

IMMUNOCHEMISTRY OF SERUM ALBUMIN—VII.

EFFECTS OF CHEMICAL MODIFICATION OF TYROSINE AND ARGININE RESIDUES ON THE CONFORMATION AND IMMUNOCHEMISTRY OF A FRAGMENT CORRESPONDING TO THE LAST THIRD OF BOVINE SERUM ALBUMIN*

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Abstract—A fragment of bovine serum albumin corresponding to the last third of the albumin molecule was previously shown, using late-course anti-albumin antisera, to possess a large amount of the immunochemical activity of the parent protein. Two chemical derivatives of this fragment were prepared, one modified at all four of its tyrosine residues and the other at four of its eight arginine residues. Circular dichroism studies showed that the derivative modified at tyrosine residues suffered no conformational changes. The derivative modified at arginine residues, however, showed a considerably altered conformation relative to the unmodified fragment. In immunochemical studies, using quantitative precipitin and precipitin inhibition studies, radioimmunoassays (Farr assay) and radioimmunoabsorption studies, both derivatives were shown to have suffered no changes in their reactivities relative to the unmodified fragment. These results indicate the exclusion of the modified residues from participation in antigenic sites of albumin.

INTRODUCTION

In a previous communication we reported the isolation and characterization of a fragment of bovine serum albumin corresponding to the sequence positions 377-571 of the albumin molecule (Habeeb & Atassi, 1976). Subsequently, in exhaustive studies of antisera from six rabbits taken serially from 7 to 398 days after initial immunization with albumin we demonstrated that this fragment accounts for a substantial portion of the immunochemical activity of the parent molecule (Sakata & Atassi, 1979a). In those studies, antisera from each of the serial bleedings from the six rabbits were studied separately, and the ability of this fragment to express the immunochemical activity of native albumin was found to increase strikingly with time, being as much as 90% with later course antisera as judged by its ability to inhibit the albumin precipitin reaction (Sakata & Atassi, 1979a).

In view of the small size of fragment 377-571 and its retention of such a large amount of the reactivity of albumin in late course antisera, this fragment is ideally suited for studying the antigenic structure of the native molecule. In the studies reported here, we have examined the effects of exhaustive chemical modification of tyrosine and arginine residues in fragment 377-571 on its conformation and immunochemistry.

MATERIALS AND METHODS

Bovine serum albumin (4 × crystallized) was obtained from ICN Pharmaceuticals Inc. Tetranitromethane was obtained from Aldrich Chemical Co., 1,2-cyclohexanedione from Baker Chemical Co., phenylglyoxal from Koch-Light Labs. and citraconic anhydride and cyanogen bromide from Eastman Chemical Co. Carrier-free ¹²⁵I was obtained from New England Nuclear Co.

Fragment 377-571 was prepared by partial tryptic hydrolysis of albumin followed by gel filtration on Sephadex G-100 and chromatography on DEAE-cellulose as previously described (Habeeb & Atassi, 1976). The purity of the preparation was determined by polyacrylamide disc gel electrophoresis, using heavily-loaded gels.

Modification of tyrosine residues in fragment 377-571

Tetranitromethane was employed to modify the tyrosine residues in fragment 377-571. A variety of experimental conditions varying the reaction medium and tetranitromethane/protein ratio were investigated in order to obtain complete nitration of all four tyrosine residues. Optimal conditions (see Results) for their modification were obtained when the reaction was performed in 0.01 M NH₄HCO₃, at a protein concentration of 1 mg/ml and a molar ratio of tetranitromethane/protein of 80. The procedure for nitration of tyrosine residues is as follows: fragment 377-571 (30 mg, 1.36 μmole) was dissolved in cold 0.01 M NH₄HCO₃ (30 ml) and the protein solution brought to room temperature. Three aliquots (450 μl, 37.46 μmoles each) of tetranitromethane (1% in 95% ethanol) were then added at 2 hr intervals with constant stirring. The reaction was allowed to continue for 2 hr after the last addition, followed by exhaustive dialysis against water at 4°C to remove excess reagent. The dialysate was freeze-dried, redissolved in 0.01 M NH₄HCO₃ (2 ml) and applied on a column of Sephadex G-75 (2.6 × 86 cm) to remove cross-linked products formed during the reaction. Under these conditions, cross-linked products of fragment 377-571

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accounted for no more than 5–10% of the total reaction product, and the monomeric fraction thus isolated was shown to be homogeneous by polyacrylamide disc gel electrophoresis. To confirm the completeness of the modification, an aliquot of the monomeric nitrated fragment was hydrolyzed and subjected to amino acid analysis.

Modification of arginine residues in fragment 377–571

The exhaustive modification of all 8 arginine residues in fragment 377–571 by phenylglyoxal and 1,2-cyclohexanedione was attempted under a variety of experimental conditions varying: (a) the reaction medium and pH, (b) the molar excesses of phenylglyoxal and cyclohexanedione, (c) the duration of the reaction, (d) the reaction temperature, and (e) the presence of various non-ionic and cationic detergents. In view of our previous observation that these dicarbonyl reagents may also react with N^2 and N^6 amino groups (Atassi *et al.*, 1976) we examined each of the derivatives prepared under the various conditions for their lysine content. As will be mentioned in Results, on amino acid analysis a diminished lysine content in these derivatives was observed. It was therefore decided that the reversible protection of lysine residues by citraconic anhydride (Habeeb & Atassi, 1970) should be performed prior to the modification of lysine residues. Citraconylation of the fragment was performed as previously described (Habeeb & Atassi, 1970).

Optimal conditions for arginine modification were determined (see Results) and the derivative used for immunochemical studies was prepared by reaction of citraconylated fragment (27 mg, 1.22 μ moles) in 0.1 *M* triethylamine (2.5 ml), pH 11, with cyclohexanedione (361 μ mole added in 0.1 *M* triethylamine, 1.62 ml). The reaction was allowed to continue for 24 hr with constant stirring at room temperature followed by removal of excess reagent by gel filtration in 0.1 *M* triethylamine on Sephadex G-25 (1.8 $\times$ 30 cm). Fractions containing protein were pooled and dialyzed against water at 4°C. Removal of the citraconyl blocking groups was performed by adjusting the pH of the dialysate to pH 3.0 with 1*N* acetic acid and allowing the deblocking to continue at room temperature for 6 hr with stirring (Habeeb & Atassi, 1970). The extent of arginine modification was examined by amino acid analysis.

Conformational measurements

Conformational changes in the derivatives of the fragment were monitored by circular dichroism (CD) measurements on a Jasco spectropolarimeter, Model J-20. All measurements were performed in 0.15 *M* NaCl/0.01 *M* sodium phosphate buffer, pH 7.2. Concentrations of protein solutions were calculated from their nitrogen contents as determined by a micro-Kjeldahl procedure and taking the molecular weight and nitrogen content for fragment 377–571 and its derivatives as 22,145 and 16.37%, respectively. Results are expressed in reduced mean residue ellipticities.

Antisera

Rabbits B8 and B9 were immunized with native albumin and rabbits B5 and B7 with fragment 377–571 according to the schedule recently described (Sakata & Atassi, 1979*a*). The times after initial immunization at which the antisera were obtained are indicated where appropriate.

Immunochemical methods

Quantitative precipitin and inhibition experiments were performed as previously described (Atassi & Saplin, 1968).

For immunoabsorption studies, albumin, the fragment and its derivatives were coupled to cyanogen-bromide-activated Sepharose CL-4B. The amount of protein coupled to the Sepharose in each case was found to be in the range of 1.0–1.2 mg/ml of packed volume. Whole immunoglobulin fractions (predominantly IgG) from anti-albumin antisera were prepared by ammonium sulfate precipitation and

chromatography on DEAE cellulose as recently described (Sakata & Atassi, 1979*a*).

Proteins were labeled with ^{125} I by the chloramine-T procedure (Hunter & Greenwood, 1962), employing a chloramine-T to protein ratio of 2 (w/w). The reaction was terminated by the addition of sodium metabisulfite. Free ^{125} I was separated from protein-bound ^{125} I by desalting on a column of Sephadex G-25 (1.5 $\times$ 15 cm) equilibrated with 0.1% rabbit hemoglobin in 0.15 *M* NaCl/0.01 *M* sodium phosphate buffer, pH 7.2, followed by dialysis of the pooled radiolabeled protein peak against 0.15 *M* NaCl/0.01 *M* sodium phosphate buffer, pH 7.2.

The binding of ^{125} I-fragment 377–571 to anti-albumin sera was determined by a Farr assay as previously described for myoglobin (Kazim & Atassi, 1978). Serum dilutions necessary for binding approximately 50% of the added ^{125} I-labeled fragment were determined by examining the binding of serial dilutions of antisera to a fixed amount of ^{125} I-labeled fragment. All antisera dilutions were made in non-immune rabbit sera. The amount of radiolabel precipitated by $(\text{NH}_4)_2\text{SO}_4$ in the presence of an identical amount of non-immune rabbit serum served as a control, and was subtracted from the amount precipitated in the presence of specific antibody.

Inhibition of the binding of ^{125} I-labeled fragment by the fragment and its derivatives was performed by preincubating various amounts of the unlabeled inhibitors with an amount of antisera appropriately diluted to bind 50% of the ^{125} I-labeled fragment to be added. Following incubation, ^{125} I-labeled fragment 377–571 was added and the reaction mixture reincubated. Bound ^{125} I-fragment 377–571 was then determined after washing precipitates obtained with $(\text{NH}_4)_2\text{SO}_4$. The incubation times, precipitation by $(\text{NH}_4)_2\text{SO}_4$, washing and counting of precipitates were performed as described earlier (Kazim & Atassi, 1978).

Quantitative immunoabsorbent titration studies of ^{125} I-labeled IgG from anti-albumin antisera were performed as recently described (Sakata & Atassi, 1979*a*). Briefly, increasing amounts of Sepharose conjugates of albumin, fragment 377–571 or its derivatives were mixed by shaking with a fixed amount of ^{125} I-IgG. Following mixing, the Sepharose was washed with 0.15 *M* NaCl/0.01 *M* sodium phosphate buffer, pH 7.2 and counted. A control adsorbent consisting of lysozyme-Sepharose was incubated with the ^{125} I-IgG under identical conditions. The ^{125} I-IgG bound to the lysozyme-Sepharose was considered to be non-specifically adsorbed and the binding of ^{125} I-IgG to albumin-Sepharose and the related adsorbents was corrected for this non-specific binding to lysozyme.

RESULTS

Modification of tyrosine residues

As mentioned in Materials and Methods, we investigated a variety of conditions for the exhaustive modification of tyrosine residues in this fragment. At molar ratios of tetranitromethane/protein of 10 or 20, we consistently modified three of the four tyrosine residues, even when the reaction was performed in the presence of 6 *M* urea. At a molar ratio of 40, only traces (less than 0.3 of a residue) of the tyrosine were left unmodified. Increasing the molar ratio of tetranitromethane from 40 to 80 did not result in any additional decrease in tyrosine content.

The reaction of fragment 377–571 with tetranitromethane under the conditions outlined in Materials and Methods resulted in the modification of practically all tyrosine residues in this fragment. Amino acid analysis of the nitrated monomeric derivative showed an insignificant amount of tyrosine remaining (Table 1). Tyrosine residues were however

Table 1. Amino acid analysis of fragment 377-571 and its derivatives^a

Amino acid	Excepted for 377-571	Found for 377-571	Found for (NO ₂) 377-571	Found for (CHD) 377-571
Tyr	4	3.86	0.27	3.90
His	4	4.82	3.85	4.19
Lys	20	20.60	19.34	19.14
Arg	8	7.93	8.10	4.07
(NO ₂) Tyr	—	—	3.70	—
(NO ₂) Tyr + Tyr	—	—	3.97	—

^a The content of all other amino acids remained unchanged.

quantitatively recovered as nitro-tyrosine and the content of all other amino acids remained unchanged (Table 1).

Modification of arginine residues

Under alkaline conditions and large molar excesses of reagent, phenylglyoxal and 1,2-cyclohexanedione react with arginine residues to give stable derivatives which do not revert to arginine on acid hydrolysis (for review, see Atassi, 1977a). We had hoped, therefore that by utilizing large molar excesses of these dicarbonyl reagents all of the eight arginine residues in fragment 377-571 could be exhaustively modified, and the modifications detected by amino acid analysis. Analytical scale modifications of the fragment in *N*-ethylmorpholine buffer, pH 8, or in 0.1 *M* triethylamine, pH 11, with molar excesses of phenylglyoxal of 10-80, resulted in a maximum modification of only four arginine residues. No improvement in the extent of modification of arginine residues was observed even when the molar excesses of phenylglyoxal was increased to 1000 and the reaction allowed to continue for 2 weeks at 37°C. When the reaction medium was made 1% with respect to each of Triton X-100, Tween-80, Brij or sodium dodecylsulfate and the reaction carried out under the conditions outlined above, there was no improvement in the number of arginine residues modified. Similar results were obtained with 1,2-cyclohexanedione.

Upon hydrolysis of the test derivatives, however, a progressive loss of lysine residues was observed with increasing amounts of cyclohexanedione and phenylglyoxal used in the reaction. This is consistent with our previous observation with lysozyme peptides (Atassi *et al.*, 1976) for which we reported the modification of N² and N⁶ amino groups by these two dicarbonyl reagents. It was therefore decided that lysine residues in the fragment should be reversibly protected by citraconylation prior to modification. Although citraconylation, due to the introduction of the negatively charged protecting group, results in extensive unfolding of protein chains (Habeeb & Atassi, 1970), even in the citraconylated fragment only 4 of the 8 arginine residues could be modified. Lysine residues were, however, quantitatively recovered. Optimal conditions for arginine modification of citraconylated fragment 377-571 were chosen (as described in Materials and Methods) and the results of amino acid analysis of the derivative are shown in Table 1. This derivative showed a decrease of 4 arginine residues and no loss of lysine residues.

Conformation of fragment 377-571 and its derivatives

Conformational changes occurring in the polypeptide backbone of fragment 377-571 upon chemical modification were monitored by CD. Figure 1 shows the CD spectra in the 204-260 nm range of fragment 377-571 and its derivatives. The reduced mean residue ellipticities at 209 and 222 nm of fragment 377-571 are similar to those reported (Reed *et al.*, 1975) for a slightly larger fragment of albumin corresponding to the sequence 377-581. No significant differences between the CD spectrum of the unmodified fragment and its nitrated derivative were observed. The derivative modified at arginine residues, however, exhibited decreased ellipticities relative to the unmodified fragment, being approximately 25 and 29% less at 209 and 222 nm, respectively. There were, however, no changes in the wavelengths of the CD minima exhibited by this derivative.

Quantitative precipitin reaction with anti-377-571

An example of the results of the quantitative precipitin reactions of fragment 377-571, (NO₂) 377-571 and (CHD) 377-571 with an antiserum prepared against fragment 377-571 are shown in Fig. 2; and a summary of the results with two additional antisera to fragment 377-571 are shown in Table 2. With none of the antisera studied did the derivatives exhibit either a shift in the equivalence point or

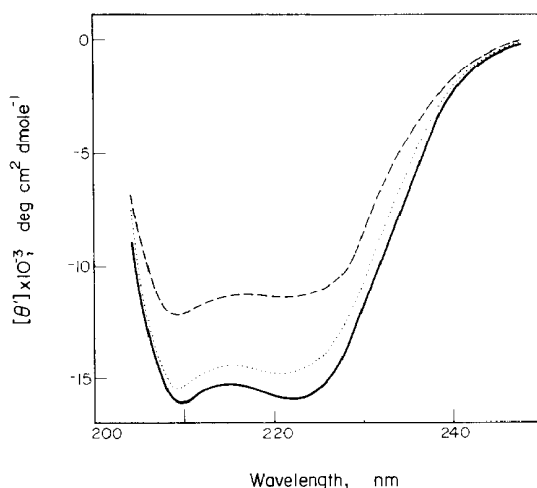


Fig. 1. CD spectra of fragment 377-571 and its derivatives. $[\theta]$ is the reduced mean residue ellipticity. (—) 377-571; (....) (NO₂) 377-571; (---) (CHD) 377-571.

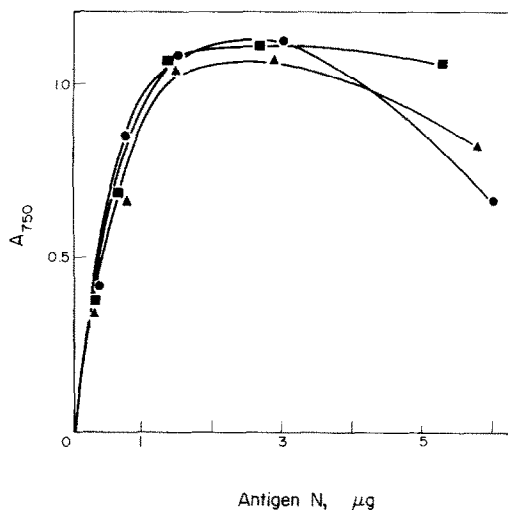


Fig. 2. An example of the precipitation reaction of 377-571 and its derivatives with antiserum B5 taken 70 days after initial immunization with fragment 377-571: (▲) 377-571; (●) $(\text{NO}_2)_2$ 377-571; (■) (CHD) 377-571.

diminished reactivity at equivalence relative to the unmodified fragment. Indeed, for the major part there was no significant difference between the precipitin curves of the derivatives and unmodified fragment.

Quantitative precipitin inhibition with anti-albumin

In studies with serial (7-398 days) antisera from 6 individual rabbits, we have previously demonstrated that fragment 377-571 inhibits the precipitin reaction of albumin with its antisera to an extent which is dependent both on the individual animal as well as the time the antiserum is obtained after immunization (Sakata & Atassi, 1979a). The fragment inhibited almost completely (90%) the precipitin reaction of albumin with late course antisera from each of the six rabbits (Sakata & Atassi, 1979a). To evaluate further the effects of modifying tyrosine and arginine residues in the fragment we compared the inhibitory activities of these derivatives with that of the unmodified fragment, using two albumin antisera from single bleedings of two rabbits that are inhibited 58% and 90% by the unmodified fragment. Each of the two derivatives showed, with a given antiserum, an equal inhibitory activity to that of the unmodified fragment. There was no significant difference either in the maximal inhibition obtained with the derivatives nor in the molar excesses relative to albumin required for 1/2 maximum inhibition.

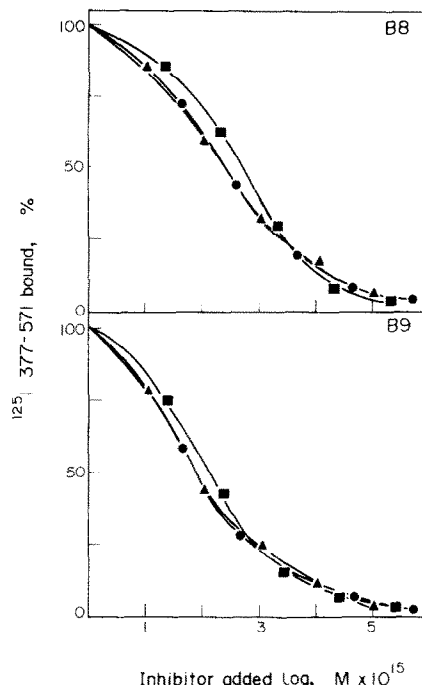


Fig. 3. Inhibition of ^{125}I -labeled 377-571 binding by unlabeled 377-571 and its derivatives. The antisera used in these assays were obtained from the 38-day bleedings of antisera prepared against albumin. In both cases the amount of antisera present bound 50% of the total ^{125}I -labeled 377-571 added (89,000 counts/min) in the absence of competing, unlabeled protein. (▲) 377-571; (●) $(\text{NO}_2)_2$ 377-571; (■) (CHD) 377-571, expressed as moles/250 μl .

Upper panel, antiserum B8; lower panel, antiserum B9.

Inhibition of antibody binding to radioiodinated 377-571 by its derivatives

In view of the likelihood that soluble complexes in the albumin immune reaction may complicate interpretation of the precipitin data (Sakata & Atassi, 1979a), we have employed additional approaches which are independent of the presence of non-precipitating complexes. The abilities of the derivatives and fragment 377-571 to inhibit in a Farr assay the binding of ^{125}I -labeled fragment 377-571 to two anti-albumin antisera are shown in Fig. 3. With both antisera, the inhibitory activity of $(\text{NO}_2)_2$ 377-571 was identical to that of the unmodified fragment, the profiles of the two inhibition curves being almost superimposable. The inhibition curve of (CHD) 377-571 showed a slight displacement from that of unmodified 377-571.

Table 2. Summary of quantitative precipitin analyses of peptide 377-571 and its derivatives with anti 377-571

Peptide	% Reaction at equivalence ^a		
	Antiserum B5 70-day bleeding	Antiserum B7 70-day bleeding	Antiserum 489 84-day bleeding
377-571	100	100	100
$(\text{NO}_2)_2$ 377-571	104	106	97.7
(CHD) 377-571	103	100	N.D.

^a Values are expressed as % precipitation at equivalence relative to the reaction of fragment 377-571 as 100%.

requiring somewhat larger amounts for comparable inhibition. However, the inhibition by (CHD) 377–571 tapered off more steeply than (NO₂) 377–571 and the unmodified fragment. Notably, both (NO₂) 377–571 and (CHD) 377–571 were able to effect complete inhibition of this binding, and at amounts comparable to those required for the unmodified fragment.

Radioimmunoabsorption of ¹²⁵I-labeled anti-albumin

A second approach which we employed to examine the effects of the modifications on fragment 377–571, and which is also independent of the presence of soluble complexes is that of quantitative radioimmunoabsorption. The 'whole' IgG fraction from antisera to albumin was labeled with ¹²⁵I and a constant amount of ¹²⁵I-labeled IgG was titrated with increasing amounts of immunoabsorbent carrying albumin, fragment 377–571, or the derivatives. An example of the titration curve with ¹²⁵I-IgG from one antiserum (38-day bleeding, rabbit B8) is shown in Fig. 4. Consistent with our previous observations (Sakata & Atassi, 1979a), relative to albumin–Sephacrose, depleting excesses of 377–571–Sephacrose adsorbed about 22% of the ¹²⁵I-IgG obtained from this antiserum (38-day bleeding). The adsorption profiles with the immunoabsorbents of (NO₂) 377–571 and (CHD) 377–571 were identical with that of the unmodified fragment. In view of the fact that similar amounts of protein were coupled to Sepharose in each of the adsorbents used here, in the absence of immunochemical differences between 377–571 and its derivatives it should be expected that the adsorption profiles of 377–571 and its derivatives would be identical. A summary of the maximal (plateau) adsorption values obtained with a 38-day antiserum from rabbit B8 and two antisera from rabbit B9, taken at two different times (38 and 398 days) after the first immunization is given in Table 3. As indicated in Table 3, adsorbents of the derivatives were equally as effective as that of 377–571 in binding to ¹²⁵I-labeled IgG.

DISCUSSION

In the chemical strategy set forth (Atassi, 1972) for the delineation of the complete antigenic structures of proteins, chemical modification is a key approach that enables the identification of antigenically essential

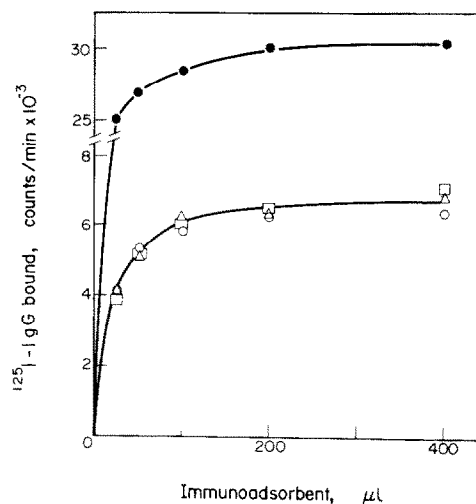


Fig. 4. An example of the radioimmunoabsorption titration of ¹²⁵I-labeled anti-albumin with increasing amounts of Sepharose coupled to albumin, fragment 377–571 and its derivatives. The IgG used in this titration was obtained from antiserum B8, 38-day bleeding. (●) albumin; (△) 377–571; (□) (CHD) 377–571; (○) (NO₂) 377–571.

amino acid residues (Atassi, 1967a, 1968; Atassi & Thomas, 1969; Atassi *et al.*, 1975, 1976; Lee *et al.*, 1975). It has been a particularly fruitful approach in the determination of the entire antigenic structures of myoglobin (Atassi, 1975, or in more detail, Atassi, 1977b) and lysozyme (Atassi, 1978, in press). The usefulness, limitations and precautions to be exercised in the application of this approach to protein immunochemistry have recently been discussed in detail, together with an extensive review of chemical modification and cleavage reactions of proteins (Atassi, 1977a). It is now well established (Atassi & Habeeb, 1969; Atassi *et al.*, 1971; Habeeb & Atassi, 1971) that, for a chemical reaction with proven specificity in the modification of a protein and in the absence of conformational changes, the nature of a chemical modification of an amino acid side chain determines its influence on biological function. Previously, we have demonstrated that nitration of an antigenically essential tyrosine residue (Atassi, 1968; Atassi & Habeeb, 1969), or the modification of an essential arginine residue by cyclohexanedione (Atassi

Table 3. Summary of the results of quantitative immunoabsorption of ¹²⁵I-labeled anti-albumin by Sepharose adsorbents of albumin, fragment 377–571 and its derivatives

Immunoabsorbent	Antiserum B8 38-day bleeding		Antiserum B9 38-day bleeding		Antiserum B9 398-day bleeding	
	¹²⁵ I-IgG bound (counts/min) ^a	Relative % ^b	¹²⁵ I-IgG bound (counts/min) ^a	Relative % ^b	¹²⁵ I-IgG bound (counts/min) ^a	Relative % ^b
Albumin	31389	100	17918	100	15086	100
377–571	6834	21.7	6359	35.5	7161	47.5
(NO ₂) 377–571	6327	20.2	6046	33.7	7304	48.4
(CHD) 377–571	7086	22.6	6197	34.5	7517	49.8

^a Maximum values obtained in the plateau region of the titration curves, an example of which is shown in Fig. 4. The total amount of ¹²⁵I-IgG added was 60,000, 41,000 and 68,200 counts/min for B8 (38-day bleeding), B9 (38-day bleeding) and B9 (398-day bleeding), respectively.

^b Expressed as a % of the maximum amount of ¹²⁵I-IgG bound to albumin–Sephacrose as 100%.

& Thomas, 1969) were sufficient to disrupt the immunochemical reactivity of the respective antigenic site in which these residues occurred.

Reaction of tetranitromethane with proteins (for review see Atassi, 1977a), in addition to nitration of tyrosine residues (Riordan *et al.*, 1966; Sokolovsky *et al.*, 1966) will also effect oxidation of thiol groups (Riordan & Christen, 1968), formation of cross-links (Doyle *et al.*, 1968; Boesel & Carpenter, 1970) and tryptophan oxidation (Cuatrecasas *et al.*, 1968; Atassi *et al.*, 1970). In view of the fact that fragment 377–571 has no thiol or indole side chains, the only side reaction that may be expected on its nitration will be cross-link formation. This was in fact the case and the monomer, which predominated and was isolated from the reaction product, was specifically and quantitatively nitrated at the four tyrosine residues. Cyclohexanedione condenses with the guanidinium group of arginine (Toi *et al.*, 1967), but may also modify amino groups (Liu *et al.*, 1968; Atassi *et al.*, 1976) and occasionally tryptophan residues (Atassi *et al.*, 1976 and for review, Atassi, 1977a). Amino group modification was prevented here by reversible acylation with citraconic anhydride.

The modified tyrosine residues in fragment 377–571 are located at positions 399, 408, 449 and 494. Only 4 of the 8 arginine residues in this fragment could be modified even under forcing conditions employing massive reagent excess, long reaction periods and unfolding solvents. Similarly, in the citraconylated fragment, only four arginine residues could be modified despite the fact that citraconylated proteins are expected to undergo considerable unfolding (Habeeb & Atassi, 1970; Singhal & Atassi, 1971; Atassi & Habeeb, 1972). These results suggested that the other 4 arginine residues are buried.

Circular dichroism measurements revealed that the fragment undergoes no conformational changes upon nitration of its 4 tyrosine residues. On the other hand, modification of 4 of the 8 arginine residues resulted in a considerable conformational change. It is interesting that modification of the arginine residues, even though it causes a change in the conformation, has no effect on the immunochemical reactivity of the fragment. This is not unusual. Thus, even though it is well established that the immunochemical reactivity of native protein antigens is highly influenced by changes in their conformation (Atassi, 1967b; Atassi & Skalski, 1969; Atassi *et al.*, 1970; Andres & Atassi, 1970) and that the immune response to a native protein is directed against its unaltered three-dimensional structure (Atassi, 1967b; Atassi & Thomas, 1969), it is now also well established that not every conformational change will influence the immunochemical properties (Atassi & Habeeb, 1969; Atassi *et al.*, 1971, 1973, 1975). The effect is dependent on the protein and on the nature and extent of the conformational change (Atassi, 1977b; Atassi & Habeeb, 1969; Atassi *et al.*, 1971).

In view of the fact that, by all the immunochemical criteria employed here, the derivatives were immunochemically identical to the unmodified fragment, it can be concluded that the modified residues are not located in antigenic sites on this fragment. The non-involvement of all 4 tyrosine residues in an antigenic site is unambiguous, whereas the case for the 8 arginine residues requires special

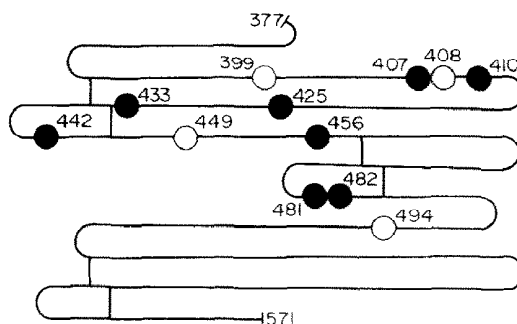


Fig. 5. A schematic line diagram given to show the relative locations of (○) tyrosine and (●) arginine residues in the primary structure of fragment 377–571. The figure is not in any way intended to represent the mode of folding of the fragment.

comment. That the 4 modified arginine residues are not part of an antigenic site is clear. However, a strong argument could be made for the non-involvement of the other four buried arginine residues. The precise determination in this laboratory of the entire antigenic structures of myoglobin (Atassi, 1975, or in more detail, Atassi, 1977b) and lysozyme (Atassi, 1978, in press) have revealed that antigenic sites can incorporate only exposed residues. Obviously buried residues, by virtue of the location of their side chain within the molecular interior, cannot form part of a protein binding site. Thus, the present findings strongly suggest that all 8 arginine residues (the 4 that are exposed and four that are buried) are not located in an antigenic site on fragment 377–571.

The distribution of tyrosine and arginine residues in the primary structure (Brown, 1975) of fragment 377–571 is shown in Fig. 5. It should be noted that these residues (Tyr 399, 408, 449, 494; Arg 407, 410, 425, 433, 442, 456, 481, 482) occur only in the first half of the fragment (sequence 399–494), while the sequence 495–571 is without either tyrosine or arginine. In view of our present results we are inclined to speculate that the antigenic reactivity in 377–571 is localized to the second half of this fragment, lying between residues 495 and 571. Other alternatives are however possible. We have previously shown that protein antigenic sites are made up roughly of 5–7 amino acid residues, either in continuous peptide linkage as for myoglobin (Atassi, 1972), or constitute spatially adjacent surface residues coming together through molecular folding (discontinuous antigenic sites: Atassi, 1978, in press; Atassi & Smith, 1978) as for lysozyme (Atassi, 1978, in press.) The possibility that amino acid residues lying between the modified residues in the first half of 377–571 may participate in such discontinuous sites should not be overlooked. Alternatively, regions 377–398, 400–406, 411–424, 426–432, 434–441, 443–448, 450–455, 457–480 and 483–493 situated between the modified tyrosine and arginine residues are each sufficiently large as to possess one or more continuous antigenic site. Further chemical modification studies of fragment 377–571 and studies of smaller peptides derived from this fragment are presently in progress in order to more precisely locate the immunochemically active regions of this fragment.

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