Enzymic and Immunochemical Properties of Lysozyme. I. Derivatives Modified at Tyrosine. Influence of Nature of Modification on Activity*

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ABSTRACT: Tyrosines 20 and 23 in hen egg-white lysozyme were modified by nitration. The derivative exhibited conformational changes evidenced by its increased susceptibility to tryptic hydrolysis and the availability of one disulfide group for reduction. The antigenic reactivity was slightly affected (77–90% relative to homologous reaction) whereas the enzymic activity decreased by 50%. Most of the antigenic reactivity was recovered upon reduction of the nitrotyrosine residues to aminotyrosine despite the fact that conformational changes still existed. The enzymic activity was recovered only slightly. Antisera against $(NO_2)_2$-lysozyme were raised in rabbits. Lysozyme and $(NH_2Tyr)_2$-lysozyme reacted less efficiently with these sera than the homologous antigen (*i.e.*, $(NO_2)_2$-lysozyme). The reaction with $(NO_2)_2$-lysozyme could not be inhibited with 3-nitrotyrosine or Gly-3-NO_2-Tyr dipeptide. The present data demonstrated that the nature of a chemical modification at a given site determines its effect on biological activity.

Changes in biological activity observed as a result of specific chemical modification and in the absence of conformational changes yield valuable information concerning protein active sites. On the other hand, absence of change in activity after modification of a residue has usually suggested nonparticipation of this residue in the active site. In the second case, however, the nature of the chemical modification must be considered. It is, therefore, necessary to determine if the modification is, in fact, chemically or sterically sufficient to impair the participation of the residue in the biological role in which it is normally involved. Perhaps a good way to assess this is to modify the amino acid residue in question in more than one way. This, of course, is not always possible due to the limitation on the number of modification reactions that achieve a high degree of specificity and because availability of a given amino acid side chain to reaction might vary with the reagent.

Tyrosyl residues can now be modified by a variety of chemical procedures. Of these, nitration with tetranitromethane (Riordan *et al.*, 1966, 1967; Sokolovsky *et al.*, 1966) appears to possess a high degree of specificity for tyrosine resulting in the introduction of a nitro group at a position *ortho* to the phenolic hydroxyl. The nitro group can then be reduced to an amino group (Wasmuth *et al.*, 1964; Sokolovsky *et al.*, 1967) with sodium hydrosulfite. Nitro and amino groups *ortho* to the phenolic hydroxyl will each influence the ionization of the latter in a different way. Accordingly, the effect of this on the biological activity should reveal whether a tyrosyl residue is involved in the function of the protein.

In the present studies, lysozyme has been nitrated at tyrosines 20 and 23. Also, the nitrotyrosine residues were reduced to 3-aminotyrosine. Enzymic and immunochemical properties are reported together with accompanying conformational changes.

Materials and Methods

Materials. Lysozyme (three-times crystallized) was obtained from Sigma Chemical Co. Lyophilized *Micrococcus lysodeikticus* cells were freeze-dried vials from Worthington Biochemical Corp. Tetranitromethane and 5,5′-dithiobis(nitrobenzoic acid) were from Aldrich Chemical Co. Tyrosine and Gly-Tyr were obtained from Mann Research Laboratories, Inc. Trypsin (three-times crystallized) was from Worthington Biochemical Corp.

Reaction of Lysozyme with Tetranitromethane. To a solution of lysozyme (22 μmol) in 0.05 M Tris–1 M NaCl buffer (pH 8.0, 22 ml) was added 220 μmol of tetranitromethane as 10% solution in 95% ethanol and the mixture was stirred magnetically for 2 hr at room temperature (22°). After reaction the mixture was dialyzed extensively against distilled water, centrifuged (0°, 0.5 hr, 5600 rpm), and excess tetranitromethane

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was removed by filtration on a column (2.2 × 15 cm) of Sephadex G-10 which was eluted with distilled water. Tubes containing the protein fraction were pooled and freeze dried. The reaction product was dissolved in 0.05 M phosphate buffer (pH 8.0) and subjected to chromatography on a CM-cellulose column (2.5 × 60 cm) using a linear salt and pH gradient. For the gradient, the mixing vessel contained 0.05 M phosphate buffer (pH 8.0, 500 ml) and the reservoir contained 0.15 M Na_2HPO_4 (1200 ml). Columns were eluted at the rate of 20 ml/hr and the fractions (5 ml each) were read at 280 mμ in a Zeiss PMQII spectrophotometer. The tubes containing the derivative fraction (see Figure 1) were pooled, dialyzed against several changes of distilled water, and freeze dried. Extent of nitration was determined spectrally and by amino acid analysis of acid and alkaline hydrolysates.

Reduction of 3-NO_2-Tyr to 3-NH_2-Tyr in Lysozyme. Reduction of $(NO_2)_2$-lysozyme[1] was accomplished with sodium hydrosulfite (Sokolovsky *et al.*, 1967). For reduction, $(NO_2)_2$-lysozyme (6.3 μmol) was dissolved in 0.5 M Tris–1 M NaCl (pH 8.0, 3 ml) and 41 molar excess of solid sodium hydrosulfite was added. The mixture was stirred magnetically at room temperature for 0.5 hr after which it was dialyzed extensively against distilled water and freeze dried. Reduction of 3-nitrotyrosine residues was confirmed spectrally and by amino acid analysis.

In order to determine any changes that might result from the effect of hydrosulfite alone, enzyme controls were prepared in which lysozyme was subjected to hydrosulfite under the same conditions that were employed in the reduction of $(NO_2)_2$-lysozyme. When treatment was complete, an aliquot was removed, precipitated, and washed with 5%t richloroacetic acid and liberated SH groups determined by 5,5′-dithiobis-(nitrobenzoic acid). The remainder was dialyzed against-distilled water and freeze dried.

Determination of the Availability of Disulfide Groups to Reduction. In order to assess chemically, conformational changes in the lysozyme derivatives, the reducibility of the disulfide groups with 2-mercaptoethanol was determined as described previously (Habeeb, 1966, 1967a) by employing 5,5′-dithiobis(nitrobenzoic acid) (Ellman, 1959).

Determination of Enzymic Activities of Lysozyme and Its Derivatives. Solutions of lysozyme containing 5, 10, 20, and 30 μg per ml in 0.06 M phosphate buffer, containing 0.09% NaCl (pH 6.2) were prepared from a stock solution of lysozyme. Enzymic activity was based on the rate of lysis of *Micrococcus lysodeikticus* and was determined by a modification of the method used by Prasad and Litwack (1963). The decrease in extinction at 450 mμ of a reaction mixture (2.5 ml of cell suspension and 0.5 ml of enzyme solution) was measured at 30-sec intervals for 3 min. The reciprocal of absorbance was plotted against time for different concentrations of enzyme. The straight lines obtained were used for plotting Δabsorbance^{-1}/min *vs.* concentration of enzyme (from 5 to 30 μg per ml). By employing this calibration curve the activity of a known amount of modified lysozyme was determined from its Δabsorbance^{-1}/min.

Preparation of 3-Nitrotyrosine. Tyrosine (0.94 mmol) was suspended in 0.05 M Tris buffer at pH 8.0 (2 ml). Tetranitromethane (10 mmol) was added as 20% solution in 95% ethanol and the mixture was allowed to stir magnetically at room temperature. Aliquots were removed at intervals, evaporated, and the extent of reaction was determined on the amino acid analyzer. Extent of nitration was: 4 hr, 45%; 52 hr, 70%; 130 hr, 97.5%. After 130-hr reaction the sample was evaporated to dryness on a rotary evaporator. Excess tetranitromethane was removed by sublimation and last traces of the reagent and salts were removed on a column (2.2 × 20 cm) of Sephadex G-10. Column was eluted with 60% ethanol. Fractions containing 3-NO_2-Tyr were combined and evaporated to dryness on a rotary evaporator.

Nitration of Gly-Tyr Dipeptide. The dipeptide Gly-Tyr (0.73 mmol) was reacted with 12 molar excess of tetranitromethane under conditions similar to those described for the nitration of tyrosine. In this case, extent of nitration was followed by taking aliquots (50 μl) of the reaction mixture at intervals which were then passed through a column (0.5 × 5 cm) of Sephadex G-10 to remove salts and excess tetranitromethane and the product was subjected to acid hydrolysis followed by amino acid analysis. After 150-hr reaction, 98.3% of tyrosine in the dipeptide was converted into 3-nitrotyrosine. The reaction product was then subjected to sublimation and gel filtration as described for 3-nitrotyrosine.

Antisera. Antibodies to lysozyme were raised in goats by the procedure described elsewhere in detail (Atassi, 1967a). Antisera from individual animals were kept separate and stored in 5-ml portions at −40°. For the immunization of rabbits, the protein, in 0.15 M NaCl (lysozyme, 10 mg in 1 ml; $(NO_2)_2$-lysozyme, 5 mg in 1 ml), was emulsified with an equal volume of complete Freund's adjuvant. The emulsion was injected intramuscularly (half of the dose) and subcutaneously. The animals were boosted 3 weeks later in an identical manner. They were bled 2 weeks following the second injection and thereafter weekly. Sera from the first three bleedings of the two rabbits immunized with the same antigen were pooled. Similarly sera from the 9–11 bleedings were combined separately. Goat antisera G9 and G10 were against lysozyme. Antiserum HM was a pool from two rabbits and was directed against lysozyme. Antiserum HN was also a pool from two rabbits but was directed against $(NO_2)_2$-lysozyme.

Analytical Methods. Electrophoresis in starch gel was at room temperature in 0.02 M sodium phosphate buffer at pH 8.0, using a potential gradient of 9.7 V/cm for 5–6 hr. Gels were stained with Amido-Black (Smithies, 1959). Spectral determinations were carried out in a Zeiss PMQII spectrophotometer and con-

[1] Abbreviations used: $(NO_2)_2$-lysozyme, enzyme nitrated at two tyrosine residues with tetranitromethane; $(NH_2Tyr)_2$-lysozyme, derivative obtained by reduction of the 3-nitrotyrosines in $(NO_2)_2$-lysozyme to 3-aminotyrosine.

tinuous spectra were done in a Cary Model 15 spectrophotometer. Double diffusion in 1% agar was by the method of Ouchterlony (1949). Precipitin and inhibition experiments have been described recently in detail (Atassi and Saplin, 1968). Nitrogen determinations were done in a micro-Kjeldahl apparatus similar to that described by Markham (1942). The concentrations of protein solutions were based on their nitrogen contents or their absorption at 280 mμ. Amino acid analysis of acid (110°, 22 or 72 hr in double-distilled, constant-boiling HCl, three-times nitrogen-flushed evacuated sealed tubes) and alkaline (saturated $Ba(OH)_2$; Ray and Koshland, 1962) hydrolysates was done on Spinco Model 120 C amino acid analyzer. Analysis for aminotyrosine on the analyzer was by the procedure employed by Sokolovsky *et al.* (1967).

Tryptic Digestion and Peptide Mapping. Before tryptic hydrolysis, the disulfide groups in lysozyme or $(NO_2)_2$-lysozyme were reduced and carboxymethylated by a procedure similar to that described by Jollès *et al.* (1963). The completeness of reduction and carboxymethylation and the specificity of the latter were confirmed by amino acid analysis.

For digestion with trypsin, the protein (10 mg) was dissolved in 0.08 M triethylaminacetic acid buffer at pH 8.0 (2.0 ml). Hydrolysis was started by the addition of 0.5% trypsin in 0.001 N HCl (10 μl). One more aliquot of trypsin solution (10 μl) was added 1 hr later. The reaction mixture was magnetically stirred and the hydrolysis was allowed to continue for 20 hr at 37° after which it was freeze dried. Peptide mapping was done by the procedure recently described (Atassi and Saplin, 1968). Specific stains for various amino acids were prepared according to the procedures summarized by Easley (1965).

TABLE I: Amino Acid Composition of Lysozyme and Its Various Derivatives.[a]

Amino Acid	Amino Acid Composition (residues/mol)		
	Lysozyme	$(NO_2)_2$-lysozyme	$(NH_2Tyr)_2$-lysozyme
3-NH_2-Tyr	0	0	1.93
Trp	5.88	5.93	5.74
Lys	6.13	5.85	6.21
His	1.02	0.902	0.86
Arg	11.2	11.3	10.9
Asp	20.8	21.6	21.3
Thr	6.31	6.33	6.12
Ser	9.76	9.88	9.58
Glu	5.15	5.26	5.41
Pro	2.01	2.21	1.89
Gly	12.0	12.0	11.91
Ala	12.1	12.2	12.2
Cys ($\frac{1}{2}$)	7.75	7.87	7.65
Val	5.86	5.93	5.83
Met	2.03	1.99	2.17
Ile	5.64	5.85	6.12
Leu	8.00	7.97	8.23
Tyr	2.95	0.974	1.04
Phe	2.96	2.93	2.99
3-NO_2-Tyr	0	2.00	0.04

[a] The results represent the average of four acid hydrolyses (two at 22 hr and two at 72 hr). Values for tryptophan were obtained from duplicate alkaline hydrolyses. Values for threonine and serine were obtained by extrapolation to zero hydrolysis time.

Results

Characterization of the Tyrosine-Modified Lysozyme. A. NITRATION OF THE TYROSINE RESIDUES. Upon chromatography in CM-cellulose the product from the nitration reaction gave essentially a single peak. No unreacted lysozyme was present (Figure 1). On starch gel electrophoresis at pH 8.0, the nitrated derivative was homogeneous and migrated as a single positively charged band with a mobility of 0.81 (relative to native lysozyme = 1). The derivative showed a peak in the visible spectrum at 350 mμ and also a shoulder between 420 and 440 mμ. Spectral determination of the extent of nitration, based on the extinction at 381 mμ (Sokolovsky *et al.*, 1966), suggested the presence of 1.83 mol of 3-nitrotyrosine in $(NO_2)_2$-lysozyme. Amino acid analyses of acid and alkaline hydrolysates of lysozyme and its nitrated derivative (Table I) showed loss of exactly two tyrosine residues by nitration. The disappearance of tyrosine was entirely accounted for by the appearance of 3-nitrotyrosine. No other amino acids were modified. It is relevant to mention here that the nitration of 2.6 tyrosyl residues in lysozyme has recently been reported (Sokolovsky *et al.*, 1967). This greater degree of nitration might have been due to the use of a higher excess (32 molar) of tetranitromethane. However, other conditions of the reaction (*i.e.*, temperature, pH, and salt concentration) were not given by the authors, and the homogeneity of their preparation was not reported.

Peptide maps of tryptic digests of reduced, carboxymethylated $(NO_2)_2$-lysozyme showed two yellow spots. In order to determine the composition of these spots, maps were prepared which were not stained with ninhydrin. The yellow color of the spots was intensified by dipping the papers in 1% HCl in ethanol. The spots were then cut out, extracted separately with 0.2 N acetic acid, and filtered on columns (0.9 × 15 cm) of Sephadex G-25. For each peptide, the tubes containing the yellow color were combined, freeze dried, and subjected to acid hydrolysis, followed by amino acid analysis. One peptide (A) had the following composition (based on molar ratio Leu:Gly of 1:2): *S*-carboxymethylcysteine, 0.88; Asp, 0.92; Ser, 0.86; Gly, 1.79; Ala, 1.42; Val, 0.81; Leu, 1.13; Tyr, 0.01; 3-NO_2-Tyr, 0.91; and Lys, 0.84. The most likely location of this peptide, based on its composition, the known sequence of lysozyme (Canfield, 1963b; Jollès *et al.*, 1963) and the locations of tryptic attack (Canfield, 1963a; Jollès *et al.*, 1963) is sequence 22–33. In this peptide, therefore, tyrosine 23 had been ni-

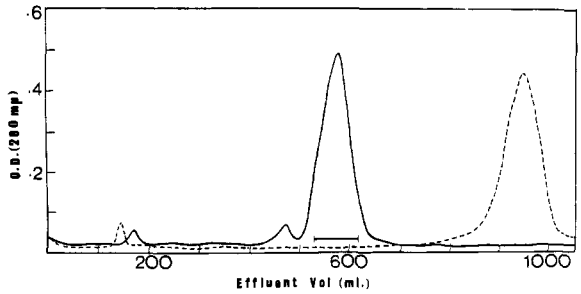

FIGURE 1. Chromatogram of the product of nitration of lysozyme on CM-cellulose (continuous line). The elution position of lysozyme from the same column, using the same gradient elution system, is shown (dashed line) for comparison. The minor components at 140 and 170 ml probably represent aggregated forms of the two proteins. The minor component appearing at 470 ml in the nitrated product probably represents a derivative modified at all three tyrosines. The tubes indicated were combined and used in the present studies. Experimental details are given in the text.

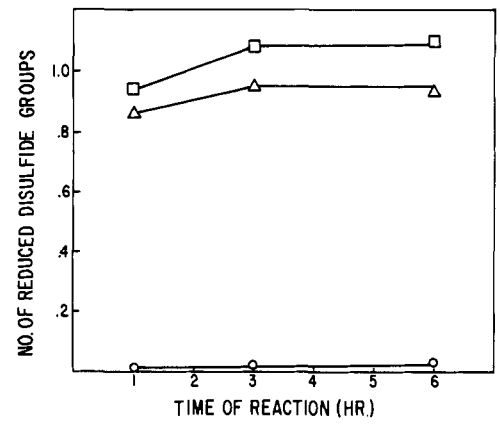

FIGURE 2: The number of disulfide groups reduced with 2-mercaptoethanol in (○) native lysozyme, (△) $(NO_2)_2$-lysozyme, (□) $(NH_2Tyr)_2$-lysozyme.

TABLE II: Reducible Disulfide Bonds and Enzymic Activity of Lysozyme and Derivatives.

Protein	% Act.	No. of Reducible Disulfide Bonds
Lysozyme	100	0.03
Hydrosulfite-pretreated Lysozyme	100	0.04
$(NO_2)_2$-lysozyme	50	0.95
$(NH_2Tyr)_2$-lysozyme	56	1.1

trated. The second peptide (B) had the following composition: Asp, 1.82; Gly, 0.73; Leu, 0.86; Tyr, 0.03; 3-NO_2-Tyr, 0.88; His, 0.95; and Arg, 1.00. The most likely location of this peptide, based on its composition, is sequence 15–21 and the modification will therefore be on tyrosine 20. This information, together with the amino acid composition of $(NO_2)_2$-lysozyme and its known chromatographic and electrophoretic homogeneity, suggests that only tyrosines 20 and 23 had been nitrated in the enzyme.

B. REDUCTION OF $(NO_2)_2$-LYSOZYME. Reduction of $(NO_2)_2$-lysozyme with sodium hydrosulfite, as described under the Experimental Section, gave a preparation which had no absorption maxima in the visible region of the spectrum. The preparation moved as a single band, with an over-all positive charge, on starch gel electrophoresis. Its mobility was greater (1.20) relative to native lysozyme. Amino acid analysis confirmed the complete reduction of 3-nitrotyrosine to 3-aminotyrosine (Table I).

Determination of Conformational Alterations. An evaluation of conformational changes in proteins containing disulfide bridges can be obtained by determining the number of disulfide groups that are available to reduction in a protein derivative as compared with those available in the native protein (Habeeb, 1966, 1967a). Figure 2 shows the number of disulfide groups reduced with 2-mercaptoethanol in native and modified lysozymes. The results are summarized in Table II. It can be seen that native lysozyme has almost no disulfide groups available to reduction under the conditions employed in this procedure. After nitration of tyrosines 20 and 23, one disulfide group becomes susceptible to reduction. Similarly, $(NH_2Tyr)_2$-lysozyme also had one disulfide bond available to reduction. It was not determined whether the reducible disulfide group was at the same location in both derivatives.

To determine the effect of hydrosulfite treatment on the disulfide bonds in lysozyme, the protein was reacted with hydrosulfite (under the same conditions used for the reduction of $(NO_2)_2$-lysozyme) and examined for any reduction of disulfide bonds (see Experimental Section). The determinations showed that only 0.02 disulfide bond/mole of lysozyme was reduced by hydrosulfite treatment. On the other hand, pretreatment of lysozyme with hydrosulfite did not lead to any increased susceptibility of disulfides to reduction with 2-mercaptoethanol since only 0.04 disulfide bond was reducible (Table II).

Conformational changes in protein derivatives may also be evaluated from their susceptibility to digestion with enzymes relative to the native protein (for review, see Rupley, 1967). Digestion with trypsin was employed here for investigating conformational changes in lysozyme derivatives. Hydrolysis was carried out on the proteins, *without* reduction and carboxymethylation of the disulfide bonds, at pH 8.0 and 40° under an atmosphere of nitrogen. The pH was kept constant on the pH-Stat by the addition of 0.05 N NaOH. Figure 3 shows the rates of alkali consumption of lysozyme, $(NO_2)_2$-lysozyme, and $(NH_2Tyr)_2$-lysozyme during tryptic digestion. It is apparent that modification of the two tyrosine residues gives rise to derivatives which are more susceptible to hydrolysis with trypsin than native lysozyme. Peptide mapping of the tryptic digests of native lysozyme and $(NO_2)_2$-lysozyme showed that no free peptides were released from the native enzyme, whereas in $(NO_2)_2$-lysozyme at least 8 peptides

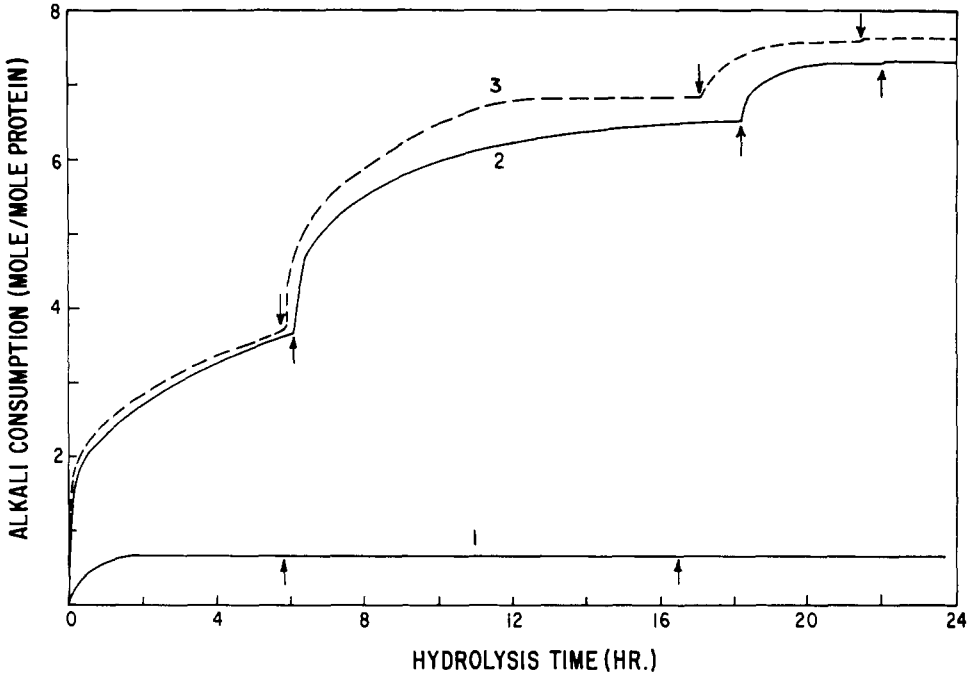

FIGURE 3: Rates of alkali consumption upon tryptic hydrolysis of (1) lysozyme, (2) $(NO_2)_2$-lysozyme, and (3) $(NH_2Tyr)_2$-lysozyme. Hydrolysis was carried out on the proteins *without* reduction and carboxymethylation of the disulfide bonds. For details see text.

(12 when the paper was heavily overloaded) were released. It was possible to identify some of the peptides from their positions on the peptide map, comparison with the assignments of Canfield (1963a) and the use of stains specific for various amino acids. The following amino acids and peptides were released in $(NO_2)_2$-lysozyme by tryptic digestion (Figure 4): lysine (possibly from positions 1 and 97); arginine (from position 14); leucine (from position 129); Asn-Arg (from sequence 113–114); Thr-Pro-Gly-Ser-Arg (from sequence 69–73) and Lys-Ile-Val-Ser-Asp-Gly-Asp-Gly-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg (from sequence 97–112); a spot which might have corresponded to one or both of the following two peptides (which often overlapped) His-Gly-Leu-Asp-Asn-NO_2-Tyr-Arg (from sequence 15–21) or Lys-Val-Phe-Gly-Arg (from sequence 1–5); and finally a spot probably corresponding to the sequence 98–112. Other spots appearing in very heavily loaded maps could not be identified. Similar peptides were also released from $(NH_2Tyr)_2$-lysozyme upon tryptic digestion. Only one spot (leucine, probably from position 129) was observed in digests of native lysozyme (Figure 4). It may be stressed here that the results do not suggest that the modification of two tyrosines causes exposure of *all* trypsin-hydrolyzable bonds (see Discussion).

Immunochemistry of the Derivatives. In agar double diffusion against antisera to lysozyme, $(NO_2)_2$-lysozyme and $(NH_2Tyr)_2$-lysozyme gave single lines which fused with the line given by lysozyme. No spurs or intersections were formed (Figure 5). In quantitative precipitin analysis, $(NO_2)_2$-lysozyme precipitated less antibody nitrogen than the native protein. The reaction of $(NO_2)_2$-lysozyme with goat antiserum G10 is shown in Figure 6. Table III summarizes the results of reaction of $(NO_2)_2$-lysozyme with antisera G9, G10, and rabbit antiserum HM. The results show that nitration of the tyrosyl residues gives a derivative which is less reactive (79, 77, and 90% with G9 and G10, and HM, respectively). Upon reduction of the 3-nitrotyrosine residues to 3-aminotyrosine, it was significant to find that most of the antigenic reactivity lost as a result of nitration was recovered after reduction. It can be seen in Table III that $(NH_2Tyr)_2$-lysozyme reacts between 95 and 99% relative to the homologous reaction. An example of these precipitin reactions with antiserum G10 is shown graphically in Figure 6. It is

TABLE III: Relative Amounts of Precipitation Formed by Lysozyme Derivatives Modified at Tyrosine with Various Antisera.[a]

Antiserum	$(NO_2)_2$-lysozyme	$(NH_2Tyr)_2$-lysozyme[b]
G9	78.8	94.8
G10	76.8	98.6
HM	90.0	97.6

[a] Values are expressed in per cent precipitation relative to homologous reaction and represent the average of three or more independent determinations which varied ±1% or less. [b] Lysozyme controls which had been pretreated with hydrosulfite, under the same condition employed for the reduction of $(NO_2)_2$-lysozyme, had an antigenic reactivity identical with that of native lysozyme.

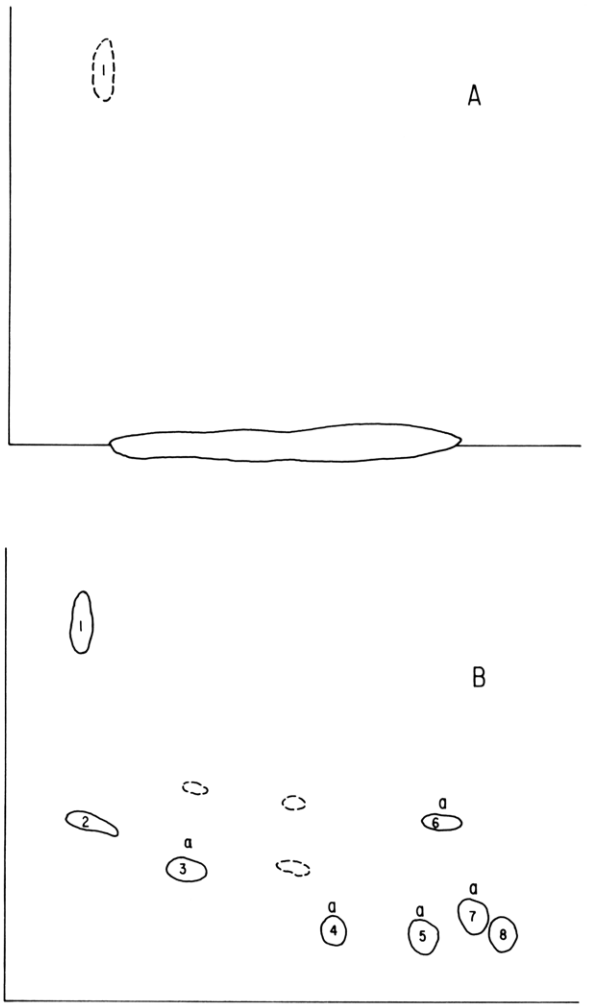

FIGURE 4: Peptide maps of tryptic hydrolysates of (A) lysozyme and (B) $(NO_2)_2$-lysozyme. Tryptic hydrolysis was carried out on the proteins *without* reduction and carboxymethylation of the disulfide bonds. The spots corresponded to the following sequences: (1) position 129 (leucine), (2) possibly sequence 98–112, (3) sequence 97–112, (4) sequence 69–73, (5) sequence 113–114, (6) sequence 15–21 and/or sequence 1–5 (which often overlapped), (7) position 14 (arginine), and (8) position(s) 1 or (and) 97 (lysine). Spots denoted by a were positive with Sakaguchi stain for arginine. Spots outlined by a broken line appeared only when papers were overloaded. For details, see text.

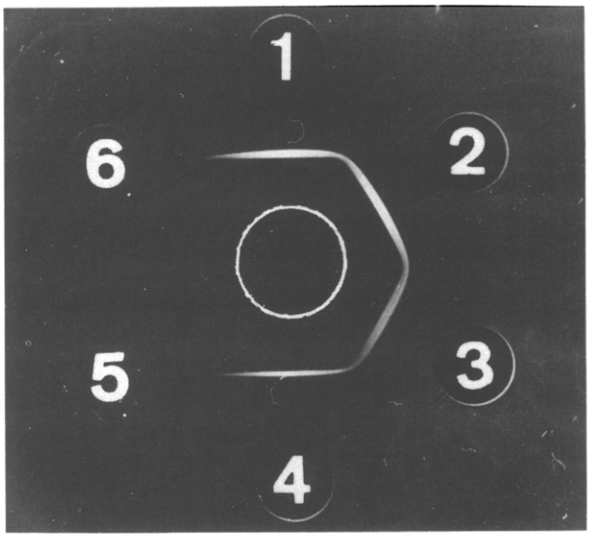

FIGURE 5: Reactions of antiserum to lysozyme (rabbit antiserum HM) with lysozyme, $(NO_2)_2$-lysozyme, $(NH_2Tyr)_2$-lysozyme, and lysozyme in wells 1, 2, 3, and 4, respectively.

relevant to mention here that the antigenic reactivities of lysozyme and of hydrosulfite-preteated lysozyme were completely identical.

To determine whether the tyrosyl residues involved in the nitration were essential parts of a reactive region[2] in lysozyme, antibodies were raised against $(NO_2)_2$-lysozyme in two rabbits. If tyrosines 20 and 23 fall into a reactive region then, it was hoped, that the nitrotyrosyl residues might be immunogenic. In agar double diffusion, lysozyme, $(NO_2)_2$-lysozyme, and $(NH_2Tyr)_2$-lysozyme, each formed a single line with antisera to $(NO_2)_2$-lysozyme showing no spurs or intersections (Figure 7). Figure 8 shows some results of quantitative precipitin analysis. Lysozyme reacted consistently less with antisera to $(NO_2)_2$-lysozyme than the homologous antigen. The reactivity of lysozyme relative to $(NO_2)_2$-lysozyme was poorer (75%) with sera from late bleedings than with early-bleeding sera (82%). The reactivity of $(NH_2Tyr)_2$-lysozyme with these sera was appreciably better than lysozyme, but nevertheless always poorer (90%) than $(NO_2)_2$-lysozyme. Upon absorption of an aliquot of each antiserum with an amount of lysozyme necessary to achieve equivalence, the supernatants showed no reaction with lysozyme and reacted only with $(NO_2)_2$-lysozyme (11%). The results are summarized in Table IV. This suggested that some antibody reactivity in antisera to $(NO_2)_2$-lysozyme was directed either against the nitrotyrosyl residues or against regions carrying these residues, or both. If nitrotyrosyl residues were immunogenic then the interaction of $(NO_2)_2$-lysozyme may conceivably be inhibited to some extent with 3-nitrotyrosine or an appropriate peptide carrying it. Therefore, 3-nitrotyrosine and Gly-3-NO_2-Tyr were prepared in a high degree of purity. In several attempts, we were not able to detect any significant inhibition with these preparations even when 100 molar excess of inhibitor to antigen was used.

Enzymic Activity of Lysozyme Derivatives. Nitration of tyrosines 20 and 23 resulted in 50% loss in enzymic activity. Reduction of the nitrotyrosine residues resulted in the recovery of some of the lytic activity. Table II summarizes the enzymic activities of the derivatives. It is significant to point out that treatment of lysozyme with hydrosulfite did not lead to any loss of enzymic activity.

[2] A distinction is made here between antigenic reactive *regions* and antigenic reactive *sites* as defined recently by Atassi and Saplin (1968).

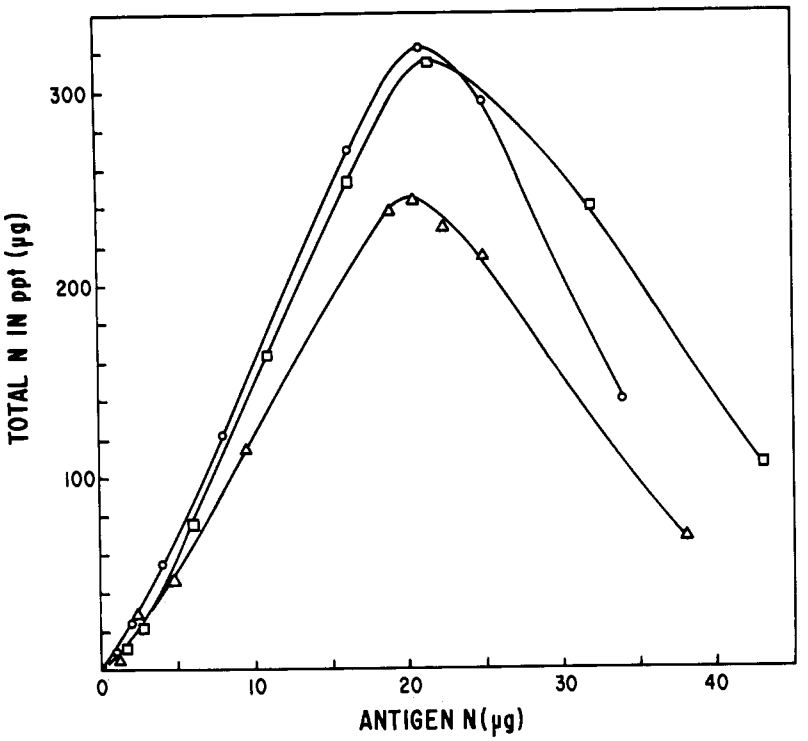

FIGURE 6: Precipitin studies on lysozyme (○), $(NO_2)_2$-lysozyme (△), and $(NH_2Tyr)_2$-lysozyme (□). Reactions were with goat antiserum G10 to native lysozyme.

Discussion

Reaction of lysozyme with tetranitromethane indicates that, under these conditions, tyrosine at position 53 is not accessible to the reagent. Tyrosine 53 is located in the short antiparallel pleated-sheet structure comprising residues 41–54 and is hydrogen bonded to threonine 43 (Blake *et al.*, 1965). This might account for its nonreactivity with tetranitromethane.[3] Evidently conformational changes take place upon modification of tyrosines 20 and 23. It is extremely difficult to determine if changes in the conformation of $(NO_2)_2$-lysozyme and $(NH_2Tyr)_2$-lysozyme are identical. Similarities in the susceptibility of a disulfide bond to reduction and release of similar peptides upon hydrolysis with trypsin suggest the presence of related configurational alterations in the two derivatives. The peptides released by tryptic digestion of the derivatives may not necessarily reflect bonds that are all accessible to tryptic attack in the two derivatives. Owing to the long duration of the digestion, bonds may be hydrolyzed in larger fragments that are initially released by cleavage of accessible bonds in the protein. Also cleavage of the first accessible bond(s) in the derivatives might lead to further alterations in conformation with resultant exposure of new bonds to tryptic attack. The results are, nevertheless, still helpful in revealing the general location of the most susceptible region in the derivative. It is noteworthy that eight out of nine peptides released were derived from one side of the molecule relative to the active-site cleft of the enzyme. The ninth peptide (peptide 69–73) was derived from the

TABLE IV: Precipitin Reaction of Antisera to $(NO_2)_2$-lysozyme with Lysozyme and Its Tyrosine-Modified Derivatives.

	Reactivity at Equivalence	
	Early Bleedings[a]	Late Bleedings[b]
Antigen	% of Total	% of Total
$(NO_2)_2$-lysozyme	100	100
$(NH_2Tyr)_2$-Lysozyme[c]	90.4	90.5
Lysozyme	82.0	75.3
	Reactivity of Antiserum after Absorption with Lysozyme	
$(NO_2)_2$-lysozyme	11	11
Lysozyme	0	0

[a] Early bleedings constitute a pool of sera from two rabbits at weeks 1–3 after the second injection. [b] Late bleedings is a pool of bleedings at weeks 9–11 from the same two rabbits. For details, see text. [c] Lysozyme and hydrosulfite-pretreated controls possessed identical antigenic reactivities.

[3] After submission of this manuscript, Hayashi *et al.* (1968) reported that tyrosines 20 and 23 are preferentially diiodinated in lysozyme. Tyrosine 53 was nonreactive.

opposite side of the molecule to the region of modification.

On nitration of the two tyrosyl residues *ortho* to the phenolic hydroxyl, the effect of the electron-withdrawing nitro group will be to increase the acidity of the phenolic hydroxyl (*cf.* pK_a values: tyrosine, 10.1 (Edelhoch, 1962); 3-nitrotyrosine, 7.2 (Sokolovsky *et al.*, 1967)). Hence it would be expected that nitration would alter the properties of the tyrosyl residues sufficiently to influence their involvement, if any, in the biological activity of a protein (Atassi, 1968). The slight decrease in antigenic reactivity of lysozyme upon nitration might suggest that one or both of tyrosines 20 and 23 are present in an antigenic region of the enzyme. However, conformational changes were observed and since these have been shown to influence the antigenic reactivity of proteins (Atassi, 1967b), it is conceivable that the decrease in antigenic reactivity might indeed be the result of conformational changes. It was therefore significant to find that reduction of the nitrotyrosyl residues to aminotyrosine resulted in an almost complete recovery of the antigenic reactivity of lysozyme. Conformational changes still persisted in $(NH_2Tyr)_2$-lysozyme and appeared to be related to those in $(NO_2)_2$-lysozyme. If the decrease in antigenic reactivity is indeed directly related to nitration and lowering of the pK_a of the phenolic hydroxyls, then complete recovery of this reactivity would be expected upon elevation of the pK_a to its original value by reduction to 3-aminotyrosine (pK_a = 10.0; Sokolovsky *et al.*, 1967). In this connection, it was recently shown (Atassi, 1968) that nitration of tyrosines 146 and 151 in sperm whale myoglobin, which are located in an antigenic reactive region on the

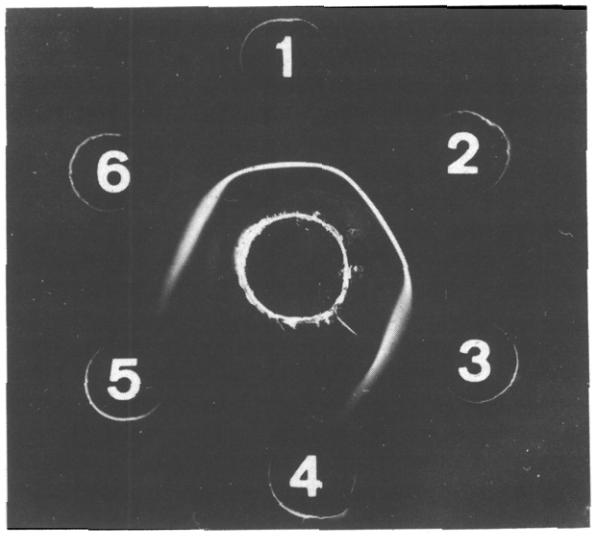

FIGURE 7: Agar double diffusion with rabbit antiserum HN to $(NO_2)_2$-lysozyme and $(NH_2Tyr)_2$-lysozyme, lysozyme, $(NO_2)_2$-lysozyme, and $(NH_2Tyr)_2$-lysozyme in wells 6, 1, 2, and 3, respectively.

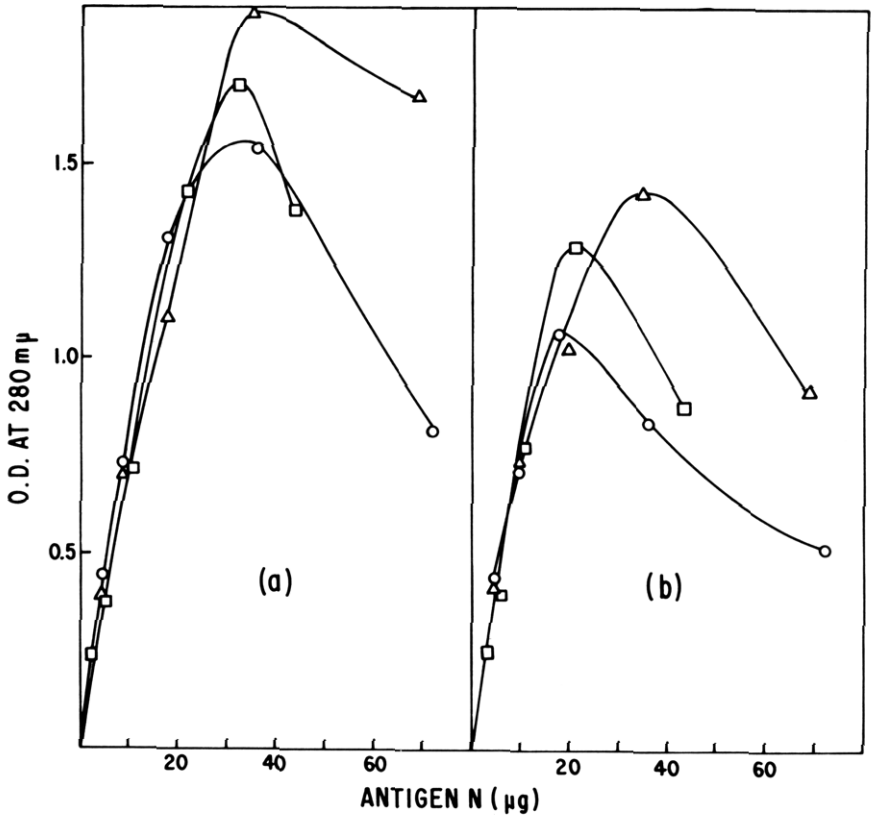

FIGURE 8: Precipitin analyses with antiserum to $(NO_2)_2$-lysozyme and (○) lysozyme, (△) $(NO_2)_2$-lysozyme, and (□) $(NH_2Tyr)_2$-lysozyme. (a) Reactions with sera from early bleedings. (b) Reactions with sera from late bleedings. For details, see text.

molecule, was sufficient for the complete removal of the reactivity of that region. However, reduction of the nitrotyrosyl residues was not carried out to test for immunochemical recovery in nitrated sperm whale myoglobin or nitrated peptide 132–153.

With antibodies against the nitrated derivative, lysozyme reacted less efficiently than the homologous antigen, especially with sera from late bleedings. The reactivity of $(NH_2Tyr)_2$-lysozyme was intermediate between lysozyme and $(NO_2)_2$-lysozyme and appeared unaltered in early or late bleedings. This, together with the results from absorption experiments, suggests that about 90% of the specificity in antisera to $(NO_2)_2$-lysozyme is directed against the molecule with the new configuration. The remainder of the specificity (11%) was directed against the nitrotyrosyl residues, either alone or in conjunction with neighboring residues of the polypeptide chain. Failure to inhibit the reaction with 3-nitrotyrosine or with Gly-3-NO_2-Tyr would favor the second alternative. It is quite likely that nitrotyrosyl residues in a protein are more immunogenic than tyrosyl residues. In this connection, it has recently been shown, for example, that nitroguanyl groups are far more immunogenic than guanyl groups when a given carrier protein was used (Habeeb, 1967b). It is noteworthy to mention that, in agar double diffusion, no spurring was obtained between lysozyme and its two derivatives when reacted with antisera to lysozyme or $(NO_2)_2$-lysozyme. Spurring should be expected but we did not observe it despite repeated attempts. At any rate, the foregoing results strongly suggest that one or both of tyrosines 20 and 23 take an active part in the binding of lysozyme with its antibodies. However, it is extremely difficult to ensure, in minute details, that conformational reorganizations in solution in $(NO_2)_2$-lysozyme and $(NH_2Tyr)_2$-lysozyme, relative to the native enzyme, are identical. A detailed investigation of the conformations of the two derivatives is in progress and will be reported later.

The great loss in enzymic activity upon nitration is not recovered on reduction of the nitrotyrosyl residues. Therefore, the loss of enzymic activity upon nitration is most likely the result of conformational changes. Since these conformational changes are still present, and if they are related, very little enzymic activity will be recovered with $(NH_2Tyr)_2$-lysozyme. None of the tyrosyl residues in lysozyme has been found to be at the binding site of the inhibitor *N*-acetylglucosamine or its dimer by X-ray crystallography (Blake *et al.*, 1965). The results obtained with the immunochemistry of the tyrosine-modified derivatives demonstrate that change in activity upon modification is dependent upon the chemical nature of the modification.

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