Antibody Combining Sites Can be Mimicked Synthetically

SURFACE-SIMULATION SYNTHESIS OF THE PHOSPHORYLCHOLINE-COMBINING SITE OF MYELOMA PROTEIN M-603

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(Received 24 October 1979)

By applying the concept of 'surface-simulation' synthesis to the combining site of the myeloma protein M-603 we were able to mimic synthetically its phosphorylcholine-binding characteristics. The synthetic surface-simulation peptide was found to bind to phosphorylcholine, whereas a control peptide that had the same amino acid composition but a different sequence showed little or no binding activity. The specificity of the binding was further confirmed by inhibition studies in which the surface-simulation peptide, but not the control peptide, inhibited the binding of ^{125}I -labelled surface-simulation peptide to phosphorylcholine. Furthermore, the surface-simulation peptide was found to completely inhibit the binding of the native myeloma protein, M-603, to phosphorylcholine. The control peptide was unable to inhibit this binding. These findings suggest that surface-simulation synthesis can be effectively employed to mimic synthetically antibody combining sites, and may in the future be a valuable tool with which to manipulate the immune response to clinically important antigens.

The concept of 'surface-simulation' synthesis was introduced by our laboratory in 1976 (Atassi *et al.*, 1976; Lee & Atassi, 1976). This concept provides the basis for an experimental approach to mimic functional surfaces on proteins that are composed of residues not linked directly in the parent protein sequence, but that are close in their three-dimensional arrangements. Through the application of surface-simulation synthesis, it was possible to elucidate the complete antigenic structure of lysozyme (for review see Atassi, 1978). Twining & Atassi (1978) were successful in simulating the antibody combining site of the human myeloma protein immunoglobulin G New. In the present paper we report the results of our efforts to simulate synthetically the phosphorylcholine-combining site of the mouse immunoglobulin A myeloma protein M-603.

Materials and Methods

Phosphorylcholine chloride (calcium salt) was from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was converted into the silver salt as described below. Carrier-free ^{125}I iodide was from New England Nuclear (Boston, MA, U.S.A.). Sepharose CL-4B was from Pharmacia Fine Chemicals

(Piscataway, NJ, U.S.A.). CNBr and CCl_3CN were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The myeloma protein M-603 was obtained from Dr. David Klapper (University of North Carolina Medical School, Chapel Hill, NC, U.S.A.). The resin and amino acid derivatives for solid-phase peptide synthesis were from Vega Fox Biochemicals (Tucson, AZ, U.S.A.). The side-chain-protecting groups used were: arginine, nitroguanidinium; glutamic acid, γ -benzyl ester; serine and tyrosine, *O*-benzyl ether. In each derivative, *t*-butyloxycarbonyl was the α -N-protecting group. All the other reagents were of the highest available purity and solvents were redistilled as indicated.

Phosphorylcholine was coupled to Sepharose through its phosphate group using CCl_3CN to effect a phosphate ester linkage. The procedure was as follows. The silver salt of phosphorylcholine was prepared by shaking an aqueous solution of phosphorylcholine (calcium salt) with a large excess of solid Ag_2CO_3 for 2 h while protected from light. After centrifugation, the supernatant was acidified with HCl to precipitate excess Ag^+ ions and re-centrifuged. The resulting supernatant was freeze-dried, washed with anhydrous redistilled methanol and dried under vacuum over KOH , then over P_2O_5 . The phosphorylcholine (silver salt) (approx. 0.4 mmol) was dissolved in dry redistilled dimethyl sulphoxide (2 ml). Sepharose (100 mg), which had

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been dehydrated with absolute ethanol and dried under vacuum over KOH and P_2O_5 , was then added to the phosphorylcholine solution, followed by anhydrous redistilled pyridine (0.4 ml) and CCl_3CN (0.5 ml, 5 mmol). The mixture was heated at $50^\circ C$ overnight with the exclusion of moisture, after which the dark-brown Sepharose was washed exhaustively with dimethyl sulphoxide and water to remove excess of reagents. Phosphorus determinations, performed by the method of King (1932), indicated $788 \mu g$ of phosphorylcholine/ml of packed volume of rehydrated Sepharose.

The peptides shown in Fig. 1 were synthesized and purified by methods previously described (Koketsu & Atassi, 1973, 1974). Surface-simulation peptide A was designed to simulate the myeloma-protein-M-603 binding site, and peptide B is a control peptide having the same amino acid composition but a different amino acid sequence from that of peptide A. The rationale for the sequences of these peptides is given in the Discussion section. After purification, each peptide showed a single spot by 'peptide mapping'. Amino acid analysis gave: peptide A, Ser, 0.95; Glu, 0.99; Gly, 3.19; Val, 1.00; Tyr, 1.91; Trp, 0.81; Arg, 1.02; peptide B, Ser, 0.95; Glu, 1.02; Gly, 2.88; Val, 0.99; Trp, 2.05; Trp, 1.08; Arg, 1.11.

Myeloma protein M-603 and peptides A and B were iodinated with ^{125}I by using chloramine-T according to the method of Hunter & Greenwood (1962). The specific radioactivities of the labelled preparations were: myeloma protein M-603, $10 \mu Ci/\mu g$; peptides A and B, $90 nCi/\mu g$.

The binding of ^{125}I -labelled peptides to phosphorylcholine-Sepharose was performed in 0.15 M-NaCl, pH 7.8. Fixed amounts of phosphorylcholine-Sepharose or uncoupled Sepharose ($10 \mu l$, packed volume) were incubated in triplicate with vari-

ous amounts of ^{125}I -labelled peptides for 18 h at $23^\circ C$ with constant shaking. After incubation, the Sepharoses were washed three times with 0.15 M-NaCl (3 ml each), and bound radioactivity was measured on a Packard γ -scintillation spectrometer. Inhibition of the binding of ^{125}I -labelled peptide A to phosphorylcholine-Sepharose was performed by preincubating phosphorylcholine-Sepharose with various amounts of unlabelled peptide A or B for 18 h at $23^\circ C$, followed by the addition of ^{125}I -labelled peptide A. Subsequent incubation times, washings and measurement of bound radioactivity were as described above. Inhibition of the binding of ^{125}I -labelled myeloma protein M-603 by the unlabelled protein, peptide A and peptide B was performed in a similar manner except that $0.01 \mu l$ (packed volume) of phosphorylcholine-Sepharose was used and the reaction was performed in the presence of a protein 'carrier' consisting of non-immune goat immunoglobulin G (3 mg/ml).

Results

The binding of ^{125}I -labelled peptide A and ^{125}I -labelled peptide B to phosphorylcholine-Sepharose is shown in Fig. 2. These results indicate that the ^{125}I -labelled surface-simulation peptide A binds significantly to phosphorylcholine-Sepharose. In contrast, under identical conditions the amount of ^{125}I -labelled control peptide B bound by phosphorylcholine-Sepharose was comparable with the 'background' values obtained with unsubstituted Sepharose. Neither ^{125}I -labelled peptide A nor ^{125}I -labelled peptide B showed binding to unsubstituted Sepharose. It can therefore be concluded that the surface-simulation peptide A interacts in a specific manner with the phosphorylcholine-Sepharose.

Amino acid residues in phosphorylcholine-combining site	61H	60H			52H	33H			35H	36H	37H
	Ser	Tyr			Arg	Tyr			Glu	(Trp)	Val
Distances in myeloma protein M-603 ($C_{(\alpha)}-C_{(\alpha)}$, in nm)	0.396		1.304		0.460		0.683		0.386		0.373
Surface-simulation peptide A	Ser	Tyr	Gly	Gly	Arg	Tyr	Gly	Glu	Trp	Val	
Distances in peptide A ($C_{(\alpha)}-C_{(\alpha)}$, in nm)	0.384		1.152		0.384		0.768		0.384	0.384	
Control peptide B	Gly	Tyr	Glu	Arg	Trp	Gly	Ser	Val	Tyr	Gly	

Fig. 1. Diagram showing the amino acid residues in the phosphorylcholine-binding site of myeloma protein M-603 and the structure of the synthetic surface-simulation peptide A designed to mimic it

The synthetic control peptide B having the same composition but a scrambled sequence relative to peptide A is also shown. Amino acid residues in myeloma protein M-603 that have been implicated in phosphorylcholine binding (Segal *et al.*, 1974; Padlan *et al.*, 1976) are shown in **bold** type. Parentheses around Trp-36H indicate that it replaces Trp-104aH for reasons outlined in the text. The $C_{(\alpha)}-C_{(\alpha)}$ distances in myeloma protein M-603 were calculated from the α -C atomic co-ordinates (Feldman, 1976). The distances in peptide A were calculated assuming a $C_{(\alpha)}-C_{(\alpha)}$ distance of each peptide bond to be 0.384 nm (3.84 Å).

To demonstrate further that the binding of ^{125}I -labelled peptide A to phosphorylcholine–Seph-
 arose was indeed specific to the particular structure of this peptide and not due to non-specific interactions, inhibition studies of this binding by unlabelled peptide A or peptide B were performed. The results of these inhibition studies are shown in Fig. 3. The maximum inhibitory activity exhibited by the unlabelled control peptide B was no more than 10% in the concentration range examined. Unlabelled peptide A, however, was a far more potent inhibitor of the binding of ^{125}I -labelled peptide A to phosphorylcholine–Seph-
 arose, the inhibition being greater than 75%.

The most convincing evidence of the ability of peptide A to bind to phosphorylcholine is its ability to completely inhibit the binding of ^{125}I -labelled myeloma protein M-603 to phosphorylcholine–Seph-
 arose (Figure 4). These inhibitions were performed under conditions whereby 5% (2000 c.p.m.) of the ^{125}I -labelled myeloma protein M-603 added was bound in the absence of inhibitor. Peptide A completely inhibits this binding. Peptide B showed no inhibition in this range whatsoever. The amount of unlabelled myeloma protein M-603 required for 50% inhibition was 0.8 ng. Although these results indicate that myeloma protein M-603 is decidedly more efficient than peptide A in binding to phosphorylcholine, the ability of this surface-simulation

Fig. 3. Quantitative inhibition by unlabelled synthetic peptide A (▲) and peptide B (●) towards the binding of ^{125}I -labelled peptide A to phosphorylcholine–Seph-
 arose. ^{125}I -labelled peptide A was incubated with phosphorylcholine–Seph-
 arose (10 μl packed volume, 50 μl final reaction volume) in the presence of various amounts (shown on the abscissa) of unlabelled peptides. The amount of ^{125}I -labelled peptide A bound in the presence of inhibitor is expressed as a percentage of the amount bound (4950 c.p.m.) in the absence of inhibitor. See the text for further details.

Fig. 2. Quantitative binding studies of ^{125}I -labelled peptide A (▲ and △) and ^{125}I -labelled peptide B (● and ○) to phosphorylcholine–Seph-
 arose (▲ and ●) and unsubstituted Sepharose (△ and ○).

Constant amounts (10 μl packed volume, 50 μl final reaction volume) of phosphorylcholine–Seph-
 arose or unsubstituted Sepharose were reacted with various amounts (shown on the abscissa) of ^{125}I -labelled peptides. See the text for details of reaction conditions and specific radioactivities of the peptides.

Fig. 4. Quantitative inhibition by unlabelled synthetic peptide A (▲) and peptide B (●) towards the binding of ^{125}I -labelled myeloma protein M-603 to phosphorylcholine–Seph-
 arose.

^{125}I -labelled myeloma protein M-603 was incubated with phosphorylcholine–Seph-
 arose (0.01 μl packed volume, 100 μl final reaction volume) in the presence of various amounts of unlabelled peptides. The amount of ^{125}I -labelled myeloma protein M-603 bound in the presence of inhibitor is expressed as a percentage of amount bound (2000 c.p.m.) in the absence of inhibitor. See the text for additional details.

peptide A to inhibit completely the binding of ^{125}I -labelled myeloma protein M-603, albeit at large excesses, strongly indicates its specificity for phosphorylcholine.

Discussion

The concept of surface-simulation synthesis was conceived to approach experimentally the fact that functional surfaces on protein molecules may be comprised of amino acid residues that are distant in their sequences yet, through molecular folding, come into close proximity in the native molecule (Atassi *et al.*, 1976; Lee & Atassi, 1976). Therefore a synthetic peptide that directly links these amino acid residues while taking into account their spatial arrangement and orientation in the native molecule may be expected to exhibit much of the same functional activity as the protein from which it was designed. In previous reports we have specified some of the basic conformational and directional requirements of surface-simulation synthesis (for summary see Atassi, 1978). The two major prerequisites for successful simulation of functional sites in proteins are (1) a knowledge of the native geometry of the site being considered and (2) identification of critical amino acid residues that directly contribute to the expression of the activity of the native protein. In the case of the accurate assignment of antigenic sites of lysozyme, both of these prerequisites had been met. The X-ray crystal structure of lysozyme was known to a high degree of resolution, and amino acid residues necessary for the expression of its immunochemical activity had been identified through exhaustive chemical-modification studies (see Atassi, 1978).

In the present study of myeloma protein M-603, the three-dimensional structure of the phosphorylcholine-binding site is known to a 0.31 nm (3.1 Å) resolution (Segal *et al.*, 1974). Also, some of the amino acid residues that directly contribute to the binding of phosphorylcholine have been inferred from the X-ray structure of crystals of Fab fragment of myeloma protein M-603 in the presence of phosphorylcholine (Segal *et al.*, 1974), and from sequence analysis of a number of other homogeneous immunoglobulins known to bind phosphorylcholine (see Padlan *et al.*, 1976). In the proposed model (Segal *et al.*, 1974; Padlan *et al.*, 1976) the major interactions between phosphorylcholine and the myeloma-protein-M-603 combining site are as follows. Ionic interactions occur between the quaternary nitrogen of phosphorylcholine and the side chains of Glu-35H and possibly Glu-59H, and the phosphate group interacts with Arg-52H. Hydrogen bonds are proposed between the haptenic oxygen atoms and the side chains of Tyr-33H and Arg-52H. Extensive van der Waals contacts are also

thought to exist between phosphorylcholine and the side chains of Trp-104aH and Tyr-33H. The interaction of phosphorylcholine with a number of other amino acids in the myeloma-protein-M-603 combining site have also been suggested (Padlan *et al.*, 1976). However, these interactions are not as well defined as the ones mentioned above. In fact, our attempts to simulate this phosphorylcholine-binding site suffered from the drawback that the precise orientations and interactions of the side chains of the proposed contact residues are not known, and we had to rely mainly on the atomic co-ordinates (Feldman, 1976) of the α -C atoms of these residues.

The amino acid residues of myeloma protein M-603 that are thought primarily to contribute to the interaction with phosphorylcholine are shown in Fig. 1. The inter-residue distances were calculated, as mentioned above, from the α -C atomic co-ordinates (Feldman, 1976). The structure of the synthetic peptide that we considered would most effectively reconstruct the phosphorylcholine-binding site, namely surface-simulation peptide A, is shown in Fig. 1. The sequence and inter-residue distances of the surface-simulation peptide A shown in Fig. 1 are aligned with the corresponding residues and distances in myeloma protein M-603. In order to approximate as closely as possible in the synthetic peptide the inter-residue distances found in myeloma protein M-603, glycine and diglycine 'spacers' were introduced where appropriate. The introduction of appropriate spacers to approximate native inter-residue distances is an essential feature of surface-simulation synthesis. When glycine spacers are used, they allow for considerable flexibility of the synthetic peptide and facilitate the rotational adjustments necessary for assuming a conformation favourable for binding.

It should be noted that the distances shown for tryptophan in myeloma protein M-603 were calculated from the atomic co-ordinates of the α -C atom of Trp-36H, not Trp-104aH. The location of the α -C atom of Trp-104aH, as determined from its atomic co-ordinates (Feldman, 1976), would appear to place the Trp-104aH side chain too far away from the rest of the phosphorylcholine-binding site for effective van der Waals contacts to be made with phosphorylcholine. Instead, Trp-36H, as proposed in a diagram by Rockey & Freed (1976), may be a more likely candidate than Trp-104aH for participation in van der Waals interactions. It should be pointed out, however, that, whether it is Trp-104aH or Trp-36H that is in contact with phosphorylcholine in myeloma protein M-603, the basic design of our surface-simulation peptide would not be significantly altered. Also, since Trp-36H has been invariably found in the aligned sequences of all of the phosphorylcholine-binding proteins examined (Pad-

lan *et al.*, 1976), we decided that this residue should be included in our synthetic simulation.

The residues Ser-61H and Tyr-60H, although not implicated by X-ray analysis to be involved in phosphorylcholine binding, were included in our surface-simulation peptide since they are in close proximity to the portion of the binding site that binds the phosphate of phosphorylcholine. The inclusion of these residues in surface-simulation peptide A affords the synthetic peptide additional opportunity for hydrogen-bonding and van der Waals contacts with phosphorylcholine. The introduction of valine in the synthetic peptide was chosen for two reasons. First, by introducing valine, the negatively charged C-terminal of the peptide was further separated from the hydrophobic indole side chain of tryptophan, which was already adjacent to the negatively charged glutamic acid. Too polar an environment around the hydrophobic tryptophan side chain might have prevented effective hydrophobic interactions by this residue. Secondly, Val-37H, which is linked to Trp-36H in myeloma protein M-603, undoubtedly contributes to a more hydrophobic micro-environment around Trp-36H, and in the synthetic peptide may even contribute to hydrophobic interactions with the methyl groups of phosphorylcholine.

Having decided on the structure of a peptide that we considered would mimic the phosphorylcholine-binding site of myeloma protein M-603, we designed a control peptide, shown in Fig. 1, that could be used as a reference to demonstrate the specificity of the binding of our surface-simulation peptide A to phosphorylcholine. A valid control that would exclude the possibility of non-specific interactions between peptide A and phosphorylcholine and demonstrate the validity of our approach was one that had the same amino acid composition but a sequence that violated all of the considerations outlined above. Peptide B, in which the inter-residue relationships of peptide A are clearly not preserved, meets this requirement.

The results from each of the three assay systems employed here to examine the ability of surface-simulation peptide A to bind to phosphorylcholine suggest that we have been successful in simulating the combining site of myeloma protein M-603. (1) ^{125}I -labelled peptide A binds significantly to phosphorylcholine-Sepharose, whereas ^{125}I -labelled control peptide B does not. (2) Peptide A inhibits the binding of ^{125}I -labelled peptide A to phosphorylcholine-Sepharose, whereas peptide B shows little or no inhibition when tested in the same concentration range. (3) Peptide A completely inhibits the binding of ^{125}I -labelled myeloma protein M-603 to phosphorylcholine-Sepharose, whereas similar amounts of control peptide B exert no effect on this binding.

The assignment of phosphorylcholine-binding activity to surface-simulation peptide A clearly relies

on a comparison of the activity of peptide A with that of the control peptide B. In our estimation, peptide B is the most suitable control for this study, since its overall amino acid composition is identical with that of peptide A. If the binding of peptide A to phosphorylcholine were due to non-specific interactions, control peptide B, having an identical amino acid composition, would be expected to behave in a similar fashion. However, the activity of peptide B was found to be insignificant in each of the three assay systems, thereby supporting the conclusion that peptide A interacts specifically with phosphorylcholine.

The ability of peptide A to compete with myeloma protein M-603 itself for binding to phosphorylcholine is a striking demonstration of the effectiveness of the surface-simulation approach. Peptide A, however, is less efficient in this binding than is the native myeloma protein M-603. This is most probably due to the fact that there are limited conformational states of this small highly solvated peptide that are suitable for binding to phosphorylcholine. Also, even though a certain fraction of the peptide molecules may assume a favourable binding conformation, the intrinsic association of this conformer with phosphorylcholine would probably be less than that of myeloma protein M-603. This should be fully expected, since it is now well established that synthetic peptides corresponding to intact antigenic sites will bind antibodies with much less affinity than the respective native protein antigens (Atassi & Saplin, 1968; Atassi, 1975, 1978). Nevertheless, in spite of these limitations, peptide A did completely inhibit the binding of ^{125}I -labelled myeloma protein M-603 to phosphorylcholine.

Our success in synthetically mimicking the phosphorylcholine-binding characteristics of myeloma protein M-603 has been very encouraging. It signifies that we may be able to approach, in an entirely unique manner, questions such as the relationship of 'contact' residues in antibody combining sites to idiotypic expression, and immunoregulation by anti-idiotypic antibodies.

We thank Dr. A. F. Rosenthal for his helpful discussions on the coupling of phosphorylcholine to Sepharose and Dr. David Klapper for supplying us with myeloma protein M-603. The technical assistance of Mr. James Davidson is gratefully acknowledged. This work was supported by Grant AI 13181 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service.

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