

## Localization and Verification by Synthesis of Five Antigenic Sites of Bovine Serum Albumin\*

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Recently we have shown that the major antigenic sites of bovine serum albumin exhibit functional equivalence progressively increasing with the time at which antibodies are obtained after the first immunization. Analysis of our recent immunochemical findings and the known covalent structure of bovine serum albumin have enabled us to predict the locations of five antigenic sites of bovine serum albumin. The predicted locations were synthesized, and immunochemical studies with late-course antisera showed them to constitute antigenic sites of native bovine serum albumin.

The antigenic structure of serum albumin has been the subject of considerable interest for over two decades (Lapresle, 1955; Porter, 1957). Recent studies from this laboratory (which are outlined below) on bovine serum albumin have enabled an extensive understanding of the antigenicity of this protein. By analysis of these immunochemical findings we are now able to predict the most likely locations for five of the antigenic sites on the bovine serum albumin molecule. The rationale for the prediction of the sites is outlined below. In this report, the predicted locations have been synthesized and their immunochemical reactions with antisera to bovine serum albumin studied.

### Methods

Solid-phase peptide synthesis, purification of the crude peptides on CM-Sephadex C-50 and establishment of purity were performed as previously described (Koketsu & Atassi, 1973, 1974). Two late-course antisera (398 days) against bovine serum albumin were obtained from rabbits B8 and B9 (Sakata & Atassi, 1978). Quantitative precipitin and inhibition experiments were performed with the whole antisera by the procedure given elsewhere (Atassi & Saplin, 1968). Pure IgG fractions from the antisera were labelled with  $^{125}\text{I}$  (Lee & Atassi, 1977). Specific antibodies to bovine serum albumin, prepared by immunoabsorption of the IgG fractions on bovine serum albumin-Sepharose, were labelled on the immunoabsorbent (Matzku & Zöller, 1977) followed by elution with 5M-guanidine hydrochloride, pH 8.5.

Proteins or peptides were coupled to CNBr-activated Sepharose CL-4B as previously described

Abbreviation used: IgG, immunoglobulin G.

\* This paper represents the 9th in the series 'Immunochemistry of Serum Albumin'. The preceding paper in the series is Sakata *et al.* (1979).

(Sakata & Atassi, 1978). The amount of peptides coupled to the adsorbents was 0.3-0.4mg/ml of packed volume. Protein adsorbents had 2.0-2.5 mg/ml of packed volume. Immunoabsorbent-titration studies, using a fixed amount of  $^{125}\text{I}$ -labelled immune IgG or  $^{125}\text{I}$ -labelled antibody and increasing amounts of immunoabsorbents, were performed in 0.1% rabbit haemoglobin in 0.15M-NaCl/0.01M-sodium phosphate buffer, pH 7.2, by the procedure described by Sakata & Atassi (1978). Four adsorbents carrying sperm-whale myoglobin, hen egg-white lysozyme, lysozyme antigenic site 1 and a 'nonsense' peptide (see Fig. 1) were used as controls to correct for non-specific binding.

### Results

The peptides synthesized in the present work are shown in Fig. 1. The synthetic peptides, after chromatography on CM-Sephadex, were homogeneous in heavily loaded peptide 'maps' and had the following amino acid compositions: for I, Glu, 1.06; Pro, 0.96; Ala, 1.02; Ile, 1.16; Leu, 0.94; Tyr, 1.93; His, 0.93; Arg, 2.20; for II, Glu, 1.04; Pro, 0.97; Ala, 0.96; Val, 0.94; Leu, 3.15; His, 1.06; Lys, 1.95; for III, Asp, 3.07; Glu, 1.02; Gly, 2.00; Ala, 2.05; Val, 0.97; Lys, 1.88; for IV, Asp, 3.02; Glu, 1.01; Gly, 1.99; Ala, 1.98; Val, 0.95; Lys, 2.02; for V, Asp, 0.97; Glu, 1.04; Pro, 0.97; Ala, 1.04; Val, 0.90; Leu, 1.28; Tyr, 0.89; Phe, 0.99; Lys, 2.00. Lysozyme antigenic site 1 was previously synthesized and reported (Atassi & Lee, 1978), and the 'nonsense' peptide has been synthesized (S. S. Twining & M. Z. Atassi, unpublished work) by the same methods (Koketsu & Atassi, 1973, 1974) in connection with studies on myoglobin. The synthetic peptides exhibited no inhibitory activity, either individually or as a mixture, towards the precipitin reaction of bovine serum albumin with its antisera when used at a 1000-fold molar excess relative to albumin. In view of the ineffective-

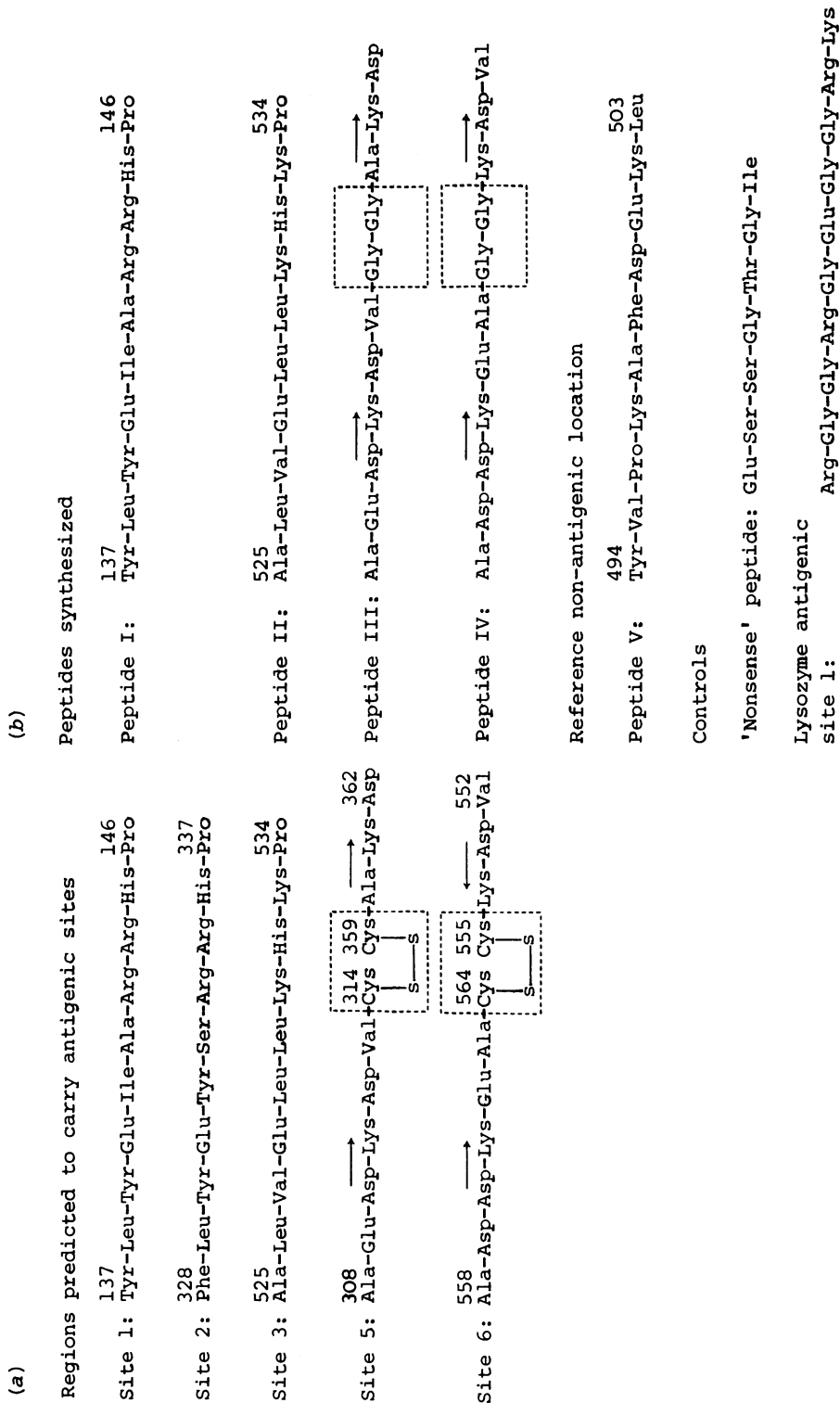


Fig. 1. (a) Structure and location of five regions predicted to carry antigenic sites of bovine serum albumin and (b) peptides synthesized and studied in the present work. Note that in peptides III and IV, diglycyl segments were used to replace the disulphide bridges on the predicted sites 5 and 6. The arrows show the directions of the peptide bonds. In (b) is also shown a region, indicated to be a non-antigenic location, that was synthesized and studied here. Lysozyme antigenic site 1 and the 'nonsense' peptide do not represent any part of the albumin molecule and were used as controls for the immunochemical studies. It is not implied that the antigenic sites comprise the entire size of the predicted regions but rather that they fall within these regions. For details, see the text.

ness of the peptides in competing with bovine serum albumin in this reaction, their activity had to be investigated by determining the ability of peptide immuno-adsorbents to bind anti-(bovine serum albumin) antibodies directly.

The amount of antibody bound by an immuno-adsorbent was determined by immuno-adsorbent-titration studies using constant amounts of  $^{125}\text{I}$ -labelled immune IgG or  $^{125}\text{I}$ -labelled antibody with increasing amounts of immuno-adsorbent. Such titration studies were carried out to determine if complete depletion of antibodies directed to the antigen on the immuno-adsorbent had occurred (Sakata & Atassi, 1978). Fig. 2 shows an example of titration curves of  $^{125}\text{I}$ -labelled antibody from one antiserum (from rabbit B9) with adsorbents of the four synthetic peptides, which we had predicted to carry antigenic sites of albumin. In several titration studies, binding results with  $^{125}\text{I}$ -labelled immune IgG and  $^{125}\text{I}$ -labelled antibody were essentially the same. The amounts of non-specific binding to adsorbents of myoglobin, lysozyme, lysozyme antigenic site 1 or 'nonsense' peptide corresponded to 3–5% of the total radioactivity bound to bovine serum albumin–Sepharose. The amount of radioactivity bound to a given volume of the control immuno-adsorbents was subtracted from that bound to an

equal volume of a given albumin or peptide immuno-adsorbent. The results with antisera from rabbits, B8 and B9 (Table 1) show that the peptides I–IV, which we had predicted to carry antigenic sites of albumin, possessed remarkable binding activity towards the bovine serum albumin antibodies in antisera from both rabbits. However, as seen in Fig. 2, it was very hard, with  $^{125}\text{I}$ -labelled antibody, to reach plateau binding with the peptides, even when 500  $\mu\text{l}$  of their immuno-adsorbents was used, and the results summarized in Table 1 are therefore lower than the plateau values. The amount of antibody to reference peptide V, indicated to be non-antigenic, was almost non-detectable. Our inability to achieve plateau binding by any of the peptide adsorbents prevented us from conducting quantitative adsorption studies.

In the course of our immuno-adsorbent studies we occasionally obtained peptide immuno-adsorbents that had little or no activity, and, furthermore, active preparations differed in their capacity to bind antibody. The loss of activity was attributed to the involvement of immunochemically essential side

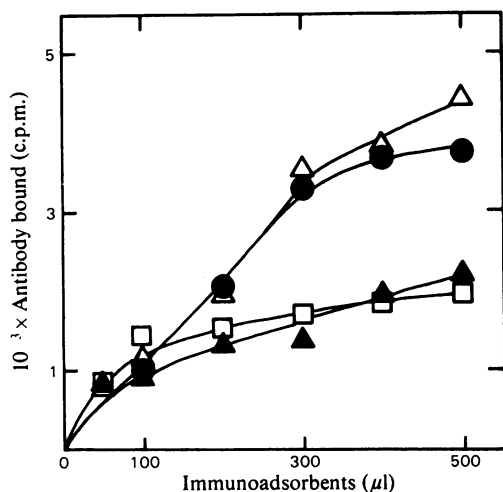


Fig. 2. An example of titration curves of fixed amounts of  $^{125}\text{I}$ -labelled antibody from rabbit B9 (398-day bleeding) with increasing amounts of peptide immuno-adsorbents  $\blacktriangle$ , Peptide I;  $\square$ , peptide II;  $\triangle$ , peptide III;  $\bullet$ , peptide IV. Each experimental point is the average of triplicate analyses which varied  $\pm 1.5\%$  or less. Each point has been corrected for non-specific binding by an equal volume of a lysozyme site 1–Sepharose which was less than 3% of the amount bound to bovine serum albumin. Note that bovine serum albumin adsorbent achieved plateau binding at 18000 c.p.m.

Table 1. Quantitative immuno-adsorption of  $^{125}\text{I}$ -labelled immune IgG from two late anti-(bovine serum albumin) sera by albumin and peptide adsorbents

Values were obtained from the amounts of label bound by 500  $\mu\text{l}$  of peptide immuno-adsorbents (see Fig. 2 for example) and are expressed as percentage of label bound relative to that bound by bovine serum albumin in the plateau as 100%. The results represent the average of determinations on at least eight different immuno-adsorbent preparations (each in triplicate), by using  $^{125}\text{I}$ -labelled immune IgG. Binding results with  $^{125}\text{I}$ -labelled antibody were essentially the same as those below. Values have been corrected for non-specific background binding, by an equal volume of myoglobin–Sepharose, lysozyme–Sepharose, lysozyme antigenic site 1–Sepharose and 'nonsense' peptide–Sepharose, which was 3–5% of the amount of radioactivity bound to bovine serum albumin.

Immuno-adsorbent	Percentage binding relative to bovine serum albumin*	
	Antiserum B8 (398-day bleeding)	Antiserum B9 (398-day bleeding)
Bovine serum albumin	100	100
Peptide I	26.4	17.4
Peptide II	18.9	20.1
Peptide III	13.9	24.4
Peptide IV	22.3	27.7
Peptide V	1.7	0.9

\* Note that the binding values by the peptide adsorbents given here are not plateau values. Therefore they do not represent the maximum amounts of antibody that can bind with a given peptide adsorbent, but rather the maximum recorded binding values (see Fig. 2).

chains in the linkage of the peptide on to Sepharose. In order to evaluate the degree of confidence in the activity of the predicted peptides, at least ten different immunoabsorbent batches (3ml each) were prepared from each peptide, and the incidence of active adsorbents obtained from each peptide was determined. These were (in number of active preparations/total number of preparations): peptide I, 13:13; peptide II, 8:11; peptide III, 11:14; peptide IV, 12:15; peptide V, 0:10.

## Discussion

Our recent studies on serial antisera (7 days to 398 days) from six different rabbits revealed that their immunochemical reactivity with bovine serum albumin fragment 377–571 increased with time after initial immunization, being as much as 90% for late-course antisera, as judged by the ability of the fragment to inhibit the bovine serum albumin precipitin reaction (Sakata & Atassi, 1978). Another fragment of bovine serum albumin (sequence 11–193) also inhibited the bovine serum albumin precipitin reaction by about 90% (Atassi *et al.*, 1976*a*) and the two fragments cross-reacted (Habeeb & Atassi, 1977*a*). These findings confirmed our suggestion (Atassi *et al.*, 1976*a*; Habeeb & Atassi, 1977*b*) that some bovine serum albumin antigenic sites [at least two (Habeeb & Atassi, 1977*a*)] are, in functional terms, potentially equivalent. The functional equivalence between two sites improves progressively with the time at which antibodies are obtained after the initial immunization (Sakata & Atassi, 1978). Therefore late-course antisera (398 days) were initially chosen for the present work, from our serial antisera (from 7 days to 398 days). We have now found (Kazim *et al.*, 1979; Sakata *et al.*, 1979) that the reactivity of fragment 377–571 resided entirely within the sequence 504–571. This has greatly simplified the problem of localizing some major antigenic sites of bovine serum albumin. Therefore, within the segment 504–571, there are regions that would be recognized by progressively later antibodies as functionally equivalent to other regions elsewhere on bovine serum albumin. If the antigenic sites comprise continuous portions of the covalent structure [defined as continuous antigenic sites (Atassi, 1978*a,b*; Atassi & Smith, 1978)], then it should be possible to localize any region(s) within the segment 504–571 that appears to repeat in general character in other parts of albumin.

So we examined the covalent structure of bovine serum albumin (Brown, 1975), bearing in mind that functional equivalence between two antigenic sites implies resemblance in binding capacity and would not necessarily require complete structural identity (Atassi *et al.*, 1976*b*; Lee *et al.*, 1976). We concluded that the regions 137–146, 328–337 and 525–534 (Fig.

1) have the potential for carrying functionally equivalent antigenic sites, if the hydrophobic amino acids at analogous positions progressively discharged equivalent binding functions as immunization continues, and if basic amino acids were ionically equivalent in terms of binding function. These three predicted regions are located in equivalent positions in the three domains of bovine serum albumin revealed by sequence studies (Brown, 1975). In view of the high similarity between the regions 137–146 and 328–337 (see Fig. 1), we considered that only one of these regions need be studied, since immunochemical activity for one will clearly implicate the other. Region 137–146 was synthesized as a representative of both. Region 525–534 is sufficiently different from the aforementioned two regions to merit its synthesis and investigation. Two other equivalent antigenic sites may also exist around disulphide bonds on the regions (308–314)–(359–362) and (558–564)–(555–552), provided that the reversal in the direction of the peptide bond between the sequences 359–362 and 555–552 (see Fig. 1) would not drastically alter their overall antigenic character. This has been shown to be permissible in certain cases (Lee *et al.*, 1976). Therefore both regions were studied by synthesis of peptides III and IV in which cystine bridges were replaced by diglycyl segments, a synthetic approach that we introduced (Lee *et al.*, 1976) during our studies on the antigenic structure of lysozyme. Finally, a reference peptide (V) corresponding to bovine serum albumin sequence 494–503, suggested as a non-antigenic region, was synthesized and studied. Two separate peptide controls consisting of lysozyme antigenic site I and a 'nonsense' peptide, which do not occur in the albumin molecule, were also studied.

The failure of these peptides to inhibit the precipitin reaction of bovine serum albumin, even at a 1000-fold molar excess, may be due to a lower affinity of immunochemically active peptides as a result of their existence in non-native conformation (Atassi & Saplin, 1968). The antibody response to native protein antigens is directed against their native conformation (Atassi, 1967; Atassi & Thomas, 1969). We have reported (Kazim & Atassi, 1977) that synthetic antigenic sites of haemoglobin did not inhibit the haemoglobin precipitin reaction, but showed remarkable abilities to bind haemoglobin antibodies by quantitative immunoabsorption. It was therefore necessary to apply this direct binding approach to the predicted synthetic antigenic sites of bovine serum albumin in view of their inability to inhibit the bovine serum albumin precipitin reaction. In titrations with <sup>125</sup>I-labelled immune IgG or <sup>125</sup>I-labelled antibody the plateau binding was not reached by the peptide adsorbents even with relatively large amounts of immunoabsorbents. The binding efficiency of the peptides may have been

impaired by their existence on the Sepharose in a conformation that does not resemble the conformation of these regions in the native molecule. Furthermore, the coupling of peptides to Sepharose would inadvertently take place via amino acid side chains that may be essential for binding with antibody. The destruction of peptide immunochemical activity as a result of involvement of immunochemically essential amino acids in the coupling to Sepharose was apparent from the fact that occasionally we obtained immunoabsorbent preparations that were almost inactive.

The remarkable binding activity of the predicted sites and the non-reactivity of the reference peptide representing an indicated non-antigenic location clearly show that the five regions predicted here represent major antigenic sites of bovine serum albumin. Taking into account that the activities determined here are not plateau values (i.e. do not represent maximum activities), the antigenic sites predicted here bind half or more of the entire antibody population elicited by native bovine serum albumin in the late antisera of the two rabbits. These results should be considered in the light that expression and immunodominance of a protein antigenic site is under genetic control (Okuda *et al.*, 1978) and varies with the immunized animal (Atassi, 1975, 1978*a,b*; Koketsu & Atassi, 1973; Lee & Atassi, 1977) and with the period antisera are obtained after immunization (Sakata & Atassi, 1978). The other antigenic sites on bovine serum albumin may either be non-equivalent or be of the type termed (Atassi, 1978*a,b*; Atassi & Smith, 1978) discontinuous antigenic sites (i.e. constituting spatially adjacent surface residues that are distant in sequence), which have been precisely determined in lysozyme (Atassi, 1978*a,b*).

In conclusion, five major antigenic sites in bovine serum albumin have been predicted and the predictions confirmed by synthetic and immunochemical studies. The predicted locations synthesized here are larger than the precisely delineated antigenic sites of sperm-whale myoglobin (Atassi, 1975) or of hen egg-white lysozyme (Atassi, 1978*a,b*) and may thus be larger than the actual antigenic sites. They were intentionally enlarged to give the prediction a greater likelihood of success by avoiding errors in prediction of boundaries.

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