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The concept and operational definition of protein epitopes

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The antigenic determinants or epitopes of a protein correspond to those parts of the molecule that are specifically recognized by the binding sites or paratopes of certain immunoglobulin molecules. Epitopes are thus relational entities that require complementary paratopes for their operational recognition. Some authors consider that the concept of epitope necessarily involves the two properties of antigenic reactivity (ability to bind to a paratope) and immunogenicity (ability to induce an immune response). Such a view creates difficulties because it makes the existence of epitopes in a protein depend on immunogenetic and regulatory mechanisms of the immunized host.

The delineation of epitopes can be achieved by antigenic cross-reactivity studies or by X-ray crystallography. Both approaches require specific criteria for deciding which residues of the antigen are in contact with the paratope and are functionally part of the epitope. The relative contribution of static accessibility, segmental mobility and induced fit to immune recognition remains controversial. Each of the methods used for analysing antigenic specificity is subject to various operational constraints originating from the type of experimental probe and from the format, sensitivity and specificity of the immunoassay used. If a protein is assumed to contain as many epitopes as the number of different monoclonal antibodies that can be raised against it, the delineation of epitopes corresponds to the summation in various hosts of the immune repertoire specific for the antigen.

Neutralization epitopes are a special subclass of the epitopes of infectious agents and toxins that are specifically recognized by antibody molecules able to neutralize the biological activity of the antigen. The identification of neutralization epitopes is important for the development of synthetic vaccines because it is this type of epitope that should be mimicked by synthesis and used as a vaccine for eliciting protective immunity. The first demonstration that synthetic peptides could elicit antibodies that neutralized viral infectivity was made by Anderer and his colleagues in the 1960s in their work with tobacco mosaic virus. Nearly 20 years passed before it was shown that antibodies to synthetic peptides were also able to neutralize the infectivity of other viruses such as foot-and-mouth disease, polio and hepatitis B viruses.

1. Definitions

The antigenic determinants or epitopes of a protein correspond to those parts of the molecule that are specifically recognized by the 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 or CDRs. In addition, some less-variable framework residues of the immunoglobulin chain have also been implicated in the structure of paratopes identified by X-ray crystallography (Amit *et al.* 1986; Sheriff *et al.* 1987). In the same way that the antibody nature of an immunoglobulin becomes established only after its complementary

44-2

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antigen has been identified, the epitope nature of a cluster of amino acids in a protein can be recognized only by means of an immunoglobulin molecule. Epitopes are thus relational entities that can be defined only in a functional and operational sense by the binding of complementary paratopes.

According to this definition, the origin of the antibody used to identify a particular epitope is irrelevant. Usually, the antibody is derived 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. For instance, such a related antigen could be a natural or synthetic peptide fragment that is able to elicit antibodies that bind the whole protein. The antibody could also be derived from a non-immunized animal or from animals such as autoimmune mice.

1.1. *Antigenicity and immunogenicity*

Some authors are reluctant to define protein epitopes only in terms of their ability to bind to paratopes, for they consider that the concept of epitope involves also the property known as immunogenicity (Atassi 1984). Immunogenicity refers to the ability of a protein to induce an immune response in a competent host, and as this capacity is controlled by various cellular regulatory mechanisms as well as by the host's immunoglobulin gene repertoire, it cannot be defined outside the host context. For instance, the immune regulatory mechanism known as tolerance to self is responsible for the usual finding that rabbit serum albumin is immunogenic in the mouse but not in the rabbit; this statement should be qualified somewhat, because drastic manoeuvres such as the use of Freund's complete adjuvant may overcome immunological tolerance and render rabbit albumin also immunogenic in the rabbit. If the existence of an epitope in a protein were made to depend on the prior demonstration of its immunogenicity, one could be confronted with the unenviable task of testing a very large number of animals of different species to find a responsive individual. To avoid such difficulties, it would seem preferable to follow the customary distinction made between the antigenic reactivity and immunogenicity of antigens and to accept that the same two properties can also be dissociated in the case of individual epitopes. In practice this means that a region in 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. For instance, peptides 1–6 and 121–127 of myoglobin would be considered as myoglobin epitopes because they are able, when injected as free or conjugated peptides, to induce antibodies that recognize intact myoglobin (Atassi & Young 1985). In contrast, Atassi (1984) argued that the regions 1–6 and 121–127 of myoglobin are not epitopes of the protein because he could not demonstrate the presence of antibodies recognizing these peptides in a number of anti-myoglobin antisera.

1.2. *How many epitopes does an antigen possess?*

Regarding the number of epitopes likely to be found on any antigen, the prevalent paradigm states that the entire accessible surface of a protein consists of a large number of overlapping epitopes (Benjamin *et al.* 1984). A less widely held view states that each protein possesses only a small number of immunodominant epitopes characterized by discrete boundaries (Atassi 1984). This latter viewpoint stems mainly from a reluctance to accept that monoclonal antibodies (MAbs) are a legitimate tool for identifying protein epitopes; protagonists of this

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viewpoint believe that the use of hybridomas tends to emphasize minor epitopes that are not immunodominant and would probably not be detected by polyclonal antisera. Immunodominant epitopes are defined as those to which the immune response is directed most frequently. As the phenomenon of immunodominance is based on comparative immunogenicity, the introduction of such a criterion leads to difficulties because the relative strength of an immune response would have to be weighed to evaluate the *bona fide* existence of an epitope.

As epitopes can only be identified operationally by binding experiments with complementary paratopes, the number of epitopes in a protein can be estimated from the total number of different MAbs it is able to induce. By means of the 'identical pairs method' of Briles & Carroll (1981) which assumes that the frequency of identical pairs of MAbs is equal to the reciprocal of the number of possible comparisons, the number of different MAbs that can be made to insulin was estimated to be at least 115 (Schroer *et al.* 1983). The insulin molecule would thus be expected to contain at least 115 epitopes. Because differences in the binding activity of individual MAbs are usually detected by their ability to discriminate between close relatives or analogues of the immunogen (Schroer *et al.* 1983; Quesniaux *et al.* 1987), the number of distinguishable epitopes is operationally determined by the availability of analogues as well as by the discrimination potential of the immunoassay. It should be stressed that the presence of sequence differences in the variable regions of two immunoglobulin molecules does not guarantee that one is dealing with two antibodies of different specificity for a particular antigen; only a binding test will show if the sequence differences affect paratope specificity, presumably because they alter critical residues that make contact with the epitope.

1.3. *Types of epitopes*

It is customary to divide epitopes into several conceptual categories that are not easily distinguished operationally. For instance, sequential epitopes have been opposed to conformational epitopes on the basis that the former are defined as random coil peptides whereas the latter are said to require a specific conformation to be recognized by their complementary paratopes (Sela 1969). In general, it is 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, it is difficult to envisage that an antibody could recognize a sequence of residues independently of its conformation and sequential epitopes should therefore not be equated to conformation-independent epitopes.

Atassi & Smith (1978) have proposed that it is more satisfactory to distinguish between continuous and discontinuous epitopes, also sometimes called contiguous and discontiguous epitopes. The label 'continuous' appears to be preferable, as it is the individual constitutive residues that can be said to be contiguous rather than the epitope as a whole; speaking of two contiguous epitopes is likely to be understood in the sense of two epitopes occupying neighbouring sites at the surface of a protein.

Continuous epitopes are defined as a short linear stretch of residues endowed with distinctive conformational features, whereas 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. In practice, the label 'continuous epitope' is given to any linear peptide fragment of a protein that is found to react with antibodies raised against

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the intact molecule; clearly this label does not mean that the linear 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, partly because of the inability of the peptides to assume the correct conformation. Furthermore, the linear peptide may represent only a part of a larger discontinuous epitope but nevertheless react with antibodies directed to the larger discontinuous structure. In addition, not every residue in a so-called continuous epitope is necessarily able to make contact with the paratope (Geysen *et al.* 1988); because some of the residues in a continuous epitope can be replaced by any of the other 19 amino acids without impairing the antigenicity of the peptide, the linear fragment may in fact be antigenically discontinuous. As most studies aimed at unravelling protein antigenicity have actually focused on the phenomenon of cross-reactive antigenicity between proteins and short peptides, our knowledge of protein epitopes concerns mainly adulterated, incomplete epitopes that have retained only part of their identity after fragmentation of the protein (Van Regenmortel 1987).

It is now widely believed that the majority of protein epitopes are discontinuous. Observation of space-filling models of globular proteins shows that very few linear stretches of residues are present at the surface of a protein (Leach 1983) and it has been calculated that no surface region of 20 Å† diameter is likely to contain only atoms from a continuous stretch of residues (Barlow *et al.* 1986). If it is assumed that all epitope–paratope interfaces in proteins cover a surface of about 700 $Å^2$, i.e. the size found in the case of lysozyme paratopes identified by X-ray crystallography (Amit *et al.* 1986; Sheriff *et al.* 1987), it follows that all epitopes are probably discontinuous.

Three further categories of epitopes have been distinguished, the so-called cryptotopes, neotopes and neutralization epitopes. Cryptotopes are hidden epitopes that become expressed only after fragmentation, depolymerization or denaturation of the antigen (Jerne 1960). Neotopes are epitopes that are specific for the quaternary structure of proteins; they arise as a result of conformational changes in the monomer induced by intersubunit bonds or by the juxtaposition of residues from neighbouring subunits, and are commonly found in viral capsids (Van Regenmortel 1966, 1982; Neurath & Rubin 1971). Neutralization epitopes correspond to those epitopes of infectious agents and toxins that are specifically recognized by antibody molecules able to neutralize the biological activity of the antigen. The identification of neutralization epitopes is important for the development of synthetic vaccines because it is this type of epitope that should be mimicked by synthesis and used for eliciting protective immunity.

2. Methods

Most of our knowledge concerning the location of epitopes in proteins has been obtained by studying the antigenic cross reactivity between the intact molecule and peptide fragments and could be derived because of the polyspecificity of antibodies, i.e. their ability to react with a variety of more or less closely related epitopes (Richards *et al.* 1975; Lane & Koprowski 1982). It is remarkable that this property of antibodies has been accepted much more slowly than the reciprocal situation, namely that an individual epitope can be recognized by a variety of different paratopes. A further insight brought about mainly by the use of MAbs is the recognition that antibodies are not only polyspecific but also heterospecific (or heteroclitic), i.e.

† 1 Å = 10^{-10} m = 10^{-1} nm.

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that they are able to bind more strongly to other antigens than to the one against which they were raised (Mäkelä 1965; Van Regenmortel 1982; Underwood 1985). This property is most easily demonstrated when MAbs are allowed to react with a series of closely related analogues of the immunogen (Al Moudallal *et al.* 1982; Harper *et al.* 1987). Heterospecificity is a reflection of the fact that the clonal selection of a B cell, which eventually leads to antibody production, can be triggered with an immunogen endowed with only moderate affinity for the B-cell receptor. As the highest possible degree of fit between epitope and paratope is not required for initiating B-cell differentiation, a higher affinity for antigens other than the immunogen is likely to be found in antibodies whenever it is looked for. 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.

The definition of specificity in antigen–antibody interactions is a particularly vexing question, especially in view of the widespread potential for cross-reactive fit between antibodies and heterologous antigens. Perfect or absolute fit between epitope and paratope is a theoretical concept and can never be established unequivocally, because it would be necessary to demonstrate that the possibility of heterospecific binding is ruled out or that further maturation of the immune response cannot occur, clearly an impossible task. Although it is commonly believed that highly specific antibodies always possess a high affinity constant for the relevant antigen, such a view is of little help for deciding whether a particular antigen–antibody interaction is specific or not. In practice, specificity is only meaningful in terms of discrimination potential. It is the particular need of the investigator to differentiate between two entities that provides him or her with the necessary criterion for deciding whether an antibody is or is not specific within the context of the task at hand. Specificity is therefore best defined operationally by the ability of a reagent to achieve the level of discrimination that is required in any particular case.

Several reviews describing the methods used to localize epitopes on proteins have appeared in recent years (Atassi 1984; Benjamin *et al.* 1984; Van Regenmortel 1984, 1986*a*; Berzofsky 1985; Jemmerson & Paterson 1986*a*). The different approaches that have been used in the mapping of protein epitopes are summarized in table 1. Methods 1–3 (table 1) are able to identify only continuous epitopes and they will therefore provide only a partial description of complex epitope structures. However, this somewhat crude analysis of protein antigenicity has been remarkably useful as many of the practical applications of immunochemical research are based on the exploitation of antigenic cross reactions between peptide fragments and proteins (Lerner 1984; Walter 1986; Van Regenmortel 1987).

Most antibodies raised against intact proteins are specific for the native conformation of the antigen and bind to peptide fragments with only low affinity. However, some antibodies raised against intact proteins react preferentially with peptide fragments derived from the antigen; such antibodies may arise because of the presence of denatured molecules in the preparation used for immunization (Lando & Reichlin 1982). On the other hand, the claim that immunization with peptides leads to a very high frequency of induction of antibodies able to recognize the *native* protein (Green *et al.* 1982; Lerner 1984) remains somewhat controversial, because usually little evidence is presented that there are no denatured protein molecules in the antigen preparation or that the protein is not partly denatured by the immunoassay. In this context, it should be stressed that proteins become at least partly denatured when they are

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Table 1. Methods used to localize epitopes in proteins

	method	reference
1.	cross-reactivity studies between peptide fragments and antiprotein antibodies	Atassi (1984)
2.	cross-reactivity studies between the protein and antipeptide antibodies	Lerner (1984)
3.	determination of critical residues in synthetic peptides by systematic replacement with other amino acids	Geysen *et al.* (1988)
4.	determination of critical residues in sets of related proteins that influence binding by MAbs	Benjamin *et al.* (1984)
5.	chemical modification studies on single residues in intact proteins	Burnens *et al.* (1987)
6.	proteolytic digestion of antigen–antibody complexes	Jemmerson & Paterson (1986*b*)
7.	competition studies with different MAbs	Berzofsky (1984)
8.	X-ray crystallography of antigen–Fab complexes	Amit *et al.* (1986)

adsorbed to a layer of plastic during a solid-phase immunoassay (Kennel 1982; Friguet *et al.* 1984).

Evidence that methods 1 and 2 (table 1) can lead to the same antigenic map of a protein was obtained for myohaemerythrin (Tainer *et al.* 1984; Geysen *et al.* 1987*b*); the results obtained in these studies demonstrate that polyclonal anti-peptide antibodies are a legitimate tool for analysing the antigenic structure of proteins. The most systematic way to look for continuous epitopes in proteins is to synthesize all possible overlapping hexa-, hepta-, octa- or longer peptides of a protein and to measure their ability to bind to anti-protein antibodies. This can be achieved rapidly by a method in which the peptides are synthesized on a linear polymer of polyacrylic acid and tested for antigenic activity while still bound to the solid phase (Geysen *et al.* 1987*a*). Although extremely rapid, this method will not identify epitopes in the *C*-terminal residues of a protein and may give results that differ from those obtained when the peptides are tested in their free form in solution. For instance, Rodda *et al.* (1986), using peptides bound to plastic rods, located the major continuous epitope of myoglobin in residues 48–55 but failed to pick up five known continuous epitopes identified in earlier work with free peptides (Atassi 1975). On the other hand, the earlier studies, which were done by inhibition of precipitation, a method likely to emphasize low-affinity binding, failed to identify the epitope subsequently found in residues 48–55.

The importance of immunoassay format on the apparent antigenic reactivity of peptides was also demonstrated in a recent study of histone H2A (Muller *et al.* 1986). The activity of different peptides was found to depend on whether they were tested as free peptides in solution, conjugated to a protein carrier or adsorbed on a plastic solid phase. Such variations, which may be attributed to differences in peptide accessibility and conformation in the different assays, illustrate the operational nature of any experimental definition of antigenicity (Van Regenmortel *et al.* 1988). It seems that peptides may be able to adopt an ordered conformation when free in solution and that this could enhance their ability to be recognized by antibodies (Schulze-Gahmen *et al.* 1985; Dyson *et al.* 1985; Williamson *et al.* 1986); it is also possible that certain conformations are induced or stabilized when the peptide is bound to a B-cell receptor or to an antibody molecule. Furthermore, it has also been shown that the same sequence of 6–7 residues can have different conformations in the different local environments of two unrelated proteins (Wilson *et al.* 1985).

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Considerable refinement in the definition of continuous epitopes can be achieved by the 'replacement set' method in which each of the residues in a synthetic peptide is, in turn, replaced by the other 19 possible amino acids (Geysen *et al.* 1988). By measuring the antigenic reactivity of all the analogues, it is possible to identify different classes of residues. Some residues are critical for binding and cannot be replaced at all without impairing the antigenicity; some residues can only be replaced by a limited number of amino acids whereas other residues can be replaced by all common amino acids without affecting binding. This latter type of residue is clearly not a contact residue of the epitope and plays a role only as far as it allows the correct positioning of neighbouring residues that do contribute to the binding energy. By analysing the pattern of replaceability linked to the retention of antibody binding in 103 continuous epitopes, Geysen *et al.* (1988) showed that on average, only five out of six residues in hexapeptides were essential for activity. In the continuous epitope of myohaemerythrin situated in residues 4–9, the *N*-terminal Ile was replaceable by any of the amino acids, indicating that the epitope in fact corresponds to residues 5–9 (Getzoff *et al.* 1987). Although the replacement set analysis is extremely useful for fine specificity studies, one should not lose sight of the fact that the solid-phase immunoassay that is used no doubt introduces a bias and that the method, in fact, measures cross reactivity with anti-protein antibodies. Only site-directed mutagenesis of the whole protein could make it possible to study the effect of substitutions in the natural environment of the intact, native antigen.

Another operational limitation of cross-reactivity studies between peptides and intact proteins should be mentioned. Peptides corresponding to inner sequences of a polypeptide chain possess ionizable groups at their *C*- and *N*-terminal ends, which are absent in the protein where these ends are involved in the formation of a peptide bond. The presence of these additional charged groups in the peptide could be expected to lower any potential antigenic cross reactivity with the intact protein. When the two ionizable groups are replaced by a carboxamide *C*-terminus and an acetylated *N*-terminus, the introduction of these groups may change the antigenicity of the peptide (Gras-Masse *et al.* 1986). In other words, leaving the extraneous charges or removing them could alter the antigenicity of the peptide sufficiently to interfere with the measurement of cross reactivity with the parent molecule.

The identification of epitopes by methods 4–6 (table 1) is most successful when MAbs are used as probes and the tertiary structure of the protein is known. Unfortunately, in most studies of this nature only two or three residues critical for binding can be identified as belonging to an epitope, which is usually of the discontinuous type. From studies with influenza virus proteins and bird lysozymes, there is evidence that single substitutions at the surface of the protein cause only local structural adjustments with no long-range conformational effects (Hornbeck & Wilson 1984; Knossow *et al.* 1984; Varghese *et al.* 1988). On the other hand, it is also known that the reactivity of epitopes can be altered by conformational changes induced by substitutions or modifications occurring at some distance from the protein surface (Ibrahimi *et al.* 1979; Al Moudallal *et al.* 1982; Cooper *et al.* 1987).

The only method that in theory could identify all the contact residues of both epitope and paratope is X-ray crystallography of antigen–antibody complexes. At present, only two epitopes of lysozyme (Amit *et al.* 1986; Sheriff *et al.* 1987) and one of influenza neuraminidase (Colman *et al.* 1987) have been analysed by this method. For the lysozyme epitope studied by Amit *et al.* (1986), the interface between antigen and antibody was found to extend over an area of about 20 Å × 30 Å, with 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

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to interact closely, i.e. within a distance of 4 Å. Although the number of residues implicated in epitopes identified by X-ray crystallography is much larger than the number of critical residues identified by other methods, it is possible that only some of the residues present in the crystallographic interface contribute to the binding energy of interaction. The stereochemical information available from these studies is entirely structural and the contribution of individual interactions to the overall functional binding remains a matter of interpretation of how to relate structure to function. For instance, the X-ray analysis provided no obvious explanation for the finding that the single substitution Gln 121 → His in lysozyme abolished completely the binding to antibody (Amit *et al.* 1986). Furthermore, as the removal of bound water from the free antigen and antibody molecules and the concomitant breaking of hydrogen bonds plays a key role, it is difficult to calculate the free energy of association of the complex.

In view of the labour-intensive nature of protein crystallography, it will not be practical to study large numbers of individual antigen–antibody complexes. Instead, it will be more informative to complement the structural information available for a particular complex with binding studies on peptide fragments and analogues of the antigen; site-directed mutagenesis of both epitope and paratope residues should also help to clarify the role of individual amino acids in the binding interaction (Roberts *et al.* 1987).

3. Mechanisms

The association of an epitope with its paratope involves the usual forces that operate in all protein interactions. If charged residues are present, their geometric, complementary arrangement generates an electrostatic field which guides the two reactants into proper alignment. This occurs at a distance of 10–100 Å and brings the two molecules close together. The subsequent stage can be described as an association in terms of overall complementary shapes that fits the usual lock and key analogy. When the two surfaces are closer than 10 Å to each other, hydrogen bonds between residues and solvent molecules are broken and hydrophobic residues become buried within the interface. During the last stage, it is likely that sidechain rearrangements occur in both epitope and paratope, a phenomenon corresponding to induced complementarity that would be facilitated in regions of the polypeptide chain that possess above average mobility. It should be emphasized that the magnitude of the motions observed in segmental mobility is of the order of 1–2 Å and that the energetic cost for binding (a few kilocalories) is therefore not necessarily prohibitive. Small motions could have a beneficial effect on the critical positioning of residues and lead to increased binding affinity (Edmundson & Ely 1986; Getzoff *et al.* 1987). The possibility of induced-fit complementarity between epitope and paratope is also suggested by the finding that segments 41–53, 65–72 and 116–129 of lysozyme, which are parts of discontinuous epitopes, possess high mobility (Karplus & Schulz 1985; Amit *et al.* 1986; Sheriff *et al.* 1987) and by the observation that peaks in the segmental mobility plots of different proteins have higher than average antigenicity (Westhof *et al.* 1984; Tainer *et al.* 1985; Geysen *et al.* 1987*b*). The correlation between segmental mobility and location of continuous epitopes indicates that the probability of obtaining anti-protein antibodies that cross react with a short, mobile peptide is highest when the immunogenic epitope region itself is mobile. Induced fit is also suggested by the observation that in the bound epitope of lysozyme studied by Sheriff *et al.* (1987), the Pro 70 and the sidechains of residues 45 and 68 appeared to have moved compared to their position in the free

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lysozyme molecule. Geysen *et al.* (1988) have argued that the high frequency with which hydrophobic residues such as Phe, Leu and Pro appear in continuous epitopes is consistent with the hypothesis that local sidechain rearrangements are the usual consequence of antibody binding (Getzoff *et al.* 1987).

Considerable mobility also exists in the CDRS of antibody molecules, but its functional role is difficult to assess because no pairs of crystal structures of free and bound antibody are available and because the high mobility of the CDRS in free antibody makes the localization of residues uncertain.

4. Predictions

The level of scientific understanding achieved in any discipline can be gauged by the accuracy of the predictions it enables one to make. In the field of protein antigenicity, it is important to distinguish predictions based only on sequence data from predictions based on a knowledge of the three-dimensional structure of proteins. The only structural information available for most proteins today concerns their amino acid sequence derived from the nucleotide sequence of the corresponding gene. Most current interest therefore centres on the validity of prediction methods that would allow the position of continuous epitopes to be deduced from certain features of the protein sequence. Regions of the protein that are predicted to be antigenic are then synthesized and the resulting peptides are used for raising antibodies. In the majority of cases the antipeptide antibodies cross react with the parent protein, and these antibodies are therefore useful reagents for isolating and characterizing gene products (Sutcliffe *et al.* 1983; Lerner 1984, Walter 1986). The high success rate of this approach is partly due to the fact that such antibodies need not necessarily recognize the native conformation of the protein to detect the protein in certain types of immunoassay. Another application of antigenicity prediction methods lies in the development of synthetic peptide vaccines (Shinnick *et al.* 1983; Arnon 1986; Steward & Howard 1987).

Parameters such as the hydrophilicity, static accessibility and mobility of short segments of polypeptide chains have been correlated with the location of continuous epitopes in proteins. Relative scales describing the structural propensity of each of the 20 amino acids have been derived and these are commonly used for constructing prediction profiles and for locating the position of epitopes (Hopp & Woods 1981; Kyte & Doolittle 1982; Karplus & Schulz 1985; Hopp 1986). As discussed elsewhere (Van Regenmortel 1987), the three parameters of hydrophilicity, static accessibility and segmental mobility are not independent variables but represent interconnected aspects of the folding pattern of globular proteins. Loops and turns, for instance, often correspond to surface projections and they also tend to contain hydrophilic residues and to possess higher than average mobility. It would serve little purpose to oppose static and dynamic explanations of antigenicity as mutually exclusive (Novotny & Haber 1986) or to give to one or other structural parameter the status of primary correlate with antigenicity (Novotny *et al.* 1987; Geysen *et al.* 1987*c*).

The predictive value of algorithms based on eight propensity scales of the amino acids has recently been compared, by using as a criterion the number of residues correctly predicted to be antigenic in four well-studied proteins (Van Regenmortel & Daney de Marcillac 1988). None of the methods achieved a high level of correct prediction, although two algorithms based on a segmental mobility scale (Karplus & Schulz 1985) and a hydrophilicity scale (Parker *et al.* 1986) were somewhat more successful than the others. The difficulties encountered

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when attempting to predict epitopes from sequence data no doubt reflect the inadequacy of a unidimensional analysis for describing the three-dimensional nature of protein epitopes. As discussed above (§3), the inclusion of non-critical, replaceable residues within the boundaries of continuous epitopes falsifies the calculated propensity scales of amino acids used for the construction of prediction profiles. This may be one of the reasons why antigenicity factors for each of the 20 amino acids calculated on the basis of their occurrence in known continuous epitopes (Welling *et al.* 1985; Geysen *et al.* 1985) were of little value for predicting epitopes in proteins (Van Regenmortel & Daney de Marcillac 1988). On the other hand, propensity factors for the occurrence of relatively non-replaceable residues in epitopes were also found to possess limited statistical significance (Geysen *et al.* 1988). Although one of the few clear-cut trends that emerged was that Arg was greatly under-represented as an antigenic residue, this finding may simply reflect sample bias; in studies of Arg-rich histones, it was found that four continuous epitopes of histones (residues 1–8 and 88–96 of H4, 130–135 of H3 and 26–35 of H2B) contained no less than eight Arg residues (Van Regenmortel *et al.* 1985).

When only critical residues within continuous epitopes were considered for evaluating the antigenic propensity of individual amino acids, a somewhat unexpected result was obtained, because no correlation was observed between these propensity factors and the classical hydrophilicity and accessibility factors (Geysen *et al.* 1988). This finding may be related to the observation (Van Regenmortel & Daney de Marcillac 1988) that none of the classical algorithms achieved a significant level of correct prediction when they were applied to the myohaemerythrin epitopes identified by the solid-phase methodology of Geysen *et al.* (1987*a*). On the basis of similar studies, Geysen *et al.* (1987*b*) had concluded that antigenicity is correlated with regions of high mobility in myohaemerythrin. However, as it is unlikely that short peptides are able to mimic constrained elements of secondary structure, these studies cannot provide information on the presence of antibodies that do recognize secondary structure. It is therefore unwarranted to conclude that the immune system is biased against the recognition of ordered structures.

Operational bias is responsible for the observation that mobile regions in proteins are preferentially recognized when short, mobile peptides devoid of any secondary structure are used as probes to localize antigenic regions. In fact, it is hard to conceive any experimental design that would measure the relative frequency of immune recognition of ordered versus unordered structures without the introduction of some bias. As studies with short peptides cannot identify the entire structure of complex epitopes, they should not be used to draw generalizations regarding which intrinsic structural features determine all possible forms of protein antigenicity. When longer peptides that have a more ordered conformation are used as probes, it can be demonstrated that regions of the protein that are structured and possess low mobility, such as α-helices, are also antigenic (Al Moudallal *et al.* 1985; Williamson *et al.* 1986). Because epitopes can only be defined by their ability to bind in a particular immunoassay, it is unavoidable that all definitions of antigenicity are operationally biased.

5. Synthetic peptide vaccines

In recent years there has been considerable interest in the possibility of using synthetic peptides as vaccines against viral, bacterial and parasitic diseases (Arnon 1987; Lerner *et al.* 1985; Brown *et al.* 1986). Although our present understanding of the mechanism of infectivity

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neutralization is inadequate (Mandel 1985), a situation which, incidentally, rules out a completely rational approach to vaccine design, many attempts have been made to achieve protective immunity by immunizing animals with synthetic peptides that mimic certain epitopes of viruses, toxins and parasites. The first demonstration that such an approach was feasible was made by Anderer (1963) and Anderer & Schlumberger (1965 *a*, *b*) in their studies of the plant virus, tobacco mosaic virus (TMV). As the early contribution of Anderer is sometimes played down by workers conversant only with animal and human viruses, it may be of interest to describe here in some detail the results he obtained. The amino acid sequence of the coat protein of TMV was established in 1960 (Anderer *et al.* 1960; Tsugita *et al.* 1960). Shortly afterwards Anderer (1963) used the *C*-terminal hexapeptide fragment of the viral protein, coupled to bovine serum albumin, for raising antibodies in rabbits. This region of the viral protein was known to have a surface orientation and Anderer found that the resulting peptide antiserum precipitated the virus and neutralized its infectivity. It is interesting to note that when the three-dimensional structure of the viral protein was elucidated several years later, the *C*-terminal residues were invisible in the map, because of considerable segmental mobility in this region of the protein (Bloomer *et al.* 1978; Van Regenmortel 1986 *b*).

In a series of detailed studies in which *C*-terminal synthetic peptides of a length of 3–6 residues were used, Anderer & Schlumberger (1965 *a*, *b*) showed that antisera raised against the synthetic peptides neutralized TMV infectivity. The biological assay consisted of mixing infectious virus with antiserum and showing that the number of local lesions obtained after inoculating tobacco plants with the mixture was greatly reduced. The antibody-neutralizing activity could be specifically abolished by prior incubation of the antiserum with the synthetic peptides. The neutralizing capacity of the peptide antisera was clearly distinguishable from the small degree of non-specific inhibition of infectivity observed with normal serum from non-immunized animals. In fact the neutralizing potency of some of the anti-peptide sera was comparable to that of anti-TMV serum. The local lesion assay in tobacco is similar to a plaque reduction test and the antibody-induced reduction in the number of lesions is clearly a neutralization phenomenon. Many characteristic features of the neutralization reaction of animal viruses have also been observed in studies with TMV (Rappaport 1957, 1959; Rappaport *et al.* 1957; Van Regenmortel 1982) and there is no doubt that Anderer and his colleagues should be credited with the discovery that synthetic peptides can elicit antibodies that neutralize viral infectivity.

Subsequently, the immunogenicity of model synthetic peptides was extensively studied by Sela and his colleagues (Sela 1969) who 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).

Since 1980, many similar studies have been undertaken with a number of animal and human viruses. Following the elucidation of the three-dimensional structures of influenza virus haemagglutinin (Wilson *et al.* 1981), poliovirus (Hogle *et al.* 1985) and rhinovirus (Rossmann *et al.* 1985), there is now considerable hope that this type of structural information may help in the development of a new generation of synthetic vaccines. At present, vaccination of animals with synthetic peptides has led to protective immunity against foot-and-mouth disease, influenza, hepatitis B, diphtheria and cholera (Arnon 1986; Brown 1986).

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6. Conclusions

Different experimental approaches used for the identification of protein epitopes lead to different views regarding the structural basis of antigenic specificity. The structural approach of X-ray crystallography points to an epitope area of about 700 $Å^2$ and to the participation of as many as 16 residues in the contact area with antibody, although it should be stressed that the contribution of individual residues to the binding energy remains uncertain. In contrast, antigenic cross-reactivity studies with peptide fragments and protein mutants seem to indicate that only 3–5 residues in the antigen are truly critical for specific binding. It is also remarkable that when non-neutralizable virus mutants are selected by MAbs, single-residue substitutions in the antigen are usually sufficient to diminish drastically or abolish antibody binding (Wiley *et al.* 1981; Knossow *et al.* 1984). In view of the range of sizes of different antigens, it is probably futile to try to define a typical epitope size even for proteins. It seems likely, for instance, that certain epitopes of a small molecule such as insulin are probably smaller than the 700 $Å^2$ epitopes of lysozyme. There is also no reason why all six CDRs of every antibody molecule must necessarily be involved in the interaction with every protein antigen.

Each of the methods used in the analysis of antigenicity is subject to various operational constraints originating in the type of probe (size of peptide, monoclonal versus polyclonal antibodies) and in the format, sensitivity and specificity of the immunoassay used. Every approach requires specific criteria for deciding, for instance, whether two residues are in contact with each other, or for assessing which residues of the antigen are functionally part of the epitope and make a contribution to the binding energy.

As our knowledge of antigenicity increases, the need for clarifying concepts and defining terms becomes more acute. At the same time, our perception of the operational constraints of immunochemical experimentation and of the difficulty of extrapolating from structure to function is heightened. When attempts are made to integrate structural data with functional measurements and biological observations, there is no alternative to the building of conceptual models. When a scientific discipline matures, it becomes obvious that our perception of the reality it describes requires increasingly sophisticated model building and conceptualizations (Goodman 1984). The task of explanatory models of antigenicity is to account for a wide variety of experimental observations such as the discrimination achieved at the level of single-residue substitutions, the limited size of the antibody repertoire, the potential recognition by the immune system of an immense number of different structures, the low frequency of antigenic cross reactions between unrelated molecules, the phenomena of tolerance and autoimmunity, and the size and range of antibody affinity constants that are commonly encountered.

Although our understanding of antigenic specificity has increased substantially in recent years, the magnitude of what remains to be explained poses exciting challenges for the future.

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