screening, to screen in duplicate and to pick only the positives that appear on the two filters.

– Another technique for detecting the bound primary antibody consists in incubating the filters after probing with ^{125}I-labelled protein A, with the horse-radish peroxidase-conjugated second antibody. In this case, only the plaques giving positive signals by the two systems are further analyzed. This ensures that false positives caused by nonspecific binding of ^{125}I protein A to the nitrocellulose filters are avoided.

– The bound primary antibody can also be detected with alkaline phosphatase-conjugated second antibody (Protoblot immunoscreening system, Promega Biotec, Madison, USA) or with a biotinylated secondary antibody followed with avidin-labelled horse-radish peroxidase (Young et al., 1985).

6.3.2. Immunochemical detection of proteins related to the human c-myc exon 1 (Gazin et al., 1986; Gazin and Galibert (1988)

Five 20–30-residue-long peptides selected from the sequence derived from the human c-*myc* exon 1 were synthesized and antisera were prepared against them by immunizing three rabbits with each of the non-conjugated peptides (chapter 4).

HeLa cells collected and resuspended as described by Green et al.

(1983) were homogenized by sonication and centrifuged during 30 min at 40 000 rpm in a Beckman 50 Ti rotor. Cellular extracts corresponding to 2×10^6 cells were loaded on 5 mm wells of SDS–PAGE (12.5%) as described by Laemmli (1970). The samples were subjected to electrophoresis in an electric field of 1400 VH (gel dimension 180×170×2 mm).

6.3.2.1. Transfer onto nitrocellulose sheet and immunodetection

After migration, the proteins were transferred onto nitrocellulose paper (Schleicher and Schüll, BA 83, 0.45 μm) as described by Burnette (1981) except that the precooled buffer (180 mM glycine – 30 mM Tris – 0.2% SDS) contained 20% methanol. Transfer was carried out for 3 h in an electric field of 30 V.

After the transfer, the membrane was placed in a plastic sealable bag and incubated twice with 50 ml PBS containing 3% BSA and 20% methanol for 3 h at 37°C with gentle shaking.

The blot was then incubated successively with antipeptide antiserum diluted 1:300 in PBS – 3% BSA – 1% sheep serum overnight at 4°C and with ^{125}I protein A (Amersham IM 144) 2×10^7 Ci/ml in PBS – 3% BSA, EDTA 0.5 mM for 2 h at room temperature. After each step, the blot was washed 3 times for 30 min with PBS – 0.1% Triton.

Finally the blot was air-dried and exposed at −70°C to Kodak XAR5 film and intensifying screen. For some experiments, immunoblots were freed of bound immunoglobulins by means of 3 M guanidine thiocyanate (twice for 1 h) and used again for incubation with another antiserum (Gazin et al., 1986).

6.3.2.2. Affinity chromatography

In order to confirm the specificity of the reaction between a polypeptide and antipeptide antibodies the following procedure described by Gazin et al. (1986) can be used. This method uses two different peptides coupled to a solid-phase and the two corresponding antisera.

Each peptide was coupled to activated CH-Sepharose 4B (Pharmacia) (see Section 3.7) or to polylysine agarose (Sigma) preactivated with 0.5% glutaraldehyde (Sigma, electron microscopy grade) for 45 min at

4°C in PBS. After extensive washing (with 500 bed volumes of demineralized water) for 10 min at 4°C, 2 mg of each peptide were added to 1 ml of wet gel. The polylysine agarose support was found to have lower non-specific adsorption than the Sepharose support.

200 µl of each antipeptide antiserum were diluted in PBS-T–3% BSA and incubated with 1.5 ml of the conjugated peptide for 18 h at 4°C with rotation. Residual antibody activity that did not bind to the peptide was characterized by ELISA and immunoblotting. In order to analyze the activity of the antibodies that bound to the peptide the same experiment was repeated with 3 ml of antiserum. A small column of peptide conjugate with adsorbed antibodies was washed with 50 bed volumes of cold PBS and 10 volumes of PBS containing 1 M NaCl. The column was reequilibrated with PBS and then incubated in a batch-wise manner for 3 h at room temperature with 3 bed volumes of PBS containing 50 µg/ml of the homologous peptide, in order to release the antibodies. The eluant was then extensively dialysed against PBS at 4°C before use in immunodetection.

By repeating the experiment with two peptides corresponding to two different regions of the protein, the specificity of detection of any polypeptide is practically guaranteed.

6.3.3. Immunodetection of an in vitro translation product : non-structural proteins of alfalfa mosaic virus (AMV) mRNA

Berna et al. (1984, 1985, 1986) have used antipeptide antibodies to specifically detect non-structural proteins coded by AMV mRNA in crude plant extracts and in a wheat germ translation system.

The [^{35}S]methionine-labelled products of AMV RNA were synthesized in wheat germ extracts (General Mills, USA) and partially purified as described by Godefroy-Colburn et al. (1985). Up to 100 µg of crude extract containing 0.1–10 ng of the proteins under study were then loaded on an SDS–PAGE (Laemmli, 1970) containing 6 to 12% polyacrylamide depending on the size of the polypeptides.

6.3.3.1. Transfer onto nitrocellulose paper

After electrophoretic migration, the proteins were transferred onto nitrocellulose sheet (Schleicher and Schüll BA 83, 0.45 μm) essentially as described by Towbin et al. (1979). Transfer was for 2 h in an electric field of 8 V/cm in a standard buffer containing 25 mM Tris, 192 mM glycine, 20% methanol. In the case of high molecular weight proteins (> 100 K) transfer was significantly improved by using a 6% polyacrylamide gel (containing 0.1% SDS and Tris–glycine buffer, pH 8.3) on the cathode side of the gel that contained the samples. This method increases the local SDS concentration and favours the migration of proteins from the gel to the nitrocellulose sheet. The final efficiency of transfer was from 85 to 100% depending on the size of the proteins (24 to 126 K).

6.3.3.2. Immunoblot procedure

The immunoreactions were carried out on the nitrocellulose sheets after saturation with 5% powdered fat-free milk in PBS (Johnson et al., 1984) during 1.5 to 2.5 h at 37°C.

Antiserum was then added at the required dilution in PBS containing 1% Tween 20 and 5% milk for 18 h at 4°C. Excess of serum was removed by four washings in PBS – 1% Tween.

The reaction of specific antibodies was detected by incubation with peroxidase-labelled sheep IgG prepared against rabbit IgG (generally diluted 1:2000) during 4 h in the dark.

After additional washing in PBS–0.05% Tween, the immunoblots were incubated with the peroxidase substrate orthodianisidine (0.5 ml saturated orthodianisidine, Sigma, in methanol and 5 μl H_2O_2 at 30 volumes in 30 ml PBS–0.05% Tween) from 15 min to several hours. For long incubation times several changes of the substrate solution were used. After stopping the reaction by rinsing with water, the blots were dried and kept in the dark.

6.3.3.3. Comments

The immunodetection was significantly improved by the use of milk in PBS instead of BSA (Fig. 6.2), by increasing the concentration of Tween

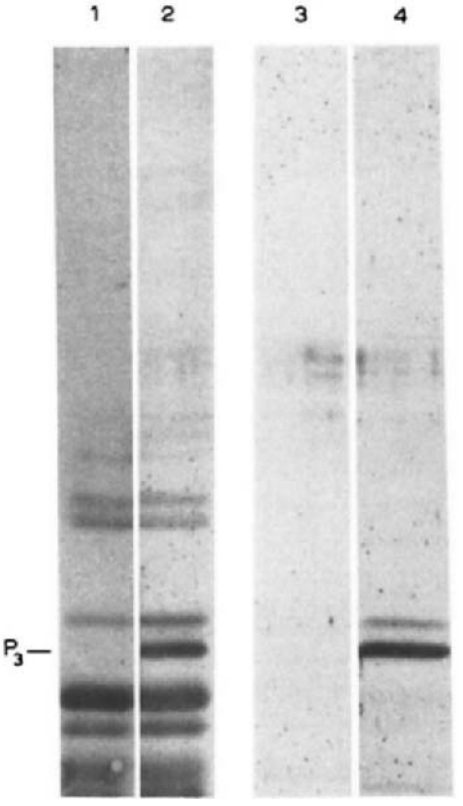


Fig. 6.2. Comparison of the influence of BSA (1,2) or skimmed milk in PBS (3,4) on the decrease of the non-specific background in immunoblotting (Berna and Godefroy-Colburn, 1988). Partially purified preparations of AMV translation products (lanes 2 and 4) and identically treated blank preparations without AMV mRNA (in lanes 1 and 3) were immunoblotted as described in 6.3.3.1 and 6.3.3.2. The presence of P3 polypeptide in the extracts was detected by using antipeptide serum diluted 1:5000.

20 up to 1% in PBS during the incubation step with specific antibodies (Fig. 6.3) and by incubating at 4–10°C instead of 20–30°C.

Generally, the dilution range for antipeptide antiserum was 1:2500 to 1:5000. The antiserum titre observed in ELISA with respect to the homologous peptide was not necessarily a good indication of how well the serum would perform in immunoblotting (Berna and Godefroy-Colburn, 1987). Different bleedings from several immunized animals had to be tested to select the most suitable one.

6.4. Discussion

Since the first studies of Walter et al. (1980) and Sutcliffe et al. (1980) demonstrating the usefulness of antipeptide antibodies for identifying DNA products, this approach has been widely used in a variety of studies in molecular and developmental biology, immunology, neurobiology and virology. Both conventional polyclonal and monoclonal antipeptide antibodies have been used for identifying gene products and for recog-

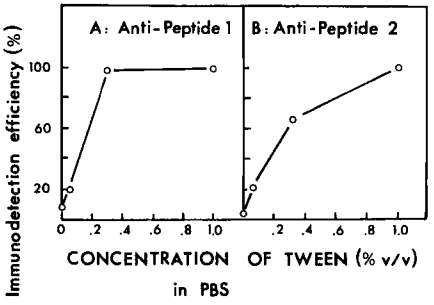


Fig. 6.3. Influence of the concentration of Tween 20 in PBS during the incubation of immunoblot with specific antiserum (Berna and Godefroy-Colburn, 1988). The immunoblotting procedure described in section 6.3.3 was used. Antipeptide antisera were diluted 1:5000 in PBS containing different concentrations of Tween. The subsequent incubation steps were in PBS containing a constant concentration of Tween (0.05%) as described in 6.3.3.2. The intensity of staining was quantified as described by Berna et al. (1986) by scanning blots at 460 nm with a Shimadzu TLC scanner (Model CS-960). The maximal absorbance was considered as 100% immunodetection efficiency and the relative staining intensity was expressed as a percentage of this maximal value. Best results were obtained by using 1% Tween (see 6.3.3.2).

nizing the cells in which these proteins are expressed. These studies clarified the functions of many proteins and also shed light on the post-translational processing of proteins. Considerable information has also been obtained regarding oncogene and protooncogene products (see 7.2.2.), MHC gene products (Choppin et al., 1986; Le Guern et al., 1987) and hormone receptors (Mulchahey et al., 1986).

A survey of the literature indicates that one of the most successful strategies for obtaining suitable reagents consists of raising antisera against the C- or N-terminal regions of the protein. Peptides of a length of 6 to 20 amino acid residues are mostly used and usually are conjugated to a carrier for immunization purposes (Tanaka et al., 1985; Walter, 1986). Somewhat longer peptides are synthesized when internal regions of the putative protein are used (Niman, 1984; Paucha et al., 1984; Neurath et al., 1985a; Tanaka et al., 1985; Gazin et al., 1986).

Antipeptide monoclonal antibodies have been used only rarely (Bellet et al., 1984; Le Guern et al., 1987). It seems that the high specificity of monoclonal antibodies for a particular epitope conformation tends to make them less suitable reagents for a type of application which requires extensive antigenic cross-reactivity (see chapter 1). In addition, the use of monoclonal antibodies in immunoprecipitation and immunoblotting experiments leads to more frequent nonspecific reactions.

Although there is a tendency not to report negative results, there is evidence that not every peptide selected on the basis of the usual prediction algorithms necessarily induces antibodies able to cross-react with the corresponding protein (Alexander et al., 1983; Altman et al., 1984). It is sound policy, therefore, to raise antibodies against several synthetic peptides corresponding to both terminal and internal regions of a protein. In addition to increasing the likelihood of obtaining suitable reagents, this will make it possible to use double antibody sandwich assays which greatly decreases the possibility of confusion due to nonspecific reactions (Walter, 1986).

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CHAPTER 7

Synthetic peptides as vaccines

7.1. Introduction

In recent years, there has been considerable interest in the use of synthetic peptides as potential vaccines against viral, bacterial and parasitic diseases (Arnon, 1980; Shinnick et al., 1983; Arnon et al., 1983; Chanock and Lerner, 1984; Lerner et al., 1985; Brown et al., 1986a). As information accumulated on the location of continuous epitopes in toxins, viruses and parasites, attempts were made to achieve protective immunity by immunizing animals with synthetic peptides that mimic certain epitopes of these agents. Following the determination, in 1960, of the amino acid sequence of the coat protein of tobacco mosaic virus (TMV), Anderer (1963) showed that short, C-terminal peptides of the viral protein could elicit antibodies that recognized the virus and neutralized its infectivity. Anderer used both natural and synthetic peptides in his work (Anderer and Schlumberger, 1965a, b, 1966a, b; Anderer et al., 1967) and should thus be credited with the discovery that synthetic peptides can elicit protective immunity against a viral infection. This principle was further elaborated by Sela and his colleagues who investigated the immunogenicity of model synthetic peptides (Sela, 1969) and showed that the bacteriophage, MS2, could be inactivated by antibodies

raised against a 20-residue synthetic segment of the viral coat protein (Langbeheim et al., 1976).

As our knowledge of the antigenic structure of viruses and other infectious agents progressed (Van Regenmortel and Neurath, 1985; Brown et al., 1986a; Pearson, 1986) it became possible to apply the same principles to the development of synthetic vaccines relevant to human and veterinary medicine. Efforts in this direction have been stimulated by the difficulties experienced in preparing conventional vaccines against certain infectious agents that present major public health problems today.

7.2. Requirements for successful vaccines

Although the dramatic successes achieved in the past by classical vaccines can hardly be overemphasized (Brunell, 1985; Babiuk, 1985), it remains true that the production and development of new vaccines today presents many difficult challenges (Melnick, 1986). Some of the contemporary problems can be summarized as follows :

(a) Increased requirement for safety. Because of high liability costs, manufacturers are faced with the difficulty of establishing the complete innocuity of killed viral vaccines and of evaluating the likelihood of genetic reversion of live, attenuated vaccines.

(b) Large scale production of vaccine preparations. Since large amounts of infectious material are needed for preparing any vaccine, burdensome containment procedures are required. Furthermore, some infectious agents do not grow in culture or grow only in unacceptable systems such as cancer cell lines that may present a danger to vaccine recipients.

(c) Antigenic variability of the pathogen. Certain infectious agents use the successful survival strategy of antigenic variation and frequently alter their neutralization epitopes by some genetic or phenotypic mechanism (Birkbeck and Penn, 1986). It is no coincidence that agents that undergo

such variation are responsible for major public health problems not yet amenable to vaccination, such as malaria, trypanosomiasis, gonococcal infections, the lentigroup of retroviruses and influenza. Ongoing antigenic variation requires the continuous development of new vaccines or alternatively, it demands a new type of approach, for instance, the unlocking of the expression of invariant but cryptic epitopes endowed with the ability to elicit neutralizing antibodies.

(d) The cost factor. The development of new, conventional vaccines entails a complex, high cost technology, which may be incompatible with the small potential market for some vaccines in developed countries or even with a large market in poor, developing countries.

The interest in synthetic vaccines derives in part from the expectation that the difficulties mentioned above may be circumvented by using new vaccination approaches. Recombinant DNA technology has made it possible to express viral proteins in bacteria, yeast and mammalian cells (Patzer and Obijeski, 1985; Berman, 1987) and such products may, in future, form the basis of stable, subunit vaccines. Another promising approach consists in introducing genes of other pathogens such as hepatitis B into vaccinia virus and using the recombinant vaccinia virus as a live vaccine (Mackett et al., 1984; Brown et al., 1986b).

The use of synthetic peptides for vaccination would have the advantage that such a vaccine is a chemical rather than a biological product, i.e., it is chemically defined and would be stable indefinitely; furthermore, the possibility of contamination with infectious agents or cellular products would be eliminated. Since it would be safe, stable and cheap, a synthetic vaccine would present many advantages. However, these advantages should not obscure the potential problems linked to the use of synthetic peptide vaccines. A vaccine containing a single epitope or a limited number of epitopes may fail because of genetic restrictions of the immune response in the vaccinated population. To overcome nonresponsiveness in certain individuals, it may be necessary to immunize with a mixture of several synthetic peptides, each one being able, in principle, to elicit a protective immune response on its own. A second impor-

tant limitation of synthetic peptides is caused by their relatively low immunogenicity. Although the use of carriers such as tetanus toxoid enhances the immunogenicity of conjugated peptides, this approach is also not devoid of problems (Audibert, 1986).

If the same carrier is used several times, sensitization may occur or suppressor T cells may be triggered. Furthermore, the specificity of the memory cells may be that of the carrier and not of the synthetic peptide. As a rule, synthetic peptides elicit protective immune responses only when administered with an adjuvant. However, several of the adjuvants used in experimental animal models, such as Freund's complete adjuvant (CFA) or aluminium salts, produce inflammatory reactions or granulomas and are therefore unacceptable for use in humans. Other adjuvants are currently undergoing clinical trials. Muramyl dipeptide (MDP) analogs which mimic the minimal adjuvant unit present in mycobacterial cell walls, and Pluronic polymers have given promising results (Audibert, 1985; Leclerc et al., 1985; Allison and Byars, 1986).

7.3. T cell epitopes

Another important issue concerns the role of T cell responses in the development of immunity against infectious agents. Although the B cell response leading to the appearance of neutralizing antibody has received by far the most attention, the T cell response leading to cytotoxic T lymphocytes (Tc) and cellular immunity is increasingly being studied at the molecular level (Lamb et al., 1985). It is clear, for instance, that Tc lymphocytes play an important role in the recovery from viral infection. B and T cells differ in the way they recognize protein antigens as shown by the fact that T cells are unable to bind to antigen in the absence of the major histo compatibility complex (MHC) class I or class II gene products (Schwartz, 1985). Furthermore, the majority of T cells do not recognize the native conformation of protein antigens (Unanue, 1984) but bind instead to linear peptide fragments of the antigen (Townsend et al., 1986; Rothbard, 1986). It is generally believed that T cells recognize the antigen only after so-called antigen processing involving fragmentation

by proteolysis, although it is also possible that the conformation of the protein antigen is simply altered in the lipophilic environment of the cell membrane (Streicher et al., 1984).

Peptide immunogens containing both synthetic B and T cell epitopes have been constructed, which shows that it may be possible to develop synthetic vaccines that stimulate both B cells and helper T cells (Leclerc et al., 1987).

Since T cells recognize antigens in the absence of their original native conformation, peptides have been particularly useful for mapping T cell epitopes. Synthetic peptides can act as a target for cytotoxic T cells and are also able to selectively stimulate lines of T lymphocytes isolated from animals immunized with a virus. In view of the limited conformational requirements of T cell epitopes, synthetic peptides are likely to be better reagents for inducing a T cell response than a more conformation-specific, B cell response. However, MHC restriction of the cellular immune response limits the potential usefulness of synthetic peptide vaccines based on T cell epitopes.

In order to be able to predict the position of T cell epitopes in proteins, attempts have been made to correlate the structural properties of such epitopes with T cell antigenicity (DeLisi and Berzofsky, 1985; Rothbard, 1986; Spouge et al., 1987; Lamb et al., 1987). Evidence has been obtained that T cell epitopes tend to correspond to amphipathic helices and avoid random coiled conformations (Spouge et al., 1987).

7.4. Antiviral synthetic vaccines

Developing peptides suitable for vaccination is a more difficult task than simply selecting peptides able to induce antibodies that will react with the virus. Although virus particles possess a large number of different epitopes, only a certain number of them are capable of eliciting antibodies that neutralize viral infectivity. In order to obtain peptides suitable for vaccination, these neutralization epitopes are the ones that have to be mimicked by synthesis. Unfortunately, our understanding of the mechanism of viral neutralization is limited (Mandel, 1985) and this

knowledge does not provide a rationale for identifying which epitopes are likely to induce neutralizing antibodies. Peptide regions likely to be involved in the process of neutralization are usually identified empirically by showing that they bind to monoclonal antibodies that neutralize viral infectivity. Synthetic peptides corresponding to such regions are expected to be able to induce neutralizing antibodies and thus to serve as potential vaccines. In view of the extensive cross-reactions between proteins and peptide fragments, it is conceivable that in order to elicit an adequate level of protective immunity, it will not be necessary to synthesize peptides that reproduce exactly the conformational features of neutral ıtion epitopes. Some of the results obtained so far with foot-and-mo ı disease, polio, hepatitis B, influenza and human immunodeficiency viruses will be briefly summarized.

7.4.1. Foot-and-mouth disease virus (FMDV)

The particles of FMDV consist of one molecule of single-stranded RNA and 60 copies of each of four structural polypeptides, VP1–VP4 (Brown, 1985). Immunization with the individual proteins showed that only VP1 was able to stimulate the production of neutralizing antibody. A synthetic peptide corresponding to residues 144–159, when injected as a KLH conjugate, was found to elicit antibodies that neutralized viral infectivity (Pfaff et al., 1982). Several peptides of VP1 coupled to KLH were investigated by Bittle et al. (1982) who found that peptide 141–160 induced the highest neutralizing activity while peptide 200–213 was less active. A single inoculation with 200 μg of the peptide 141–160 conjugate was sufficient to protect guinea pigs against a subsequent challenge with the virus. The neutralizing titre raised by this peptide was about one order of magnitude lower than that obtained with inactivated virus but was several orders of magnitude higher than that elicited by intact VP1. This indicates that it may sometimes be easier to obtain the correct immunizing conformation of a neutralization epitope with a synthetic peptide than with the complete protein. Presumably, this was due to the fact that the monomer did not fold properly when deprived of the scaffolding provided by the surrounding structural proteins. The optimal peptide length needed for

obtaining the best immunogenic response is still a matter of debate (Parry et al., 1985; Meloen et al., 1987).

The role of the carrier protein in the immune response to FMDV peptides has also been examined (Francis et al., 1985). Classical studies with hapten–protein conjugates (Mitchison, 1971; Rajewsky et al., 1969) have shown that an optimal boosting effect is obtained with the hapten coupled to the same carrier as the one used for priming. However, in the FMDV system, the response was not carrier restricted. Guinea pigs primed with peptide 141–160 KLH could be boosted with peptide 141–160 coupled to KLH or to tetanus toxoid, or with uncoupled peptide (Francis et al., 1985). In recent studies, it was shown that immunization with uncoupled peptides of FMDV could also induce antibodies capable of neutralizing the virus (Di Marchi et al., 1986; Francis et al., 1987).

7.4.2. Poliovirus

Poliovirus particles are made up of a single RNA molecule and of 60 copies of each of the four structural proteins VP1–VP4. The virus occurs in three distinct serotypes, 1, 2 and 3, which do not cross-neutralize, i.e., antibodies against one serotype do not neutralize either of the other two (Emini et al., 1985). The atomic structure of type 1 poliovirus has recently been established by X-ray crystallography (Hogle et al., 1985). Three neutralization epitopes were identified by the isolation of mutants that are resistant to neutralization by monoclonal antibodies and by mapping the sequence changes in these mutants (Hogle et al., 1986). Since all the substitutions occurred in exposed loops that are readily accessible to antibody binding, it was concluded that the mutation sites are probably located within a number of different viral epitopes. Site 1 was located on VP1 mainly between residues 89–103; site 2 comprised residues 220–222 of VP1 and residues 164–171 of VP2, while site 3 comprised residues 286–290 of VP1 and residues 58–60, 70 and 71 of VP3 (Minor et al., 1986). Sites 2 and 3 are thus neotopes, i.e., epitopes arising from the quaternary protein structure (see chapter 1, section 1.3). In view of our knowledge of the three-dimensional structure of poliovirus, the stage has been set for mimicking such neotopes by peptide synthesis

along the lines followed by Atassi in the case of discontinuous epitopes of lysozyme (Atassi et al., 1976). Several other peptides of VP1 have also been reported to elicit neutralizing antibodies in rabbits (Chow et al., 1985).

Synthetic peptide 93–103 of VP1, corresponding to site 1, was able after conjugation to BSA to elicit a significant serotype-specific level of neutralizing antibodies in rabbits (Emini et al., 1983). In addition, several other peptides which do not correspond to the sites identified in the mutation studies and which are located in the interior of the mature virion were able to prime the immune system of rabbits. After a priming injection with the peptides, a single inoculation of a sub-effective dose of virus was able to induce a neutralizing response. Since the priming peptides are not located at the surface of the protein, the immunizing activity of the corresponding regions in the virus implies that a conformational change occurs which leads to the exposure of these residues. Additional studies are required to elucidate some of the puzzling aspects of these results.

Site 1 of type 3 poliovirus was also studied using a synthetic peptide corresponding to residues 89–104 of VP1, with additional cysteine residues at the N and C termini. This peptide, when coupled to a carrier protein and administered with CFA, was able to elicit neutralizing antibody in 18 out of 19 immunized animals (Ferguson et al., 1985). The ability of antipeptide sera to neutralize variants presenting mutations within site 1 was also tested. Single substitutions in the region 93–98 were sufficient to render the virus resistant to neutralization by some of the antisera, a finding similar to earlier observations made with antisera raised against FMDV peptides (Rowlands et al., 1983). This illustrates one of the difficulties of producing an effective vaccine based on a single peptide sequence, since a highly restricted protective response centered on one epitope would be easily evaded by the selection of antigenic variants of the virus.

Monoclonal antibodies raised against peptide 89–104 were found to react differently in direct ELISA, depending on whether the antigen was free peptide, coupled peptide or the virus (Minor et al., 1985). As is commonly observed in such studies, the monoclonal antibodies recognized

different conformations of the peptide and only some of these conformations approximated to the one occurring in the native virus particle. The influence of immunoassay format on the antigenic activity of peptides is well-substantiated (Muller et al., 1986) and it is thus important to test the activity of antipeptide antibodies in assays that preserve the relevant epitope conformation (McCullough et al., 1985).

7.4.3. Influenza virus

The particles of type A influenza virus are enclosed by a lipid envelope to which haemagglutinin (HA) and neuraminidase surface antigens are attached by sequences of hydrophobic amino acids at one end of the molecule (Air and Laver, 1985). The HA accounts for about 25% [f] the virus protein and is responsible for the attachment of virus to cells. Although antibodies to the HA neutralize the infectivity of the virus, they do not protect the host against recurrent outbreaks of influenza. This is due to the fact that the HA undergoes continuous antigenic variation which takes two different forms. The first one is a continuous antigenic drift produced by the gradual accumulation of single amino acid substitutions in any one of four major antigenic sites of the HA molecule (Wiley et al., 1981). Antigenic drift can be mimicked artificially in the laboratory by growing the virus in the presence of neutralizing monoclonal antibodies. This allows mutants to be selected which mostly contain a single substitution in one of the antigenic sites (Wilson et al., 1981; Caton et al., 1982). The infectivity of such mutants can still be neutralized by antibodies specific for any one of the non-mutated antigenic sites. It is the cumulative effect of mutations in each of the neutralization antigenic sites that eventually produces epidemiologically successful variants capable of infecting individuals immune to viruses previously in circulation.

The second type of antigenic variation occurring in influenza is known as antigenic shift. This is generated by genetic reassortment in a mixed infection involving a human influenza virus and an avian or equine strain of the virus. This leads to the sudden emergence of a completely new human HA subtype possessing many alterations in all the antigenic sites.

Following the elucidation of the atomic structure of HA by X-ray crys-

tallography (Wiley et al., 1981), four major antigenic sites were identified at the surface of the molecule by the selection of non-neutralizable antigenic variants by means of monoclonal antibodies.

In view of our extensive knowledge of the antigenic properties and three-dimensional structure of the HA (Wiley et al., 1981), influenza virus appeared to be an excellent model for studying the potential role of synthetic peptides in vaccination. Since viral strains infective in humans are also infectious for mice, it is possible to evaluate the immune response against the synthetic peptides in vitro as well as in vivo and to study antigenic cross-reactivity as well as active and passive protection. Several laboratories have investigated the immunogenicity of synthetic HA peptides.

In one series of experiments, 20 partially overlapping peptides representing over 75% of the HA sequence were synthesized, coupled to KLH and used to immunize rabbits (Green et al., 1982). Of the 20 peptide antisera, 18 were found to react in ELISA with HA and intact virus, although none of the peptides reacted with anti-HA antibodies present in influenza virus antisera. As discussed in chapter 1 (Section 1.4.2), the high frequency with which the antipeptide antibodies were found to react with HA may have been caused by partial denaturation of the HA molecule following its adsorption to the solid-phase. These results, therefore, do not represent convincing evidence for the claim that synthetic peptides corresponding to virtually any part of a protein are able to induce antibodies that cross-react with the native protein. When the aim is to obtain neutralizing antibodies, it is important to pay particular attention to the ability of the peptides to induce antibodies that react with the pathogen in a liquid phase immunoassay. Such an assay is more likely to correspond to the in vivo situation where an infectious virus particle encounters neutralizing antibodies.

Several groups have investigated the ability of synthetic HA peptides to induce protective immunity against influenza. A peptide corresponding to residues 91–108 of HA, after conjugation to tetanus toxoid, was shown to partially protect immunized mice against challenge infection with the virus (Muller et al., 1982b). This region, which does not correspond to one of the four antigenic sites identified by Wilson et al. (1981),

is conserved in the HA of several influenza strains, and the antipeptide antibodies were indeed found to elicit protection against several different viral strains. This finding is of considerable importance since it suggests that synthetic vaccines could, in principle, lead to cross-protection against different serotypes and that they may thus overcome one of the limitations of conventional vaccines against antigenically variable viruses such as influenza. The findings of Muller et al. (1982b) were confirmed by Wilson et al. (1984) who showed that the peptide 75–110 of HA could induce monoclonal antibodies reacting with HA; the major activity within this peptide was found to be located in the sequence 98–106 which is contained within the 91–108 peptide studied by Muller et al. (1982b).

Interestingly, since the sequence 98–106 is relatively inaccessible in the trimer interface of HA (Wilson et al., 1981, 1984), the results imply that a conformational change must occur in the trimeric structure prior to antibody binding. The monoclonal antibodies raised against peptide 75–110 were found to have a lower affinity for the complete HA molecule than for a series of model peptides (Wilson et al., 1986). These results agree with other observations indicating that antipeptide antibodies usually react at least 10–100-fold less well with the parent protein than with the peptide itself (Neurath et al., 1982; Hirayama et al., 1985).

Several groups have also studied the region 140–146 of HA which forms antigenic site A and corresponds to a protruding loop at the surface of the trimeric HA structure found on the intact virus. Immunization with linear peptides (residues 139–146 and 123–151) corresponding to this region did not lead to antibodies cross-reacting with the virus (Jackson et al., 1982; Shapira et al., 1984, 1985). However, immunization with a longer peptide corresponding to residues 138–164 led to the induction of antibodies that recognized the virus and provided partial protection against challenge infection with the virus (Shapira et al., 1984).

7.4.4. Hepatitis B virus (HBV)

Conventional vaccines against HBV are prepared from the viral surface

antigen, HBsAg, obtained from human blood of chronic carriers. As a result, these vaccines are very expensive and not readily available. This explains the concerted efforts of many laboratories to develop synthetic vaccines against HBV (Neurath and Kent, 1985). Synthetic peptides corresponding to nearly the entire HBsAg sequence have been studied and the regions 117–137 and 138–149 were consistently found to elicit antibodies cross-reacting with HBsAg (Hopp, 1981; Dreesman et al., 1982; Neurath et al., 1982; Prince et al., 1982; Bhatnagar et al., 1982). Since the only animal species in which protection against HBV can be investigated is the chimpanzee, the testing of potential synthetic vaccines is particularly cumbersome. Nevertheless, partial protective effects have been reported in chimpanzees immunized with the peptide 110–137 of HBsAg (Gerin et al., 1983). The sequence 139–147, which represents a major hydrophilic area within HBsAg and which seems to be able to induce both a B cell and T cell response (Steward et al., 1986), has been proposed as an important component of any synthetic hepatitis B vaccine (Howard et al., 1986).

It has recently been established that HBsAg particles contain not only the envelope S-protein of 226 residues but also longer polypeptides comprising additional residues at the N-terminal side (Neurath and Kent, 1987). The middle (M) protein comprises 281 residues, i.e., an additional 55 residues at the N-terminal end of S-protein that are encoded by the pre-S2 region of HBV DNA. The large (L) protein comprises 400 residues, i.e., an additional 119 residues encoded by the pre-S1 region (Tiollais et al., 1985). Both the M and L proteins appear to be underrepresented in HBsAg particles. In humans, HBV was shown to elicit not only antibodies to the S protein but also antibodies that recognized the M and L proteins (Neurath et al., 1984b). There is evidence that the inclusion of pre-S2 sequences in HBV vaccines may improve their efficacy (Milich et al., 1985; Neurath et al., 1986b). Recently two synthetic peptides corresponding to epitopes of the pre-S region were shown to elicit virus neutralizing antibodies (Neurath et al., 1986c; Itoh et al., 1986).

7.4.5. Human immunodeficiency virus (HIV)

HIV is the human retrovirus responsible for the acquired immune deficiency syndrome (AIDS). The complete nucleotide sequence of the HIV genome has been determined and the antigenic properties of the envelope glycoprotein gp160 have been extensively studied. This gp160 protein is processed into an amino terminus subunit, gp120, and a carboxyl transmembrane subunit, gp41 (Wong-Staal and Gallo, 1985).

The serum of AIDS patients has been shown to contain antibodies that neutralize the virus (Weiss et al., 1985; Robert-Guroff et al., 1985; Clavel et al., 1985). Presumably, infection occurred in such patients before the development of neutralizing antibody. Since the envelope glycoproteins are the most immunogenic components of HIV (Allan et al., 1985; Barin et al., 1985; Montagnier et al., 1985), several laboratories have focused their attention on the antigenic properties of these surface proteins and attempted to localize the position of continuous epitopes by the synthetic peptide approach. Kennedy et al. (1986) showed that an 18-residue peptide of gp41 (corresponding to residues 735–752 of the precursor envelope protein) was able to elicit antibodies in rabbits which recognized the gp160 in radioimmunoprecipitation tests. This peptide had been selected on the basis of its high hydrophilicity and predicted β-turn conformation and was coupled to KLH for immunization purposes.

Wang et al. (1986) showed that a 21-residue-long synthetic peptide of gp 41 (residues 586–606 of the precursor glycoprotein) was able to reproducibly detect specific antibodies in the serum of AIDS patients. In this study, ten overlapping synthetic peptides of a length of 19–21 residues were screened for reactivity with pooled AIDS serum. These ten peptides spanned a region of 102 residues located between two hydrophobic areas of gp41. Only the 21-mer peptide (residues 586–606) gave a strong positive signal with the serum of AIDS patients when the peptide was used as the solid-phase antigen in indirect ELISA. The enzyme immunoassay using the peptide as antigen gave positive results with all the sera that were reactive with the gp41 of HIV in electrophoretic immunoblot analysis.

The ability of a single peptide to detect antibodies in virtually all AIDS patients was probably due to the fact that this peptide corresponds to a highly conserved region of the gp41 envelope protein. In view of its sensitivity and specificity, this assay appeared to be preferable to assays utilizing whole disrupted virus or *E.coli*-derived recombinant products as the solid-phase antigen.

In a subsequent study (Chanh et al., 1986), the 21-mer peptide (residues 735–752) studied by Kennedy et al. (1986) as well as a 30-mer peptide corresponding to residues 503–532 of the gp160 precursor protein were studied for their capacity to elicit antibodies able to neutralize HIV. These two peptides, which correspond to regions of the gp41 and gp120 proteins respectively, were shown to induce antibodies in rabbits which reduced the replication of HIV as measured by the level of reverse transcriptase activity in culture supernatant fluids (Chanh et al., 1986). These results indicate that synthetic peptides are able to mimic neutralization epitopes of the gp41 and gp120 of HIV and that the synthetic vaccine approach may thus become feasible also in the case of AIDS. However, it should be stressed that these in vitro neutralization data need to be backed by in vivo protection data.

7.5. Antibacterial synthetic vaccines

The use of synthetic peptides for inducing an immune response to bacterial toxins has been studied in the case of diphtheria and cholera toxins. A synthetic peptide corresponding to residues 188–201 of diphtheria toxin was found to be able to elicit a neutralizing immune response against the toxin and to protect guinea pigs (Audibert et al., 1981). When this peptide was covalently attached to a multichain poly(DL-alanine) and to MDP, it induced an effective neutralizing response in mice when administered in aqueous physiological solution (Audibert et al., 1982).

The cholera toxin consists of two polypeptide chains, the A subunit which is responsible for its biological effect and the B subunit which binds to cell receptors. Synthetic peptides corresponding to residues 8–

20 and 50–64 of the B chain, when conjugated to tetanus toxoid, were able to elicit antibodies that neutralized the biological activity of the intact cholera toxin as well as that of the heat-labile toxin of pathogenic strains of *E. coli* (Jacob et al., 1983, 1984, 1985). Both toxins possess considerable sequence homology. It seems, therefore, that synthetic peptides could lead to a general vaccine suitable for protection against various forms of bacterial diarrhoea.

7.6. Antimalaria vaccine

Considerable efforts are currently being made to develop a vaccine against malaria (Miller, 1985; Coppel, 1986). The parasite *Plasmodium falciparum* is responsible for the disease in man. The life cycle of the parasite consists of (1) a sporozoite stage that is injected by the mosquito vector and initiates infection in man, (2) an asexual erythrocyte stage which causes the disease, and (3) a sexual stage when the infection is transmitted to mosquitoes. Different antigens are expressed during the various stages of the life cycle. An effective sporozoite vaccine would interrupt the life-cycle of the parasite, block infection and prevent transmission of the disease.

The sequence of a major surface protein of the sporozoite, the circumsporozoite (Cs) protein, has been derived from gene cloning studies. The Cs protein was found to contain a repeating tetrapeptide sequence $(NANP)_n$ which was recognized by human sera collected from a region endemic with malaria (Zavala et al., 1985a). A synthetic 12-residue peptide, $(NANP)_3$, when conjugated to tetanus toxoid, was able to induce in rabbits a specific response against *P. falciparum* (Zavala et al., 1985b).

Since antibodies to $(NANP)_3$ were also shown to neutralize the infectivity of the parasite, there is considerable hope that synthetic peptides may become the first successful vaccine against malaria (Nussenzweig and Nussenzweig, 1986).

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Appendix A

Sources for chemicals

Acetonitrile :	J.T. Baker; Rathburn; BDH
Amino acid derivatives :	Peptide Institute ; Bachem (S); Novabiochem; Chemical Dynamics; Propeptide; CRB
BHA and/or MBHA resins :	USB; Peninsula; Applied Biosystems; Peptide Institute; Bachem (USA); UCB
Boc AA-chloromethyl resin :	Peninsula; Bachem (USA); Novabiochem
Boc AA-Pam resins :	Applied Biosystems; Du Pont; Neosystem; Novabiochem
Chloromethyl resin :	Bio Rad
p-Cresol :	Aldrich; Merck
Dichloromethane :	Merck; Riedel de Haën
Diisopropylcarbodiimide :	Aldrich
p-Dimethylaminopyridine :	Aldrich
Diisopropylethylamine :	Aldrich; Merck
Dimethylformamide :	Fluka; Riedel De Haën
Dimethyl sulfide :	Aldrich
Ethane dithiol :	Aldrich
Fmoc polyamide resins :	CRB; Milligen
Fmoc polystyrene resins :	Novabiochem
Hydrogen fluoride :	Prodair; Matheson
N-hydroxybenzotriazole :	Aldrich
Ion Exchange resins :	Merck; Pharmacia; Whatman; IBF
Ninhydrin :	J.T. Baker
Polyamide resins :	CRB; Milligen
Resins for gel chromatography :	Merck; IBF; Pharmacia
Trifluoroacetic acid :	Aldrich
Trifluoromethanesulfonic acid :	Aldrich

Appendix B

Sources for apparatus

Manual SPP synthesizer :	Peninsula
Semi automatic SPP synthesizer :	Bachem (S) ; Du Pont de Nemours ; LKB
Automatic SPP synthesizer :	Applied Biosystems ; Beckman ; Biosearch ; Du Pont de Nemours ; LKB ; Milligen
Multi channel semi automatic SPP synthesizer :	Neosystem
Multiple peptide synthesis :	CRB (Geysen Technology) ; Biosearch (Houghten Technology) ; Du Pont de Nemours
HF Lines :	Peptide Institute ; Multiple peptide systems
HPLC systems :	Hewlett-Packard ; Varian ; Waters ; Beckman ; Gilson ; Kratos ; Shimatzu
HPLC columns :	Vydac ; Brownlee ; Waters
Amino acid analyzers :	Pico-tag (Millipore) ; Amino-tag (Varian)

Appendix C

Addresses of suppliers

Aldrich Chemical :	In USA : PO Box 355, Milwaukee, WI 53201, USA In France : 27, Fossé des Treize, 67000 Strasbourg, France
Applied Biosystems :	In USA : 850 Lincoln Centre Drive, Foster City, CA 94404, USA In France : 13 rue de la Perdrix, 95948 Roissy Charles de Gaulle, France
Bachem :	3132 Kashiwa street, Torrance, CA 90505, USA
Bachem (S) :	Hauptstrasse 144, CH-4416 Bubendorf, Switzerland
BDH Chemicals :	Poole, BH12 4NN, England
Beckman :	In USA : 1117, California Ave, Palo Alto, CA 94304, USA In France : 52–54, Chemin des Bourdons, 93220 Gagny, France
Bio-Rad :	2200 Wright Avenue, Richmond, CA 94804, USA
Biosearch - New Brunswick :	2980 Kerner Bd, San Rafael, CA 94901, USA
Chemical Dynamics :	PO Box 395, 3001 Hadley Road, South Plainfield, NJ 07080, USA
C.R.B. :	Button End Industrial Estate, Houston, Cambridgeshire CB2 5NX, England
Du Pont - Molecular Genetics Instruments :	In USA : BRMC, Chandler Mill, Wilmington DE 19898, USA In Europe : Postfach 401240, 6072 Dreieich, W. Germany
Fluka AG :	CH-9470 Buchs, Switzerland
Gilson :	In USA : Box 27, 3000 West Beltline, Middleton, WI 53562, USA In France : BP 45, 95400, Villiers-le-Bel, France
Hewlett-Packard Europe :	150, route du Nant - d'Avril, 1217 Meyrin 2, Genève, Switzerland
IBF :	35, Av. Jean Jaurès, 92390 Villeneuve la Garenne, France
J.T. Baker Chemicals :	In USA : 222 Red School Lane, Phillisburg, NJ 08865, USA In France : Sochibo, 8, rue des Frères Caudron, BP 18, 78142 Velizy Cedex, France
LKB Instruments :	In Europe : ABS - 16125 Bromma, Sweden, and BP 29, 69, rue de Paris, 91404 Orsay, France

Matheson :	PO Box 85, 932 Paterson Plank Road, Rutherford, NJ 07073, USA
Merck Schuchardt :	In Europe : Frankfurter Strasse 250, 6100 Darmstadt, W. Germany, and 5/9 rue Anquetil, BP No. 8, 94130 Nogent sur Marne, France
Multiple peptide systems :	P.O. Box 5000, Solana Beach, CA 92075, USA
Neosystem :	21, rue de la Rochelle, 67100 Strasbourg, France
Novabiochem :	Weidenmattweg 4, Postfach CH-4448 Laüfelfingen, Switzerland
Peninsula Laboratories :	In USA : 611 Taylor Way, Belmont, CA 94002, USA In Europe : Box 62, St Helens Merseyside, WA9 3AJ, England
Peptide Institute :	4-1-2 Ina, Minoh-Shi, Osaka 562, Japan
Pharmacia :	In USA : 800 Centennial Avenue, Piscataway, NJ 08854, USA In France : 8, Square Newton, 78390 Bois d'Arcy, France
Prodair :	Centre Paris Pleyel, 93521 Saint-Denis, France
Propeptide :	BP 12, 91710 Vert-Le-Petit, France
Rathburn Chemicals :	Walkerburn, Scotland
Riedel-de Haën :	Aktiengesellschaft, Wunstorfer Strasse, D-3016 Seelze 1, Hannover, W. Germany
Shimatzu :	In Japan : 1, Nishinokyo - Kuwabaracho, Kyoto 604, Japan In France : Touzart Matignon, 8, rue Eugène Henaff, 94400 Vitry sur Seine, France
United States Biochemical :	In USA : PO Box 22400, Cleveland, OH 44122, USA In France : Touzart Matignon, 8, rue Eugene Henaff, 94400 Vitry sur Seine, France
UCB Bioproducts :	Chemin du Foriest, 1420 Braines-L'Alleud, Belgium
Varian :	In USA : 220 Humboldt Court, Sunnyvale, CA 94089, USA In France : Quartier de Courtaboeuf, BP 12, 91941 Les Ulis Cedex, France
Waters - Milligen (divisions of Millipore) :	In USA : PO Box 255, Bedford, MA 01730, USA In France : BP 307, 78054 Saint Quentin, Yvelines Cedex, France

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