Binding with lysozyme of antibodies against surface-simulation peptides representing the lysozyme antigenic sites

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(Received 18 November 1981/Accepted 9 December 1981)

Previously it had been shown that native lysozyme has three discontinuous antigenic sites (comprising spatially adjacent residues that may be distant in sequence) that were mimicked by surface-simulation synthetic peptides that had the capacity to bind the bulk (97–99%) of the antibody response against native lysozyme. In the present work these three surface-simulation synthetic peptides were coupled to succinoylated bovine serum albumin, and the conjugates were injected into rabbits. Antibodies against each peptide reacted, as expected, only with that peptide, but it was also found that the antibodies could bind with lysozyme, and the complete specificity of this binding was rigorously established. The advantages of these findings in conformational and immunological investigations are outlined.

In the final stages of our determination of the antigenic structure of lysozyme, it was found that its antigenic sites comprise residues that are not directly linked by peptide bonds but are spatially adjacent by virtue of the three-dimensional folding of the molecule (Atassi, 1978; Atassi & Lee, 1978*b*). For the synthetic proof of the discontinuous nature of the lysozyme antigenic sites (as defined by Atassi & Smith, 1978) we introduced the concept of surface-simulation synthesis (Atassi *et al.*, 1976; Lee & Atassi, 1976). By this concept, protein functional sites composed of residues that are spatially close but that may not be in direct peptide linkage in the protein sequence can be synthetically mimicked by a peptide incorporating the active residues with appropriate spacing and directionality. The peptide thus generated does not exist in the native protein, but attempts to mimic a surface region of it [hence the term 'surface-simulation' synthesis (Lee & Atassi, 1976)]. This concept provided a versatile experimental approach for synthetically mimicking protein binding sites with diverse activities, and was also proposed as a powerful tool for studying protein conformation (Atassi, 1978; Atassi & Lee, 1978*b*). The latter depended on making antibodies to surface-simulation peptides representing selected surface areas of a protein molecule and then studying the binding of these antibodies with the native protein. However, although it is a standard procedure in immunology that antibodies to small peptides can be made by immunizing with conjugates of the peptides on a high-molecular-weight

carrier, it is yet to be shown that antibodies against surface-simulation synthetic peptides will bind with the native protein. The present work describes studies with antibodies raised against three surface-simulation peptides corresponding to the three discontinuous antigenic sites of lysozyme (Atassi, 1978).

The synthetic peptides are: site 1 peptide, Arg-Gly-Gly-Arg-Gly-Glu-Gly-Gly-Arg-Lys (Atassi & Lee, 1978*a*); site 2 peptide, Phe-Gly-Lys-Lys-Asn-Thr-Asp (Lee & Atassi, 1977*b*); site 3 peptide, Lys-Asn-Arg-Gly-Phe-Lys (Lee & Atassi, 1977*a*). The synthesis, purification and characterization of the surface-simulation peptides representing lysozyme antigenic sites 1, 2 and 3 have been described in detail in the respective references. Egg-white lysozyme (three-times crystallized) and rabbit haemoglobin (twice crystallized) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine serum albumin (four-times crystallized) was from ICN Pharmaceuticals (Covina, CA, U.S.A.).

Goat antisera (G9 and G10) against lysozyme were prepared as previously described for myoglobin (Atassi, 1967). The same procedure was employed for the preparation of antibodies against polymerized bovine cytochrome *c* (Reichlin *et al.*, 1970). Preparation of the immunoglobulin G fraction from various antisera has been described (Lee & Atassi, 1977*a*). Labelling of immunoglobulin G fraction by ¹²⁵I was by the chloramine-T method (Hunter & Greenwood, 1962). Peptides were coupled to succinoylated (3-carboxypropionylated) bovine

serum albumin after activation of its carboxy side chains by conversion into the reactive *p*-nitrophenyl ester groups (Atassi *et al.*, 1981). The extent of coupling was determined by amino acid analysis of acid hydrolysates (Atassi & Saplin, 1968). Antibodies to the peptides were raised in outbred New Zealand White rabbits by immunizing two rabbits each with a given peptide conjugate (1 mg) as an emulsion in complete Freund's adjuvant by using the schedule indicated in Fig. 1. Antisera against the sites were in the following rabbits: site 1, rabbits L31 and L45; site 2, rabbits L33 and L34; site 3, rabbits L35 and L43. Serial bleedings were obtained at 2- or 3-week intervals, and antisera from selected bleeds were screened for anti-peptide antibodies by binding with lysozyme adsorbed on round-bottomed wells of poly(vinyl chloride) plates as described elsewhere (Sakata & Atassi, 1981). Bovine ribonuclease A, rabbit haemoglobin and bovine cytochrome *c* were used as controls for non-specific binding. Lysozyme and peptide immunoabsorbents were prepared as previously described (Twining & Atassi, 1979) and contained 1.5 mg of lysozyme/ml of packed volume or 0.5 mg of peptide/ml. Quantitative immunoabsorbent titration study of ^{125}I -labelled immunoglobulin G fractions was performed in 0.1% rabbit haemoglobin as reported previously (Sakata & Atassi, 1979). Inhibition of the binding of ^{125}I -labelled antibodies to adsorbents of lysozyme was performed by preincubation with various amounts of anti-lysozyme antisera (G9 and G10), anti-(polymeric cytochrome *c*) or pre-immune goat antisera as previously described (Twining & Atassi, 1978).

The albumin-peptide conjugates had the following peptide contents (in mol of peptide/mol of albumin): site 1 conjugate, 18.4; site 2 conjugate, 17.7; site 3 conjugate, 30.0. Antisera against these conjugates from each rabbit were screened for the presence of antibodies that will bind with lysozyme. It was found that anti-lysozyme antibodies (at least in terms of binding) could be detected in antisera as early as 14 days and persisted at least up to 125 days after the initial immunization with a given peptide-albumin conjugate. Later bleedings were not studied. Fig. 1 gives an example of the results with serial antisera, from one rabbit each, against the three peptide-albumin conjugates. The specificity of the binding of these antibodies with lysozyme was suggested from the fact that they did not bind with unrelated proteins such as rabbit haemoglobin, bovine ribonuclease and bovine cytochrome *c*. Also, pre-immune sera or sera from rabbits that had been immunized with bovine serum albumin, sperm-whale myoglobin or polymeric bovine cytochrome *c* showed no binding activity with lysozyme. Furthermore, antisera from rabbits that had been immunized with albumin conjugates of the synthetic

Fig. 1. Time-course study of the binding of rabbit anti-peptide antibodies with lysozyme and unrelated control proteins

Antibodies were determined by reaction with ^{125}I -labelled protein A by binding with lysozyme or control protein on poly(vinyl chloride) plate adsorbents. ○ and ●, Serial antibodies against synthetic site 1 conjugate (rabbit L45); △ and ▲, serial antibodies against site 2 conjugate (rabbit L33); □ and ■, serial antibodies against site 3 conjugate (rabbit L43). Open symbols represent binding of the antibodies with lysozyme; black symbols represent the average binding values of the respective antibodies with the control proteins, rabbit haemoglobin, bovine ribonuclease A and bovine cytochrome *c*. Vertical arrows indicate days on which injections with each peptide conjugate were given.

antigenic sites of myoglobin (Atassi, 1975) did not bind to lysozyme.

Further examination of the binding was performed by immunoabsorbent titration studies. The specificity of the binding was indicated (Table 1) from the finding that antibodies to a given site bound only with immunoabsorbents of that peptide. Also, control adsorbents of ribonuclease A, cytochrome *c* and antigenic site 1 of myoglobin (Atassi, 1975) had no binding activity with these antisera. The protein controls were chosen because, like lysozyme, they are very basic proteins and therefore their inability to bind would serve to reassure that the binding of anti-peptide antibodies by lysozyme-Sepharose is not caused by non-specific charge effects. Furthermore, lysozyme adsorbents could not bind ^{125}I -labelled antibodies against polymeric bovine cytochrome *c* (results not shown).

The specificity of the binding was further confirmed by inhibition studies. The binding of anti-peptide antibodies to lysozyme adsorbents was fully inhibited by free lysozyme. More importantly, the binding was almost completely inhibited by goat

Table 1. *Immunoabsorbent binding studies of anti-site antibodies with site, lysozyme and control protein adsorbents*
 Titrations were performed with a fixed amount of the ^{125}I -labelled immunoglobulin G (5×10^4 c.p.m.) from one anti-serum to each peptide conjugate. The results below summarize the binding values obtained with 500 μl of each immunoabsorbent. The results have been corrected for non-specific binding (usually 3–5%) by an equal volume of uncoupled Sepharose. Each value is the average of triplicate analyses, which varied $\pm 1.5\%$ or less.

Immunoabsorbent	Anti-(site 1 peptide) (L45)	Anti-(site 2 peptide) (L33)	Anti-(site 3 peptide) (L35)
Site 1	4480	30	0
Site 2	10	5100	0
Site 3	0	0	3900
Lysozyme	4510	5180	3950
Ribonuclease	20	30	10
Cytochrome c	0	20	0
Site 1 of myoglobin	10	0	0

anti-lysozyme antisera whose antibody specificity is known to be directed to these three surface-simulation antigenic sites (Atassi, 1978). Conversely each anti-peptide antiserum inhibited the binding of ^{125}I -labelled anti-lysozyme antibodies (from goat G9) to lysozyme–Sepharose by an amount that correlated well with the amounts of antibodies directed to that site in antiserum G9 (Atassi, 1978). The reaction of lysozyme with goat anti-lysozyme antibodies was inhibited by 95.8% by a mixture of antisera to the three peptides. Fig. 2 shows the results of inhibition with one anti-lysozyme antiserum (goat G9), and the results with the other antiserum tested (goat G10) were essentially the same. It is noteworthy that the binding of anti-peptide antibodies with lysozyme adsorbents was not inhibited by the following: cytochrome c, ribonuclease A, antisera against sperm-whale myoglobin, antisera against bovine serum albumin or antisera against polymeric bovine cytochrome c. Clearly antibodies against the surface-simulation peptides bind to lysozyme with complete specificity.

The concept of surface-simulation synthesis was introduced (Atassi *et al.*, 1976; Lee & Atassi, 1976) and developed during, and was vital for, the determination of the entire antigenic structure of lysozyme (Atassi, 1978; Atassi & Lee, 1978*b*). The antigenic sites of this protein are of the discontinuous type (Atassi, 1978; Atassi & Smith, 1978). These studies also revealed the presence of directional and conformational requirements attending surface-simulation synthesis (Lee & Atassi, 1977*a,b*; Atassi & Lee, 1978*a*). This concept has afforded a new and versatile dimension for synthetically mimicking a variety of protein binding sites, and was suggested as a potentially powerful tool for studying protein conformation (Atassi, 1978; Atassi & Lee, 1978*b*).

The potential application of surface-simulation synthesis to monitor protein conformation depends on the preparation of antibodies to synthetic surface-stimulation peptides that are designed to

Fig. 2. *Inhibition by various antisera of the binding with lysozyme–Sepharose of ^{125}I -labelled immunoglobulin G against the synthetic sites*

O, Inhibition of the binding of anti-(site 1 peptide) antibodies (L45, 125-day bleed) by goat anti-lysozyme antiserum G9; Δ , inhibition of the binding of anti-(site 2 peptide) antibodies (L34, 125-day bleed) by anti-lysozyme antiserum G9; $\square$, inhibition of the binding of anti-(site 3 peptide) antibodies (L43, 93-day bleed) by anti-lysozyme antiserum G9. $\bullet$ represents the inhibitions (average values) by non-immune goat serum, rabbit antisera against bovine serum albumin and rabbit antisera against polymeric bovine cytochrome c. Inhibition with another anti-lysozyme antiserum (G10) gave essentially similar results to those shown here with antiserum G9.

mimic surface areas of a protein molecule, and the ability of these antibodies to recognize and react with those surface regions in the native protein molecule. Such antibodies could then be used as conformational probes to double-check the three-dimensional structure of a protein in solution

(Atassi, 1978). This would also constitute an effective approach to monitor the acquisition of correct residue arrangements of preselected regions of a protein (or fragment) on renaturation of a denatured protein or re-formation of previously reduced disulphide bonds. Because this approach would afford a direct region-by-region scanning of the proximity of several residues on the surface, it may be useful to those interested in the mechanism of protein folding or in the prediction of protein conformation from its sequence.

Even though it has been well established (Atassi *et al.*, 1976; Lee & Atassi, 1976, 1977*a,b*; Atassi, 1978; Atassi & Lee, 1978*a,b*) that antibodies against a native protein having discontinuous antigenic sites will bind with appropriately designed surface-simulation peptides mimicking the antigenic sites, it remained to be shown that the reverse cross-reaction could take place. This is indeed quite critical, in view of the fact that a surface-simulation synthetic peptide does not, after all, exist in the native protein. The present findings clearly establish that antibodies to properly designed surface-simulation peptides will recognize and bind with the native protein. The approach would therefore afford much more valuable and precise information than that derived from chemical (e.g. availability of side chains to chemical modification), spectral or other physical measurements in solution. Thus, for example, the present antibodies report simultaneously on the spatial interrelationships of 16 surface residues in lysozyme. Of course, in principle, antibodies could be made to several surface-simulation peptides representing surface areas that jointly encompass the entire surface of a protein molecule. These antibodies should recognize and bind with those surface areas even though the latter do not represent antigenic sites when the native protein is used as the immunogen. Such a battery of antibodies would provide the most powerful conformational probes available to monitor the three-dimensional structure of a protein in solution. The ability to make antibodies to surface areas of a protein molecule that

are preselected and mimicked by surface-simulation synthesis presents a versatile and powerful tool in exploiting therapeutic applications for helper, suppressive, tolerogenic and allergenic sites on free proteins and those of other invasive agents.

This work was supported by the Mayo Foundation and in part by Grant AM-18920 from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U.S. Public Health Service.

References

- Atassi, M. Z. (1967) *Biochem. J.* **102**, 478–487
- Atassi, M. Z. (1975) *Immunochemistry* **12**, 423–438
- Atassi, M. Z. (1978) *Immunochemistry* **15**, 909–936
- Atassi, M. Z. & Lee, C.-L. (1978*a*) *Biochem. J.* **171**, 419–427
- Atassi, M. Z. & Lee, C.-L. (1978*b*) *Biochem. J.* **171**, 429–434
- Atassi, M. Z. & Saplin, B. J. (1968) *Biochemistry* **7**, 688–698
- Atassi, M. Z. & Smith, J. A. (1978) *Immunochemistry* **15**, 609–610
- Atassi, M. Z., Lee, C.-L. & Pai, R.-C. (1976) *Biochim. Biophys. Acta* **427**, 745–751
- Atassi, M. Z., Kazim, A. L. & Sakata, S. (1981) *Biochim. Biophys. Acta* **670**, 300–302
- Hunter, W. M. & Greenwood, F. C. (1962) *Nature (London)* **194**, 495–496
- Lee, C.-L. & Atassi, M. Z. (1976) *Biochem. J.* **159**, 89–93
- Lee, C.-L. & Atassi, M. Z. (1977*a*) *Biochem. J.* **167**, 571–581
- Lee, C.-L. & Atassi, M. Z. (1977*b*) *Biochim. Biophys. Acta* **495**, 354–368
- Reichlin, M., Nisonoff, A. & Margoliash, E. (1970) *J. Biol. Chem.* **245**, 947–954
- Sakata, S. & Atassi, M. Z. (1979) *Biochim. Biophys. Acta* **576**, 322–332
- Sakata, S. & Atassi, M. Z. (1981) *Mol. Immunol.* **18**, 961–967
- Twining, S. S. & Atassi, M. Z. (1978) *J. Biol. Chem.* **253**, 5259–5262
- Twining, S. S. & Atassi, M. Z. (1979) *J. Immunol. Methods* **30**, 139–151