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THE PHYSICAL BASIS FOR INDUCTION OF PROTEIN- REACTIVE ANTIPEPTIDE ANTIBODIES

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PERSPECTIVES AND OVERVIEW

It is now generally accepted that small peptide fragments of proteins, usually coupled to a larger protein carrier, may elicit an antibody response in experimental animals. Many antibodies raised in this way are capable of reacting with the cognate sequence of the intact protein molecule. This important new class of reactions promises new approaches to vaccine production that may obviate some of the problems associated with vaccines that use whole cells, viruses, or molecules. These aspects have recently been reviewed (14, 52, 66).

In contrast, antibodies can also be raised against the protein molecule, which, in some cases, will also recognize small peptide sequences from the protein. This occurs with lesser frequency than the reverse case (antipeptide

antibodies recognizing protein) (52) because of the spatial juxtaposition in the intact protein of portions of the peptide chain that are far removed from each other in sequence. If several noncontiguous regions of the polypeptide are required for antibody recognition, then it is most likely that the presentation of only one of the regions as a peptide will not result in cross-reaction. These epitopes (antigenic determinants) are termed discontinuous and are a dominant feature of antiprotein antibody reactions. In contrast, for antipeptide antibodies, all epitopes must of necessity be continuous. Antibodies to those epitopes in proteins that approximate continuous sites also react with peptides of the cognate sequences. Protein antigens and their reactions have been reviewed by several authors (8, 9, 23, 66).

The recent discovery of hybridoma technology, which leads to the formation of monoclonal antibodies, has allowed more specific probing of the immune system. A single type of antibody molecule is produced instead of the more common spectrum of polyclonal antibodies produced in a normal immunization. The potential for specificity provided by the combination of antipeptide and monoclonal antibody technology has exciting possibilities for studies of immunochemistry at the molecular level. There have been many excellent reviews of monoclonal antibody technology (including 45, 48, 57, 77).

A distinction needs to be made between antigens and immunogens. An immunogen is a substance that will elicit antibody production; an antigen is a substance that will react with an antibody. Thus, if a peptide is presented to an antibody-producing system, it may elicit antibody formation, i.e. be immunogenic. The antibodies so formed may then be capable of reacting with the immunogenic peptide and/or with the cognate sequence in the protein, which are thus antigens.

This review is limited to questions concerning the molecular basis for the formation and reaction of antipeptide antibodies. In other words, it is concerned with peptides as immunogens. The intriguing question is why the peptide, disordered as it is, should be capable of eliciting antibodies that are reactive with the ordered protein molecule. This apparent paradox, and approaches toward its solution, are addressed in this review. We do not pretend to be exhaustive, since there have been many excellent reviews in recent years covering various aspects of relevance here.

ANTIPEPTIDE AND ANTIPROTEIN ANTIBODIES

Continuous and Discontinuous Determinants

RECOGNITION OF PEPTIDES BY ANTIPROTEIN ANTIBODIES A considerable amount of work has been done on the localization of epitopes of protein

immunogens and antigens by the use of short peptide fragments corresponding to the sequences of parts of the protein (recently reviewed in 86, 87). There are problems associated with this approach. The first problem is that most antibodies raised against a protein immunogen will be against discontinuous epitopes. Indeed, Barlow et al (7) have claimed that all protein epitopes must be discontinuous to some extent. This means that only antibodies against epitopes that include a substantial continuous sequence of polypeptide will cross-react with peptide fragments of the protein.

Conceptually, the recognition of small peptide fragments of proteins by antibodies to the native protein is not hard to imagine. If a given epitope is substantially continuous, then it seems reasonable that the corresponding peptide, even though largely unfolded in solution, could bind to antibody raised against the protein by a simple induced-fit mechanism (87). Previous studies have suggested that interactions with the antibody may introduce order into a peptide (16, 20, 25, 73, 84) in a mechanism similar to that envisaged in the binding of peptide hormones to their receptors. There is no need for a specific conformation to occur in a peptide hormone free in solution, because the specificity resides completely in the receptor (52). This mechanism has recently been rationalized in terms of a shift in the conformational equilibria of the peptide in solution (24).

RECOGNITION OF PROTEINS BY ANTIPEPTIDE ANTIBODIES A phenomenon far more difficult to explain is the recognition of the cognate protein by an antibody raised against a peptide. Dogma holds that a short peptide has no strongly preferred conformation in water solution. If, indeed, the conformational ensemble of the peptide contains only a random set of unfolded forms, then the antibodies raised against the peptide should recognize only unfolded forms. Yet, in many instances, the cognate sequence in the folded protein is recognized. We must postulate either that the sequence in the protein can be deformed by the antibody to fit its binding site (a process that is likely to be much more costly in energy terms than the induced fit of a small peptide) or alternatively that the antibody was actually raised to a peptide conformation that approximated that of the protein. It is possible that either or both of these alternatives may operate in different systems.

In studies of antipeptide antibodies and their reactions, the method of assay may influence the experimental results. Assays of reactivity may be carried out in solution or with various components immobilized on plastic plates (the enzyme-linked immunosorbent assay, ELISA). Some systems behave differently in solution and in ELISA assays, apparently because of local unfolding, which has occasionally been observed in the immobilized

proteins (91, 96). However, there are many systems for which no difference has been observed between solution and solid-phase assays (e.g. 33).

The issue of the deformability or mobility of protein epitopes is discussed briefly in a later section. It has recently been established that short peptides can indeed have conformational preferences for structured forms in water solution; this is also discussed in more detail below. A third intriguing possibility is that the conformation of the peptide on the carrier protein or on the B-cell receptor is made similar to that of the cognate sequence in the native protein by a nonspecific mechanism, the stabilization of folding in a protein environment.

REPLACEMENT NETS One convenient tool for rapid screening of epitopes is the replacement net method (36, 42). A whole protein or peptide immunogen sequence can be screened in overlapping short peptides. When the binding sites are established, solution or solid-phase methods allow replacement of all amino acid residues at each position in the peptide epitope with all 20 amino acids. The binding of antibody is assessed, and those residues where binding is abolished by amino acid replacement are termed "essential." When a conservative replacement of a residue allows some binding, the residue is termed "selected" (35).

Molecular Basis for Antibody Binding to a Protein

DISTRIBUTION OF ANTIGENIC SITES Current views of the distribution of antigenic sites in proteins have recently been reviewed (9, 82, 86, 87). Most studies correlate the behavior of a particular protein antigen-antibody system with various structural features of the protein antigen as a means of identifying epitopes on the protein. A more direct approach has recently been reported: mapping of protein epitopes using chemical modification of free and antibody-bound antigen (15).

A correlation has been observed between the segmental mobility of portions of a protein antigen and the antigenicity of the corresponding peptides (89) or protein regions (93). Westhof et al (89) estimated segmental mobility from temperature factors derived from high-resolution X-ray crystal structures; this procedure must be used with caution, since temperature factors can be influenced by crystal packing effects and thus rendered useless for the estimation of mobility. This problem does not arise in NMR experiments (e.g. 58). Novotny & Haber (62) have proposed that antigenicity is correlated not with chain mobility, but with surface exposure of residues. A new theory is based on an estimation of the extent to which a segment of the protein chain protrudes from the surface (7, 85). The argument is that since all antiprotein antibodies probably recognize discontinuous epitopes to some extent, then the longest continuous stretch

of protein chain in a primary antibody recognition site in the intact protein will be the most visible to the antibody when the protein antigen is denatured or presented in the form of short peptides. The sequences most likely to contain continuous stretches of protein chain will occur on the surface of the protein as protruding loops. Another predictive method invokes hydrophilicity, which is also associated with residues at the surface of the protein (41). Parker et al (63) recently found that hydrophilicity of amino acid side chains correlates best with antigenicity when it is considered in combination with other factors such as mobility or surface accessibility.

Getzoff, Geysen, and coworkers (35, 37) have recently explored these hypotheses systematically using replacement nets to define the epitopes of antiprotein antibodies and interpreting the results in terms of the three-dimensional structure of the protein. The results suggest that the entire protein surface is antigenic, but the probability of finding antibodies to a given site depends on local stereochemistry. The least reactive domains consist of tightly packed regions of low mobility, generally concave grooves in the molecular surface. By contrast, the most reactive domains are characterized by high local mobility, by convex surface shape, and often by negative electrostatic potential. Solvent exposure of side chains vital to the antibody-binding reaction was found to be less important, since many of the essential amino acid residues have buried side chains. Getzoff et al (35) postulated a possible molecular mechanism for the antigen-antibody binding in these cases: Initial binding to solvent-exposed amino acid residues may promote local side-chain displacement and thereby allow the participation of other, previously buried, residues.

DO CONFORMATIONAL CHANGES OCCUR ON ANTIBODY BINDING? The postulate that conformational changes in proteins are induced by antibody binding has a long history. Crumpton (22) observed that antibodies to apomyoglobin cause conformational changes when bound to metmyoglobin, expelling the heme from the holoprotein. Deformation of the antigen by the antibody has also been invoked to explain poliovirus neutralization kinetics (54), altered binding affinities for the binding of second antibodies to antigens (53), and inhibition of enzymes by antibodies (3).

Edmundson & Ely (31) recently directly observed a conformational change induced by an antibody in an antigen. They determined crystal structures for immunoglobulin light-chain (Bence-Jones) dimers from the patient Mcg bound to chemotactic peptides. In one of the binding sites on the dimer the dipeptide *N*-formyl methionyl tryptophan was present in the energetically unfavorable *cis* configuration, which was induced by the presence of the antibody.

In this context, the determination of the structure of protein antigen-antibody complexes is of great interest. Two such structures have recently been determined by X-ray crystallography (2, 19). The 2.8-Å crystal structure of hen egg white lysozyme bound to an antilysozyme antibody (2) differed little from X-ray structures of the native protein. In contrast, the 3-Å unrefined structure of a complex between an antineuraminidase antibody and neuraminidase (19) suggested (a) an unusual pairing of the variable regions of the light and heavy chains of the immunoglobulin, possibly resulting from the movement of the two regions relative to each other upon antigen binding, and (b) local perturbation of the antigen at the center of the epitope. The interaction was described as "having the character of a handshake" (19). The difference observed for these two structures illustrates the dictum that "the only rule [in immunological systems] is that there is no rule" (52). Undoubtedly both structural change and structural conservation will be observed as additional X-ray structures of antibody-protein complexes become available.

A further factor that has been given little attention is the mobility or deformability of the antigen-binding site of the antibody (19, 44). Structures of additional antigen-antibody complexes will be required to establish whether deformability of the antigen-binding site is generally important in antigen-antibody union.

RECOGNITION BY ANTIPEPTIDE ANTIBODIES

Order-Disorder Paradox

The foregoing discussion bears tangentially on the main topic of this review, namely the mechanism by which an antibody raised against a short peptide can react with the folded protein. The surprising thing is that this cross-reaction probably occurs in the majority of cases (see e.g. 33, 61). It is therefore of great importance for antigen-antibody reactions, as well as for other fields discussed later in this review.

Restated, the problem can be termed the "order-disorder paradox" (27, 29, 98): How can antibodies raised against a highly disordered state (the small peptide) recognize a highly ordered state (the folded protein)? This paradox arises with antipeptide antibodies but not with antiprotein antibodies.

In the following sections we address this paradox and describe experimental approaches that have been used in recent attempts to elucidate it. First, we stress that antipeptide antibodies are, in principle, very different from those raised against intact proteins. Antiprotein antibodies are induced by largely ordered protein structures. Then, depending on the experiment, they are tested for cross-reactivity against an ordered target

(protein) or an experimentally disordered target (peptide fragment). Conversely, antipeptide antibodies are made against a largely disordered structure and are tested for cross-reactivity with a more ordered protein target.

Molecular Basis for Recognition by Antipeptide Antibodies

REACTION OF ANTIPEPTIDE ANTIBODIES WITH COGNATE SEQUENCES IN PROTEINS Several factors appear to be important in determining whether an antipeptide antibody will bind to the cognate sequence in the folded protein. The length of the immunizing peptide is critical: A minimum length of six residues is required to raise antibodies to a peptide; for the antibodies to react with the cognate protein sequence, the minimum length is ten residues (83). Antibodies to accessible epitopes have a better chance of binding the protein (52). The orientation and method of attachment to the carrier may also be important (26). Segmental mobility of the cognate sequence in the native protein may also be an essential aspect of recognition of the protein by the antipeptide antibody. We have discussed above the hypotheses that mobility and many other parameters may be used for the mapping of protein epitopes by cross-reaction of peptides with antiprotein antibodies. We suggest that protein mobility may have a great deal more validity as an explanation for the cross-reaction of antipeptide antibodies with proteins. A considerable amount of experimental work supports this proposal (40, 81).

Whatever the mechanism of induction of antibody by a flexible peptide, it is extremely unlikely that the conformation in the immunogenic peptide will correspond precisely to the conformation of the cognate sequence in the native protein. Thus for optimal binding to the antibody, conformational rearrangement of the antigenic site on the protein is necessary. Rearrangement may involve only relatively minor movement of side chains or, at the other extreme, movement of the backbone conformation as well. The greater the free energy that must be expended in protein conformational change, the weaker the antibody-protein binding will be. If the epitope is in a region of high conformational flexibility, then deformation of the protein can be achieved with minimal free energy penalty. Indeed, in the most favorable cases, normal thermal fluctuations of the protein segment may sample the conformations recognized by the antibody. If, on the other hand, the cognate sequence in the protein is of restricted mobility, then the free energy expended in distortion of the protein to fit the antibody combining site will be manifest in reduced binding affinity.

Some experimental observations fit well with these concepts. Antipeptide antibodies raised against peptides corresponding to helical regions in calcium-saturated calmodulin (34) did not react with the protein in the pres-

ence of calcium, although they reacted with calcium-free calmodulin. This behavior implies that the cognate sequences in calmodulin change from antigenic, mobile structures in the calcium-free state to less antigenic, relatively rigid helical structures in the presence of calcium.

Is it possible that other parameters such as hydrophobicity, surface accessibility, or continuity of the protein chain in loops protruding from the surface could better explain protein recognition by antipeptide antibodies? Hydrophobicity of residues in the protein chain correlates well with surface accessibility (63). One would expect, too, that antipeptide antibodies raised against peptides derived from surface regions of the protein would be more likely to recognize the protein. Thus hydrophobicity and surface exposure may be a reasonable guide for a priori selection of immunogenic peptides (88). However, this does not mean that all parts of the determinant must be hydrophilic and exposed to the surface. For example, binding of both monoclonal antipeptide and antiprotein antibodies (33) and polyclonal antiprotein antibodies (35, 37) to myohemerythrin indicated as essential certain amino acid residues whose side chains were shown as buried in the X-ray structure of the protein (78).

FOLDING OF IMMUNOGENIC PEPTIDES IN SOLUTION There have been two approaches toward resolving the order-disorder paradox. One approach has been an investigation of the mobility of the cognate regions of the protein, as discussed in the previous section. Another approach is to investigate the possibility that short immunogenic peptides are not as unstructured in solution as was previously thought.

We address first the question of the so-called random coil structure of peptides in water solution. The structure of short peptide fragments of proteins in water solution is not identical to that of the cognate sequence in the folded protein. The fragments do not even have a single structure, but consist of an ensemble of many conformations (90), mostly extended chain (12, 28). They may have conformational preferences in solution, but these are once again only a part, and sometimes quite a small part, of the whole ensemble of conformations. In some cases, preferred conformations may be structures other than those observed in the X-ray structure of the native protein (27).

The evidence for these structured forms has come primarily from proton nuclear magnetic resonance (NMR) experiments on solutions of the peptides (27–30, 56, 94). These experiments have several advantages for the detection of even quite small percentages of structured forms. In contrast to many other forms of spectroscopy, e.g. circular dichroism, proton NMR provides information, once resonances have been assigned, about structure and dynamics at specific sites in a peptide or protein. Several NMR

parameters provide information about molecular structure. The most important parameters in the context of peptides are the nuclear Overhauser effect (NOE), temperature coefficients of amide proton resonances, amide proton exchange rates, and coupling constants, particularly the $^3J_{\text{NH}}$ coupling constant between the $\text{C}^{\alpha}\text{H}$ and NH protons of the same amino acid residue. Chain reversals associated with β -turns and helices result in certain characteristic short intrachain proton-proton distances, which can be detected through NOE measurements. Complementary information about hydrogen bonding interactions can often be obtained from amide proton exchange rates or temperature coefficients. None of the above criteria for NMR detection of structured forms in short peptides is sufficient evidence for turn or helix conformations in solution without corroborative evidence from other NMR parameters or from other techniques such as circular dichroism (47).

Residues 98–106 of influenza virus hemagglutinin The immunology of the influenza virus has been the subject of intense research activity in recent years (see e.g. 51, 52). In particular, the virus coat protein hemagglutinin, which carries some of the principal antigenic determinants, has been studied in considerable detail (92, 97). In one series of experiments, a 36-amino acid peptide corresponding to residues 75–110 of the HAI chain of influenza virus hemagglutinin (Figure 1) was found to be strongly immunogenic. One particular region of the peptide was found to be immunodominant for monoclonal antipeptide antibodies (96). This region has been singled out for structural study in solution by ^1H NMR.

An NMR investigation of the conformation of the peptide YPYDVPDYA (residues 98–106 of the hemagglutinin HAI chain) (27) revealed a distinct conformational preference in water solution for a β -turn involving the residues YPYD. This preference was indicated by a lowered temperature coefficient for the Asp4 amide proton and by distinctive NOE connectivities between the backbone protons of the amino acid residues in the turn, as shown schematically in Figure 2. A particularly felicitous feature of this system is the built-in control arising from the occurrence of *cis-trans* isomerism about the Tyr-Pro peptide bond. The β -turn structure occurs only in the *trans* isomer; the *cis* isomer shows no

75 80 85 90 95 100 105 110
HCDGFQNEKWDLFVERSKAFSNCYPYDVPDYASLRS

Figure 1 Amino acid sequence of the 36-residue immunogen (residues 75–110) from the HAI chain of influenza virus hemagglutinin (96). The region studied by NMR (residues 98–106) (27) is indicated.

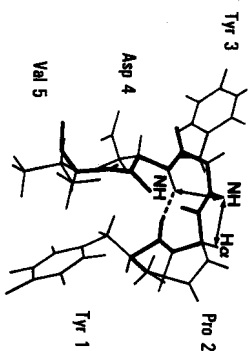


Figure 2 Computer graphics diagram of residues 98-102 of the HA1 chain of influenza virus hemagglutinin, showing inferred β -turn structure (27) in water solution. NOE connectivities are indicated, and the Tyr1 CO-Asp4 NH hydrogen bond is shown by a dotted line.

evidence of structure formation and thus provides an intrinsic unfolded control.

Further experimentation on five-amino acid peptides derived from YPYDVPPDYA has provided much information on the sequence dependence of β -turn formation and on the factors that stabilize the β -turn in water (28). With proline kept constant at position 2 of the turn, the residue at position 3 is the primary determinant of the turn's stability. Hydrophobic amino acids at this position strongly destabilize the β -turn. The highest populations of forms that contain turns occur for the peptides YPGDV and YPYDV, approximately 50% of the *trans* isomer was estimated to be in a β -turn conformation at 5°C in YPGDV. For peptides of the series YPX_XDV, where X represents all L-amino acids except tryptophan and proline, the temperature coefficient, used as a measure of the β -turn population, correlates with the statistical probabilities of β -turn formation determined from protein crystal structures (17) (Figure 3). Hydrophilic and hydrophobic side chains at position 3 show different correlations, probably because of a statistical bias in the Chou & Fasman (17) probabilities: β -turns generally occur on the solvent-exposed surface of proteins, where hydrophilic side chains are more probable (50). For small peptides, solvent exposure would be approximately equal for all side chains, so no bias toward hydrophilic side chains would be expected at position 3 of the β -turn (28).

The immunological behavior of the peptide YPYDVPPDYA has also been examined in detail (42, 43, 95). The aspartate at position 4 of the peptide (position 101 in the hemagglutinin molecule) was found essential for binding of the monoclonal antibodies, and the alanine at position 9 of the peptide (position 106 of the protein) is selected. The first two residues of the peptide, Tyr-Pro, do not appear to be essential for binding to the antibody (95), which may indicate that the β -turn is not necessary for antibody binding. Nevertheless it is probably significant that most of the antibodies appear to have been raised against this region of the 36-residue immunizing peptide (96). Thus the conformational preference observed

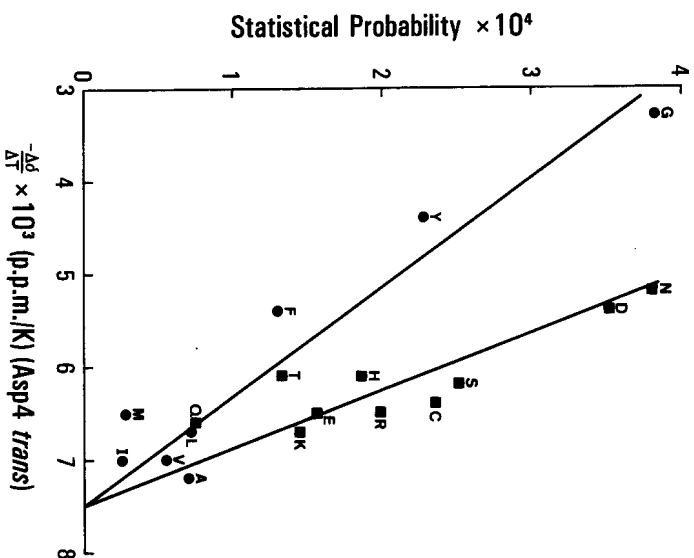


Figure 3 The statistical probability of β -turn formation (17) plotted against temperature coefficient for the *trans* Aspartate amide proton for the peptide series YPX_XDV. Circles represent peptides for which residue 3 is hydrophobic (plus glycine); squares represent peptides for which residue 3 is hydrophilic (from 28).

may have a role in the immunogenicity of the peptide, which is quite distinct from the antigenicity of the peptide in later interactions with antibody. Different structural requirements for immunogenicity and antigenicity were previously invoked for proteins (23).

It appears that the structure observed in solution for the short synthetic peptide is not present in the intact hemagglutinin trimer (97). No β -turn involving residues YPYD is evident in the X-ray structure. The cognate sequence is present in the trimer interface, which makes it inaccessible to antibody in the native molecule. It appears that the binding observed in ELISA assays was to some form of the protein other than the one that appears in the crystal structure; hence the protein may display conformational mobility or flexibility, at least in selected regions, in these solid-phase assays (95). In addition, these antibodies detected a major pH-induced conformational change in solution; such a structural event may have a role in the biologically important fusion process in the endosome (91).

Myohemerythrin C-helix peptide The immunological properties of myohemerythrin have been the subject of considerable attention (35, 37, 81). The intact protein has several antigenic regions for anti-protein antibodies (35, 37). Studies on synthetic peptide fragments of myohemerythrin and their immunogenicity for antibodies reactive with the native protein provided the first demonstration of the correlation between segmental mobility and antigenicity for antipeptide antibodies (81). The high-resolution X-ray structure of myohemerythrin (78) has shown that the protein consists of a four-helix bundle. The most immunogenic of the synthetic peptides corresponding to the four myohemerythrin helices appears to be the C-helix peptide, residues 69–87 of the native protein (T. Fieser, unpublished observations). This region also has higher than average segmental mobility in the intact protein (81).

The C-helix peptide has also been the subject of NMR studies designed to detect structure in water solution. The NMR spectrum (29, 30) of the C-helix peptide EVVPHKKMHKDFLEKIGL is well resolved and, importantly, indicates that the peptide is monomeric even at low temperatures. A set of NOE connectivities observed in the C-terminal half of the peptide (30) was more indicative of turn conformations than of regular α -helix (99). The circular dichroism spectrum of the C-helix peptide under similar conditions (30) showed no negative ellipticity at 222 nm, the diagnostic wavelength for helix. Thus it was concluded that no regular helix was present in the peptide in water solution under these conditions. On addition of trifluoroethanol, however, the circular dichroism spectrum revealed about 50% regular helical content in the peptide. The pattern of NOE connectivities in the NMR spectrum of the peptide under these conditions showed that regular helix is formed in the C-terminal half of the peptide, between residues 10 and 18 (residues 79–86 in the protein).

The observed NOE connectivities indicate that the folded conformations have local structure and short-range order in water solution. Long-range order is absent, as shown by both NMR and CD experiments. However, the peptide adopts a number of transient local conformations, a series of turnlike structures between residues 10 and 18, all of which interconvert rapidly by way of the predominantly extended-chain random-coil form (Figure 4*a*). This ensemble is termed the "nascent helix" since it is readily stabilized in an ordered, helical conformation. NMR and CD experiments indicate that the peptide forms a helix with long-range order in water-trifluoroethanol mixtures, but only in the C-terminal half of the peptide (Figure 4*b*). The conformation of the peptide in the native myohemerythrin (residues 69–86) is helical throughout its length (Figure 4*c*).

In recent work with monoclonal antibodies, Fieser et al (33) concluded that the epitope consists of the same residues (DFLEKI) that are encom-

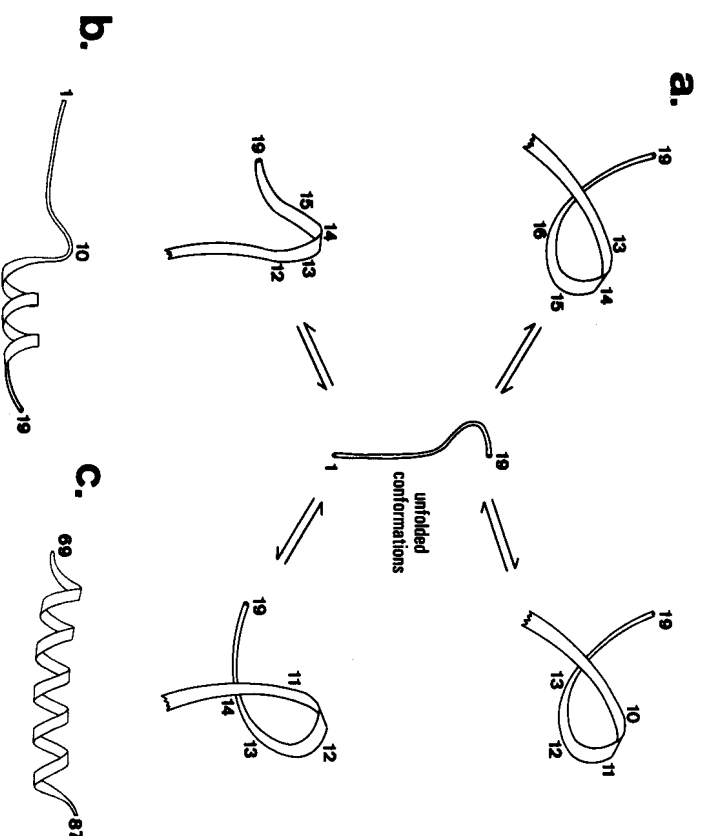


Figure 4 Schematic representations of the myohemerythrin C-helix peptide structure: (a) In water solution, a nascent helix, or series of turn conformations in equilibrium with extended chain, forms at the C-terminal end. (b) In water-trifluoroethanol mixtures, the C-terminal half of the peptide is helical. (c) In the intact protein, the entire sequence is helical (from 30).

passed by the nascent helix (Figure 5). This region also comprises one of the epitopes for polyclonal antibodies to native myohemerythrin (35).

Other peptides Several other examples have been reported of structured peptides that consist of or contain immunogenic or antigenic regions either for antipeptide or anti-protein antibodies. These include an immunogenic repeat region (sequence ...NANP...) of the circumsporozoite protein of

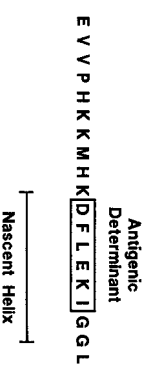


Figure 5 Sequence of myohemerythrin C-helix peptide showing location of the antigenic determinant (33) and of the nascent helix in water solution (30).

the malaria parasite (6, 32). Two groups have calculated possible structures; Gibson & Scheraga (38) predicted the formation of locally ordered structures in solution, while Brooks et al (13) suggested that the most stable structure in solution will have only long-range order (13). NMR studies of a 12-amino acid peptide representing three repeats of the unit NPNNA revealed a detectable population of structured forms (A. Satterthwait, P. E. Wright & H. J. Dyson, unpublished observations). Another example is a peptide spanning the antigenic domain of the *Herpes simplex* virus glycoprotein gD-1 (94), in which two regions of secondary structure coinciding with antibody recognition sites were found by NMR analysis. These regions include a helical turn and a short helical structure in equilibrium with random coil. A third example (56) is an antigenic region of myelin basic protein that appears to contain a β -turn. No backbone NOE connectivities were found, so the nature of the structures remains equivocal.

Selection of immunogenic peptides Intuitively, it seems likely that peptide sequences that have the greatest propensity to fold in water solution should provide the best immunogens to raise protein-reactive antibodies. Unfortunately, a priori prediction of sequences with high probability of folding is difficult, since most numerical predictive methods use data derived from protein crystal structures and are therefore not always applicable to small peptides in solution. The predicted probability of β -turn formation in peptide sequences has recently been correlated with immunogenicity of peptides from uropathogenic *Escherichia coli* pili (69, 70, 74). This correlation has been used as a basis for a method for selection of immunogenic peptide sequences that depends on a high predicted frequency of β -turn formation (60).

Schulze-Gahnen et al (76) have generated antibodies against a peptide with designed secondary structure for use as possible probes of secondary structure in proteins. They designed an immunogenic peptide with a high probability of adopting a β -turn structure between two segments of anti-parallel β -sheets, i.e. a β -hairpin. The claimed specificity of the immune response to this β -turn must be viewed with caution, since only four residues were conserved in the control peptide; the amino acid replacement, rather than loss of secondary structure, may explain the decreased affinity for the control peptide. Evidence for β -hairpin formation by the immunogenic peptide was based on circular dichroism measurements. Since the peptide is known to aggregate in water solution, the conclusions of Schulze-Gahnen et al are dubious. This study illustrates one of the pitfalls in structure determination for small peptides in water; if structure formation accompanies aggregation, then it is extremely difficult to make

meaningful statements about the relationship between peptide conformational preferences and properties such as immunogenicity or antigenicity.

Role of the Carrier Protein

The most common technique for eliciting anti-peptide antibodies involves coupling of the peptide to a carrier protein. Recently it has been observed (4, 26) that the carrier protein can have considerable effects on the immunological properties of the peptide. Bahraoui et al (4) found that a nine-amino acid peptide sequence from scorpion toxin II was coupled to bovine serum albumin (BSA). Anti-peptide antibodies were elicited that bound with high affinity to the BSA-coupled peptide and to the cognate region of the intact protein but did not bind to the free peptide. This observation argues for some role for the carrier protein in the immunogenicity of the coupled peptide; perhaps it stabilizes a conformation in the bound peptide that is not highly populated in the free peptide in solution. The orientation of the peptide coupled to a carrier protein also influences the binding of anti-peptide antibodies (26). An octapeptide sequence derived from the α chain of human acetylcholine receptor was coupled through either an N- or C-terminal link to a carrier protein and used as an immunogen. The resulting antisera reacted at comparable titers to the uncoupled immunizing peptides, but did not cross-react with the identical but oppositely linked peptide.

NMR experiments to determine the conformation of immunogenic peptides coupled to a model carrier protein are in progress in our laboratory. One conformation has been observed that differs from that of the free peptide in water solution; it appears to resemble the conformation found in the native protein from which the peptide sequence was derived (S. Eibina & P. E. Wright, unpublished observations).

A Plausible Model for Anti-peptide Antibody Induction

Several immunogenic and/or antigenic peptides have now unexpectedly shown marked conformational preferences in water solution. It appears, too, that anti-peptide antibodies are most reactive with the cognate sequence in the folded protein when the epitope is accessible and mobile. These two observations can go a long way to explain the order-disorder paradox. We reemphasize that it is important to distinguish between the immunogenicity and the antigenicity of peptides, and that in the majority of cases the peptide is presented to the immune system attached to a larger protein molecule, the carrier.

We postulate the following mechanism for induction of anti-peptide antibodies capable of binding with high affinity to the cognate sequence

in the native protein. First, we propose that the best peptide immunogens are likely to have a high folding propensity, i.e. that the amino acid sequence strongly favors formation of a particular secondary structure. Thus a highly preferred conformation of the free peptide in water solution will often be detectable by NMR or other measurements. It seems improbable that the immune system can directly select one conformation, even if it is the most populated, from the ensemble of rapidly interconverting structures present in a small linear peptide in water solution. Rather, we suggest that the most highly preferred conformation of the free peptide in water is stabilized on the surface of the carrier protein or becomes stabilized when the peptide binds to the B-cell receptor. In other words, we suggest that the secondary structure encoded by the amino acid sequence of the peptide becomes stabilized in the protein environment of the carrier or the B-cell receptor. If a particular secondary structure is strongly specified by the amino acid sequence of the peptide, then it is likely that the peptide will adopt the same secondary structure in the B-cell receptor and within the cognate folded protein from which it is derived. In this case, the resulting antibody might be expected to recognize the cognate sequence in the folded protein. This mechanism is shown schematically in Figure 6. Of course, the exact three-dimensional structures of the receptor-bound peptide and the cognate sequence in the folded protein are unlikely to be identical even if the secondary structures are the same, so that flexibility in the protein still has a role in optimizing the antibody-protein interaction. An important component of this model is that many strongly immunogenic peptides are characterized by their high tendency to fold into secondary structures. Thus there is an intriguing possibility that many immunogenic peptides represent initiation sites for protein folding. The implications of this proposal are discussed in the following section. Note that we do not expect that all immunogenic peptides must have the potential to adopt regular secondary structures; it is not difficult to understand

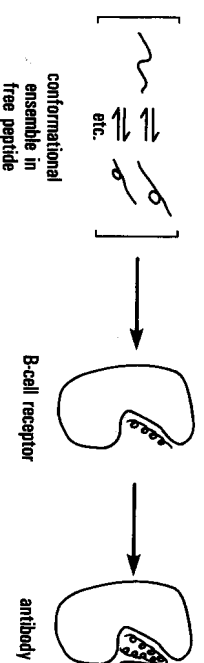


Figure 6 Schematic representation of a plausible mechanism for induction of protein-reactive antibodies by peptide immunogens. The conformational ensemble in the free peptide includes (in this case) a nascent helix, which is stabilized into a regular helix on the B-cell receptor. The conformation in the native protein is also a helix, which can thus be recognized by antibodies to the peptide.

how exposed protein loops of irregular structure and high mobility can be recognized by antipeptide antibodies. Rather, the above model suggests a plausible mechanism by which antipeptide antibodies may be induced that recognize such regular secondary structures as α -helix or β -turns.

Stabilization of secondary structure in a peptide upon its binding to a protein is not without precedent. Calmodulin, for example, is known to bind with very high affinity to a number of linear peptides and to stabilize them in helical conformations (21, 55). Of greater immunological interest is a substantial body of (largely circumstantial) evidence that implicates formation and stabilization of amphiphilic helices in T-cell recognition of immunogenic peptides (1, 9, 65).

In this context, studies of the antigenic region A of influenza virus hemagglutinin (75) may be relevant. This region contains amino acid residues 140–146 of the HA1 polypeptide (92). It has not been possible to induce protein-reactive antibodies using peptides containing this sequence, even though the sequence is a highly accessible and probably quite mobile loop in the three-dimensional structure (97). Antibodies were induced with peptides in which a loop conformation was enforced through formation of a disulfide bond (75). The resulting antibodies cross-react, although weakly, with native virus. (Reaction with isolated native hemagglutinin was not reported.) The cross-reaction may be an indication of a conformation-specific reaction in which the free or carrier-bound linear peptide has no conformational preference and is incapable of inducing antibodies to the loop conformation. Alternatively, the linear peptide may have a strong conformational preference incompatible with the loop structure in the native protein.

Implications for Other Fields

PROTEIN FOLDING The observation that short regions of peptides in water solution adopt ordered structures has important implications for the initiation of protein folding. A possible first step in protein folding is the formation of local secondary structures from one or more regions of the polypeptide chain. A role for local secondary structures, including β -turns and helices, in initiation of protein folding has been an integral part of several folding models (reviewed in 46, 67, 68). The main objection to such models in the past has been the lack of experimental evidence for formation of secondary structure by linear peptides and unfolded proteins. Thus the identification of local secondary structure in immunogenic peptides and in other systems (10, 47, 79, 80) is of considerable importance, since it clearly establishes that local folded structures do occur in peptide fragments of proteins in water solution and that these structures are stabilized by local interactions dictated by the amino acid sequence. The number

and nature of the secondary structures now observed implies their existence in the polypeptide chain of a protein under folding conditions, where they might be expected to have an important role in directing subsequent folding events. Thus it now appears that a combination of studies using antipeptide antibodies and structure determinations on short peptides in solution by NMR can be used to probe initiation sites for protein folding.

Antibodies were previously used to study protein folding (71, 72). Stable local structures, which may differ from those in the final folded protein, can be recognized by antibodies, and have been postulated as initiation sites in protein folding (16, 71, 72, 84). Recent work with monoclonal antipeptide antibodies (11) has shown that interaction between antibodies and partially folded protein intermediates can occur. Kinetic measurements indicate that intermediates are formed with structures sufficiently similar to that in the native protein to be recognized by antibodies. Subsequent conformational changes in the partially folded region result in the formation of a fully native structure. This behavior provides a powerful method for following later events in protein folding.

SYNTHETIC VACCINES The potential usefulness of the antipeptide antibody technology in the production of synthetic vaccines is enormous. The development of a synthetic immunogen to elicit antibodies that neutralize viruses or toxins but that have no harmful side effects still remains a challenge, since reaction of an antipeptide antibody with the cognate sequence in a viral protein, for example, will not necessarily result in neutralization of the virus. Several cases have been found in which antipeptide antibodies do, however, have neutralizing effects (5, 18, 39, 59, 70, 74). The possibility of using peptides as synthetic vaccines has recently been reviewed (49), and some of the problems with such vaccines have been outlined (64). It remains to be seen whether the technology can be generalized to human and animal populations.

ACKNOWLEDGMENTS

We thank Dr. T. Fieser for helpful discussions and Dr. I. A. Wilson for a critical reading of the manuscript.

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STRUCTURAL AND MICROANALYTICAL IMAGING OF BIOLOGICAL MATERIALS BY SCANNING MICROSCOPY WITH HEAVY-ION PROBES

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PERSPECTIVES AND OVERVIEW

The interactions of fast ions with solid matter can provide much information about the structure and chemical composition of the bombarded