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Delineation of the Third Antigenic Site of Lysozyme by Application of a Novel 'Surface-Simulation' Synthetic Approach Directly Linking the Conformationally Adjacent Residues Forming the Site*

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We have previously shown that an antigenic site in native lysozyme resides around the disulphide bridge 30–115 and incorporates Lys-33 and Lys-116 and one or both of Tyr-20 and Tyr-23. These residues fall in an imaginary line circumscribing part of the surface of the molecule and passing through the spatially adjacent residues Tyr-20, Arg-21, Tyr-23, Lys-116, Asn-113, Arg-114, Phe-34 and Lys-33. The identity of the site was confirmed by demonstrating that the synthetic peptide Tyr-Arg-Tyr-Gly-Lys-Asn-Arg-Gly-Phe-Lys (which does not exist in lysozyme but simulates a surface region of it), and an analogue in which glycine replaced Tyr-23, possessed remarkable immunochemical reactivity that accounted entirely for the expected reactivity of the site in native lysozyme. Tyr-23 is not part of the site, and its contribution was satisfied by a glycine spacer. The novel approach presents a powerful technique for the delineation of antigenic (and other binding) sites in native proteins and confirms that these need not always comprise residues in direct peptide linkage.

Previous studies from this laboratory on the antigenic structure of native hen egg-white lysozyme (which are presented in detail in the Discussion) have shown that an antigenic site (the term is used here according to the definition given by Atassi & Saplin, 1968) is situated around the disulphide bridge 30–115 (Atassi *et al.*, 1973). The side chains of Lys-33 and Lys-116 are essential parts of the site (Lee *et al.*, 1975). Also, one or both of Tyr-20 and Tyr-23 may fall within, or extremely close to, an antigenic site (Atassi & Habeeb, 1969; Atassi *et al.*, 1971). Since the three-dimensional structure of lysozyme is known (Blake *et al.*, 1967; Imoto *et al.*, 1972), its analysis revealed that the aforementioned four side chains, with other intervening residues, fall in an imaginary line circumscribing part of the surface of the globular protein. The surface-encircling line was described by the following residues: Tyr-20, Arg-21, Tyr-23, Lys-116, Asn-113, Arg-114, Phe-34 and Lys-33. By resorting to an unorthodox and novel approach devised recently in our laboratory (Atassi *et al.*, 1976*c*), a peptide was synthesized in which the foregoing eight residues were directly linked via peptide bonds, with intervening spacers where appropriate. In another peptide analogue, Tyr-23 was replaced by a glycine residue. The two peptides were purified and characterized, and their immunochemistry was studied. Since this approach affords peptides that do not exist in the protein but attempts to copy a surface region of the native molecule, we propose the term 'surface-simulation' synthesis to describe it.

* This paper represents the 17th article in the series 'Enzymic and Immunochemical Properties of Lysozyme'. The preceding paper in the series is Atassi *et al.* (1976*c*).

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Experimental Procedure

Lysozyme of hen egg-white was a three-times-crystallized preparation from Sigma Chemical Co. (St. Louis, MO, U.S.A.). It was homogeneous by starch-gel, polyacrylamide-gel and disc electrophoresis. The Merrifield resin for solid-phase peptide synthesis was a chloromethylated co-polystyrene with 2% divinylbenzene cross-links and had 1.31 mequiv. of Cl/g. The resin and the amino acid derivatives were obtained from Cyclo Chemical Corp. (Los Angeles, CA, U.S.A.). The following side-chain-protecting groups were used: asparagine, *p*-nitrophenyl ester; tyrosine, *O*-benzyl ether; lysine, ε-benzyloxycarbonyl dicyclohexylammonium salt; arginine, *guanidino*-nitro, and the t-butyloxycarbonyl was employed for the protection of all α-NH_2 groups. The purity of each derivative was checked by t.l.c. Preparation of 1 M-HCl in acetic acid and purification of organic solvents were done by the procedures already described (Koketsu & Atassi, 1973, 1974).

Antisera G9 and G10 were early-course antisera against native lysozyme prepared in goats by the method previously described (Atassi, 1967*a*).

Esterification of t-butyloxycarbonyl-ε-benzyloxycarbonyl-lysine (after stripping of the dicyclohexylammonium salt of the lysine derivative by citric acid) on to the resin, coupling of each new residue on to the growing peptide chain and cleavage of the completed peptides from the resin were carried out as described in detail elsewhere (Koketsu & Atassi, 1973, 1974). The peptides were hydrogenated in 80% (v/v) acetic acid at 60°C over 10% Pd on charcoal for 48h. The crude synthetic peptides were subjected to ion-exchange chromatography on columns (2.5cm× 70cm) of DEAE-Sephadex A-50. Columns were eluted at the rate of 40ml/h with a linear concentration gradient at a constant pH of 4.8. For the gradient, the mixing vessel contained 0.1M-acetic acid/pyridine buffer (1 litre) and the reservoir contained 1M-acetic acid/pyridine buffer (1 litre). Samples (0.5ml) of the effluent fractions (5.2ml) were freeze-dried, redissolved in water (1.5ml) and their absorbances determined at 230nm.

Spectral determinations, peptide 'mapping' and amino acid analysis of acid hydrolysates (triplicate hydrolyses each at 22, 48 and 72h) were done as described in detail previously (Atassi & Saplin, 1968). Concentrations of protein and peptide solutions were obtained from their nitrogen contents, which were determined by the procedure described previously (Atassi *et al.*, 1971). The theoretical nitrogen contents of lysozyme and the two synthetic peptides were calculated from their sequences and were: lysozyme, 18.80%; peptide I (see Fig. 1), 20.64%; peptide II, 22.50%. The calculated molecular weights of lysozyme and the synthetic peptides were: lysozyme, 14306; peptide I, 1288.6; peptide II, 1182.5. Procedures for quantitative precipitin and inhibition experiments have been described previously (Atassi & Saplin, 1968).

Results

The peptides obtained by cleavage from the resin were invariably heterogeneous by peptide 'mapping', showing five or six spots, of which one was the major product. On cutting out the ninhydrin-positive spots from the 'map', extracting the colour with water, clearing the extracts by centrifugation and reading the absorbance at 570nm, the major spot in the crude products accounted for 55–65% of the total ninhydrin colour. Such degrees of purity are unsatisfactory for immunochemical studies. Chromatography on DEAE-Sephadex resolved the crude peptide I into four components, and the major peak was eluted at 198–270ml. Crude peptide II gave four components by column chromatography, with the major peak being eluted at 146–203ml. Fractions belonging to each peak were combined and freeze-dried. By elution of the ninhydrin-positive colour from heavily loaded 'maps', the major peak in each case had purity of 99% or better. Amino acid compositions of the pure synthetic peptides were: for peptide I, Asp, 1.03; Gly, 2.07; Tyr, 1.83; Phe, 1.03; Lys, 2.03; Arg, 2.01; for peptide II, Asp, 1.02; Gly, 3.02; Tyr, 0.90; Phe, 1.02; Lys, 1.91; Arg, 2.11. For each peptide, the composition agreed well with that expected from its sequence (Fig. 1).

Each of the present synthetic peptides inhibited the reaction of lysozyme with its antisera (see Fig. 2 and Table 1). With antiserum G9, replacement of Tyr-23 by a glycine residue improved the inhibitory activity of the site substantially. This substitution, on the other hand, had no meaningful effect on the reaction with antiserum G10. Table 1 shows that, with each of the antisera studied, the inhibitory activity of peptide II was almost equal to the maximum expected reactivity of the site as derived from chemical modification studies of intact lysozyme. Neither of these two peptides had any inhibitory activity towards the reaction of sperm-whale myoglobin with its antisera, even at a 3000-molar excess relative to myoglobin. In addition, myoglobin synthetic antigenic regions of equal size to peptides I and II had no inhibitory effect on the lysozyme immune reaction (for example see Fig. 2). These findings confirmed that the behaviour of the synthetic peptides I and II was due to a specific immune reaction.

Discussion

Immunochemical and conformational studies on specific chemical derivatives of lysozyme previously reported from this laboratory have enabled the definition of the roles of several amino acid locations in the antigenic structure of lysozyme. Of direct relevance here was the finding that nitration of Tyr-20 and Tyr-23 in lysozyme caused a decrease of the antigenic reactivity of the derivative by 21 and 23% with antisera G9 and G10 respectively (Atassi & Habeeb, 1969). Conversely, with antisera to the derivative lysozyme reacted 75–82% relative to the homologous reaction (Atassi & Habeeb, 1969). On reduction of the nitrotyrosine residues to aminotyrosine, the antigenic reactivity with antisera to lysozyme was completely recovered (99–100%). From these findings and the conformational investigations it was concluded that one or both of Tyr-20 and Tyr-23 is located in, or very close to, an antigenic site in lysozyme (Atassi & Habeeb, 1969; Atassi *et al.*, 1971). Recently, from three homogeneous succinylated (3-carboxypropionylated) derivatives of lysozyme that suffered no conformational changes, we showed that modification of Lys-33 and Lys-116 effected a decrease of the antigenic reactivity by 32

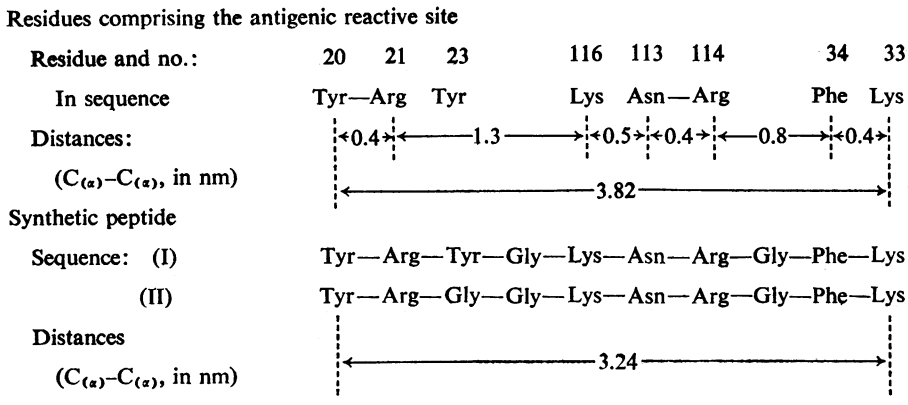

Fig. 1. *Diagram showing the conformationally adjacent residues constituting the antigenic site and their location in the primary structure of lysozyme*

Residues Tyr-20, Tyr-23, Lys-116 and Lys-33 have been directly demonstrated to be parts of an antigenic site in native lysozyme (see the text). These four residues describe very well a line that circumscribes a part of the protein surface. The surface-encircling line will pass by necessity through the intervening residues shown in the upper part of the diagram. The separation between the residues are given in $C_{(\alpha)}$–$C_{(\alpha)}$ distances. In the lower part of the diagram the sequences of the two peptides synthesized to simulate the surface region of the molecule are shown and the length given assumes an ideal $C_{(\alpha)}$–$C_{(\alpha)}$ distance of 0.36 nm (3.6 Å). In peptide II a glycine spacer replaces Tyr-23. Note that the $C_{(\alpha)}$–$C_{(\alpha)}$ distances are not indicative of the separation between the amino acid side chains, since the latter can move over a wide range and need not be oriented in one line on binding with antibody.

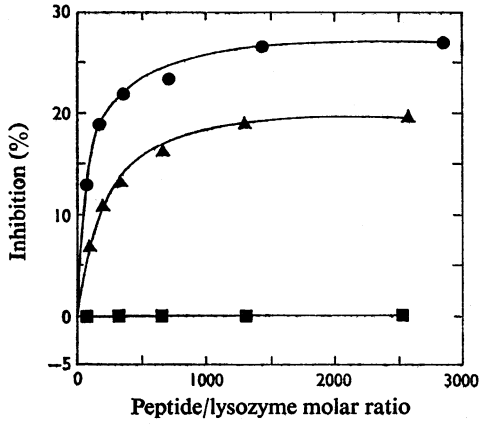

Fig. 2. *Quantitative inhibition by the pure synthetic peptides of the precipitin reaction of native lysozyme with antiserum G9*

▲, Peptide I; ●, peptide II; ■ represents behaviour of a control synthetic peptide corresponding to sequence 15–22 of sperm-whale myoglobin and which comprises an antigenic site in myoglobin.

and 33% with antiseta G9 and G10 respectively and implicated the presence of both residues in an antigenic site (Lee *et al.*, 1975). A disulphide peptide corresponding to sequence 22–33–(Cys-30–Cys-115)–115–116 possessed a substantial inhibitory activity towards the immune reaction of native lysozyme (Atassi *et al.*, 1973). These findings demonstrated that an antigenic site is present around the disulphide bridge 30–115 and that Lys-33 and Lys-116 are essential residues for the site, which could also incorporate one or both of Tyr-20 and Tyr-23. The decreases in antigenic reactivity on modification of Lys-33 alone or of Tyr-20 and Tyr-23 were considerably less than that obtained on modification of both Lys-33 and Lys-116. Such findings are characteristic when the modified residues occur at or near the end of the site (Atassi *et al.*, 1975).

Examination of the three-dimensional structure of lysozyme revealed that the residues Tyr-20, Tyr-23, Lys-116 and Lys-33 can be accommodated (with other intervening residues) in an imaginary line (or plane) circumscribing part of the surface topography of the protein. The surface-encircling line passes through the following residues: Tyr-20, Arg-21, Tyr-23, Lys-116, Asn-113, Arg-114, Phe-34, Lys-33. The distances between the contiguous residues of the site are shown in Fig. 1. Recently we introduced a novel and an unorthodox approach for delineation of antigenic sites in proteins by direct linkage of the relevant conformationally adjacent residues constituting the site into a single peptide (Atassi *et al.*, 1976*c*). For the present site it was estimated that a spacer between Tyr-23 and Lys-116 and another between Arg-114 and Phe-34 were necessary

Table 1. *Inhibitory activity of the pure synthetic peptides*

Values are expressed in maximum percentage inhibition by a peptide of the precipitin reaction of native lysozyme with two different goat antisera. Results represent the averages of three or six replicate determinations, which varied ±0.8% or less.

	Antiserum G9		Antiserum G10	
Peptide	Max. inhibitory activity (%)	Molar ratio at ½ max. inhibition*	Max. inhibitory activity (%)	Molar ratio at ½ max. inhibition*
Peptide I	20.0	160	25.6	670
Peptide II	27.8	80	28.8	650
Max. expected reaction of the site	28.8†		29.9†	

* These values represent peptide/lysozyme molar ratio at 50% of the maximum inhibition.

† These values represent the average decrease in antigenic reactivity of lysozyme obtained on modification of Tyr-20 and Tyr-23 or of Lys-33 and Lys-116. For details see text.

to achieve the correct separations between their side chains (Fig. 1). Further, since in the native protein Tyr-23 is much less exposed than Tyr-20, it was decided to investigate whether the contribution of Tyr-23 can be satisfied by a glycine spacer. Two peptides were therefore synthesized (Fig. 1) which linked the residues of the site directly by peptide bonds.

The immunochemical findings showed that the approach worked remarkably well. In fact, the synthetic site accounted for almost all the contribution of this site to the reaction of the native protein with each antiserum. Obviously it is not entirely possible to duplicate in the synthetic peptides the exact distances of separation between the consecutive residues of the site (Fig. 1). The short peptides will exist mostly in unfolded conformational states in solution. The immune response to native protein antigens is directed against their native three-dimensional structure (Atassi & Thomas, 1969). For appropriate binding with antibody, the conformation of a reactive site must approximate its shape in the native protein (Atassi, 1967*b*; Atassi & Saplin, 1968). The probability of finding this favourable conformational state will improve with increase in peptide concentration (Atassi & Saplin, 1968). Also, antibody will induce somewhat its own required conformation on a free antigenic site (Atassi, 1975). These factors explain the large peptide excess required. Since these peptides do not even exist in lysozyme, their mere reactivity becomes the more remarkable. The findings also demonstrate that Tyr-23 is not necessary for the reactivity of the site. In fact, with one antiserum (G9) it seems to exert a detrimental effect on the reactivity of the site, owing to an unfavourable mode of folding or to steric hindrance (or both), with that antibody. Its replacement by glycine obviated this problem.

This is the last of the three antigenic sites of lysozyme to be described. The other two sites have been delineated either by synthesis of several disulphide peptides (Atassi *et al.*, 1976*a*) or by direct linking of surface residues of the site (Atassi *et al.*, 1976*c*). These are the first such sites to be defined in proteins (Lee & Atassi, 1975; Atassi *et al.*, 1976*b*,*c*), even though it was proposed quite early (Atassi & Saplin, 1968) that an antigenic reactive site can be composed of regions or residues that are close in three-dimensional structure but distant in sequence. The present work clearly shows that the spatially adjacent residues of the site can act as if in direct linkage. Alternatively, the antigenic site may be composed of residues that are in fact in direct peptide linkage, as in myoglobin (Atassi, 1975). Whether one type of antigenic site or the other, or a mixture of both, will exist in a given protein will obviously depend on the protein, and may be determined to a great extent by the stabilization of the structure by internal disulphide cross-links. Finally, the present approach affords perhaps the most powerful and convincing chemical evidence for the correctness of the three-dimensional structure of lysozyme determined by X-ray-crystallographic studies (Blake *et al.*, 1967; Imoto *et al.*, 1972). It should also be extremely useful in the study of certain other protein binding sites, but can be applied only after considerable chemical narrowing down has been achieved and with the full knowledge of the three-dimensional structure.

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