

ENZYMIC AND IMMUNOCHEMICAL PROPERTIES OF LYSOZYME—VI CONFORMATION, ENZYMIC ACTIVITY AND IMMUNOCHEMISTRY OF DERIVATIVES MODIFIED AT ARGININE RESIDUES

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Abstract—Modification of the arginine residues in lysozyme with cyclohexanedione has been carried out under two different conditions. The first derivative (CHD-Lysoz I) was prepared by reaction in 0.1 *N* NaOH. However, it was found that control lysozyme preparations subjected to these reaction conditions lost all enzymic activity, although their antigenic reactivity was not affected. To avoid this complication, a second derivative (CHD-Lysoz II) was prepared by reaction in 0.1 *M* triethylamine. In fact, lysozyme pretreated with this solvent retained entirely its enzymic activity and its antigenic reactivity. In both CHD-Lysoz I and CHD-Lysoz II, 10 arginine residues were modified. The two derivatives possessed identical conformations, as revealed from their optical rotatory dispersion and circular dichroism behaviors and from the availability of the disulfide bonds to reduction and susceptibility to chymotryptic attack, but were greatly unfolded relative to lysozyme. The two derivatives had no enzymic activity and very little antigenic reactivity with antisera to lysozyme. Conversely, with antisera to CHD-Lysoz I, lysozyme reacted only partially (60 per cent), and these sera showed appreciable (28 per cent) cross-reaction with CHD-human serum albumin. Interpretation of the enzymic and immunochemical data was complicated by the fact that too many (10) arginine residues were modified and that appreciable conformational changes were present. Preparation of a derivative with a least number of modified arginine residues was therefore desirable, and this was afforded by reaction of lysozyme with phenylglyoxal (PG-Lysoz). In PG-Lysoz, one arginine residue was modified and it was located at position 61. The derivative had identical optical rotatory dispersion and circular dichroism parameters with those of lysozyme. However, minor conformational changes were present, and these were only revealed by the slight increase in disulfide reducibility (0.26 bonds) and by a small increase in susceptibility to tryptic attack (from 0.16 bonds in lysozyme to 0.97 bonds in PG-lysozyme). The enzymic activity of PG-Lysoz was suppressed slightly (to 83 per cent) but the pH optimum was unaltered. With antisera to lysozyme, PG-Lysoz showed equal antigenic reactivity to that of lysozyme. Conversely, with antisera to PG-Lysoz, the reactions of lysozyme and PG-Lysoz were identical. Also, the two proteins had equal reactivities with antisera to CHD-Lysoz I (58–60 per cent relative to homologous antigen).

It was, therefore concluded that arginine-61 is not located in an antigenic site of lysozyme. Also, the findings demonstrate that not all conformational changes will influence antigenic reactivity. The change in the latter will be dependent on the protein and on the nature of the conformational reorganization.

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INTRODUCTION

It is essential, for the complete elucidation of the antigenic structure of globular proteins, to study a variety of long and overlapping peptides obtained from various regions in the molecule. The exhaustive mapping out of the antigenic structure of sperm whale myoglobin was possible only by the extensive utilization of this approach (Atassi, 1967a; Atassi and Saplin, 1968; Atassi and Singhal 1970a, b; Singhal and Atassi, 1970), together with the preparation of a number of specifically modified proteins and peptides (Atassi, 1967b, 1968, 1969; Atassi and Caruso, 1968; Atassi and Thomas, 1969). With lysozyme, it has not been possible to employ this approach due to the complication that lysozyme is a 'tight' protein completely inaccessible to proteolytic attack, although some success has been obtained with pepsin (Fujio *et al.*, 1968a, b; Shinka *et al.*, 1967). In the absence of a better alternative, various investigators are, therefore, studying the unfolded preparation in which the disulfides have been reduced and carboxymethylated. However, native lysozyme and the unfolded, reduced-carboxymethylated derivative are immunochemically unrelated (Gerwing and Thompson, 1968; Young and Leung, 1970). In our work, we have been primarily interested in the elucidation of the antigenic structure of the *native* protein and its relationship to the three-dimensional structure as we have accomplished for myoglobin. Accordingly specifically modified derivatives are being prepared and their conformation, immunochemistry and enzymic properties studied. In previous communications we reported on the role of the tyrosine (Atassi and Habeeb, 1969; Atassi *et al.*, 1971), tryptophan (Habeeb and Atassi, 1969) and lysine residues (Habeeb and Atassi, 1970, 1971). We wish to report now on the role of the arginine residues.

Specific modification of the guanido group of arginine can be accomplished by condensation with 1,2-cyclohexanedione (Toi *et al.*, 1967) or with phenylglyoxal (Takahashi, 1968). This paper describes the reaction of lysozyme with CHD and with PG¹ under a variety of conditions so that the preparation of the least drastically modified derivative is achieved. The conformation of these derivatives, together with their immunochemistry and their enzymic behavior, are exhaustively studied.

MATERIALS AND METHODS

Materials

Lysozyme (three times crystallized) was obtained from Sigma Chemical Co. and was found to be homogeneous by starch gel, acrylamide gel and disc electrophoresis. *Micrococcus lysodeikticus* cells were freeze-dried vials from Worthington Biochemical Corp. CHD was from Baker Chemical Co. (Phillipsburg, N.J.) and PG from Koch-Light Laboratories Ltd. Trinitrobenzene sulfonic acid was from Eastman Organic Chemicals; 5,5'-dithiobis (nitrobenzoic acid) from Aldrich Chemical Co. Trypsin-TPCK (twice crystallized) and α -chymotrypsin (three times crystallized) were from Worthington Biochemical Corp. Crystalline human serum albumin was purchased from Calbiochem.

Reaction of lysozyme with 1,2-cyclohexanedione

Reaction was carried out under two different conditions.

A. *CHD-Lysoz I*. This was prepared by reaction in 0.1 *N* NaOH. A 10 molar excess of CHD per arginine residue was used. The procedure has been described elsewhere (Atassi and Thomas, 1969) for modification of arginine residues in apomyoglobin. To take into account the effect of alkaline treatment on lysozyme, a control sample (100 mg) was prepared in which lysozyme was subjected exactly to all the conditions and manipulations used in the foregoing preparation except that in this case CHD was not added.

B. *CHD-Lysoz II*. This preparation was carried out in triethylamine. Lysozyme (200 mg) was dissolved in 0.1 *M* triethylamine (20 ml). To this was added a solution (5 ml) of CHD (100 mg) in 0.1 *M* triethylamine. The reaction mixture (pH 10.9) was stirred magnetically at room temperature for 16 hr, after which it was adjusted to pH 8.0 with 1 *N* HCl, dialyzed extensively against distilled water and freeze-dried. A control sample (100 mg) of lysozyme was prepared in a similar manner, except that in this case CHD was not added.

Reaction of human serum albumin with 1,2-cyclohexanedione

This was carried out under the conditions employed for CHD-Lysoz I. Amino acid analysis of duplicate acid hydrolysates of human serum albumin and CHD-HSA, showed that 15 arginine residues were modified by reaction with CHD.

Reaction of lysozyme with phenylglyoxal

In order to achieve the preparation of a homogeneous derivative modified at the minimum possible number of arginine residues, reaction was first carried out on an analytical scale under a variety of conditions. These experiments are described under 'Results.' For the preparative scale reaction, lysozyme (500 mg) was dissolved in 0.02 *M* *N*-ethylmorpholine-acetate buffer at pH 8.0 (30 ml). An aliquot of phenylglyoxal solution (220 mg in 8.0 ml) in the same *N*-ethylmorpholine-acetate buffer was added. The reaction mixture was stirred magnetically at 0°C for 50 min after which it was filtered on a column (70 × 2.5 cm) of Sephadex G10 which was eluted with water at 0°C. The effluent fractions (3 ml each) comprised two ultraviolet absorbing peaks, the first one being the protein peak and the second one containing phenylglyoxal. Fractions containing the protein peak were combined, dialyzed extensively against distilled water and then freeze-dried.

Determination of enzymic activity

Enzymic activity of lysozyme and derivatives was determined from the rate of lysis of *Micrococcus lysodeikticus* (Atassi and Habeeb, 1969). Variation of enzymic activity with pH was performed in 0.066 *M* phosphate buffers, and activity was expressed in terms of per cent relative to maximum activity of lysozyme at pH 6.2 as 100 per cent.

*Abbreviations: CHD, 1,2-cyclohexanedione; PG, phenylglyoxal; CHD-Lysoz, lysozyme modified at ten arginine residues with CHD; PG-Lysoz, lysozyme modified at one arginine residue with PG; CHD-HSA, human serum albumin modified at 15 arginine residues.

Evaluation of conformational changes

The accessibility of the disulfide bonds to reduction with 2-mercaptoethanol, in native lysozyme and derivatives was determined as described in detail elsewhere (Atassi and Habeeb, 1969; Habeeb, 1966, 1967a). Susceptibility of peptide bonds to tryptic or chymotryptic attack was determined on lysozyme and derivatives *without* reduction and carboxymethylation of the disulfide bonds (Atassi and Habeeb, 1969).

Optical rotatory dispersion and circular dichroism studies were carried out on solutions of proteins in water containing 0.07–0.15 mg per ml. Measurements were made with a Cary Model 60 Spectropolarimeter, equipped with a Model 6001 circular dichroism accessory. Experimental procedure and quantitative treatment of data have been described in detail elsewhere (Atassi and Singhal, 1970b). Optical rotatory dispersion data are reported in reduced mean residue rotation $[m']$, corrected for the refractive index dispersion of water, n . The values of the mean residue molecular weight employed in the present work for lysozyme and derivatives are shown in Table 1. The Moffitt–Yang parameter, b_0 , was calculated using their equation (Moffitt and Yang, 1956) and taking λ_0 as 212 nm. In analogy to $[m']$, CD data are given here as reduced molar ellipticities, $[\theta']$, by correcting for the refractive index dispersion of water n . Units of $[\theta']$ are in deg. cm² per d-mole.

Antisera

Procedures for preparation of antisera in rabbits (Atassi and Habeeb, 1969) and in goats (Atassi, 1967b) have already been described. Goat antisera G9 and G10 were against lysozyme. The following antisera were raised in rabbits and were against: HM, lysozyme; HD, CHD-Lysoz and HG, PG-Lysoz. Antisera from individual animals were kept separate and stored in 8 ml portions in the frozen state at –40°C.

Analytical methods

Starch gel electrophoresis was in 0.02 M sodium phosphate buffer at pH 8.0 (Atassi and Habeeb, 1969). Disc electrophoresis was performed in 7.5 per cent acrylamide gel using Tris–glycine buffer, pH 8.9 (Habeeb, 1969). Sedimentation experiments were carried out at 20°C and 59,780 rev/min, in sodium phosphate buffer (pH 7.5 and ionic strength of 0.1), in a Spinco, model E ultracentrifuge using a synthetic boundary cell. Three protein concentrations were used for the calculation of $S_{20,w}^0$. The extent of amino group modification was determined by the trinitrobenzene sulfonic acid method (Habeeb, 1967b) and by reaction with fluorodinitrobenzene (Atassi and Saplin, 1966). Protein samples were subjected to acid (Atassi and Habeeb, 1969) or alkaline (Ray and Koshland, 1962) hydrolysis and assayed on a Bio-Cal BC 200 amino acid analyzer. Tryptic hydrolysis of reduced-carboxymethylated lysozyme, or derivatives, was done as described elsewhere (Atassi and Habeeb, 1969). Peptide maps (Atassi and Saplin, 1968) were treated with specific stains (Easley, 1965) for various amino acids. Double diffusion in 1 per cent agar was by the method of Ouchterlony (Ouchterlony, 1949). Precipitin and absorption experiments have already been described in detail (Atassi and Saplin, 1968; Atassi and Habeeb, 1969).

Determination of protein concentration

Concentrations of protein solutions were determined from their nitrogen contents. Nitrogen analyses were carried out by a micro-Kjeldahl procedure (Markham, 1942) and by using Nessler's reagent standardized with ammonium sulfate. Three replicate analyses were done on each solution and they varied ± 0.3 per cent. The nitrogen contents of lysozyme and derivatives were calculated from their amino acid composition and are shown in Table 1.

Table 1. Molecular weights, mean residue weights and nitrogen contents of lysozyme and derivatives

Protein	Mol. wt.	M _R	Nitrogen Content (%) ^a
Lysozyme	14,306	110.90	18.80
PG-Lysoz	14,508	112.47	18.54
CHD-Lysoz ^b	15,246	118.19	17.64

^aValues were calculated from the amino acid composition of these derivatives.

^bCHD-Lysoz denotes both CHD-Lysoz I and CHD-Lysoz II since they possessed identical amino acid compositions.

RESULTS

Reaction of lysozyme with cyclohexanedione and characterization of the derivatives

Reaction of lysozyme with CHD was performed under two different conditions. This was due to our finding that treatment of lysozyme with 0.1 *N* NaOH, usually employed for reaction of proteins with CHD, resulted in complete inactivation of the enzyme and that this complication is avoided by reaction in 0.1 *M* triethylamine. Reaction of lysozyme with CHD in 0.1 *N* NaOH or in 0.1 *M* triethylamine yielded apparently identical derivatives that had $S_{20,w}^0 = 2.285$ and gave essentially a single positively charged band, with some slight trailing, in starch gel or disc electrophoresis. The trailing disappears entirely on electrophoresis in the dissociating solvent of sodium dodecyl sulfate–sodium phosphate at pH 7.2 (Maizel, 1966). The two derivatives had 10.0 modified arginine residues each, and they also possessed identical enzymic and immunochemical properties. Attempts to prepare partially modified preparations were complicated by the aggregation, so that heterogeneity may have been from differently modified derivatives as well as from their aggregates. A different modification reaction was therefore more desirable and this was accomplished with phenylglyoxal.

Reaction of lysozyme with phenylglyoxal and characterization of the derivative

Preliminary experiments were first carried out on an analytical scale in order to determine conditions most suitable for specificity and that will yield homogeneous preparations modified at the minimum number of arginine residues. Reaction was examined on 10 mg of lysozyme in 0.02 *M* *N*-ethylmorpholine-acetate buffer (1 ml) at pH 8.0 under two different temperature conditions (i.e. room temperature and 0°C). For the reaction at room temperature, 1% PG (1 ml) was used and in reaction at 0°, 0.5% PG (1 ml) in the same buffer was

employed. Aliquots were removed from each reaction mixture at intervals of 15, 30 and 60 min, passed through a column (10×1 cm) of Sephadex G10 and examined by starch gel electrophoresis and amino acid analysis after acid hydrolysis. Reaction at room temperature was unsatisfactory giving preparations that were too extensively modified. Only aliquots removed from the 0°C reaction at 30 or 60 min gave a homogeneous reaction product and had 0.9 and 1.05 modified arginine residues respectively. Large scale preparations were, therefore, carried out under these conditions for about 1 hr.

The large scale preparation gave, in starch gel electrophoresis, essentially a single band which migrated with a mobility of 0.90 (relative to lysozyme = 1.00), but some trailing was present. In disc electrophoresis, the trailing took the form of discreet minor bands (three or four). The multiple banding disappeared completely in phosphate-sodium dodecyl sulfate buffer indicating that the observed heterogeneity is due to tendency of PG-Lysoz to aggregate and that these aggregates dissociate in the presence of sodium dodecyl sulfate.

Amino acid analysis of PG-Lysoz showed that 1.06 arginine residues had reacted with phenylglyoxal and no other amino acids were modified. However, the possibility of amino group modification had to be taken into account (Takahashi, 1968). Determination of free amino groups by the trinitrobenzene-sulfonic acid method (Habeeb, 1967b) or by reaction with fluorodinitrobenzene, followed by amino acid analysis (Atassi and Saplin, 1966) showed that 99.3–100 per cent of amino groups were free, confirming that the reaction here with PG was entirely specific for arginine.

Location of the modified arginine residue was determined by peptide mapping of tryptic hydrolysates of reduced, S-Carboxymethylated PG-Lysoz and compared with the corresponding digests from lysozyme. Staining with ninhydrin showed little or no difference between the peptide patterns of lysozyme and PG-Lysoz. One spot corresponding most likely to sequence 62–68 (by comparing these maps with the pattern reported by Canfield (1963a) was only present in traces (and only in heavily loaded maps) in PG-Lysoz. The disappearance of peptide 62–68 might suggest the modification of either arginine-61 or arginine-68. However, peptide 69–73 was present in PG-Lysoz maps with an equal intensity to the corresponding peptide in Lysozyme. Peptide 46–61 does not stain with ninhydrin (Canfield, 1963a) but carries tyrosine at position 53. Staining with Pauly stain for tyrosine should therefore, reveal this peptide. The results are shown in Fig. 1. On staining with Pauly stain for tyrosine and histidine, lysozyme maps showed three spots: A, B_I and C. From a previous report (Atassi and Habeeb, 1969), peptide A was shown to correspond to sequence 22–33 and peptide C to sequence 15–21. Therefore, peptide B_I, which carries the third tyrosine of lysozyme, must correspond to sequence 46–61. In PG-Lysoz the position of peptide B_I changes to position B_{II} (Fig. 1). In addition, peptide B_{II} was cut out from ten peptide maps that had been lightly stained with Pauly's reagent. The spots were extracted with 0.1 M acetic acid and the extract was cleared by centrifugation and freeze-dried. The residue was dissolved in 0.1 M acetic acid (0.5 ml), applied onto a column (1×10 cm) of Sephadex G25 and the first ultraviolet absorbing material (emerging in the void volume) was freeze-dried. The residue was dissolved in 1 ml of constant boiling HCl, hydrolysed and its amino acid

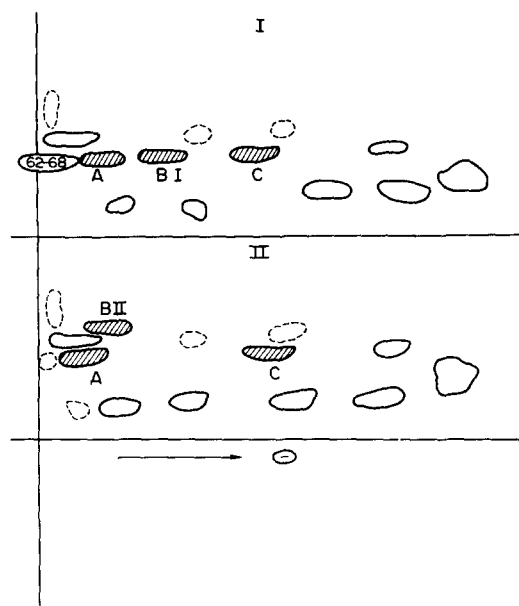


Fig. 1. Tracing of the peptide maps of Lysozyme (I) and PG-Lysoz (II). The maps show only the Sakaguchi positive and the Pauly positive (shaded) spots. Spot A reacts with Pauly stain only. All the other spots give a positive reaction with Sakaguchi stain for arginine. Note that peptide 62–68 and peptide B_I, corresponding to sequence 46–61, disappear in PG-Lysoz. A new spot B_{II}, corresponding to sequence 46–68 appears in this derivative.

composition determined. It had the following composition (based on Gly = 3.0): Trp, not determined; Arg, 1.15; CM-Cys, 0.96; Asp, 5.98; Thr, 1.86; Ser, 1.89; Glu, 1.01; Gly, 3.0; Ile, 1.62; Leu, 0.85; Phe, 0.10; and Tyr, 0.31. By comparison of this composition with the amino acid sequence of lysozyme (Canfield, 1963b), the most likely location for this peptide is sequence 46–68. These findings lead to the conclusion that arginine-61 had been modified in PG-Lysoz.

Conformation of the derivatives

The accessibility of the disulfide bonds to reduction in the CHD derivatives is shown in Table 2, together with susceptibility to hydrolysis by α -chymotrypsin. An appreciable increase in the accessibility of the disulfide bonds was obtained in CHD-Lysoz I and in CHD-Lysoz II. Susceptibility to chymotryptic attack was even more greatly increased. Lysozyme controls, pretreated with 0.1 *N* NaOH, showed some conformational changes (evidenced by a slight increase in disulfide reducibility and in susceptibility to hydrolysis with chymotrypsin) while those pretreated with 0.1 *M* triethylamine suffered no conformational changes. In PG-Lysoz, the disulfide reducibility was somewhat increased (0.26 bonds) and it was also slightly more susceptible to tryptic attack (0.97 bonds) than native lysozyme (0.14 bonds). Reaction conditions that were used for the preparation of PG-Lysoz did not cause any change in these parameters in control preparations of lysozyme. Table 2 summarizes the results for PG-Lysoz.

Table 2. Enzymic activity, disulfide reducibility and susceptibility to proteolytic hydrolysis of lysozyme and its derivatives

Protein	Enzymic activity (%)	No. of reducible disulfides ^a	Susceptibility to α -chymotrypsin ^b	Tryptic susceptibility ^b
Lysozyme	100	0.02	0.16	0.14
Lysozyme pretreated with 0.1 N NaOH	0	0.25	1.40	—
CHD-Lysoz I	0	1.85	11.03	—
Lysozyme pretreated with 0.1 M triethylamine	100	0.02	0.09	—
CHD-Lysoz II	0	1.73	10.92	—
Control Lysozyme ^c	100	0.02	—	0.14
PG-Lysoz	83	0.26	—	0.97

^aValues are obtained after 3 hr reduction with 2-mercaptoethanol.

^bAlkali consumption values (in mole NaOH/mole protein) were determined after leveling off at 8 hr.

^cThis denotes a preparation of lysozyme which had been subjected to all the conditions used in the preparation of PG-Lysoz, except that in this case PG was not added. For experimental details, see text.

In optical rotatory dispersion measurements, the rotatory behavior of PG-Lysoz was identical with that of native lysozyme (Table 3). The two CHD derivatives had identical optical rotatory dispersion parameters (Table 3), but they were appreciably less rotatory than lysozyme. The rotatory behaviors of lysozyme controls were indistinguishable from that of native lysozyme.

In circular dichroism studies, the $[\theta']$ values at 208 nm and at 220 nm for PG-Lysoz and for lysozyme were identical (Table 3). The CHD derivatives showed lower ellipticity values. CHD-Lysoz I and CHD-Lysoz II had similar circular dichroism behavior (Table 3). Lysozyme controls for the present reaction conditions had circular dichroism parameters identical with those of native lysozyme.

Table 3. Optical rotatory dispersion and circular dichroism parameters of lysozyme and derivatives^a

Protein	ORD Parameters			CD Parameters	
	$[m']_{233}$	$[m']_{199}$	b_0	$[\theta']_{221}$	$[\theta']_{208}$
Lysozyme	$-4,170 \pm 140$	$+16,500 \pm 500$	-205 ± 5	-7080 ± 300	-8990 ± 400
PG-Lysoz	$-4,090 \pm 100$	$+16,170 \pm 550$	-196 ± 8	-7000 ± 350	-8870 ± 410
CHD-Lysoz I	$-2,456 \pm 70$	$+11,940 \pm 400$	-125 ± 6	-6160 ± 280	-7470 ± 360
CHD-Lysoz II	$-2,460 \pm 85$	$+12,010 \pm 750$	-126 ± 5	-6180 ± 290	-7490 ± 380

^aDeterminations were carried out on the protein solutions in water. Lysozyme controls subjected to the various reaction conditions, employed in the preparation of each of the three derivatives, had identical parameters to those of native lysozyme.

Enzymic activity of the derivatives

Both CHD derivatives had no enzymic activity (Table 2). Lysozyme controls which had been treated with 0.1 *N* NaOH (reaction conditions for CHD-Lysoz I) show complete loss of enzymic activity. On the other hand lysozyme controls, pretreated with 0.1 *M* triethylamine (conditions used for preparation of CHD-Lysoz II), showed complete retention (100 per cent) of the lytic activity. The enzymic activity of PG-Lysoz was studied at several pH intervals between pH 5–8, in order to determine if any change in the pH optimum had occurred as a result of modification of arginine-61. The results showed that the pH optimum for PG-Lysoz was similar to that of lysozyme (i.e. pH 6.2). The lytic activity at pH 6.2 was 83 per cent relative to that of native lysozyme. Controls subjected to the same conditions as those used in the preparation of PG-Lysoz showed intact (100 per cent) enzymic activity.

Immunochemistry of the derivatives

A. CHD-Derivatives. In agar double diffusion experiments with antisera to lysozyme, both CHD derivatives gave a single precipitin line which spurred with the line given by lysozyme. Quantitative precipitin analysis (Fig. 2 and Table 4) confirmed that both CHD derivatives reacted equally but very poorly with antisera to lysozyme. It is relevant to mention that both control lysozyme preparations exhibited antigenic reactivities which were identical with the reactivity of native lysozyme.

Antisera against CHD-Lysoz I gave, in agar double diffusion, single precipitin lines with all the present derivatives. However, the lines due to lysozyme and

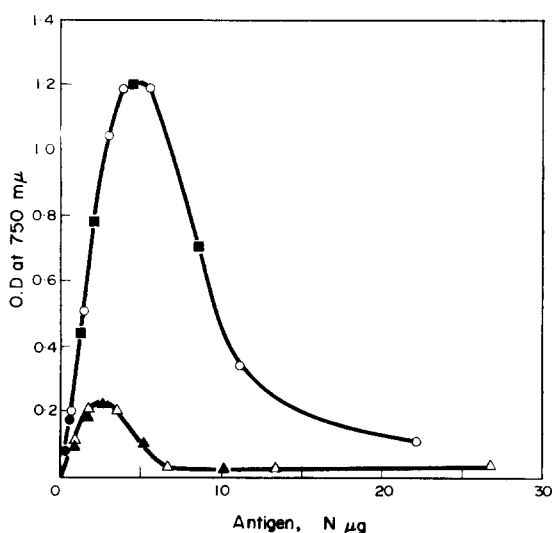


Fig. 2. Quantitative precipitin reaction of goat antiserum G10 against lysozyme with: ○, lysozyme; ■, alkaline-pretreated lysozyme control; △, CHD-Lysoz I and ▲, CHD-Lysoz II. Note that treatment with 0.1 *N* NaOH (conditions used for the preparation of CHD-Lysoz I) does not affect the antigenic reactivity of lysozyme. For experimental details, see text. The antiserum was diluted 1:3 with 0.15 *M* NaCl before use in this reaction.

PG-Lysoz were weaker than, and also formed a spur with, the CHD-Lysoz line, the spur being a continuation of the latter. In quantitative precipitin analysis with these antisera, equal but lower reactivities were obtained with lysozyme and PG-Lysoz, relative to the homologous reaction (Fig. 3). With these antisera, CHD-Lysoz I and CHD-Lysoz II possessed equal reactivities. It is significant that CHD-human serum albumin showed appreciable reaction (28 per cent, relative to homologous antigen) with antisera to CHD-Lysoz I. Also, absorption of these sera with CHD-HSA resulted in the removal from the sera of 30 per cent of their reactivity with the homologous antigen. The immunochemical results are summarized in Table 4.

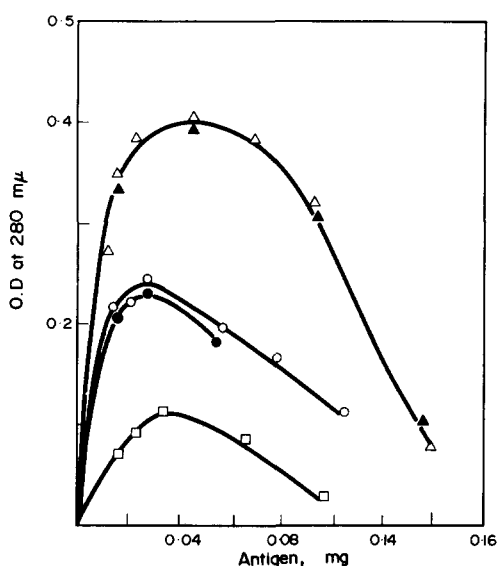


Fig. 3. Precipitin reaction of antiserum to CHD-Lysoz I with: Δ , CHD-Lysoz I; $\blacktriangle$, CHD-Lysoz II; $\circ$, Lysozyme; $\bullet$, PG-Lysoz; $\square$, CHD-HSA.

B. PG-Lysoz derivative. With antisera to lysozyme this derivative gave, in agar double diffusion experiments, a single line which fused completely with the line due to lysozyme showing no spurs or intersections. In quantitative precipitin analysis, it had equal reactivity to that of the native protein. Figure 4 shows an example of the precipitin reaction of PG-Lysoz with goat antiserum G10 and the reactivity of this derivative with several antisera to lysozyme is shown in Table 4. It can be seen that, with each antiserum, lysozyme and PG-Lysoz showed identical reactivities. Also, absorption of the sera with PG-Lysoz removes all (100 per cent) the reactivity of these sera with lysozyme and absorption with lysozyme removes reactivity with PG-Lysoz.

With antiserum to PG-Lysoz, in agar double diffusion, lysozyme and PG-Lysoz showed lines of complete identity. Also in quantitative precipitin analysis with this antiserum, lysozyme and PG-Lysoz were indistinguishable (Figure 5). The two CHD derivatives reacted poorly with this antiserum (19–20 per cent relative to PG-Lysoz). The results are summarized in Table 4. Absorption of these sera with lysozyme removes reactivity with PG-Lysoz entirely.

Table 4. Immunochemical studies with antisera to lysozyme or its derivatives

Antiserum	Reaction with antiserum at equivalence ^a				
	Antisera to lysozyme			Anti PG-Lysoz	Anti CHD-Lysoz I
	G9	G10	HM	HG	HD
<i>Protein</i>					
Lysozyme	100	100	100	99	60
PG-Lysoz	100	99	99	100	58
CHD-Lysoz I ^b	8	19	20	19	100
CHD-Lysoz II ^b	9	21	22	20	98
CHD-HSA	0	0	0	0	28

^aValues represent percent precipitation relative to homologous antigen and are averages of three replicate analyses which varied $\pm 1.3\%$ or less.

^bControl preparations for reaction conditions of CHD-Lysoz I and CHD-Lysoz II had equal antigenic reactivities to those of native lysozyme.

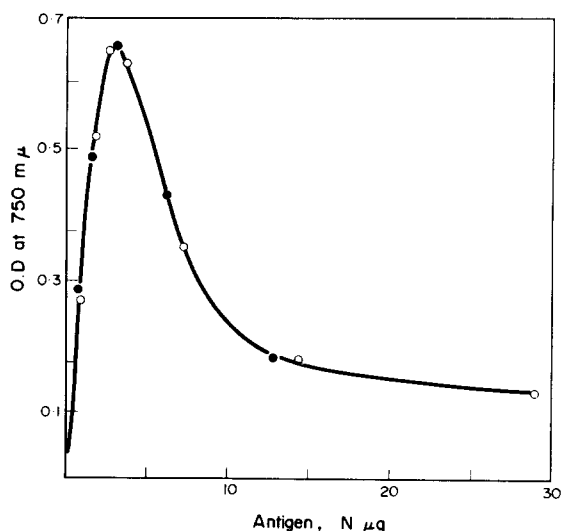


Fig. 4. Quantitative precipitin reaction of lysozyme (○) and PG-Lysoz (●) with goat antiserum G10 against lysozyme. The antiserum was diluted 1:5 with 0.15 M NaCl before use in this reaction.

DISCUSSION

Reaction of CHD with lysozyme was specific for arginine under both sets of experimental conditions employed in the present work. CHD condenses with guanido groups of arginine to give N⁵-(4-oxo-1,3-diaza spiro-[4,4] non-2-ylidene)-L-ornithine or CHD arginine (Toi *et al.*, 1967). Atassi and Habeeb (1969) have demonstrated that changes in biological activity of a modified protein are dependent upon the nature of the modification. With the present arginine modifications, the positive charge of the side chain of arginine is lost and in addition, the bulky group introduced at the site of modification will exert a considerable

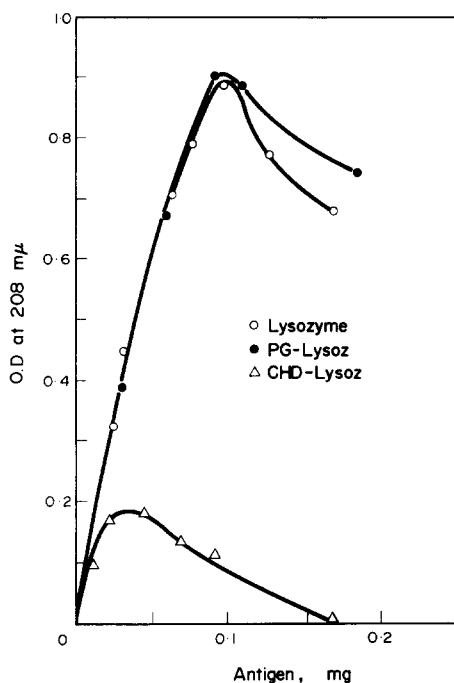


Fig. 5. Precipitin reaction of antiserum to PG-Lysoz with: ○, lysozyme; ●, PG-Lysoz and △, CHD-Lysoz I.

steric influence. These modifications will therefore be sufficient to reveal the involvement of the arginine residues in the enzymic and immunochemical properties. Takahashi (1968) reported that PG may react with ϵ -amino groups in proteins. In the present work, none of the amino groups in PG-Lysoz had reacted. Also exhaustive peptide mapping of tryptic hydrolysates of the reduced, carboxymethylated derivatives strongly confirmed that no group, other than arginine-61, had been modified in this derivative. It is interesting that arginine-61 reacted preferentially with PG, especially in view of the fact that of the other ten arginines, eight are completely exposed (Blake *et al.*, 1965, 1967).

Appreciable conformational changes were observed upon modification of ten arginine residues with CHD. These conformational changes were similar and independent of the conditions employed in the preparation of the two CHD derivatives. Modification of lysozyme at arginine-61 with PG induced some slight conformational changes that were completely undetectable by optical rotatory dispersion and circular dichroism measurements and they were only revealed by the slight increase in disulfide reducibility and susceptibility to tryptic attack. The insensitivity of the rotatory behavior of lysozyme to small changes in conformation has been previously reported (Habeed and Atassi, 1970).

Complete loss of enzymic activity upon modification of ten arginine residues might have resulted from conformational changes. However, it seems possible that arginine-114 is present at the binding site of the enzyme (Blake *et al.*, 1967). The small decrease (17 per cent) in enzymic activity obtained upon modification

of arginine-61 must have been caused by the slight conformational changes and steric hinderance at the binding site. Arginine-61 is adjacent to tryptophan-62 (Canfield, 1963b) which is present at the binding site of the enzyme (Blake *et al.*, 1967; Hayashi *et al.*, 1965). Recently, the modification of some arginine residues in lysozyme was reported (Davies and Neuberger, 1969), by reaction with 2,3-butane-dione and the preparation was 25 per cent as active as the native protein. However, the homogeneity of this preparation, the specificity of the modification reaction, and the locations of the modifications were not determined by these authors. Also, no studies were carried out to investigate any conformational changes resulting from the modification.

The decrease in antigenic reactivity observed with the CHD derivatives could suggest the involvement of one (or more) of the arginine residue(s) in the interaction of lysozyme with its antibodies. However, conclusions are complicated by the presence of conformational changes. It is now well established that conformational changes influence the antigenic reactivity of proteins (Atassi, 1967c, 1970; Atassi and Skalski, 1969; Atassi and Thomas, 1969; Habeeb, 1967c). It is relevant to point out here that antisera against the CHD derivatives exhibited, in addition to specificity against antigenic sites in lysozyme, a haptenic specificity against CHD-arginine as demonstrated by reaction of these sera with CHD-human serum albumin.

Lysozyme and PG-Lysoz were immunochemically indistinguishable in reaction with antisera to lysozyme. In addition, the two proteins exhibited equal abilities to react with antisera to PG-Lysoz, although some slight differences were observed in the region of antigen excess. Also, the reactivity of each antigen can be completely removed by absorption with the other protein. These findings, therefore, clearly demonstrate that the PG-arginine side chain in position 61 was not in an immunogenic location. Conformational differences between lysozyme and PG-Lysoz, although small, were detectable. Apparently these conformational changes did not exert an influence on the antigenic reactivity of the protein, perhaps because they were only localized. These findings demonstrate that not all conformational changes will result in a measurable effect on the antigenic reactivity. Other examples of this observation have recently been reported (Atassi and Habeeb, 1969; Atassi *et al.*, 1971). The change in antigenic reactivity will be dependent on the protein and on the nature of the conformational reorganization.

In conclusion, the present data show that modification of ten arginine residues in lysozyme with CHD induces large changes in conformation which are accompanied by complete loss of enzymic activity and a drastic decrease in antigenic reactivity. Modification of arginine-61 with phenylglyoxal results in small, and possibly localized, conformational changes and the derivative suffered only a slight loss of enzymic activity while the antigenic reactivity remained unaffected. It was therefore concluded that arginine-61 is not an essential part of an antigenic site in lysozyme.

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