The Precise and Entire Antigenic Structure of Native Lysozyme*

By M. ZOUHAIR ATASSI† and CHING-LI LEE

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

(Received 8 July 1977)

The exact boundary, residue, conformational and directional definitions of the three antigenic sites of native hen's egg-white lysozyme are described. The results clearly reveal that the three antigenic sites account quantitatively for the total antigenic reactivity of the protein. Thus the entire antigenic structure of lysozyme has now been precisely determined and is briefly discussed here, together with the power of the surface-simulation synthetic concept.

In the preceding paper (Atassi & Lee, 1978) the boundary, conformational and directional refinements of antigenic site 1 of hen's egg-white lysozyme were found by 'surface-simulation' synthesis. We have reported the precise definition of the other two antigenic sites of the protein, also by surface-simulation synthesis (Atassi *et al.*, 1976c; Lee & Atassi, 1977a,b). Therefore we have now achieved the precise definition of the entire antigenic structure of native lysozyme. In the present paper, only a summary of the main features of the antigenic structure of lysozyme will be presented. An extensive analysis of the strategy and conclusions relating to antigenic structures of proteins has appeared (Atassi & Habeeb, 1977) and presents a comprehensive review of the work on the antigenic structure of lysozyme just as we commenced surface-simulation synthesis.

Experimental

All experimental procedures used here were identical with those described in the preceding paper (Atassi & Lee, 1978).

Results and Discussion

Do the three antigenic sites of lysozyme account for its total immune reaction?

In evaluating whether the antigenic structure elucidated in our laboratory does in fact represent the entire antigenic profile of the protein, a vital piece of evidence is to determine the extent of the lysozyme immune reaction that can be accounted for by the total reactivities of the three antigenic sites. This is most critical in view of the fact that our

delineation culminated in the production and synthesis of antigenic sites that are 'unconventional' in character.

A most direct way to answer this question can be derived from the total inhibitory activities of the three sites towards the lysozyme immune reaction. Table 1 summarizes the results obtained with rabbit and goat antisera. With goat antiserum G9 and the IgG fraction of antiserum G10, the total of immunochemical reactivities of the three sites accounted for 101.5 and 97% respectively. With the IgG fractions from rabbit antisera L7 and L21, the combined inhibitory activities of the three sites were 95.3 and 95.5% respectively. Note the improvement in the inhibitory activity (Table 1) when the IgG fractions of the antisera were used. This has been discussed in detail earlier in the preceding paper (Atassi & Lee, 1978) and in connection with the behaviour of the other two antigenic sites (Lee & Atassi, 1977a,b). Perhaps it should be noted again here that the IgG fractions accounted for 99–100% of the immune reaction of the respective antisera.

Another approach to quantitative accounting of the total immunochemical contribution of the sites is to determine the fraction of lysozyme antibodies that can be specifically bound by the synthetic surface-simulation sites. Table 2 summarizes the binding results of ¹²⁵I-labelled antibodies by immunoabsorbents of the three sites. In a single passage into an immunoabsorbent of lysozyme, 97.0% of the total radioiodinated antibody was removed. Passage of the unadsorbed antibody through a second immunoabsorbent column removed the remainder of the label. On the other hand, by a single passage of a sample of the labelled antibodies through only one of the site-Sepharose columns, the three sites effected a calculated total removal of 93% of the lysozyme antibodies. When this was followed by passage through a lysozyme immunoabsorbent, 3–4% of the remaining unbound label could be removed. Com-

Abbreviation used: IgG, immunoglobulin G.

* This paper represents the 21st in the series 'Enzymic and Immunochemical Properties of Lysozyme'. The preceding article in the series is Atassi & Lee (1978).

† To whom correspondence should be addressed.

Table 1. *Quantitative accounting of the three surface-simulation synthetic sites for the total antigenic reactivity of lysozyme*

The values are given in maximum percentage inhibition of the quantitative precipitin reaction of lysozyme by each of the synthetic sites independently. The sites were immunochemically independent (see Table 2). The identities of sites 1, 2 and 3 are shown in Fig. 1. The IgG fractions accounted for 99–100% of the total immune reaction of the respective parent antisera. Values for site 1 were obtained from Atassi & Lee (1978), those for site 2 from Lee & Atassi (1977b), and those for site 3 from Lee & Atassi (1977a), except for reaction of site 3 with the IgG fraction of antiserum L21, which was not previously determined. It was therefore determined here for the sake of completeness.

Site	Maximum percentage inhibition			
	Goat antisera		Rabbit antisera	
	G9	G10	L7	L21
(a) Reactions with whole antisera				
Site 1	32.5	33.3	22.2	21.3
Site 2	35.7	32.3	12.7	18.2
Site 3	33.3	28.8	19.2	22.2
Total	101.5	94.4	54.1	61.7
(b) Reactions with the IgG fractions of the antisera				
Site 1		32.3	33.4	31.4
Site 2		34.5	28.6	28.9
Site 3		30.3	33.3	35.2
Total		97.1	95.3	95.5

Table 2. *Total binding of lysozyme antibodies by immunoadsorbents of the three surface-simulation synthetic sites*

Results are expressed as percentages of ^{125}I -labelled antibody (from antiserum G10) bound by the synthetic sites or by lysozyme. For the structural identity of the sites see Fig. 1. The peptides were immunochemically independent, since specific antibody eluted from one site-immunoadsorbent was not bound by an immunoadsorbent carrying another site, but was bound completely on re-passage on to the site-Sepharose that was originally used for its isolation. Results refer to a single passage on a given immunoadsorbent. Results for independent binding were obtained by passage of a portion of the antibody solution on one of the immunoadsorbents indicated, and those for serial passage refer to serial passage of the same sample of the antibody solution on the immunoadsorbent of site 1, then site 2, then site 3. Values for site 1 were from Atassi & Lee (1978), those for site 2 from Lee & Atassi (1977b) and those for site 3 from Lee & Atassi (1977a). For details see the text.

	Percentage of applied antibody bound by immunoadsorbent					Binding by three sites relative to lysozyme (%)
	Lysozyme	Site 1	Site 2	Site 3	Total	
Independent binding by each immunoadsorbent	97.0	26.9	36.5	29.5	92.9	95.8
Binding by passage through sites serially					94.8	97.7

parison of first-passage values from site-Sepharose with that from lysozyme-Sepharose clearly demonstrates that the three sites can bind 96% of the total antibody relative to that bound by lysozyme. Serial passage of a single sample through all three sites (site 1, then site 2, then site 3) removed 94.8% of the antibody, which corresponded to 97.7% relative to the amount removed by lysozyme. It is critical to point out that the sites were immunochemically independent, since antibody eluted from one site-

immunoadsorbent could not bind with another site-immunoadsorbent, but was adsorbed quantitatively on re-passage on to the site-Sepharose that was initially used for its isolation.

The fact that the surface-simulation synthetic sites (Fig. 1) can account for 96–100% of the total immune reaction of native lysozyme provides a most convincing and powerful demonstration of the correctness of the delineation. The impact of these results becomes even more remarkable when it is

pointed out that it has been shown (Atassi, 1972; Koketsu & Atassi, 1973, 1974) that an intact antigenic site with no extraneous amino acids would usually react less than when it is an integral part of a longer peptide. The extraneous residues or segments are frequently important for the correct folding of the site (Atassi, 1972).

Summary of the main features of the antigenic structure of lysozyme

Native lysozyme carries three antigenic sites. The

identities of the sites are summarized in Fig. 1, and Plates 1 and 2 show their locations in the three-dimensional structure.

Site 1. This antigenic site is constructed by the side chains of the spatially contiguous five surface residues (Atassi & Lee, 1978): Arg-125, Arg-5, Glu-7, Arg-14, Lys-13. The extended dimension of the site from Arg-125 to Lys-13 is 3.0 nm. These residues bind with antibody as if in direct peptide linkage. In fact the reactivity of this site is fully satisfied by the surface-simulation synthetic peptide Arg-Gly-Gly-

Site 1

Site 2

Site 3

Fig. 1. Three antigenic sites representing the entire antigenic structure of lysozyme

The diagram shows the spatially contiguous residues constituting each antigenic site and their numerical positions in the primary structure. The distances (in nm) separating the consecutive residues and the overall 'extended' dimension of each site are given, together with the dimension of each surface-simulation synthetic site. The latter assumes an ideal $C_{(\alpha)}$ -to- $C_{(\alpha)}$ distance of 0.362 nm. Site 1 was precisely defined in the preceding paper (Atassi & Lee, 1978). The precise boundary, conformational and directional definitions of sites 2 and 3 were described earlier [Lee & Atassi (1977b) and Lee & Atassi (1977a) respectively]. The three sites account quantitatively for the entire (96–100%) antigenic reactivity of lysozyme (see the text for details).

Arg-Gly-Glu-Gly-Gly-Arg-Lys, which does not exist in native lysozyme. The surface-simulation synthetic site exhibits a directional preference (Arg-125 to Lys-13), which appears to be independent of the species of the immunized animal, at least with the rabbits and goats so far tested. The site has a restricted conformational freedom, as demonstrated by its sensitivity to variation of the residue spacing in surface-simulation synthesis. The intactness of the disulphide bond 6-127 in native lysozyme is critical for the integrity of this site (Atassi *et al.*, 1973, 1976b).

Site 2. This site also consists of spatially contiguous surface residues. These are: Trp-62, Lys-97, Lys-96, Asn-93, Thr-89, Asp-87 (Atassi *et al.*, 1976c; Lee & Atassi, 1977b). The extended length of the site from Trp-62 to Asp-87 (C_{α} -to- C_{α} distance) is 2.73 nm. As with site 1, site 2 also forms an imaginary line circumscribing part of the surface topography of the protein. This line passes through the residues forming the site that bind with antibody as if in direct peptide-bond linkage. Thus the surface-simulation synthetic peptide Phe-Gly-Lys-Lys-Asn-Thr-Asp, which does not exist in lysozyme, carries the full reactivity of the site (Atassi *et al.*, 1976c; Lee & Atassi, 1977b). With the antisera so far studied, the antigenic site exhibits a preferred 'direction' in surface-simulation synthesis (Trp-62 to Asp-87) towards the goat antisera and none towards the rabbit antisera. The antigenic site is subject to conformational restrictions indicated by spacing-between residues requirements. The intactness of the disulphide bonds 64-80 and 76-94 is critical to bring together the various constituent residues of the site (Atassi *et al.*, 1976a). This antigenic site overlaps with the enzymic active site because they both share Trp-62 (Lee & Atassi, 1975; Atassi *et al.*, 1976a).

Site 3. Like sites 1 and 2, this site is also constituted of spatially adjacent surface residues. The antigenic site comprises five residues, which are: Lys-116, Asn-113, Arg-114, Phe-34, Lys-33 (Lee & Atassi, 1977a). These residues describe an imaginary line that circumscribes part (2.1 nm in C_{α} -to- C_{α} distance from Lys-116 to Lys-33) of the molecule. They act functionally towards the antibody as if they are in direct peptide bond linkage. Accordingly, the surface-simulation synthetic peptide (which does not exist in lysozyme) having the structure Lys-Asn-Arg-Gly-Phe-Lys carries the full immunochemical reactivity of the site (Lee & Atassi, 1977a). With the two rabbit and two goat antisera so far studied the antigenic site exhibited a preferred direction (Lys-116 to Lys-33), since the reverse surface-simulation synthetic sequence was immunochemically inefficient. The intactness of the disulphide bond 30-115 is critical for the integrity of this antigenic site in lysozyme (Atassi *et al.*, 1973). Antigenic site 3 overlaps with the hexasaccharide substrate-binding site

at the carbonyl group of Phe-34 and the side chain of Arg-114 (Lee & Atassi, 1977a).

Potential of surface-simulation synthesis

It is evident that elucidation of the antigenic structure of lysozyme would not have been possible without our introduction of 'surface-simulation' synthesis. However, the power of this unorthodox concept should not be confined to determination of protein antigenic sites and it may be of value to outline here very briefly some of its potential applications.

We have pointed out (Atassi *et al.*, 1976c; Lee & Atassi, 1976) that the results from this approach on the three antigenic sites of lysozyme afford the most powerful and convincing chemical evidence for the correctness of the three-dimensional structure of lysozyme as derived from the X-ray crystallographic studies (Blake *et al.*, 1967; Imoto *et al.*, 1972). Thus, for example, the three antigenic sites of lysozyme (Fig. 1) report on the spatial interrelationships of a total of 16 surface residues all at once.

The utility of this approach should not be limited to exploitation of antigenic sites. Other parts of the surface could be copied into appropriate surface-simulation synthetic peptides linking conformationally contiguous residues, which can then be coupled to a suitable carrier and the conjugate used for immunization. The antibodies thus prepared will recognize and react with those regions in the native protein, even though the regions are not antigenic sites when the native protein is used as an immunogen. These antibodies could be used as conformational reagents to double-check the three-dimensional structure of a protein. The approach could also be used as a probe to monitor the acquisition of correct residue alignments by various preselected parts of the surface of a protein molecule (or a fragment thereof) upon renaturation of a denatured protein or refolding of a derivative having previously reduced disulphide bonds. Furthermore, should methods for predicting protein conformation from its sequence ever become more reliable, then the predicted three-dimensional structure could be readily double-checked by antibodies to surface-simulation synthetic peptides designed from the predicted structure.

The concept should also find application in studies on subunit interactions in oligomeric proteins and, in principle, on other interactions involving proteins. These could include the synthetic construction of a substrate-binding site of an enzyme and the interaction sites of some proteins with lipids, carbohydrates and other prosthetic groups. A related application of the concept will be in protein-receptor interactions, which will open up untapped avenues in the molecular elucidation of many biochemical and

EXPLANATION OF PLATE 1

Lysozyme model showing the relative positions of the residues constituting antigenic sites 1 and 2

The side chains of the residues of the sites are outlined and those making up site 1 are coloured in blue, whereas the residues constituting site 2 are shown in red. The preferred 'direction' of site 1 (at least by surface-simulation synthesis) is Arg-125 to Lys-13. Site 2 had a preferred 'direction' only with goat antisera (Trp-62 to Asp-87), but exhibited no directional preference with rabbit antisera.

EXPLANATION OF PLATE 2

Lysozyme model showing the position of antigenic site 3 on the molecule relative to sites 1 and 2

The side chains of the residues comprising the sites are outlined. The residues constituting site 3 are in yellow. This view is taken by rotating the model 125° anticlockwise on the vertical axis relative to the view shown in Plate 1. From this perspective only parts of site 1 can be seen, which are the residues Lys-13, Arg-5 and Arg-125, shown in blue. Also, of site 2 only Trp-62 can be seen and is shown in red. Site 3 showed the same directional preference (Lys-116 to Lys-33) towards rabbit and goat antisera.

immunological phenomena. Obviously, the three-dimensional structure of the protein under study must be known in detail. Furthermore, the concept can only be applied after the careful chemical identification of the residues involved in binding of a protein with the receptor, antibody or other protein interactions.

The most intriguing question is whether an antibody combining site can be reconstructed or copied by surface-simulation synthesis. This will have far-reaching implications in immunology. Findings from our laboratory (Atassi & Zablocki, 1977) indicate that this may have indeed been accomplished by synthesizing two surface-simulation synthetic peptides that are complementary to antigenic sites 2 and 3 (Fig. 1) of lysozyme. It was quite remarkable that these synthetic complementary copies behaved immunochemically in all respects like specific antibody combining sites. Thus, by the synthesis of the antibody combining site, the surface-simulation concept has made a major advance in protein immunochemistry. Although the concept came as a by-product of our determination of the antigenic structure of lysozyme, it should present a major new asset in protein chemistry.

Conclusions

This is the second antigenic structure of a protein to be precisely defined. The entire antigenic structure of sperm-whale myoglobin has also been elucidated (Atassi, 1975; or in more detail, Atassi, 1977). However, it is critical to note that the antigenic structure of lysozyme (like that of myoglobin) was determined with early-course (3–4 weeks after the first immunization) antisera raised only in goats and rabbits. It is unknown whether the antigenic structure that is recognized will change on prolonged immunizations. The primary antibody response to a globular protein is directed against its native three-dimensional structure (Atassi & Thomas, 1969), and proteolytic fragmentation of the protein may play a relatively more significant role in the antigenic expression in late-course antisera (Atassi & Thomas, 1969).

Many general conclusions relating to antigenic structures of proteins were derived from our accurate mapping out of the antigenic structure of myoglobin (Atassi, 1972, 1975, 1977). All of these conclusions are applicable to lysozyme equally as well. These include: the small size and sharp boundaries of the antigenic sites, their presence only in a limited number, their surface location (see Plates 1 and 2), their sensitivity to conformational changes and many other features [for further details see Atassi (1975, 1977)].

The five antigenic sites of myoglobin are made up of residues in direct peptide-bond linkage, whereas

the three sites of lysozyme each constitutes spatially contiguous residues that were frequently very distant in sequence. Even though we had previously suggested (Atassi & Saplin, 1968) the existence of such antigenic sites in proteins, their identification in lysozyme is the first such example in protein immunochemistry. However, the antigenic sites both in myoglobin and in lysozyme are sensitive to conformational changes in the respective proteins, with those of lysozyme showing, as expected, a higher sensitivity. Accordingly, it is totally inadequate to identify the antigenic sites of myoglobin by the terms 'linear', 'sequential', or 'primary' or some such terms, although identifying the antigenic sites of lysozyme by the terms 'spacial', 'conformational' etc. It may be more suitable to state that the antigenic sites of myoglobin consist of conformationally distinct continuous surface portions of its structure and those of lysozyme are made up of conformationally (or spatially) contiguous surface residues that are mostly not in direct peptide bond linkage. Whether the antigenic sites in a protein will belong to one type or another or a mixture of both will obviously depend on the protein, and may be determined to a great extent by the presence of internal cross-links (Lee & Atassi, 1976). In this regard, we caution that immunization with a protein that had been chemically cross-linked is likely to alter its antigenic expression.

It is relevant to comment on the antibody combining site that will be complementary to the antigenic sites of lysozyme. The antigenic sites 1, 2 and 3 require that the 'extended' antibody combining sites should be at least 3.0, 2.7 and 2.1 nm respectively. This is quite similar to the dimensions of the antigenic sites of sperm-whale myoglobin (Atassi, 1975, 1977), which range between 2.0 and 2.4 nm. The size of the antibody combining sites required for the two proteins is somewhat larger than the combining site for haptens as determined by X-ray crystallography. This is seen in the combining site of the Fab' fragment of protein New (a human IgG) towards a hydroxyl derivative of vitamin K₁ (Poljak *et al.*, 1973; Amzel *et al.*, 1974), of the Fab' fragment of a myeloma protein from mouse (McPc 603) towards phosphocholine (Padlan *et al.*, 1973) and a Bence-Jones protein (λ chain) dimer (Ely *et al.*, 1973; Schiffer *et al.*, 1973). The latter revealed a conical cavity 1.0 nm deep with an opening of 1.5 nm connected to a pocket of 1.7 nm and the part of the combining site involved in binding depended on the size and structure of the bound hapten. Therefore the antibody combining site can vary to provide maximum complementarity to the antigenic site with which it binds. With proteins, unlike haptens, the antigenic site will be expected to fill the entire combining site. Thus the above dimensions found for antibody combining sites accommodating haptens may represent an artificial situation and the combining sites towards protein antigenic

sites will be larger. This should be readily achieved by the flexibility of the hypervariable region.

Examination of the antigenic sites of lysozyme (Fig. 1) shows that they are very rich in basic amino acids. This was also seen in myoglobin, but we caution against premature generalizations. Obviously, interactions with antibody are predominantly polar in nature, with considerable stabilizing effects being contributed by hydrophobic interactions and some hydrogen-bonding, especially with site 2. However, it should be emphasized that the basicity of the sites cannot be the only rationalization for their antigenicity, because other alignments of basic conformationally contiguous residues, which are non-antigenic, can be generated on the surface. Clearly, the arrangement of the residues in the imaginary surface-encircling line bearing the aligned residues is highly critical. It is well to caution here that the sequence and three-dimensional features that confer immunogenicity on given parts or surface areas of a protein molecule are still not too clear (Atassi, 1975).

The work was supported by a grant (A113181) 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

- Amzel, L. M., Poljak, R. J., Saul, F., Varga, J. M. & Richards, F. F. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1427–1430
- Atassi, M. Z. (1972) *Specific Recept. Antibodies, Antigens Cells: Int. Convocation Immunol. Proc.* 3rd 118–136
- 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. & Habeeb, A. F. S. A. (1977) in *Immunochemistry of Proteins* (Atassi, M. Z., ed.), vol. 2, pp. 177–264, Plenum, New York
- Atassi, M. Z. & Lee, C.-L. (1978) *Biochem. J.* **171**, 419–427
- 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. (1977) *J. Biol. Chem.* **252**, 8784–8787
- Atassi, M. Z., Habeeb, A. F. S. A. & Ando, K. (1973) *Biochim. Biophys. Acta* **303**, 203–209
- 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., Lee, C.-L. & Pai, R.-C. (1976c) *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
- Ely, K. R., Girling, R. L., Schiffer, M., Cunningham, D. E. & Edmundson, A. B. (1973) *Biochemistry* **12**, 4233–4237
- 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
- Lee, C.-L. & Atassi, M. Z. (1975) *Biochim. Biophys. Acta* **405**, 464–474
- 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
- Padlan, E. A., Segal, D. M., Spande, T. F., Davies, D. R., Rudikoff, S. & Potter, M. (1973) *Nature (London) New Biol.* **245**, 165–167
- Poljak, R. J., Amzel, L. M., Avey, H. P., Chen, B. L., Phizackerly, R. P. & Saul, F. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 3305–3310
- Schiffer, M., Girling, R. L., Ely, K. R. & Edmundson, A. B. (1973) *Biochemistry* **12**, 4620–4630