Boundary Refinement of the Lysozyme Antigenic Site Around the Disulphide Bond 6–127 (Site 1) by ‘Surface-Simulation’ Synthesis*

By M. ZOUHAIR ATASSI† and CHING-LI LEE

Department of Immunology, Mayo Medical School, Rochester, MN 55901, U.S.A.

(Received 8 July 1977)

1. We have previously shown that an antigenic site (site 1) in native lysozyme resides around the disulphide bond 6–127 and, by classical synthesis of nine disulphide peptides, the antigenic site was accurately narrowed down to the structure Cys₍₆₎–Arg₍₁₄₎–[Cys₍₆₎–Cys₍₁₂₇₎–Gly₍₁₂₆₎–Arg₍₁₂₈₎]. Only a few residues on this disulphide peptide were proposed to be involved in the reactivity with antibody. However, this lacked direct verification and the role of Arg-128 remained uncertain. 2. In the present work, several peptides were designed and synthesized by the surface-simulation concept devised in our laboratory. These enabled the precise definition of the site as well as the investigation of its conformational and directional requirements. 3. The results showed that the antigenic site (site 1) is made up of the spatially contiguous surface residues: Arg-125, Arg-5, Glu-7, Arg-14, Lys-13. The surface-simulation synthetic peptide Arg-Gly-Gly-Arg-Gly-Glu-Gly-Gly-Arg-Lys (which does not exist in native lysozyme, but copies a surface region of it) accounted entirely for the maximum expected reactivity of the site (i.e. about one-third of the total antigenic reactivity of lysozyme). An immunoadsorbent of the peptide also removed about one-third of the total lysozyme antibodies. 4. The antigenic site exhibited restricted conformational freedom. The achievement of the full reactivity of the site by surface-simulation synthesis requires the appropriate choice of spacer separation between its reactive residues. The surface-simulation synthetic site exhibits the same mono-directional preference (Arg-125 to Lys-13) for the rabbit and goat antisera so far tested. The site describes a line which encircles a part (3.01 nm in C_(α)-to-C_(α) distance from Arg-125 to Lys-13) of the surface of the molecule.

In previous communications on the antigenic structure of hen egg-white lysozyme (see the Discussion section for detail), we have reported (Atassi *et al.*, 1973) that an antigenic site [the term is used here to denote conformationally adjacent residues that may be distant in sequence (Atassi & Saplin, 1968)] resides around the disulphide bond 6–127. Our studies on the immunochemistry of specific chemical derivatives of native lysozyme enabled us to narrow down this site to a conveniently small size. Accurate delineation of the site was then achieved (Atassi *et al.*, 1976b) by studying the immunochemistry of nine synthetic disulphide peptides, representing various sequences on the two sides of the disulphide bond 6–127. It was found (Atassi *et al.*, 1976b) that the smallest synthetic unit that retained the full expected reactivity of the site was the disulphide peptide Cys₍₆₎–Arg₍₁₄₎–[Cys₍₆₎–Cys₍₁₂₇₎–Gly₍₁₂₆₎–Arg₍₁₂₈₎]. Only selected residues on this peptide were actively involved in binding with anti-

body. A three-dimensional organization of these residues was proposed (Atassi *et al.*, 1976b) from examination of the three-dimensional structure of lysozyme. We devised the novel and unorthodox surface-simulation synthetic approach (Atassi *et al.*, 1976d) by which conformationally contiguous surface residues constituting an antigenic site were directly linked, via peptide bonds with intervening spacers where appropriate, into a single peptide. Such a peptide does not exist in the native protein, but copies a surface region of it, hence the term ‘surface-simulation’ synthesis (Lee & Atassi, 1976). This afforded a most powerful approach to study protein antigenic sites (and obviously other types of binding sites) and has enabled us to define the precise boundaries, conformational restrictions and directional requirements of the other two antigenic sites 2 (Atassi *et al.*, 1976d; Lee & Atassi, 1977b) and 3 (Lee & Atassi, 1977a) of lysozyme.

To define more precisely and without speculation the boundaries as well as the conformational and directional requirements of antigenic site 1 it has become necessary to apply the surface-simulation synthetic approach for the reinvestigation of this site. In the present work, several surface-simulation

Abbreviation used: IgG, immunoglobulin G.

* This paper represents the 20th in the series ‘Enzymic and Immunochemical Properties of Lysozyme’. The preceding paper in the series is Lee & Atassi (1977b).

† To whom correspondence should be addressed.

synthetic peptides representing various parts of the surface region around the indicated site were synthesized, purified and characterized and their immunochemistry studied in detail.

Experimental

Materials

Hen egg-white lysozyme was a three-times crystallized preparation from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Its homogeneity was confirmed by starch-gel, polyacrylamide-gel and disc electrophoresis. The resin for solid-phase peptide synthesis (a chloromethylated co-polystyrene with 1% divinylbenzene cross-links containing 1.19 mequiv. of Cl/g) was from Vega-Fox Biochemicals (Tucson, AZ, U.S.A.). The amino acid derivatives for peptide synthesis were from Beckman Instruments (Palo Alto, CA, U.S.A.). The side-chain-protecting groups used were: arginine, guanidino-nitro; glutamic acid, γ -benzyl ester; lysine, N^{ϵ} -benzyloxycarbonyl dicyclohexylammonium salt; and the t-butoxycarbonyl group was used for the protection of all α -amino groups. The purity of each derivative was checked by t.l.c. Reagents and solvents for peptide synthesis were prepared and purified as previously described (Koketsu & Atassi, 1973, 1974a). Carrier-free ^{125}I , Sepharose 4B, CNBr and *p*-benzoquinone were obtained from the sources previously given (Lee & Atassi, 1977a).

Synthesis and purification of the peptides

The procedures for solid-phase synthesis have been described (Koketsu & Atassi, 1973, 1974a). The crude synthetic peptides were purified by chromatography on columns (2 cm $\times$ 70 cm) of DEAE-Sephadex A-50 (Pharmacia, Piscataway, NJ, U.S.A.) by using a linear concentration gradient at a constant pH of 4.8 and 22°C. For the gradient, the mixing chamber contained 0.05 M-acetic acid/pyridine buffer (1 litre) and the reservoir contained 1.0 M-acetic acid/pyridine buffer (1 litre). The elution was at the rate of 30–40 ml/h. Effluent fractions (5.2 ml) were analysed as previously described (Pai & Atassi, 1975).

Immunochemical and analytical methods

Early-course antisera G9 and G10 were raised in goats, and antisera L7 and L21 were raised in rabbits, each against native lysozyme by the procedure previously described (Atassi, 1967a). Quantitative precipitin and inhibition experiments were done as described elsewhere (Atassi & Saplin, 1968). The peptide molar excess relative to lysozyme at half the maximum inhibition was determined from double-reciprocal plots (Lee & Atassi, 1977a).

The lysozyme-Sepharose immunoabsorbents, as well as control immunoabsorbents carrying glycine, histidine or sperm-whale myoglobin, prepared by CNBr activation (March *et al.*, 1974) or by *p*-benzoquinone activation (Brandt *et al.*, 1975) of Sepharose 4B, were those previously described (Lee & Atassi, 1977a,b). The peptide immunoabsorbent used here, prepared by the two activation procedures, had 3.2–2.7 mg/ml packed volume.

The procedures for the preparation of the IgG fraction of the antisera, the preparation of specific lysozyme antibodies on lysozyme-Sepharose, the radioiodination of the specific antibodies and the binding of ^{125}I -labelled antibody by immunoabsorbents were as already given in detail (Lee & Atassi, 1977a,b).

The concentrations of protein and peptide solutions were based on their nitrogen contents. The procedure for nitrogen determination as well as other analytical procedures used here have been described (Atassi *et al.*, 1976a; Lee & Atassi, 1977a).

Results

Purification and characterization of the synthetic peptides

The peptides synthesized here are given in Fig. 1. The rationale for their synthesis is given in the Discussion section. The peptides obtained after cleavage and deprotection were heterogeneous by peptide 'mapping', showing a major spot and four to six minor spots. By cutting out the ninhydrin-positive spots from the 'map', elution of the colour with water and reading the A_{570} of the eluate, we showed that the major spots accounted for 50–65% of the total ninhydrin colour. Such impure preparations are unsuitable for immunochemical studies. No meaningful purification could be obtained by gel filtration on columns (2 cm $\times$ 70 cm) of Sephadex G-10. Chromatography on DEAE-Sephadex resolved each synthetic product into several components. The elution patterns for peptides I–IV is shown in Fig. 2. The major components indicated in Fig. 2 were subjected to two or three rechromatography experiments on similar columns. After each chromatography, the effluent fractions (every other tube) were monitored by high-voltage electrophoresis (Atassi & Saplin, 1968) to ensure the optimum pooling of fractions containing the major component. The major component thus obtained was homogeneous by peptide 'mapping' and was 99% pure or better as determined by the colour intensity of the eluted ninhydrin-positive spots from heavily loaded 'maps'. The amino acid composition of the material in each major peak (see Table 1) was in excellent agreement with that expected from its sequence. Table 1 also gives the theoretical nitrogen contents and

Fig. 1. Spatially contiguous surface residues constituting the antigenic site and their numerical position in the primary structure of lysozyme (a)

The values give the distances (in nm) separating the consecutive residues of the site and are given as $C_{(\alpha)}$ -to- $C_{(\alpha)}$ distances together with the overall extended dimension of the site. (b) The primary structures of the surface-simulation synthetic peptides that were designed to copy the site and investigate its directional and conformational requirements are also given. For the rationale behind the design of the peptides, see the text. The $C_{(\alpha)}$ -to- $C_{(\alpha)}$ distance for the surface-simulation synthetic site assumes an ideal $C_{(\alpha)}$ -to- $C_{(\alpha)}$ distance of 0.362 nm. The residues shown in (IV) were demonstrated in the present paper to comprise the antigenic site and this surface-simulation peptide carried the full expected immunochemical reactivity of the site.

molecular weights of the peptides which were calculated from the amino acid sequences. The calculated molecular weight and nitrogen content of lysozyme are 14306 and 18.80% respectively (Atassi *et al.*, 1971).

Reactions of the IgG fractions, specific antibodies and 125 I-labelled antibodies

The IgG fractions prepared from lysozyme antisera accounted in quantitative precipitin analysis for 99–100% of the total immune reactions of the respective antisera. The specific antibody preparation, obtained from the IgG fraction (of antiserum G10) on a lysozyme immunoabsorbent, had 98% of the total reactivity of the original antiserum. The antibody content constituted 11.1% of the total IgG. After radioiodination, the reactivity of the antibody with native lysozyme was entirely unaffected.

Inhibitory activity of the pure synthetic peptides

Each of the synthetic peptides showed an inhibitory activity towards the quantitative precipitin reaction of lysozyme with its antisera. Fig. 3 shows an example of the inhibition curves with one antiserum (G9) and the results with four different antisera are summarized

in Table 2. Peptides I and II differed only in the fact that they had exactly the reverse sequences (Fig. 1). Of these, peptide II was immunochemically more efficient with all the antisera studied. The conformational restraints of the site were investigated by altering the distance separating Arg-5 from the *N*-terminal arginine. Peptide IV showed the highest immunochemical reactivity with all the antisera. When the distance separating the first two arginine residues from the *N*-terminal was increased by one glycine spacer (peptide III), the immunochemical reactivity decreased only slightly with antiserum G9 (from 32.5 to 26.8%). However, a much larger decrease in reactivity was observed with the other three antisera.

The possibility of improving the immunochemical efficiency of the peptides by studying the reaction of peptide IV with the IgG fractions of the antisera, instead of the whole antisera, was examined. The results are summarized in Table 3: the molar excess of peptide relative to lysozyme required to achieve half the maximum inhibition was greatly decreased. Not only was there an improvement in the binding efficiency of the peptide, but in fact there was an appreciable increase in the maximum inhibitory activity exhibited by the peptide, especially with antisera L7 and L21. Finally, the maximum inhibitory

Fig. 2. Chromatographic patterns of the synthetic peptides I, II, III and IV

Chromatography was on DEAE-Sephadex A-50 (2cm $\times$ 70cm) with a concentration gradient at a constant pH of 4.80 (see the text for details). Fraction volume was 5.2ml. Samples (0.5ml) of the effluent fractions were freeze-dried, redissolved in water (1.5 ml) and their A_{230} determined. Tubes indicated by a bar corresponded to the main peak.

Table 1. Amino acid compositions, molecular weights and nitrogen contents of the pure synthetic peptides

Values are given in mol of amino acid/mol of peptide and represent the average of six acid hydrolyses on each peptide (duplicates at 22, 48 and 72h). Molecular weights and nitrogen contents of the peptides were calculated from their structures (see Fig. 1).

Amino acid	Peptide I		Peptide II		Peptide III		Peptide IV	
	Calc.	Found	Calc.	Found	Calc.	Found	Calc.	Found
Glu	1	0.97	1	0.94	1	1.03	1	0.99
Gly	6	5.87	6	5.99	7	6.82	5	4.99
Lys	1	1.07	1	1.07	1	1.02	1	1.01
Arg	3	2.93	3	2.96	3	3.02	3	2.97
Mol.wt.	1068.3		1068.3		1125.4		1011.3	
Nitrogen content (%)	27.1		27.1		27.4		27.7	

activity of peptide IV was in excellent agreement with the maximum expected reaction of the site in native lysozyme (i.e. about one-third of the total immune reaction of the protein).

Binding of ¹²⁵I-labelled antibody by peptide immuno-adsorbent

The fraction of lysozyme antibodies that can bind specifically with peptide IV was determined by an immuno-adsorbent carrying peptide IV. Table 4 shows that the amount of ¹²⁵I-labelled specific antibodies (from antiserum G10) bound to CNBr-prepared immuno-adsorbents of peptide IV was 26.9% of the total antibodies. Identical results were obtained on immuno-adsorbents of peptide IV prepared by activation with benzoquinone, which bound 27.3 ± 1.1% of the total specific antibody. All (97.0%) ¹²⁵I-labelled

antibody to lysozyme could be adsorbed out on lysozyme-Sephadex. The fraction of the antibody that was bound by an immuno-adsorbent carrying peptide IV was only slightly lower than the value of the inhibitory activity (32.3%) by the free peptide on the immune reaction of lysozyme with the IgG fraction of that antiserum. The amount of specific binding by the peptide-immuno-adsorbent was corrected for non-specific adsorption of ¹²⁵I-labelled antibody on controls of glycine-Sephadex or myoglobin-Sephadex. This accounted for 1.5–2.8% of the total label added. Another set of control experiments gave a comparable extent of non-specific binding of ¹²⁵I-labelled non-immune goat IgG on lysozyme-Sephadex or peptide-Sephadex. The final control value shown in Table 4 is an average of duplicates of each of these experiments.

Discussion

Immunochemical behaviour of the peptides

The somewhat high molar excess of peptides, relative to lysozyme, required to achieve maximum inhibitory activity is not unusual and was entirely expected. This has already been discussed in detail in relation to the behaviour of the other two surface-simulation synthetic antigenic sites of lysozyme (Atassi *et al.*, 1976*d*; Lee & Atassi, 1976, 1977*a,b*) and of the synthetic intact antigenic sites of sperm-whale myoglobin (Koketsu & Atassi, 1973, 1974*a,b*; Pai & Atassi, 1975; Atassi & Pai, 1975). The large molar excess of peptide required can in part be attributed to conformational factors in view of the fact that, in solution, these peptides will be expected to exist in greatly unfolded conformational states. The immune response to native protein antigens is directed against their native three-dimensional structure (Atassi, 1967*b*; Atassi & Thomas, 1969). For proper interaction with antibody, the native mode of folding of an antigenic site is needed (Atassi, 1967*b*; Atassi & Saplin, 1968; Atassi, 1970; Habeeb & Atassi, 1971), and the probability of finding such a conformational

Fig. 3. Example of the quantitative inhibition by the pure synthetic peptides of the precipitin reaction of native lysozyme with one antiserum (G9)

●, Peptide I; □, peptide II; ▲, peptide III; ■, peptide IV; △, behaviour of a control synthetic peptide corresponding to residues 15–22 of sperm-whale myoglobin and which constitutes an antigenic site in myoglobin (Koketsu & Atassi, 1974*a*; Atassi, 1975). For the reactivity of the peptides with other antisera see Tables 2 and 3.

Table 2. Inhibitory activity of the pure synthetic peptides

Results are given as maximum percentage inhibition by the peptide of the quantitative precipitin reaction of native lysozyme with whole antisera. Each value is the average of at least three or six replicate determinations, which varied by ±0.7% or less. Values in parentheses indicate the peptide/lysozyme molar ratio at 50% of the maximum inhibition. G9 and G10 are goat antisera, L7 and L21 are rabbit antisera, each against native lysozyme.

Peptide	Antiserum ...	Maximum inhibitory activity with whole antisera (%)			
		G9	G10	L7	L21
I		25.1 (160)	12.8 (300)	6.67 (400)	8.50 (380)
II		30.0 (100)	23.3 (260)	9.00 (320)	10.7 (310)
III		26.8 (100)	18.6 (270)	7.7 (320)	9.12 (800)
IV		32.5 (90)	33.3 (150)	22.2 (260)	21.3 (280)

Table 3. *Inhibitory activities of peptide IV with whole antisera and with their IgG fractions: comparison with the expected reaction of the site*

Results are expressed as maximum percentage inhibition by peptide IV of the precipitin reaction of native lysozyme with a given antiserum or its respective IgG fraction. Each value is the average of six replicate determinations, which varied by $\pm 0.8\%$ or less. Values in parentheses indicate the peptide: lysozyme molar ratio at 50% of the maximum inhibition. G9 and G10 are goat antisera, L7 and L21 are rabbit antisera, each against native lysozyme. Abbreviation: n.d., not determined.

Antiserum	Maximum inhibitory activity (%)		
	Whole antiserum	IgG fraction of antiserum	Expected reaction of the site*
G9	32.5 (90)	n.d.	27.2
G10	33.3 (150)	32.3 (55)	32.1
L7	22.2 (260)	33.4 (80)	n.d.
L21	21.3 (280)	31.4 (85)	n.d.

* Obtained from Atassi *et al.* (1976b).

Table 4. *Adsorption of specific goat ^{125}I -labelled antibody on lysozyme by immunoadsorbent carrying synthetic peptide IV*

The specific ^{125}I -labelled antibody fraction from goat antiserum G10 was isolated on a lysozyme immunoadsorbent before use in these studies (see the text for details). The immunoadsorbents used in the experiments below were prepared by CNBr activation. Identical results were obtained with immunoadsorbents prepared by *p*-benzoquinone activation. The value for percentage of antibody bound is corrected for the amount of label bound in the control experiment. The 'control' value is the average of control experiments (each set up in duplicates) using glycine-Sepharose, histidine-Sepharose and myoglobin-Sepharose. Also, another set of controls was used with ^{125}I -labelled non-immune goat IgG. The amount of non-specific background binding in the various controls ranged between 1 and 3% of the total label applied (for details, see the text). The amount of antibody applied on each immunoadsorbent was 75 600 c.p.m.

Expt. no.	Amount of antibody bound (c.p.m.)	Antibody bound (%)
1	23 100	27.7
2	21 900	26.1
Control	2180	
Average		26.9

state for a free intact antigenic site in solution will improve with increase in peptide concentration (Atassi & Saplin, 1968).

The improvement in the immunochemical efficiency of the peptides when the IgG fractions of the antisera

Fig. 4. *Covalent structure of the antigenic site that we have previously delineated by the classical synthesis of nine disulphide peptides around the disulphide bond 6-127*
The residues underlined by a solid line were proposed to be directly involved in the binding with antibody, whereas the residue underlined by a dotted line may come in contact with antibody. The classical synthetic approach left some uncertainty about the active involvement of Arg-128 in the site. See the text for details (taken from Atassi *et al.*, 1976b).

were used has also been reported for antigenic sites 2 and 3 of lysozyme synthesized by the 'surface-simulation' strategy (Lee & Atassi, 1977a,b). It may have been indicative of proteolysis and/or binding of the synthetic sites by serum proteins. Similar observations have previously been made with fragments of lysozyme (Atassi *et al.*, 1973), of bovine serum albumin (Habeeb *et al.*, 1974; Atassi *et al.*, 1976c; Habeeb & Atassi, 1976) and with the synthetic antigenic sites of myoglobin (Atassi, 1977).

Previous assignment and synthesis of the site

From our previous conformational and immunochemical studies on derivatives of the intact protein, it was shown that Asp-119 and Leu-129 were not parts of an antigenic site in native lysozyme (Atassi *et al.*, 1974; Atassi & Roseblatt, 1974; Atassi *et al.*, 1975). Also, modification of Trp-123 (Atassi & Zablocki, 1976) by reaction with 2,3-dioxo-indoline-5-sulphonic acid (Atassi & Zablocki, 1975) or Met-12 (as well as Met-105) (Atassi *et al.*, 1976b) by reaction with β -propiolactone demonstrated that these residues were not located in an antigenic site. The peptide Cys₍₆₎-Lys₍₁₃₎-(Cys₍₆₎-Cys₍₁₂₇₎)-Gly₍₁₂₆₎-Arg₍₁₂₈₎ had previously been found (Atassi *et al.*, 1973) to carry substantial antigenic reactivity, which, with two other disulphide-containing peptides, jointly accounted for almost all (90%) of the antigenic reactivity of native lysozyme. These results indicated that an antigenic site resides around the disulphide bond 6-127. On one side of the disulphide bond, the antigenic site clearly begins after Trp-123 and ends at or before Arg-128. On the other side of the disulphide bond, the second part of the antigenic site should end at or close to Met-12.

With the accomplishment of this degree of delineation by chemical approaches, it became feasible to achieve the final narrowing down by a synthetic approach. The preceding results were critical in that they pointed to the appropriate regions to be synthesized for immunochemical investigations. Accord-

ingly, we synthesized and studied the immunochemistry of nine disulphide peptides corresponding to various overlaps of the sequences 3–14 and 125–129 on the two sides of the disulphide bond 6–127 (Atassi *et al.*, 1976b). These studies enabled us to describe the covalent structure of the antigenic site (Fig. 4), and from examination of the three-dimensional structure we proposed (Atassi *et al.*, 1976b) that the residues Arg-14, Lys-13, Glu-7, Ala-10, Gly-126, and Arg-128 have the spatial possibility to form the antigenic site. Also, the sulphur atom of Cys-6 may come in contact with the antibody-combining site. Furthermore, Met-12 (as well as Met-105), which in the three-dimensional structure (Blake *et al.*, 1967; Imoto *et al.*, 1972) is completely buried within the interior of the molecule, does not participate in interaction of lysozyme with its antibodies (Atassi *et al.*, 1976b) and could be replaced in a synthetic peptide by a glycine residue without an immunochemical effect.

The above delineation of antigenic site 1 by chemical modification, and finally by a classical synthetic concept, presented the maximum that could be achieved by the conventional approaches of protein chemistry. Although it afforded an excellent and, until then, unequalled degree of delineation of an antigenic site composed of conformationally contiguous residues, it came a little short of an unequivocal proof. Thus the residues presumed to be involved in direct binding of the synthetic disulphide-containing site (Fig. 4) with antibody were deduced from examination of the three-dimensional structure of native lysozyme. No matter how compelling this evidence may have been, the fact remained that the identity of the exact residues constituting the antigenic site was at best hypothetical. A more precise definition of the antigenic site was therefore desirable.

Rationale for the synthesis of the peptides

We have developed (Atassi *et al.*, 1976d) a novel and unorthodox 'surface-simulation' synthetic approach for the precise definition of antigenic (and other binding) sites in proteins. In this concept the spatially adjacent residues of the site are directly linked, via peptide bonds with intervening spacers where appropriate, into a single peptide which does not exist in the native protein, but mimics a surface region of it. This unusual concept was termed 'surface-simulation' synthesis (Lee & Atassi, 1976). This has already proved to be a powerful strategy that enabled us to define the precise structures of antigenic site 2 (Atassi *et al.*, 1976d; Lee & Atassi, 1977b) and antigenic site 3 (Lee & Atassi, 1976, 1977a) in lysozyme, both of which were composed of conformationally contiguous surface residues. Our introduction and successful application of the 'surface-simulation' synthetic concept has now made

Table 5. Distances separating Arg-5 from Arg-125 or Arg-128 and design of spacers

The distances (in nm) are from $C_{(\alpha)}$ to $C_{(\alpha)}$. The number of required glycine spacers in surface-simulation synthesis is based on an ideal $C_{(\alpha)}$ -to- $C_{(\alpha)}$ peptide-bond distance of 0.362 nm. For details see text.

Separation	$C_{(\alpha)}$ -to- $C_{(\alpha)}$ distance (nm)	Required glycine spacers	No. of spacers used in synthesis
Arg-5 to Arg-125	0.93	2.57	2 and 3
Arg-5 to Gly-126 to Arg-128	1.63	4.50	4

the re-examination of antigenic site 1 imperative so that the precise picture at the residue level will be formulated for the entire antigenic structure of lysozyme. It has been shown (Atassi *et al.*, 1976a) that lysozyme has only three major antigenic sites. Further unequivocal evidence for this is presented in the following paper (Atassi & Lee, 1978).

The residues previously proposed to constitute the antigenic site describe an imaginary line that circumscribes part of the surface of the molecule. A careful re-examination of a constructed lysozyme model showed that Ala-10 is not very likely to be a part of an antigenic site described by this imaginary line. Therefore we decided to investigate whether a glycine spacer between Arg-14 and Glu-7 will fulfil the requirement. Similarly, we investigated whether the possible involvement of Cys-6 can be satisfied by a glycine spacer. Finally, synthesis of the site (Fig. 4) by a classical strategy could not adequately differentiate whether the antigenic site required Arg-125 or Arg-128 (Atassi *et al.*, 1976b), possibly because the folding of the structure shown in Fig. 4 may conceivably fulfil either requirement. Therefore the distances between Glu-7 on the one hand and Arg-128 or Arg-125 on the other were measured and are shown in Table 5, which also gives the number of glycine spacers to be incorporated into the surface-simulation synthetic peptides if Arg-125, or alternatively Arg-128, is part of the antigenic site. If Arg-125 is part of the site, then about two glycine spacers are required. On the other hand, if Gly-126 and Arg-128 are essential parts of the site, as we have proposed (Atassi *et al.*, 1976b), then approximately four glycine spacers are required. Previously, we had shown for antigenic sites 2 and 3 that the correct spacing between the residues is critical in the design of surface-simulation synthetic sites (Lee & Atassi, 1977a,b). In addition to this conformational restriction, the synthetic surface-simulation sites exhibited a preferred 'direction'. To determine the appropriate synthetic direction, peptides I and II of exactly reverse sequences were synthesized with three glycine spacers. Three spacers were initially selected, since

they represented an average distance situation for the involvement of Arg-125 or Arg-128. The determination of the most favourable direction can then be followed by design and synthesis of peptides having distances of separation corresponding to Arg-125 or Arg-128 and in the correct synthetic direction. This strategy affords the rationale for the peptides synthesized and studied here (Fig. 1).

Accurate definition and conformational restrictions of the site

The findings reported here indicate that the surface-simulation synthetic sequence expressed in peptide II was immunochemically more reactive with each of the four antisera than the structure of the reverse sequence represented by peptide I. Having thus found the correct 'direction' of the surface-simulation synthetic site, the distance separating the first two arginine residues from the *N*-terminal end was varied. It is significant that the inclusion of four glycine spacers (peptide III) gave a peptide having a lower immunological reactivity than the peptide that carried two glycine spacers between the *N*-terminal arginine and the residue corresponding to Arg-5 (peptide IV). In fact, rabbit antisera L7 and L21 and goat antiserum G10 were extremely sensitive to the alterations in the spacing between the reactive residues. On the other hand, a smaller but significant difference was exhibited in reaction with antiserum G9. From these findings, it can be clearly concluded that the residue constituting a critical part of the antigenic site is Arg-125 and not Arg-128. Furthermore, it is evident that antigenic site 1, like the other two antigenic sites of lysozyme (Lee & Atassi, 1977a,b) exhibits stringent restrictions on the conformational degrees of freedom.

Obviously, antigenic site 1 has a preferred direction on the surface of the globular protein. We have found similar directional preferences for antigenic site 2 (Lee & Atassi, 1977b) and antigenic site 3 (Lee & Atassi, 1977a) of lysozyme. These results are co-ordinated in the following paper (Atassi & Lee, 1978). The findings are significant in view of the fact that in our previous studies, which showed (Lee *et al.*, 1976; Lee & Atassi, 1976) that antigen-antibody interaction is effected only through the side chains, the question of directionality was not considered. The directional sensitivity is further striking when it is remembered that the backbone does not even exist in the native protein, that on the surface of the protein all directions are presumably equivalent, and that in solution the protein has an unrestricted rotational freedom. However, the orientations of the side chains will be different in the two synthetic directions which may affect the free energy of binding (Lee & Atassi, 1977b). Directionality therefore is a function of side-chain orientation.

Finally, it is sometimes believed that the maximum inhibitory activity of a peptide does not necessarily express its true immunochemical reactivity. Consequently, we investigated the ability of an immuno-adsorbent carrying the most inhibitory synthetic peptide (peptide IV) to bind radioiodinated antibodies to lysozyme. The amount of antibody (from antiserum G10) bound by the peptide-Sepharose column constituted 26.9% of the total antibody population. This value was similar to, although somewhat lower than, the maximum inhibitory activity (32.3%) of the free peptide with the IgG fraction of the antiserum. This difference is not too meaningful, especially in view of the fact that excellent agreements were obtained between the inhibitory activity and the antibody-binding ability for each of the synthetic surface-simulations of antigenic site 3 (Lee & Atassi, 1977a) and site 2 (Lee & Atassi, 1977b) of lysozyme. Thus, as previously concluded (Lee & Atassi, 1977a,b), the inhibition values do in fact provide a faithful measure of the immunochemical reactivity of the peptide. This agreement is not confined to lysozyme and has been reported for the synthetic antigenic sites of myoglobin [Atassi & Koketsu (1975); for a comprehensive review see Atassi (1977)] and for two large inhibitory fragments of bovine serum albumin (Atassi *et al.*, 1976c; Habeeb & Atassi, 1976).

In summary, for the antisera studied here, antigenic site 1 of native lysozyme is constructed (Fig. 1) precisely by the five surface residues: Arg-125, Arg-5, Glu-7, Arg-14, Lys-13. The synthetic surface-simulation site (Fig. 1, peptide IV) shows a mono-directional preference (Arg-125→Lys-13) which appears to be species-independent, since it was the same for the two rabbit and the two goat antisera tested. The site is subject to conformational restrictions and requires the correct residue spacing in its synthetic surface-simulation.

The work was supported by a grant (AI 13181) from the National Institute of Allergy and Infectious Diseases, and in part by a grant (AM 18920) from the Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service.

References

- Atassi, M. Z. (1967a) *Biochem. J.* **102**, 478-487
- Atassi, M. Z. (1967b) *Biochem. J.* **103**, 29-35
- Atassi, M. Z. (1970) *Biochim. Biophys. Acta* **221**, 612-622
- Atassi, M. Z. (1975) *Immunochemistry* **12**, 423-438
- Atassi, M. Z. (1977) in *Immunochemistry of Proteins* (Atassi, M. Z., ed.), vol. 2, pp. 77-176, Plenum, New York
- Atassi, M. Z. & Koketsu, J. (1975) *Immunochemistry* **12**, 741-744
- Atassi, M. Z. & Lee, C.-L. (1978) *Biochem. J.* **171**, 429-434

- Atassi, M. Z. & Pai, R.-C. (1975) *Immunochemistry* **12**, 735–740
- Atassi, M. Z. & Roseblatt, M. C. (1974) *J. Biol. Chem.* **249**, 482–487
- Atassi, M. Z. & Saplin, B. J. (1968) *Biochemistry* **7**, 688–698
- Atassi, M. Z. & Thomas, A. V. (1969) *Biochemistry* **8**, 3385–3394
- Atassi, M. Z. & Zablocki, W. (1975) *Biochim. Biophys. Acta* **386**, 233–238
- Atassi, M. Z. & Zablocki, W. (1976) *J. Biol. Chem.* **251**, 1653–1658
- Atassi, M. Z., Perlstein, M. T. & Habeeb, A. F. S. A. (1971) *J. Biol. Chem.* **246**, 3291–3296
- Atassi, M. Z., Habeeb, A. F. S. A. & Ando, K. (1973) *Biochim. Biophys. Acta* **303**, 203–209
- Atassi, M. Z., Roseblatt, M. C. & Habeeb, A. F. S. A. (1974) *Immunochemistry* **11**, 495–500
- Atassi, M. Z., Suliman, A. M. & Habeeb, A. F. S. A. (1975) *Biochim. Biophys. Acta* **405**, 452–463
- Atassi, M. Z., Lee, C.-L. & Habeeb, A. F. S. A. (1976a) *Immunochemistry* **13**, 7–14
- Atassi, M. Z., Koketsu, J. & Habeeb, A. F. S. A. (1976b) *Biochim. Biophys. Acta* **420**, 358–375
- Atassi, M. Z., Habeeb, A. F. S. A. & Lee, C.-L. (1976c) *Immunochemistry* **13**, 547–555
- Atassi, M. Z., Lee, C.-L. & Pai, R.-C. (1976d) *Biochim. Biophys. Acta* **427**, 745–751
- Blake, C. C. F., Mair, G. A., North, A. C. T., Phillips, D. C. & Sarma, V. R. (1967) *Proc. R. Soc. London Ser. B* **167**, 365–377
- Brandt, J., Andersson, L.-O. & Porath, J. (1975) *Biochim. Biophys. Acta* **386**, 196–202
- Habeeb, A. F. S. A. & Atassi, M. Z. (1971) *Biochim. Biophys. Acta* **236**, 131–141
- Habeeb, A. F. S. A. & Atassi, M. Z. (1976) *J. Biol. Chem.* **251**, 4616–4621
- Habeeb, A. F. S. A., Atassi, M. Z. & Lee, C.-L. (1974) *Biochim. Biophys. Acta* **342**, 389–395
- Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C. & Rupley, J. A. (1972) *Enzymes 3rd Ed.* **7**, 665–868
- Koketsu, J. & Atassi, M. Z. (1973) *Biochim. Biophys. Acta* **328**, 289–302
- Koketsu, J. & Atassi, M. Z. (1974a) *Immunochemistry* **11**, 1–8
- Koketsu, J. & Atassi, M. Z. (1974b) *Biochim. Biophys. Acta* **342**, 21–29
- Lee, C.-L. & Atassi, M. Z. (1976) *Biochem. J.* **159**, 89–93
- Lee, C.-L. & Atassi, M. Z. (1977a) *Biochem. J.* **167**, 571–582
- Lee, C.-L. & Atassi, M. Z. (1977b) *Biochim. Biophys. Acta* **495**, 354–368
- Lee, C.-L., Pai, R.-C. & Atassi, M. Z. (1976) *Immunochemistry* **13**, 681–687
- March, S. E., Parikh, I. & Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 149
- Pai, R.-C. & Atassi, M. Z. (1975) *Immunochemistry* **12**, 285–290