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Enzymic and Immunochemical Properties of Lysozyme. Evaluation of Several Amino Group Reversible Blocking Reagents*

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ABSTRACT: Reactions of lysozyme with several reversible blocking reagents for amino groups have been studied. Derivatives obtained by complete modification of the amino groups by reaction with tetrafluorosuccinic, maleic, or citraconic anhydride or by reaction with diketene were electrophoretically heterogeneous. In each case modification of some hydroxyl groups was also obtained. Complete loss of enzymic activity accompanied the complete modification of the amino groups by any of these reagents. Removal of the maleyl blocking groups in ML_7Lys resulted in a grossly electrophoretically heterogeneous preparation, with recovery of 90% of free amino groups, 83% of enzymatic activity, and almost all the ability to react with antisera to lysozyme. Similarly removal of blocking groups in derivatives prepared by reaction with tetrafluorosuccinic anhydride or with diketene was not complete yielding, in each case, heterogeneous preparations with incomplete recovery of enzymic and immunochemical properties. With CT_7Lys, complete deblocking of amino groups was obtained at pH 4.2 in 3 hr. Deblocked derivatives could be prepared which showed little or no electrophoretic heterogeneity, and absolutely complete restoration of enzymic activity and antigenic reactivity. Conformational changes associated with complete blocking of amino groups were not large and complete reversion to native conformation was obtained on deblocking with CT_7Lys, but not with ML_7Lys. The results show that, of the reversible amino group blocking reagents studied here, citraconic anhydride is the most satisfactory.

Reversible blocking of amino groups is a valuable tool for protecting these groups from side reactions which might, in certain cases, take place during modification of some functional groups. Also, such protecting groups are useful for rendering hydrolysis with trypsin specific for cleavage at arginine residues. Many such reversible blocking reagents have been reported. However, a careful investigation of their applicability and comparison of their specificity, ease of removal, homogeneity of the blocked and deblocked derivatives, and changes in their conformation and their biological activities has not been carried out.

In the present work, lysozyme has been chosen as the protein model for such investigation. Thus the reactions of lysozyme with maleic anhydride (Butler *et al.*, 1967), citraconic anhydride (Dixon and Perham, 1968), tetrafluorosuccinic anhydride (Braunitzer *et al.*, 1968), or diketene (Marzotto *et al.*, 1967, 1968) have been studied. The specificity and reversibility of the reactions and the homogeneity of all reaction products were examined. Enzymic and immunochemical properties of the lysozyme derivatives and of lysozyme preparations obtained by removal of blocking groups are reported together with accompanying conformational changes.

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Materials and Methods

Materials. Lysozyme (three-times crystallized) was obtained from Sigma Chemical Co. Lyophilized *Micrococcus lysodeikticus* cells were in vials from Worthington Biochemical Corp.; maleic anhydride from Fisher Scientific Co.; citraconic anhydride from Eastman Organic Chemicals; tetrafluorosuccinic anhydride from K & K Laboratories, Inc.; diketene and 5,5′-dithiobis(nitrobenzoic acid) from Aldrich Chemical Co.

Reaction of Lysozyme with Maleic Anhydride and Removal of Blocking Groups. The anhydride was added as a stock solution (50 mg/ml) in dioxane to a solution of lysozyme (2% in 0.1 M phosphate buffer, pH 8.0) and the amount varied between 0.25 and 4 mole of reagent per free amino group in order to obtain preparations with variable number of amino groups. Reaction was at room temperature for 30 min after which the proteins were dialyzed against water and freeze-dried. The maximum concentration of added dioxane during reaction was 8% which causes no changes of conformation of lysozyme (Hamaguchi and Kurono, 1963).

For removal of blocking groups, ML_7Lys[1] (20 mg) was uniformly dispersed in 4 ml of 0.05 M acetate buffer (pH 3.5) and the suspension was stirred magnetically at 37°. At intervals, a 0.75-ml sample was removed and diluted with 4.25 ml of 0.06 M sodium phosphate buffer containing 0.9% NaCl (pH 6.2). Samples from this solution were used to determine free amino groups by TNBS, enzymic activity and for disc electrophoresis. A control was performed with lysozyme at pH 3.5 and 37° to determine the effect of conditions of incubation on enzymic activity and immunochemical reactivity with antisera to lysozyme.

Reaction of Lysozyme with Citraconic Anhydride. For reaction, lysozyme (1.96 g) was dissolved in water (50 ml) and the pH was adjusted to 8.20. Aliquots (100 μl) of citraconic anhydride were added to the magnetically stirred protein solution, at 30-min intervals. A total of 800 μl of citraconic anhydride was added. Reaction was at room temperature and a pH of 8.2 was maintained by the addition of 5 N NaOH on the pH-Stat. When addition of citraconic anhydride was complete, the reaction mixture was allowed to stir at room temperature for 2 more hr at pH 8.20. The solution was then dialyzed at 0° against several changes of water which had been preadjusted to pH 8.5–8.8 with NH_4OH and finally freeze-dried. Removal of blocking groups was studied at 40° and different pH conditions as described under Results.

Reaction of Lysozyme with Diketene and with Tetrafluorosuccinic Anhydride and Removal of Blocking Groups. Conditions for reaction with diketene were similar to those described by Marzotto *et al.* (1968). Removal of the acetoacetyl blocking groups was carried out at 25° in 2% hydroxylamine-HCl (pH 7.0). Reaction with tetrafluorosuccinic anhydride and removal of the blocking groups were done according to the procedure described by Braunitzer *et al.* (1968).

Determination of Extent of Modification of Amino Groups. Free Amino groups were determined by trinitrobenzenesulfonic acid (Habeeb, 1966a, 1967a). Solutions of unmodified lysozyme served as standard and contained 100% free amino groups as determined by TNBS.

Determination of Esterification of Phenolic Hydroxyl Groups. The spectrophotometric method of Riordan and Vallee (1964) which depends on lability of ester linkage to hydroxylamine was used. A solution of ML_7Lys and CT_7Lys (0.7 mg/ml) in 0.06 M borate buffer (pH 7.5) was treated with an equal volume of 0.2 M hydroxylamine-HCl preadjusted to pH 7.5. Absorbance of treated and untreated modified lysozymes were measured at 280 mμ at intervals of 0–18 hr.

Determination of Esterification of Hydroxyamino Acids in Modified Lysozyme. The number of ester bonds in derivatives was determined by hydroxylamine and $FeCl_3$ as described previously (Habeeb and Atassi, 1969) using a calibration curve of glycine methyl ester hydrochloride as a standard.

In order to examine the homogeneity of the reaction product obtained by deesterification, 10 mg of derivative in 5 ml of 0.15 M NaCl was incubated at 37° with 5 ml of 2 M hydroxylamine HCl adjusted to pH 10. A 2-ml sample was removed, at intervals of 1, 2, 4, 8, and 24 hr, the pH adjusted to 7.0, and the sample was dialyzed against water followed by Tris-glycine buffer (0.043 M Tris and 0.046 M glycine (pH 8.9) and then subjected to disc electrophoresis on polyacrylamide gel. A control was included in which lysozyme was subjected to treatment with hydroxylamine followed by disc electrophoresis to evaluate any changes due to conditions of incubation.

Evaluation of Conformational Changes. Optical rotatory dispersion measurements were made with a Cary Model 60 spectropolarimeter on solutions in water containing 0.06–0.1 mg/ml. Measurements on each protein were at several concentrations employing cells with light paths of 5, 10, and 20 mm. The spectropolarimeter was purged with dry nitrogen for 30 min before scanning of each sample. Base-line scans were done before and after each protein sample. Each protein sample was scanned at least five times at each concentration and the rotations at various wavelengths were the average from these scans. Optical rotatory dispersion data are expressed in reduced mean residue rotation, $[m']$, corrected for the refractive index dispersion of water. Experimental procedure and quantitative treatment of data has been described in detail elsewhere (Atassi and Singhal, 1970). The Moffitt–Yang parameter, b_0, was calculated using their equation (Moffitt and Yang, 1956) and taking λ_0 as 212 mμ.

The susceptibility of the disulfide bonds to reduction with 2-mercaptoethanol in modified lysozyme compared to native lysozyme was used to evaluate conformational changes (Habeeb, 1966b, 1967b; Atassi and Habeeb, 1969). Accessibility of peptide bonds to tryptic hydrolysis was determined according to the procedure previously described (Atassi and Habeeb, 1969).

Analytical Methods. Disc electrophoresis was performed in 7.5% acrylamide gel using Tris–glycine buffer (pH 8.9) (0.043 M Tris and 0.046 M glycine) as described previously (Habeeb, 1969). Enzymic activity was determined from the rate of lysis of *M. Lysodeikticus* as described elsewhere (Atassi and Habeeb, 1969). Sedimentation experiments were carried out at 20° and 59, 780 rpm in a Spinco, Model E ultracentrifuge using a synthetic boundary cell. Three protein concentrations were used for the calculation of $s^0_{20,w}$. Samples were dissolved in sodium phosphate buffer (pH 7.5) and ionic

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: ML, maleyl; CT, citraconyl; TFSu, tetrafluorosuccinyl; the subscript used with these indicates the number of amino groups modified; Lys, lysozyme; TNBS, trinitrobenzenesulfonic acid.

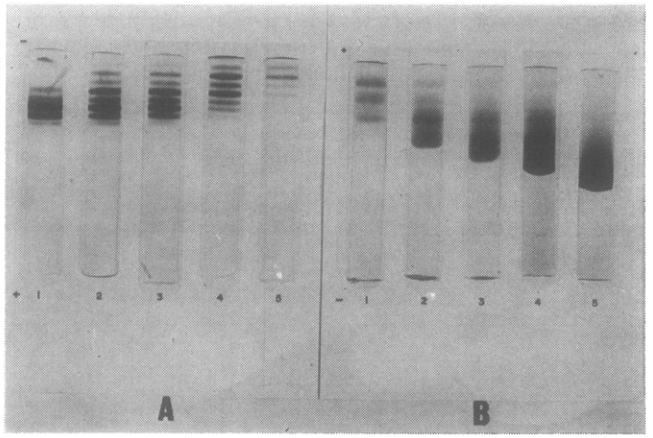

FIGURE 1: Disc electrophoretic pattern of ML_7Lys on demaleylation by incubation at 37° and pH 3.5 at various time intervals. (A) Anode at bottom of tube; 1, 0 hr; 2, 3 hr; 3, 6 hr; 4, 1 day; 5, 2 day. (B) Anode at top of tube; 1, 1 day; 2, 2 days; 3, 3 days, 4, 4 days and 5, lysozyme.

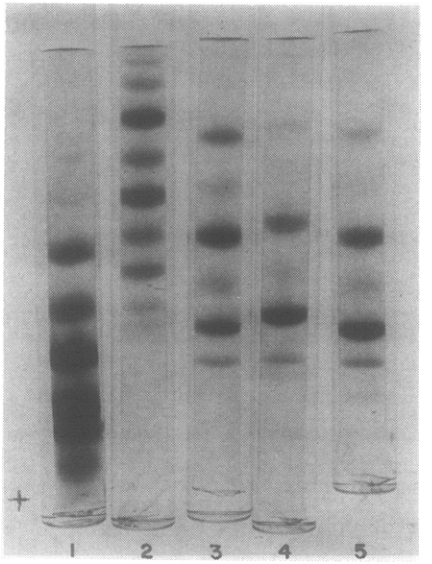

FIGURE 2: Disc electrophoresis of CT_7Lys after treatment with 1 M hydroxylamine at pH 10 for various times. 1, 0 hr; 2, 1 hr; 3, 2 hr; 4, 4 hr and 5, 8 hr.

strength 0.1. Concentrations of protein solutions were derived from their nitrogen contents determined by Kjeldahl digestion and followed by Nessler's reagent standardized with ammonium sulfate. Three replicate analyses were performed on each solution and they varied ±0.5%.

Immunochemical Methods. Preparation of rabbit antisera to lysozyme has already been described (Atassi and Habeeb, 1969). Goat antisera were prepared as described by Atassi (1967). The sera were kept separate and stored in 5-ml aliquots at −40°. Quantitative precipitin experiments have been described elsewhere (Atassi and Saplin, 1968).

Results

Derivatives Obtained by Reaction with Maleic Anhydride. Complete reaction of the amino groups of lysozyme occurred when the molar ratio of anhydride/number of free amino groups was 2 or more. Disc electrophoresis of the derivatives showed multiple discrete bands even when all seven free amino groups (six lysine residues) were maleylated (Figure 1). No O acylation of tryosine residues was detected in ML_7Lys or CT_7Lys. On the other hand, two to three residues of hydroxyamino acids were esterified in ML_7Lys. Disc electrophoresis of the product of deesterified ML_7Lys showed electrophoretic heterogeneity and decreased anodic mobility. A lysozyme control incubated for up to 24 hr in 1 M hydroxylamine HCl (pH 10) did not suffer any changes in its electrophoretic mobility.

Unmasking of the amino groups in ML_7Lys was carried out in acetate buffer at pH 3.5 and monitored by disc electrophoresis from 3 hr up to 5 days. The electrophoretic pattern of deblocked ML_7Lys showed extensive electrophoretic heterogeneity. Even after 4 or 5 days of demaleylation the electrophoretic pattern of lysozyme was approached but not recovered (Figure 1) and the recovery of free amino groups (as determined by TNBS) reached a maximum of 90% after 5 days.

Reaction of Lysozyme with Citraconic Anhydride. A. HOMOGENEITY OF THE PRODUCT AND SPECIFICITY OF REACTION. Acylation was carried out under conditions that will yield complete masking of the amino groups and this was confirmed by reaction with TNBS. The derivative obtained at this stage was homogeneous by electrophoresis on starch gel but showed appreciable heterogeneity on disc electrophoresis.

To determine the specificity of the reaction, CT_7Lys (15 mg) was dissolved in 1 M hydroxylamine HCl (10 ml) preadjusted to pH 10. Solution was allowed to stand at 40° and aliquots (2 ml) were withdrawn at intervals of 1, 2, 4, 8, and 24 hr and precipitated with 5% trichloroacetic acid, recovered on the centrifuge, dissolved in 0.5 ml of Tris–glycine buffer (pH 8.9) and dialyzed against several changes of the same buffer. Disc electrophoresis of each sample (Figure 2) showed the appearance of several less electronegatively migrating bands indicating removal of citraconyl groups due to deesterification at some hydroxy amino acids. Lysozyme controls subjected to similar treatment showed no change in electrophoretic mobility. Determination with hydroxylamine–$FeCl_3$ indicated that two to three residues of hydroxyamino acids were esterified in CT_7Lys.

B. REMOVAL OF CITRACONYL GROUPS FROM BLOCKED AMINO GROUPS. Portions of CT_7Lys (20 mg each), dissolved in 0.05 M acetate buffers (5 ml each) at pH 4.2, 5.2, or 6.4, or in 0.05 M phosphate buffers at pH 7.2 or 8.0, were incubated at 40°. Aliquots (1 ml) were removed at intervals and the free amino group content was determined by the TNBS method. Results are shown in Figure 3 and indicate that complete deblocking of amino groups was achieved within 3 hr at pH 4.2. Disc electrophoresis of recovered material showed heterogeneity which was minimal after 24 hr exposure to pH 4.2. Exposure of this deblocked preparation (*i.e.*, at pH 4.2 for 24 hr) to 1 M hydroxylamine HCl at pH 10 for 1 hr, abolished electrophoretic heterogeneity almost completely (Figure 4). However, the main band was only slightly less electropositive than lysozyme (its mobility was 0.98 relative to native lysozyme = 1.00). It is relevant to comment here on the case of removal of the citraconyl blocking group from masked amino groups. It can be seen in Figure 3 that the masking group is easily

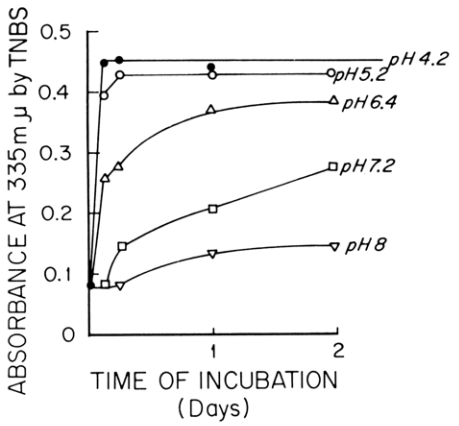

FIGURE 3: Removal of citraconyl blocking group at different pH values.

removable at pH 5.2. Unmasking at this pH leveled off in 6 hr at a maximum of 6.7 amino groups deblocked per mole of lysozyme. At pH 6.4 deblocking proceeded at a slower rate, but in 6 hr an average number of 4 amino groups was unmasked. After 2 days at pH 6.4 an average number of 5.9 amino groups was unmasked. Of major interest, however, is the finding that citraconyl blocking groups are removed at neutral pH at quite an appreciable rate. Even at pH 8.0, the rate of removal of the masking group was still detectable on prolonged durations of exposure to this pH at 40° (Figure 3). Therefore, the best procedure for storage of citraconyl proteins was in the freeze-dried state at 0° or lower. No unmasking of amino groups was observed on long (several months) standing in the freeze-dried state.

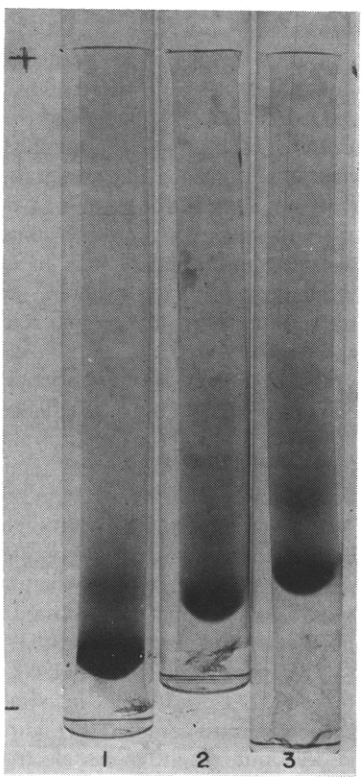

FIGURE 4: Disc electrophoresis pattern of acid (pH 4.2) deblocked CT_7Lys after treatment with 1 M hydroxylamine HCl (pH 10). 1, Lys; 2, deblocked CT_7Lys treated with hydroxylamine, and 3, deblocked CT_7Lys.

TABLE I: Optical Rotatory Dispersion Parameters of Lysozyme Derivatives with Masked or Unmasked Amino Groups.

Protein	$[m']_{233}$	b_0
Lysozyme	−4080 ± 50	−205 ± 5
ML_7Lys	−4140 ± 90	−188 ± 10
Deblocked ML_7Lys	−4040 ± 80	−187 ± 10
CT_7Lys	−3930 ± 100	−228 ± 12
Deblocked CT_7Lys	−4050 ± 100	−203 ± 4

Comparison of Physical and Biological Parameters to Evaluate Reversibility of Maleyl and Citraconyl Groups. The suitability of a reversible blocking reagent cannot be based only on the extent of recovery of free amino groups and on the homogeneity of the preparations upon deblocking. Other parameters were therefore studied and the results on ML_7Lys and CT_7Lys will be outlined.

A. EVALUATION OF CONFORMATIONAL CHANGES. In optical rotatory dispersion measurements all samples showed negative rotation with a minimum at 233 mμ. The rotatory behaviors of ML_7Lys, CT_7Lys, and their corresponding deblocked derivatives were quantitatively similar (Table I).

Conformational changes can also be investigated by the accessibility of the disulfide bonds to reduction with mercaptoethanol. Lysozyme has no reducible disulfide bonds in neutral solutions (Atassi and Habeeb, 1969). The accessibility of the disulfide bonds in each of ML_7Lys and CT_7Lys was appreciably increased (Table II). Conformational changes were also determined by susceptibility of the derivatives to trypsin. The results are summarized in Table II. In each of ML_7Lys and CT_7Lys accessibility to tryptic attack was greatly increased. On removal of the blocking groups in ML_7Lys, the resulting preparation was slightly accessible to trypsin whereas deblocking of CT_7Lys yielded a preparation which was completely inaccessible to tryptic attack. These chemical methods therefore strongly suggest that conforma-

TABLE II: Conformational Studies on Lysozyme and Derivatives by Chemical Methods.

Protein	No. of Reducible Disulfide Bonds	Susceptibility to Trypsin. Consumption of NaOH (Mole/Mole of Protein)
Lysozyme	0.03	0.15
ML_7Lys	0.33	7.83
CT_7Lys	0.88	8.45
Deblocked ML_7Lys	0.11	1.80
Deblocked CT_7Lys	0.04	0.13

TABLE III: Antigenic Reactivity of Blocked and Deblocked Lysozyme Derivatives.

	Reactivity of Equivalence[a]				
Antiserum	Lysozyme	ML_7Lys	CT_7Lys	Deblocked ML_7Lys	Deblocked CT_7Lys[b]
G9	100	51	42	94	99
G10	100	49	46	95	100
HM	100	55	40	98	100

[a] Values are expressed in per cent precipitation relative to homologous reaction. [b] The antigenic reactivity of lysozyme controls subjected to these conditions of deblocking was identical with that of the native protein.

tional changes take place upon complete blocking of amino (and other) groups with maleyl or citraconyl groups. In the case of CT_7Lys, the protein acquires its native conformation completely upon deblocking. It is relevant to mention here that these conformational changes were not too clear from the optical rotatory dispersion measurements which confirms that the rotatory behavior of lysozyme is not too sensitive to conformational changes (Atassi *et al.*, 1970).

B. SEDIMENTATION COEFFICIENTS OF MODIFIED LYSOZYMES. Each of ML_7Lys and CT_7Lys showed a single sharp boundary indicative of a monodisperse system. The values for $s^0_{20,w}$ were: ML_7Lys, 2.15 S; and CT_7Lys, 2.09 compared to a value of 1.91 S for lysozyme (Sophianopoulos *et al.*, 1962). Aggregation, therefore, did not accompany these modifications.

C. ENZYMIC ACTIVITY OF BLOCKED AND DEBLOCKED DERIVATIVES. There was almost 95% loss of enzymic activity accompanying modification of an average of four amino groups. Retrieval of enzymic activity on deblocking of ML_7Lys is shown in Figure 5. A maximum of 83% of the enzymic activity was recovered when 90% of the amino groups were demaleylated. The extreme heterogeneity of the products preclude any definite conclusion on the relationship between free amino groups and enzymic activity. It is conceivable that the active species may represent only a fraction of the whole population.

Little or no enzymic activity (2–4%) could be detected in CT_7Lys. On deblocking, the enzymic activity was completely recovered, in contrast to 83% recovery obtained on demaleylation of ML_7Lys (Figure 5). Lysozyme subjected to conditions of demaleylation showed no changes in electrophoretic mobility, enzymic activity, or its ability to react with antisera to lysozyme.

D. IMMUNOCHEMISTRY OF THE DERIVATIVES. The efficiency of removal of various blocking groups was also monitored immunochemically. ML_7Lys reacted between 49 and 55% with antisera to lysozyme and upon removal of the blocking groups the antigenic reactivity increased to 94, 95, and 98% with antisera to lysozyme G9, G10, and HM, respectively. The reactivity of CT_7Lys was 40–46% with antisera to lysozyme. Upon deblocking the reactivity was recovered to 99–100% relative to the homologous reaction. The results are summarized in Table III.

Derivatives obtained with Tetrafluorosuccinic Anhydride and with Diketene. Lysozyme modified with tetrafluorosuccinic anhydride showed a great degree of electrophoretic heterogeneity. $TFSu_7Lys$ was esterified at two to three hydroxyl groups, had no enzymic activity, and its antigenic reactivity with antisera to lysozyme was 42–48%. Deblocking for 4 days gave electrophoretically heterogeneous preparations with an average of 4.6 unmasked amino groups, 64% enzymic activity, and reacted 73–78% with antisera to lysozyme.

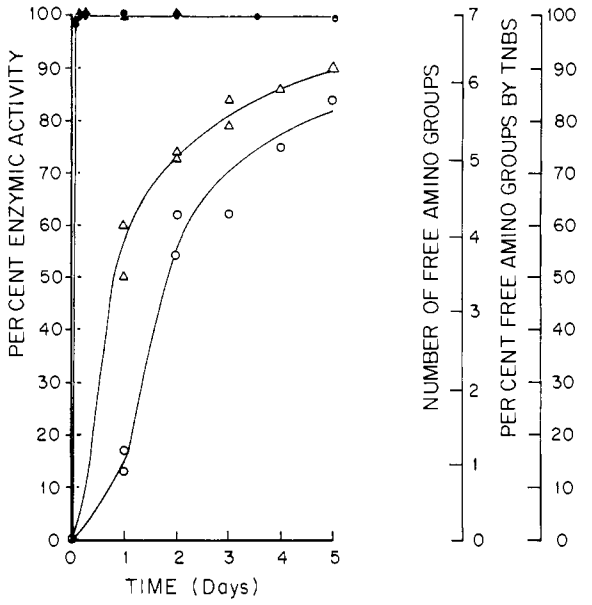

FIGURE 5: Recovery of free amino groups and enzymic activity as a function of time of deblocking of ML_7Lys (open symbols) and CT_7Lys (closed symbols). (○,●) Enzymic activity and (△,▲) free amino groups.

Complete blocking of amino groups with diketene gave a preparation that was also modified at five to six hydroxyl groups, exhibited high electrophoretic heterogeneity, no enzymic activity, and reacted 39–44% with antisera to lysozyme. Deblocking for 3 days resulted in heterogeneous preparations that had an average of 5.6–5.9 unmasked amino groups, 82–89% enzymic activity, and reacted 78–81% with antisera to lysozyme.

Due to the incomplete deblocking, the extreme heterogeneity of deblocked preparations, and because enzymic and immunochemical properties were not completely restored, no further investigations were performed on these derivatives.

Discussion

It is necessary to mention at the outset that the present work shows that none of the reagents used here possessed absolute specificity for amino groups. No tyrosine residues were modified with maleic and citraconic anhydrides which is similar to results obtained with succinylation (Habeeb *et al.*, 1958). However, some hydroxyamino acid residues were esterified by these reagents. It is quite likely that the esterification is not uniform and is responsible for the heterogeneity on disc electrophoresis. Partial and even complete modification of the amino groups of lysozyme was accompanied by a marked electrophoretic heterogeneity. On the other hand, Freedman *et al.* (1968) reported no esterification of hydroxy-

amino acids by maleic anhydride when used in 43-fold molar excess per lysine residue in rabbit immunoglobulin and purified antibodies. However, these investigators did not test for the homogeneity of their product.

Completely modified or even partially maleylated lysozyme derivatives showed a decrease in enzymic activity which amounted to 95% with modification of an average of four amino groups or higher. It is not certain whether the loss in enzymic activity is due to conformational changes which, although too little, might be quite sufficient to effect an almost complete loss of activity, or is the result of replacing a positively charged ammonium group of lysine by a negatively charged carboxyl group with concomitant changes in noncovalent interactions in the neighborhood of the active site. Modification of all ϵ-amino groups of lysine in lysozyme by guanidination, which did not affect the charge (Geschwind and Li, 1957), resulted in no loss of enzymic activity. On the other hand, acetylation with acetic anhydride (leading to loss of charge of amino groups) resulted in a heterogeneous product (Yamasaki *et al.*, 1968a). All the derivatives were devoid of activity when assayed against *M. lysodeikticus* at pH 7 (Yamasaki *et al.*, 1968b).

The conformational changes that accompanied complete modification of the amino groups by reaction of lysozyme with maleic or citraconic anhydride were surprisingly small. Thus they were not detectable by optical rotatory dispersion, slightly so by reducibility of the disulfides and only well by susceptibility to tryptic attack. Susceptibility to tryptic attack in deblocked ML_7Lys but not of deblocked CT_7Lys indicated lack of reversion to native conformation in deblocked ML_7Lys.

One of the significant aspects of the present work was the careful investigation of the reversibility of the masking groups. In derivatives prepared by reaction of lysozyme with tetrafluorosuccinic anhydride or with diketene, complete unmasking of amino groups was not achieved giving preparations that were highly heterogeneous and which showed only partial retrieval of enzymic and immunochemical properties.

Demaleylation of ML_7Lys was not complete (after 5 days at pH 3.5) and was accompanied by extensive electrophoretic heterogeneity despite the recovery of 90% of the free amino groups, 83% of the enzymic activity, and almost complete reactivity with antisera to lysozyme. In addition, native conformation was not completely recovered (see Table II). Freedman *et al.* (1968) showed that a completely maleylated anti-*p*-azobenzenearsonate antibody recovered 88% of the free amino groups on demaleylation for 4 days. Despite the complete recovery of combining sites, the average binding constant was restored to 72% of its original value. However, heterogeneity of the demaleylated antibody was not investigated by Freedman *et al.* (1968). Unless the product of demaleylation is examined critically for homogeneity, the recovery of the native protein from maleyl derivatives by demaleylation is questionable.

In contrast, derivatives prepared by reaction of lysozyme with citraconic anhydride gave on deblocking homogeneous preparations with recovery of 100% of free amino groups, enzymic activity, immunochemical properties, and native conformation.

The usefulness of a reversible blocking reagent depends on the ease of removal of the masking groups under conditions that will not lead to denaturation, the homogeneity of the deblocked preparation, and extent of recovery of the native conformation and the biological properties. Clearly, with the reagents studied here, only citraconic anhydride derivatives yielded, upon deblocking, preparations that satisfied all the foregoing criteria. It is extremely likely that these conclusions are of wide applicability and are not confined only to lysozyme, since recent investigations with sperm whale myoglobin (Atassi and Singhal, 1970) gave identical results.

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