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Invited Paper

Use of Monoclonal Antibodies in the Study of the Conformation and Conformational Alterations in Proteins

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The conformational flexibility of many of the native proteins facilitates their attainment of new reversible three-dimensional structures under well-specified conditions. Such structures appear to be of great importance in biological processes involving biologically active proteins, such as enzymes, receptors or protein hormones. Recently it was shown that the above conformational alterations can be followed not only by physicochemical methods but also immunochemically by the use of monoclonal antibodies (mAbs). mAbs are specific proteins, each capable of recognizing a corresponding well-characterized region (epitope) on the surface of a protein antigen. They are thus useful as tools in the study of the structure and properties of protein surfaces. Of particular interest is the fact that it is possible to prepare mAbs with predetermined specificities towards the different epitopes of a given protein. Such mAbs can thus be used to detect conformational alterations occurring at the different segments of a protein antigen. In the present review article we describe our own work and that of others on the use of mAbs as probes to detect the conformation and conformational alterations occurring in proteins exposed to different conditions, such as heat, adsorption onto solid surfaces, interaction with ligands and protein–protein interaction. The use of mAbs in the detection of protein folding intermediates is also described.

Powerful X-ray diffraction techniques developed for protein analysis have made it possible to elucidate the three-dimensional structure of approximately 300 proteins to date. In all of them it was noticed that the bending of the polypeptide backbone, leading to the formation of α-helices, β-pleated sheets and characteristic loops, brings about contact between amino acid residues located originally at relatively large distances from one another along the peptide chain. Because of the tertiary structure thus obtained, native protein molecules possess a characteristic "topography" when surveyed from the outside and a characteristic core when surveyed from the inside. The characteristic three-dimensional structure of a native protein seems to be derived from the primary amino acid sequence of the protein, since polypeptides possessing this primary sequence can coil, under appropriate conditions, into the native three-dimensional structure. Exposure of proteins to extreme conditions (high temperatures, extreme pH values or denaturating agents) is usually accompanied by disruption of the three-dimensional structure characterizing the native protein. The resulting denatured proteins possess physical, chemical, and biological properties which differ markedly from those of the native protein. Recently accumulated evidence shows that the three-dimensional structure of proteins is not rigid, but undergoes subtle reversible conformational fluctuations. Flexibility of amino acid side chains, mobility of peptide segments, and movement of structural domains as rigid bodies, have been demonstrated.[1] Physical methods (X-ray or neutron diffraction, NMR, fluorescence spectroscopy, hydrogen-deuterium exchange) have proved particularly useful in the determination of the amplitudes, time spans, and energies involved in these conformational oscillations. The conformational flexibility of many of the native proteins facilitates their acquisition of new reversible three-dimensional structures under well-specified conditions. Such structures seem to be of great importance in biological processes involving biologically active proteins, such as enzymes, receptors or protein hormones in which the conformational alterations in solution can be readily followed, not only by physicochemical methods but also immunochemically by the use of monoclonal antibodies (mAbs).

In the present review we describe the work of ourselves and others on the use of mAbs as probes to detect the conformation and conformational alterations occurring in proteins exposed to different conditions (heat, adsorption onto solid surfaces, interaction with ligands, protein–protein interaction). The use of mAbs in the detection of protein folding intermediates is also described.

Monoclonal Antibodies as Conformational Probes

The cell hybridization technique developed by Köhler and Milstein[2] enables the preparation of murine monoclonal antibodies (mAbs) capable of recognizing corresponding single well-characterized regions (epitopes) on the surface of the protein antigens used to elicit the immunological response. It thus seems that mAbs might serve as useful tools in the study of the structure and properties of protein surfaces. All antigenic determinants of proteins appear to be topographical, i.e., composed of

structures on the protein surface which may be contained within a single segment of the amino acid sequence or assembled from residues far apart in the amino acid sequence but brought together on the surface by the folding of the protein in its native conformation.[3] It is thus pertinent to note that mAbs are not only directed towards corresponding amino acid residues of the antigen, but seem also to recognize well-defined conformations of protein segments. Any alteration in the conformation of such a segment is usually accompanied by a marked change in the characteristic binding of the mAb with its protein antigen. It is obvious, however, that the use of mAbs as conformational probes of proteins will be reliable only if general information is available as to the antigenic determinant recognized by the mAb, and if no conformational alterations are induced in the antigen as a result of its interaction with the mAb.

The three-dimensional structure of an antigen-antibody complex was recently determined for the first time by X-ray crystallography using hen egg white lysozyme (HEL) and the Fab fragment of a mAb (D1.3) at a resolution of 2.8 Å.[4] The antigenic determinant was shown to be topographic, with the interface between antigen and antibody extending over an area having maximum dimensions of 30×20 Å. Complex formation with the mAb D1.3 did not produce any significant distortion in the structure of the lysozyme. This was also confirmed by the simultaneous binding of two different mAbs to the lysozyme[5] and the retention of enzymatic activity by the HEL-mAb D1.3 complex.[6] It is worth noting that mAbs towards different enzymes have been obtained which do not affect their catalytic activity.[7]

The three-dimensional structure of the HEL-D1.3 Fab complex suggests that the interaction between the enzyme and the mAb can be described by a "lock and key" model, in which complex formation is essentially an association between two rigid molecules. An absence of marked conformational changes (>2 Å) upon complex formation is, in fact, characteristic of nearly all protein-protein complexes studied to date at high resolution, in which the structure of the unbound form of at least one of the component proteins is also known. These complexes include bovine trypsin–pancreatic trypsin inhibitor;[8] porcine kallikrein A–bovine pancreatic trypsin inhibitor,[9] and carboxypeptidase A–potato inhibitor.[10]

The three-dimensional structure of another enzyme antibody complex, that formed between neuraminidase and a corresponding mAb, was recently elucidated by X-ray crystallography.[11] Unlike in the case of HEL mAb D1.3 Fab, the specific interaction was accompanied here by significant conformational changes in both the antigen and antibody. Conformational changes in proteins as a result of interaction with their corresponding antibodies have also been reported by others.[12] It is reasonable to assume that such changes occur in a region of the protein characterized by significant flexibility. If this is the case, one might explain the observed conformational alterations by assuming an "induced fit mechanism," whereby the antigen and/or the antibody achieve greater complementarity by undergoing conformational changes.

It is still too early to predict, on theoretical grounds, when the interaction between a protein antigen and its corresponding mAb proceeds according to the lock and key mechanism, and when it favors the induced fit mechanism. It is obvious, however, that determination of the parameters characterizing the binding of a protein antigen with its mAb in solution should supply valuable information on the conformational characteristics of the interacting protein antigen epitopes.

Recognition of the region of interaction of a protein antigen with a given mAb is necessary if the conformational alterations occurring under various conditions in that region are to be detected. Antigenic determinats can be delineated by means of a physical approach using X-ray diffraction methods as illustrated above, or by an immunochemical approach in which direct or competitive assays are used to determine the reactivity of the mAb with isoenzymes or natural variants, and with natural or synthetic fragments of the target proteins. It has recently become clear that antibodies against most regions of a protein can be induced by immunization with short synthetic peptides. This has permitted the generation of mAbs with predetermined specificity[13] which constitute excellent tools for probing surface conformations of proteins at precise locations.

Detection of Conformational Changes Induced by Ligands

The activity of many enzymes, and particularly of allosteric enzymes, is drastically modified by the binding of coenzymes, ligands, modulators, cofactors or inhibitors. Coenzymes are involved in many transfer reactions and may act by changing the molecular dynamics of specific biocatalysts.

Conformational alterations resulting from the binding of the β_2-subunit of *Escherichia coli* tryptophan synthase with its ligands were detected and analyzed by Djavadi-Ohaniance et al.[14] using mAbs as specific probes. Five mAbs recognizing five different epitopes of the β_2-subunit of *E. coli* tryptophan synthase were prepared and their apparent binding constants with the enzyme subunit determined in solution. Fixation of the coenzyme pyridoxal 5-phosphate and the substrate L-serine by the β_2-subunit modified the affinity constants of most of the mAbs for the enzyme, thus demonstrating the existence of extended conformational rearrangements of the

protein. Association of the α-subunit with the β_2-subunit, brought about an increase of the tryptophan synthase activity and abolished the serine deaminase activity of β_2; this was accompanied by important conformational changes readily detected with appropriate mAbs. The above changes are not limited to a small region near the ligand binding site of the protein but rather affect an extended area of the protein surface, as shown by the observation that each ligand modifies the association constant of at least four mAbs recognizing distinct and non-overlapping epitopes.

Conformational transitions induced by the binding of metal ions to specific sites of proteins have been monitored by various physicochemical methods. These transitions can also be localized by immunochemical methods. Specific mAbs directed against the calcium-stabilized structure of human prothrombin have been prepared and characterized by Lewis et al.[15] One of the mAbs was found to bind with prothrombin only in the presence of $CaCl_2$. It did not interact with abnormal prothrombin lacking the γ-carboxy-glutamate residues known to interact with metal ions. Replacement of the calcium cation by Mn^{2+}, Mg^{2+}, and Co^{2+} promoted binding. The conformational transitions in prothrombin occurring as a result of the metal binding have been monitored using circular dichroism and fluorescence techniques.[16,17] The above findings were corroborated by the earlier studies of Tai et al.[18] who used polyclonal antibodies in their immunochemical studies. Antigenic differences could be detected in bovine prothrombin in the presence or absence of Ca^{2+} with antibodies raised in rabbits and fractionated by affinity chromatography.

The value of mAbs as conformational probes is further illustrated by their use in the detection of the calcium-induced conformational change(s) in a C-reactive protein (a protein appearing in the serum in the acute phase of certain infections). Three out of four mAbs against C-reactive protein bind to the protein antigen in the presence of Ca^{2+}, but not in the presence of EDTA, indicating that the conformation of the antigenic determinant recognized by these mAbs is calcium-dependent. The Ca^{2+}-independent binding of the fourth mAb, however, suggests that the conformation of the epitope recognized by this mAb is independent of bound calcium.

Monoclonal antibodies possessing similar binding characteristics to those described above, and directed towards the various domains of protein C have been recently described by Wakabayashi et al.[19] The three mAbs which react with protein C only in the presence of Ca^{2+} were found to bind to the light chain of protein C, i.e. the chain possessing γ-carboxy-glutamic acid residues. Other mAbs which recognize the conformation of a protein in a medium devoid of Ca^{2+}, but not that in a medium containing Ca^{2+}, have been described by Dixit et al.,[20] who prepared and studied the characteristic of two such mAbs directed against thrombospondin.

The induction of different calcineurin conformations by metal ions could also be demonstrated by suitable mAbs.[21] The experimental data showed that the active conformational states of calcineurin are metal-ion-dependent, that the mAb VA_I affects the Ni^{2+}-induced conformational change of the enzyme, and that the β-subunit of calcineurin plays a critical role in the expression of Ni^{2+}-stimulated phosphatase activity. Conformational changes induced in myosin by Ca^{2+} could also be detected with appropriate mAbs. Particularly useful in the studies of Shimizu et al.[22] was a mAb capable of detecting conformational alteration in the light chain of myosin LC2.

Three mAbs that interact with carboxypeptidase-A with relatively high binding constants but without affecting the enzyme activity, were used in our laboratory[23] for the detection of subtle conformational alterations occurring in the enzyme molecule when exposed to various conditions. Of particular interest was the finding that the three mAbs can detect subtle reversible conformational alterations occurring on removal of zinc from the active site of the enzyme by 1,10-phenanthroline. Reconstitution of the enzymatic activity by addition of zinc to the apoenzyme led to full recovery of the antigenic characteristics of carboxypeptidase-A recognized by the three mAbs.

Use of Monoclonal Antibodies in the Detection of Protein Folding Intermediates

The "translation" of the amino acid sequence of a newly synthesized polypeptide chain into the well-ordered "native" three-dimensional structure of a functional protein is commonly referred to as "protein folding." This was clearly illustrated by the classical experiment of Anfinsen et al.[24] who demonstrated that reduced and urea-denatured pancreatic ribonuclease readily undergoes renaturation in an oxygen containing solution on removal of the denaturing agent. It should be pointed out, however, that the detailed mechanism involved in molecular renaturation and denaturing is still only poorly understood, though obviously of great interest to the molecular biologist. Up to quite recently the conformational processes occurring during renaturation or denaturation were studied mainly from a theoretical point of view as very few experimental techniques were available to tackle the problem. Rapid developments in the various physical methods, particularly X-ray and NMR, as well as the recently developed capacity for substituting different amino acid residues in proteins by site directed mutagenesis, have opened up new possibilities for the experimental study of the

process and the intermediate conformers appearing during protein renaturation and denaturation. Protein folding seems to proceed through a series of structural intermediates from short stretches of local, barely stable, secondary structures to compact "domains" that interact to yield the final tertiary structure. At each step on this pathway, the structural intermediates appear to undergo conformational rearrangements. In these protein folding studies mAbs can contribute as probes for detection of the early intermediates. The potential usefulness of mAbs in this connection was illustrated by the pioneer work of Blond and Goldberg[25] on the β_2 subunit of *E. coli* tryptophan synthase. Two mAbs were directed against the native β_2-subunit of the enzyme, and their association with either the intact β_2-subunit or its isolated domains was investigated using fluorescence energy transfer between the tryptophan residues of the antibodies and a dansyl group covalently linked to the antigen. Denaturation of the β_2-subunit was induced by a concentrated guanidine hydrochloride solution, and renaturation initiated by extremely rapid dilution of the denaturating agent. Association of both mAbs with the antigen occurred within a few seconds of intitiating the renaturation, while complete refolding of the β_2 subunit required several minutes. It was concluded that immunoreactive intermediates appear to be formed at an early stage of the folding process.

The β_2-subunit of *E. coli* tryptophan synthase is composed of two independently folding domains; F_1 (N-terminal) and F_2 (C-terminal) which can be isolated following mild proteolytic treatment of β_2 by trypsin. The effects of domain assembly on the conformations of the F_1 and F_2 domains were analyzed using six mAbs which recognize six different epitopes of the native β_2-subunit.[26] It was found that the association of the F_1 and F_2 domains within β_2 is accompanied by structural changes within the two domains, as detected by variations in their affinity constants for the mAbs.

The building of the quaternary structure of proteins composed of several protein subunits is often accompanied by corresponding subtle conformational changes in each of the subunits. Monoclonal antibodies can be used here to detect suspected conformational alterations. Such alterations have been observed, for example, in the case of glutamine synthetase, an enzyme composed of 12 identical subunits. Some of the mAbs prepared against this enzyme[27] were found to bind only to the monomeric form, whereas others recognize the full dodecameric form.

An interesting use of mAbs is in the detection of the assembly of mouse muscle acetylcholine receptors in vivo.[28] The receptor consists of α, β, γ, and δ subunits. With the aid of the mAbs prepared, one α-subunit species that may be an assembly intermediate has been identified.

Detection of Conformational Changes Induced by Heat and Absorption onto Solid Supports

The susceptibility of the tertiary structure of proteins to heat, to denaturants such as urea and guanidine hydrochloride, and to extreme pH value, is well-known. Such conformational alterations have been readily detected by means of polyclonal antibodies directed either to the native or to the denatured corresponding protein antigen. There is also considerable evidence that the binding of proteins to plastic surfaces, or to other organic or inorganic surfaces, may cause partial denaturation and lead to changes in protein conformation and antigenicity.[29–33] One might even predict that the specific environment to which enzymes, receptors and other biologically active proteins are exposed when embedded in native membranes could affect their conformation. Milne et al.[34] have recently demonstrated that mAbs can also be used to distinguish between the lipid dependent and reversible conformational states of human apolipoprotein B.

Vaidya et al.[32] reported marked modifications in the antigenic behavior of creatine kinase (CK) and lactate dehydrogenase (LDH) after their adsorption onto polystyrene plates, as revealed by their corresponding mAbs. Hybridomas producing anti-CK and anti-LDH antibodies were screened by an enzyme-linked immunosorbent assay (ELISA) with antigen coated onto plastic wells. Of seven antibodies that were positive for each isoenzyme of CK, four failed to bind to the native enzyme in solution. Moreover, none of the mAbs prepared against the various LDH isoenzymes, interacted with the corresponding enzyme in solution. These results strongly support the suggestion made that the conformation of protein-antigen in the solid phase may differ from that in solution. Observations similar to those of Vaidya et al.[32] were recorded in our own laboratory where it has been found that the mAb MF 30 against human lactate dehydrogenase isoenzyme 5 (HLDH5) was found to interact with HLDH5 adsorbed onto the polystyrene microtiter plate, but not with the enzyme in solution.[35] In contrast, mAb 2/66 prepared against porcine lactate dehydrogenase 5 (PLDH5) reacted equally well with HLDH5, when in solution and when adsorbed onto polystyrene. Following its denaturation by heating to 70 °C HLDH5 no longer reacted with mAb 2/66. The findings obtained are summarized in Table 1. Our data obtained suggest that the mAbs MF30 and 2/66 recognize two different antigenic sites of HLDH5. The antigenic site which is recognized by 2/66, and is present in the native enzyme both in solution and when adsorbed onto polystyrene, disappears on heating. The other antigenic determinant is recognized by mAb MF30 when the enzyme is adsorbed onto a polystyrene surface both before and after heat treatment.

Table 1. Qualitative Description of the Binding of the mAbs 2/66 and MF30 with Native and Heat Treated Human or Porcine Lactate Dehydrogenase Isoenzyme 5 (HLDH5 or PLDH5) when in Solution or Adsorbed onto Polystyrene

Monoclonal antibody	Solution[a]				Adsorbed onto polystyrene surface[b]			
	HLDH5		PLDH5		HLDH5		PLDH5	
	Nat.[d]	Ht.[d]	Nat.	Ht.	Nat.	Ht.	Nat.	Ht.
2/66[c]	+	−	+	−	+	−	+	−
MF30[c]	−	−	−	−	+	+	−	+

a) The native and heat-treated enzymes were allowed to interact with the corresponding monoclonal antibodies in solution. b) Interaction was allowed to take place between the native or heat-treated enzymes adsorbed onto polystyrene and corresponding antibodies kept in solution. c) 2/66 and MF30 denote the monoclonal antibodies obtained against porcine and human LDH5 isoenzymes, respectively. d) Nat. stands for native enzyme; Ht. stands for enzyme heated in PBS, pH 7.5, for 45 s at 70 °C (see Ref. 35).

Subtle conformational alterations in the antigenic region of carboxypeptidase-A(CPA) between the amino acid residues 209—218 following the adsorption of CPA onto polystyrene were also detected by our group with the aid of three mAbs directed towards this antigenic region.[23,36] The mAbs interacted with CPA with relatively high binding constants without affecting the enzyme's activity. Binding of the three mAbs with CPA adsorbed onto the polystyene plates was characterized by apparent binding constants which were higher by one or two orders of magnitude than the corresponding binding constants characterizing their interaction in solution. The conformational alterations occurring in CPA following its treatment with urea were also readily detectable by the mAbs employed.

Detection by mAbs of subtle conformational changes in proteins following their physical adsorption to solid supports has also been reported by the French school. Friguet et al.,[30] using mAbs directed against different epitopes of the β_2-subunit of *E. coli* tryptophan synthase, have shown that some of the antibodies bind very slowly to the native β_2-subunit in solution, but recognize the antigen quite rapidly when adsorbed onto a microtiter plate. Their observation imply that the ELISA test commonly used in screening for specific hybridomas may lead to the retention of antibodies not specific for the native conformation of the antigen.

Also investigated by our group was the interaction of mAbs raised against native hen egg white lysozyme (HEL) with the enzyme after its partial irreversible denaturation by heating. This antigen/antibody system was chosen because it has been well-characterized by the Pasteur group.[4] Irreversibly heat-denatured HEL (D. Kenett, unpublished results) was recognized by the mAbs D44.2 directed against the "loop" region of the native enzyme and by the mAb D1.3 directed against the amino and carboxyl termini of the enzyme. The mAb D74.3, which binds to an

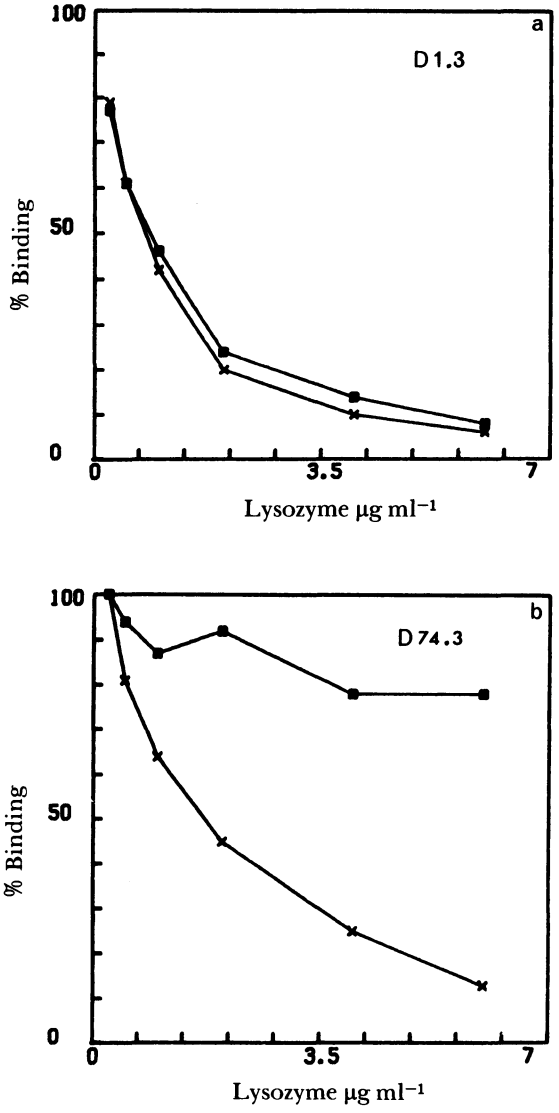

Fig. 1. Binding of the mAb D 1.3 (Fig. 1a) or mAb D 74.3 (Fig. 1b) onto HEL adsorbed on microtiter polystyrene plates in the presence of increasing concentrations of native (-X-X-) or partially heat denatured (-□-□-) (120 min at 95 °C, pH 5) enzyme.

epitope within the active site region of HEL, reacted only weakly with the denatured enzyme which lost most of its catalytic activity after heating (see Fig. 1). The active site of lysozyme is located in a cleft between two globular lobes which oscillate btween an open and a closed conformation during the catalytic cycle. As revealed by the mAbs, the active site appears to be more prone to conformational change than the rest of the molecule probably because of the hinge bending motion.

Monoclonal Antibodies as Topographical Probes

Monoclonal antibodies usually recognize a single well-characterized "surface" of a protein antigen. One might, therefore, expect to find them useful in building up a topographic picture of protein antigens, providing information about their orientation, attachment, ligand binding sites and carbohydrate attachment sites. They might be particularly useful in the elucidation of the molecular "surfaces" involved in subunit assembly, and the protein "surfaces" involved in the binding of a given protein with other proteins or with macro- or micro-environmental components.

The use of mAbs in the study of subunit assembly may be illustrated by the work of Moradi-Amel et al.[37] on the structure of mitochondrial F_1-ATPase. Four stable mAbs against pig heart mitochondrial F_1-ATPase were prepared. Three of them interact with the β-subunits and one binds strongly to the α-subunits. Quantitative analysis of the interaction of these mAbs with their corresponding subunits led to the prediction that mitochondrial F_1-ATPase consists of three α- and three β-subunits.

As an example of the use of mAbs in the topographical analysis of proteins, we might cite the elucidation of the position of the immunoglobulin (IgD) molecules within lymphocyte membranes using mAbs.[38,39] Treatment of intact cells with trypsin abolished the binding of mAb 11-6.3, but did not affect the binding of mAb 10-4.22. The mAb 11-6.3 was found to bind to the Fab portion of IgD, while mAb 10-4.22 bound to the Fc portion. It was thus conculded that IgD is enclosed within the lymphocyte cell membrane via its Fc portion.

The use of mAbs against subunits of human chorionic gonadotropin (hCG) in order to examine the orientation of the hormone in its complex with gonadal receptors, has been illustrated by Moyle et al.[40] hCG is a glycoprotein composed of α- and β-subunits which interact with gonadal receptors to stimulate steroid synthesis. The purified mAbs obtained were iodinated and used to determine which antigenic sites on hCG remained free in the hCG-receptor complexes. The resulting data indicated that portions of the β-subunit were buried (i.e. failed to bind with radiolabeled antibody), whereas other portions remained exposed (i.e., they bound with radiolabeled antibody). Those antibodies which interacted with portions of hCG that became inaccessible in the hCG-receptor complex also blocked the biological action of hCG, whereas those that interacted with exposed sites had little or no effect on its activity. A similar approach was used in order to determine the orientation of a human leukocyte interferon molecule on its cell surface.[41] Using a mAb against a synthetic interferon fragment, it was possible to show that the carboxyl terminus of the interferon remains accessible to the mAb even after its interaction with the corresponding cell surface receptor.

The use of mAbs as probes of the α-bungarotoxin (αBTx) and cholinergic-binding regions of the acetylcholine receptor (AcChR)[42] revealed the non-equivalence of the two αBTx binding regions of the AcChR molecule. Furthermore, it was possible to identify epitopes on the αBTx binding region that, when bound, produce differential effects on the binding of the agonist carbamyl-choline and the antagonist d-tubocurarine.

Concluding Remarks

We have shown here that mAbs might serve as useful probes in the detection of given protein conformations and conformational alterations. The high specificity of mAbs towards given antigen determinant conformations was recently illustrated by the preparation of "abzymes," i.e. mAbs that can catalyze a chemical reaction because they have been made to recognize the transition state of the reaction under consideration.[43] The value of mAbs as conformational probes will no doubt increase as more detailed information is acquired as to the structure of the antigenic epitopes which they recognize. In this respect, the production of mAbs of predetermined specificity by means of selected peptides might be of considerable importance.

Since mAbs prepared against a given protein recognize different antigenic sites of the antigen, it should be possible to use them in detecting the effect of amino acid substitution by site-directed mutagenesis on the conformation of different molecular regions. They may thus pave the way for the preparation of highly stable proteins under selected conditions.

In the section dealing with the use of mAbs as topographical probes, we have shown that they can be used in the elucidation of the quaternary structure of proteins, receptors and more complex biological systems. Their application in this area will undoubtedly increase in the future.

The use of mAbs as conformational probes is relatively new. Their use in conjunction with other methods in studies on structure and function is promising.

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