Conformational Studies on Modified Proteins and Peptides

IV. CONFORMATION OF LYSOZYME DERIVATIVES MODIFIED AT TYROSINE OR AT TRYPHTOPHAN RESIDUES*

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SUMMARY

Conformational investigations have been carried out on derivatives of lysozyme in which tyrosines 20 and 23 were nitrated (NT2-lysozyme), or in which the nitrotyrosine residues had been reduced to aminotyrosine (AT2-lysozyme). Also, a derivative modified at the 6 tryptophan residues by reaction with 2-nitrophenyl sulfenyl chloride (NP6-lysozyme) was studied. In optical rotatory dispersion measurements, NT2-lysozyme and AT2-lysozyme showed equal rotations at the negative 233-nm minima and at the positive 199-nm maxima. Also, they had identical b values. These two derivatives were more rotatory than lysozyme. On the other hand, NPS6-lysozyme showed an appreciable degree of unfolding evidenced by a decrease of all its optical rotatory dispersion parameters. Measurements of the reduced molar ellipticities showed that the present three derivatives, like lysozyme, give a negative circular dichroism band at 208 nm and a shoulder around 220 nm. Results were in agreement with optical rotation measurements. The rotatory behavior of lysozyme and the derivatives showed no change in the pH range 7 to 3. The conformational changes revealed by optical rotatory dispersion and circular dichroism measurements were also shown by increase in disulfide reducibility. Both NT2-lysozyme and AT2-lysozyme exhibited appreciable disulfide reducibility (one bond) relative to lysozyme (0.03 bond), and the great unfolding in NPS6-lysozyme was also confirmed by the large increase in accessibility of its disulfide bonds (2 bonds). Changes in conformation obtained on binding of each of these proteins with sodium dodecyl sulfate were monitored by disulfide accessibility. It was shown that NT2-lysozyme and AT2-lysozyme assume 'relaxed' conformation with identical disulfide accessibility (two bonds). The conformation of lysozyme also became 'relaxed', although to a lesser extent than the tyrosyl derivatives, on binding with sodium dodecyl sulfate. In contrast to these, NPS6-lysozyme, assumed a

*constrained' conformation on binding with sodium dodecyl sulfate. It was concluded that conformational changes take place upon modification of the tyrosine or tryptophan residues and that NT2-lysozyme and AT2-lysozyme had identical conformations. From these results and the previously reported immunochemical behaviors of these derivatives, it may be concluded that one (or both) of tyrosines 20 and 23 is located in an antigenic reactive site in lysozyme. The results also indicated that, although the antigenic reactivity of native proteins is highly influenced by changes in conformation, not every conformational change will exert an effect on the antigenic reactivity. This should be dependent on the protein and the nature of the conformational change.

Conformational changes that might take place upon chemical modification of proteins usually influence their biological properties. However, change in a biological property may also be the direct result of the modification of an amino acid side chain. The modification itself might, in fact, be chemically or sterically sufficient to impair the participation of the residue in the biological role in which it is normally involved. It is, therefore, usually difficult to determine whether change in a biological property is directly caused by chemical modification of the side chain of a given amino acid or is simply the by-product of the conformational change. A great help in this connection would be to modify the amino acid in question in more than one way, if possible. We have recently reported (1) the preparation of lysozyme derivatives modified at tyrosines 20 and 23 in more than one way. In one derivative, tyrosines 20 and 23 were nitrated and in the other the nitrotyrosine residues were reduced to aminotyrosine. The enzymic and immunochemical properties of these derivatives were investigated in detail. An appreciable decrease in the antigenic reactivity was obtained on nitrination of tyrosines 20 and 23. This reactivity was entirely recovered on reduction of the nitrotyrosine residues to amino-tyrosine. On the other hand, the decrease in enzyme activity caused by nitrination was not recovered on reduction of the nitro-tyrosine residues. Both derivatives showed conformational
lysozyme, lysozyme nitrated with the use of tetranitromethane is possible to determine unequivocally whether change in activity, is an indication of a direct involvement of the modified residue in its chemical properties of the derivative. In addition, similar conformational investigations were carried out on a lysozyme derivative in which all 6 tryptophan residues in lysozyme were modified by reaction with 2-nitrophenyl sulfenyl chloride. This derivative has been reported (2) to have no enzymic activity and very little antigenic reactivity. From these studies, it was possible to determine unequivocally whether change in activity, for each derivative, is caused by the conformational change or is an indication of a direct involvement of the modified residue in the biological function.

**EXPERIMENTAL PROCEDURE**

**Materials**—Lysozyme (three times crystallized) was obtained from Sigma and its homogeneity was confirmed (1, 2) by starch gel and acrylamide gel electrophoresis. Sodium dodecyl sulfate (SDS) was from Matheson, Coleman and Bell and 5,5'-dithiobis-(nitrobenzoic acid) (7) as described by Habeeb (8).

**Preparation of Lysozyme Derivatives Modified at Tyrosines 20 and 23**—The 6 tryptophan residues in lysozyme were modified by reaction with 2-nitrophenyl sulfenyl chloride. Method of preparation and the exhaustive characterization of the derivative have been described in detail elsewhere (2), in conjunction with the enzymic and immunochemical properties of the derivative.

**Optical Rotatory Dispersion and Circular Dichroism Measurements**—ORD and CD experiments were carried out on solutions of proteins (containing 0.08 to 0.35 mg of protein per ml) in water (glass double distilled) at 25°C. Measurements were made with a Cary model 60 spectropolarimeter, equipped with a model 6001 circular dichroism accessory.

Measurements on each protein were made at several concentrations employing cells with light paths of 0.5, 1, 5, and 10 mm. For measurement below 220 nm, only 0.5- and 1-mm cells were used with maximum damping (Pen period 30), very low scan speeds (30 s per nm) and a full range of 0.1 to 0.2 degree. Solvent baseline scans were performed before and after each protein sample. Each sample was scanned at least five times at each concentration and the rotations at various wave lengths were the average from these scans. ORD data are reported in reduced mean residue rotation at a corrected for the refractive index dispersion of water, n. Experimental procedure and quantitative treatment of data has been described in detail elsewhere (3). The values of the mean residue molecular weight employed in the present work for lysozyme and derivatives are shown in Table I. The Moffitt-Yang parameter, b0, was calculated with their equation (4) and taking λs as 212 nm.

The circular dichroism accessory records data directly in terms of ellipticity, θ, in degrees and was calibrated as described by Aataei (5). In analogy to [θ], CD data are given here as reduced molar ellipticities, [θ], by correcting for the refractive index dispersion of water n (i.e., [θ] = [θ] n/m² + 2). Units of [θ] are in deg cm² per decimole.

Change of protein conformation with pH was studied by the addition of increasing amounts of acid (2 N HCl) or alkali (2 N NaOH) to a solution of protein in water. After each change in pH, the protein solution was centrifuged (4000 rpm, 1 hour, 0°C) and aliquots were then removed for triplicate nitrogen analyses. Therefore, the exact protein concentration at each pH value was determined directly, thus avoiding some of the complications arising from the precipitation of certain derivatives upon change in pH.

**Determination of Effect of SDS Binding on Susceptibility of Disulfide Bonds to Reduction**—A solution (2.5 ml) of lysozyme or derivative (1.5 to 1.7 μmoles) in Tris-glycine buffer at pH 7.0 (6), was mixed with SDS to give a 7 M excess of SDS per mole of protein (i.e. 1 mole of SDS per free amino group). The protein-SDS complex was stirred at room temperature for 20 min, after which it was diluted to 10 ml with Tris-glycine buffer, pH 7.0, followed by addition of 0.25 M 2-mercaptoethanol (2.5 ml). Aliquots (1.5 ml each) were withdrawn at intervals of 15, 30, 45, 60, 90, 120, and 180 min. Protein was precipitated immediately with 5% trichloroacetic acid (10 ml), centrifuged, and washed five times on the centrifuge with 5% trichloroacetic acid (3 ml each). Sulphydryl content was determined with 5,5'-dithiobis (nitrobenzoic acid) (7) as described by Habeeb (8).

**Determination of Protein Concentration**—Concentrations of protein solutions were based on their nitrogen contents which were calculated from their amino acid compositions. Nitrogen contents of solutions were determined by a microkjeldahl procedure and by using Nessler's reagent standardized with ammonium sulfates. Three or four replicate analyses were done on each protein solution and they varied ±0.3%. Table I shows the molecular weights, nitrogen contents, and mean residue weights for lysozyme and the present three derivatives.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Molecular weight</th>
<th>Nitrogen content (%)</th>
<th>Mean residue weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>14306</td>
<td>18.8</td>
<td>110.9</td>
</tr>
<tr>
<td>NT₂-lysosyme</td>
<td>14370</td>
<td>18.9</td>
<td>111.4</td>
</tr>
<tr>
<td>AT₄-lysosyme</td>
<td>14388</td>
<td>19.0</td>
<td>110.9</td>
</tr>
<tr>
<td>NPS₂-lysosyme</td>
<td>14865</td>
<td>18.7</td>
<td>115.2</td>
</tr>
</tbody>
</table>

1 The abbreviations used are: ORD, optical rotatory dispersion; CD, circular dichroism; EDS, sodium dodecyl sulfate; NT₂-lysosyme, lysozyme nitrated with the use of tetrathionemethane at 2 tyrosine residues (tyrosines 20 and 23, see Reference 1); AT₄-lysosyme, derivative obtained by reduction of the 2 residues of 3-nitrotyrosine in NT₂-lysosyme to 3-aminotyrosine; NPS₂-lysosyme, lysozyme modified at all 6 tryptophan residues with 2-nitrophenyl sulfenyl chloride.

**RESULTS**

**Optical Rotatory Dispersion Measurements**—Each of the three lysozyme derivatives studied here showed a negative rotation.
FIG. 1 ORD spectra of (1) NPS₄-lysozyme, (2) lysozyme, (3) NT₄-lysozyme, (4) AT₄-lysozyme. Measurements were carried out on the solutions in water.

TABLE II

ORD parameters of lysozyme and derivatives

<table>
<thead>
<tr>
<th>Derivative</th>
<th>pH</th>
<th>[m']₂₃₃</th>
<th>[m']₉₀</th>
<th>b₀</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>6.50</td>
<td>-4250</td>
<td>+15,600</td>
<td>-205</td>
</tr>
<tr>
<td>NT₄-lysozyme</td>
<td>6.76</td>
<td>-4875</td>
<td>+17,910</td>
<td>-256</td>
</tr>
<tr>
<td>AT₄-lysozyme</td>
<td>6.52</td>
<td>-4890</td>
<td>+18,800</td>
<td>-259</td>
</tr>
<tr>
<td>NPS₄-lysozyme</td>
<td>6.29</td>
<td>-3605</td>
<td>+12,850</td>
<td>-275</td>
</tr>
</tbody>
</table>

Measurements were carried out on the solutions in water. For experimental details, see text.

Minimum at 233 nm and a positive rotation extremum 199 nm. The ORD spectra of lysozyme, NT₄-lysozyme, AT₄-lysozyme and NPS₄-lysozyme are shown in Fig. 1. It can be seen that NT₄-lysozyme and AT₄-lysozyme had similar ORD spectra and each was more rotatory than native lysozyme both at the negative minimum and at the positive maximum. The b₀ values (Table II), which were almost identical for NT₄-lysozyme (−256) and AT₄-lysozyme (−260), were also higher than the corresponding value for lysozyme (−205). On the other hand NPS₄-lysozyme was less rotatory than lysozyme at the negative minimum at 233 nm and at the positive maximum at 199 nm.

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Fig. 2. Effect of change in pH on the rotatory behavior of lysozyme and derivatives. a, change in [m']₂₃₃ values; b, corresponding change in b₀ values. ○, lysozyme; △, NT₄-lysozyme; ▲, AT₄-lysozyme; □, NPS₄-lysozyme. Measurements could not be carried out on AT₄-lysozyme above pH 7.5 and on NPS₄-lysozyme above pH 8.5 due to their spontaneous and continuous precipitation.

Since the ionization properties of the phenolic hydroxyl in nitrotyrosine and aminotyrosine are different (cf. pKₐ, tyrosine 10.1 (9); 3-nitrotyrosine, 7.2 (10); 3-aminotyrosine 10.0 (10)), then the conformations of NT₄-lysozyme and AT₄-lysozyme might change in different ways on variation of pH. It was therefore decided to investigate the effect of pH on the conformation of these derivatives. The change in the rotatory behavior of lysozyme and its three derivatives as a function of pH are shown in Fig. 2. In the upper half of the diagram the values of [m']₂₃₃ are plotted, while the lower half of the diagram shows the corresponding b₀ values. From these, it can be seen that lysozyme undergoes little or no conformational change in the pH range 3.0 to 8.0. A small decrease in the value of [m']₂₃₃ was apparent around pH 9.0 which coincides with the isoelectric point of lysozyme. The value of [m']₂₃₃ for NPS₄-lysozyme remained virtually unaltered between pH 3 to 8.5, while the b₀ values decreased steadily. Both NT₄-lysozyme and AT₄-lysozyme showed little or no change in the value of [m']₂₃₃ or in their b₀ values between pH 3 to 7. Their b₀ values were almost identical throughout this range. The small difference in their [m']₂₃₃ disappeared completely around pH 7.0 as they seemed to possess identical mean residue rotations around neutrality. Measurements at pH values higher than 7.5 could not be performed on AT₄-lysozyme, since at higher pH values solutions of this derivative suffered spontaneous and continuous precipitation.

Circular Dichroism Measurements—Conformational changes were also investigated by CD measurements. Lysozyme and the three derivatives showed negative ellipticity bands at 208
TABLE III

CD parameters of lysozyme and derivatives

Measurements were carried out on the solutions in water. For experimental details, see text.

<table>
<thead>
<tr>
<th>Derivative</th>
<th>$\sigma^0_{222}$</th>
<th>$\sigma^0_{280}$</th>
<th>$\sigma^0_{222}/\sigma^0_{280}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>8,680</td>
<td>7330</td>
<td>0.845</td>
</tr>
<tr>
<td>NT$_1$-lysozyme</td>
<td>11,570</td>
<td>8970</td>
<td>0.775</td>
</tr>
<tr>
<td>AT$_2$-lysozyme</td>
<td>10,920</td>
<td>8490</td>
<td>0.777</td>
</tr>
<tr>
<td>NP$_S$-lysozyme</td>
<td>6,680</td>
<td>6050</td>
<td>0.906</td>
</tr>
</tbody>
</table>

Fig. 3. CD spectra of NPS$_S$-lysozyme (1), lysozyme (2), AT$_2$-lysozyme (3), and NT$_1$-lysozyme (4). Measurements were carried out on the solutions in water.

nm and a shoulder around 220 nm. However, the ellipticities for NPS$_S$-lysozyme at both wavelengths were greatly suppressed relative to the corresponding values of lysozyme. On the other hand, these ellipticity values for NT$_1$-lysozyme and AT$_2$-lysozyme which were similar, were higher than the values of the native protein (Table III). These findings were in agreement with the aforementioned ORD results. The CD spectra of lysozyme and its three derivatives are shown in Fig. 3. These results, together with those from ORD studies suggest a considerable degree of unfolding in NPS$_S$-lysozyme.

Effect of Binding with SDS on Accessibility of Disulfide Bonds to Reduction—In aqueous solution and in the absence of dissociating agents, none of the four disulfide bonds in lysozyme is accessible to reduction with mercaptoethanol (1, 2, 6). This is also shown in Fig. 4, where it can be seen that lysozyme, in the absence of SDS, had only 0.03 disulfide bond that was reducible. On the other hand, NT$_1$-lysozyme and AT$_2$-lysozyme exhibited almost identical disulfide accessibility, each showing one susceptible disulfide bond after reduction for 120 min. NPS$_S$-lysozyme exhibited an even greater disulfide accessibility as shown in Fig. 5. Almost two and a half disulfide bonds were reducible in this derivative.

The effect of binding with SDS on the mode of folding of these proteins and thus on the accessibility of the disulfide bonds was quite revealing. Upon binding with SDS, lysozyme, NT$_1$-lysozyme, and AT$_2$-lysozyme showed a great increase in the accessibility of the disulfide bonds to reduction. Thus an average of one disulfide bond becomes accessible in lysozyme, and in
NT$_2$-lysozyme and AT$_2$-lysozyme two disulfide bonds become reducible. This suggests that binding with SDS induces 'relaxed' conformations in each of lysozyme, NT$_2$-lysozyme, and AT$_2$-lysozyme. The accessibility of the disulfide bonds to reduction in these three proteins, in the presence and absence of SDS is shown in Fig. 4. It is noteworthy that the behaviors of NT$_2$-lysozyme and AT$_2$-lysozyme are almost identical in the presence or absence of SDS. The behavior of NPS$_5$-lysozyme is shown in Fig. 5. It can be seen that upon binding with SDS, the accessibility of the disulfide bonds to reduction is greatly decreased. For example after 15 min of reduction, NPS$_5$-lysozyme had 2.3 reducible disulfides in the absence of SDS and this decreased to 0.26 disulfide upon binding with SDS. Even when reduction was continued for longer durations, the disulfide accessibility, upon binding of this derivative with SDS did not exceed 1.3 reducible bonds. These results clearly indicate that NPS$_5$-lysozyme (in contrast with lysozyme and its tyrosyl derivatives) assumes a 'constrained' or tighter conformation upon binding with SDS.

**DISCUSSION**

Considerable conformational differences between lysozyme and its three derivatives were revealed by the present ORD and CD measurements. The rotatory properties of NPS$_5$-lysozyme suggest a great degree of unfolding in this derivative. However, the observed increase in the $b_0$ values might be hard to reconcile with the decrease in the values of $[\mu']$ and $[\theta']$ relative to lysozyme. This may be partly caused by the contribution of the new aromatic side chains as a result of introducing the aromatic nitrophenyl sulfenyl groups. Also the unfolding will cause some exposure of the tryptophan residues which might generate some aromatic side chain optical activity. The increase in the $b_0$ value as the pH is lowered from 8.5 to 3.0 lends further support that this was caused by optical activity of side chains that have been exposed as a result of increased unfolding in acid medium.

The rotatory behaviors of the tyrosyl derivatives of lysozyme were quite different from that of NPS$_5$-lysozyme. The increases observed in the mean residue rotations at 223 and 199 nm were also accompanied by a corresponding increase in the $b_0$ value. These results suggested that NT$_2$-lysozyme and AT$_2$-lysozyme were more folded than lysozyme and this conclusion was also confirmed by a corresponding increase in the ellipticity. Both tyrosyls 20 and 23 are exposed and exist in a nonhelical region (11) and it therefore was not expected that their modification to nitrotyrosine or aminonitrotyrosine should induce a more folded conformation. The third tyrosine in lysozyme, which is located at position 53 (12), has been shown to be inaccessible to reaction with tetranitromethane (1) or to iodination (13). Tyrosine 53 is located in the short antiparallel pleated sheet structure comprising residues 41 to 54 and is engaged in hydrogen bonding (11) which might account for its nonreactivity. It is highly significant that NT$_2$-lysozyme and AT$_2$-lysozyme showed identical ORD and CD parameters suggesting that these two derivatives had closely related, if not identical, conformations. The rotatory behaviors of lysozyme and its three derivatives exhibited almost no change on decrease of pH from neutrality to around pH 3.0, indicating that lysozyme and its three derivatives undergo little or no acid denaturation expressed as unfolding on decrease of pH to 3.0. In contrast, myoglobin, which has no disulfide bonds, suffers a sharp and considerable degree of unfolding between pH 3.7 and 3.5 as revealed by its ORD and CD behavior (14).

Further investigation of differences in conformation were carried out by determining the susceptibility of the disulfide bonds to reduction in presence and absence of SDS. Conformational changes in proteins containing disulfide bonds can be evaluated by monitoring the accessibility of the disulfide bonds to reduction as compared to those available in the native protein (8, 15). In this approach there already yielded valuable information on some derivatives of lysozyme (1, 2, 16). Also, conformational differences between lysozyme and $\alpha$-lactalbumin were first revealed, and their existence suggested, because of differences in their disulfide accessibility (6). The existence of these conformational differences have since been further confirmed by small angle diffraction measurements (17) and by the demonstration that remarkable differences exist in the reactivity of the carboxyl groups in the two proteins (18). The polypeptide chain in lysozyme is folded in such a way so as to produce a high degree of rigidity and unavailability of its disulfide bonds to reduction (1, 2, 6). In NPS$_5$-lysozyme, the large increase in disulfide accessibility was caused by a great degree of unfolding and this was in agreement with the conclusions obtained from ORD and CD measurements. The conformational changes that occurred upon modification of the tyrosol residues were accompanied by an appreciable increase in disulfide accessibility. It is relevant to point out that disulfide reducibility was almost identical in the two derivatives, NT$_2$-lysozyme and AT$_2$-lysozyme, again suggesting similar conformational changes.

Binding of proteins with SDS induces conformational changes in proteins and has been shown by various investigators (19-23) to protect certain proteins against urea denaturation. Habeel (8) has shown that the binding of SDS to bovine serum albumin resulted in a relaxed conformation with increased susceptibility of disulfide bonds to reduction. Binding of some nonhelical proteins with SDS has also been reported (24) to promote ordered structures to a considerable degree. It appears that the effect on highly helical proteins is much less than that on nonhelical proteins (25-28). In the present work, lysozyme appeared to assume a relaxed conformation upon binding with SDS. In the tyrosyl derivatives, NT$_2$-lysozyme and AT$_2$-lysozyme, a relaxed conformation is also observed in each case. However, it should be pointed out here that the S 4 complexes of these two tyrosyl derivatives showed identical disulfide accessibility. In contrast, is the unique behavior of NPS$_5$-lysozyme which undergoes some tightening on binding with SDS resulting in a 'constrained' conformation.

The similarity in conformation of NT$_2$-lysozyme and AT$_2$-lysozyme, concluded from ORD and CD data and from disulfide accessibility in the presence and absence of SDS is in agreement with previous reports on the susceptibility of these derivatives to tryptic attack (1). Whereas lysozyme is completely inaccessible to hydrolysis with trypsin, the two tyrosyl derivatives and NPS$_5$-lysozyme showed an appreciable degree of accessibility (e.g., alkali consumptions in 5 hours of trypptic digestion were: lysozyme, 0.16; NT$_2$-lysozyme, 3.70; AT$_2$-lysozyme, 3.73; and NPS$_5$-lysozyme, 4.96 mole of NaOH per mole of protein). Not only were the number of peptide bonds cleaved on tryptic hydrolysis identical in NT$_2$-lysozyme and AT$_2$-lysozyme, but also similar peptides were released from the two derivatives (1), and these did not resemble the peptides released by the trypptic hydrolysis of NPS$_5$-lysozyme.

The foregoing information clearly points to the conclusion that the tyrosyl derivatives NT$_2$-lysozyme and AT$_2$-lysozyme have
closely similar, if not identical conformations. It is, of course, likely that minor differences in conformation may exist that cannot be detected by the present methods. On the other hand, NPS
-lysozyme is more unfolded.

Enzyme and immunochemical studies have been carried out on these derivatives. NPS
-lysozyme has no enzymatic activity (2), and much lower activity was observed in NT
-lysozyme (50%) and AT
-lysozyme (56%) relative to lysozyme (1). Precipitin reactions were performed at pH 7.5. With antisera to native lysozyme, NPS
-lysozyme reacted poorly (18%), and an appreciable decrease in reactivity (down to about 80%) was observed for NT
-lysozyme (1). Also, lysozyme reacted with antisera to NT
-lysozyme, to the extent of 75 to 82% relative to the homologous reaction. On reduction of the nitrotyrosine residues to aminotyrosine, the antigenic reactivity with antisera to lysozyme was completely recovered (99 to 100%). Conformational changes still persisted in AT
-lysozyme and, from the foregoing discussion, appear to be almost identical with those in NT
-lysozyme. The decrease in antigenic reactivity, therefore, is directly related to the modification of tyrosyl side chains and not a by-product of conformational changes. Nitration of the tyrosine residues ortho to the phenolic hydroxyl will effect an increase in the acidity of this OH group, because of the electron-withdrawing influence of the nitro group (cf. pK values, 3-nitrotyrosine, 7.2 (Reference 10); tyrosine, 10.1 (Reference 9)). If the decrease in antigenic reactivity is indeed directly related to nitration and lowering of the pK of the phenolic hydroxyl, then complete recovery of this reactivity would be expected when the pK is increased to its original value by reduction to 3-amino
tyrosine (pK = 10.0 (Reference 10)). Atassi (29) has shown that nitration is sufficient to completely eliminate the reactivity of the COOH-terminal antigenic reactive region in myoglobin. Loss in enzymic activity must be caused by the conformational alteration, which is still present in all three derivatives.

In conclusion, the foregoing results show that nitration of tyrosines 20 and 23 in lysozyme and their subsequent reduction to aminotyrosine give two derivatives that possess very similar, if not identical conformations. Modification of the tryptophan residues yields a derivative which is greatly unfolded. From these results and the previously reported immunochemical findings (1), it can be concluded that one or both of tyrosines 20 and 23 is located in an antigenic reactive region in lysozyme. It is now well established (30-33, 14) that the antigenic reactivity of proteins is highly influenced by changes in the conformation of the antigen. Also, the primary antibody response is directed against the native, three-dimensional structure of a protein antigen (33). However, the present findings clearly show that not every conformational change will be expected to exert an influence on the antigenic reactivity. This will rather depend on the protein and the nature of the conformational change.

REFERENCES

CHEMISTRY AND METABOLISM OF MACROMOLECULES:
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