Denaturation of Globular Proteins

II. THE INTERACTION OF UREA WITH LYSOZYME*

JOHN R. WARREN‡ AND JULIUS A. GORDON

From the Department of Pathology, University of Colorado School of Medicine, Denver, Colorado 80220

SUMMARY

The extent of urea interaction with lysozyme in aqueous solution at various concentrations of urea was shown to equal that previously reported by us for bovine serum albumin (GORDON, J. A., AND WARREN, J. R., J. Biol. Chem., 243, 5663 (1968)). In the absence of supporting electrolyte, in contrast to albumin, the progressively greater interaction of urea with lysozyme from 3 M to 9 M urea was unaccompanied by significant change in optical rotatory parameters \( [m^D_{578} = -52°, b_0 = -142] \) or intrinsic viscosity \( [\eta^{[20]} = 5.0 \text{ cc g}^{-1}] \) of the protein. Lysozyme in water did show a decrease in its intrinsic viscosity to 2.8 cc g\(^{-1}\) and a 10-fold increase in enzymic activity upon the addition of 0.2 M NaCl. Exposure of lysozyme in aqueous 0.2 M NaCl to 8.1 M urea then increased the intrinsic viscosity of the protein to 4.6 cc g\(^{-1}\).

Additional studies showed that, when measured by optical rotation, solubility, and sulphydryl group determination, lysozyme in water was resistant to reduction by 0.09 M 2-mercaptoethanol or 0.02 M dithiothreitol. The further addition of urea induced unfolding of the protein with a forward rate constant dependent upon the 3.9th power of urea, as measured by changes in optical rotation, and a final intrinsic viscosity of 18.7 cc g\(^{-1}\). The appearance of around eight sulphydryl groups for each completely unfolded lysozyme molecule was shown to be independent of nonspecific solvent, pH, or oxidation-reduction potential effects. The number of urea molecules interacting with each reduced, unfolded lysozyme molecule (1 urea molecule per 2.8 amino acid units) increased over that seen with unreduced, folded lysozyme (1 urea molecule per 3.5 amino acids).

The native stability toward mercaptans observed with lysozyme in aqueous acetamide was consistent with the smaller solute-protein interaction and the known poor denaturing ability of this amide.

The behavior of lysozyme in aqueous urea and acetamide is consistent with the following conclusions: (a) like serum albumin, the extent of solute-protein interaction is paralleled by some degree of protein perturbation, the latter reflected by lysozyme in change of intrinsic viscosity or disulfide bond stability (or both); (b) unlike serum albumin, the disruption of lysozyme conformation upon interaction with 8 M urea alone is small by the criteria of optical rotatory dispersion and viscometry; (c) the ratio of interaction of urea with reduced and unfolded lysozyme is nearly identical with that found previously with unfolded serum albumin, suggesting common sites of interaction such as peptide groups.

In a recent paper we reported that the denaturation of bovine serum albumin in aqueous solutions of urea appears to involve interaction of urea with the protein (1). The extent of urea interaction with albumin at different concentrations of urea, as determined by ultrafiltration, was seen to parallel the degree of denaturation of the protein, as judged from changes in optical rotatory dispersion. Unlike serum albumin and most other globular proteins, the conformation of lysozyme shows little or no change when routinely exposed to concentrated urea solutions (2-4). Lysozyme is therefore thought to be very "resistant" to urea. If urea-protein interaction is a common factor in the mechanism of protein denaturation, one might expect lysozyme to reveal little or no interaction with urea even at high concentrations of this denaturant. Thus, a study of lysozyme in urea solutions by the technique of ultrafiltration was instituted to evaluate further the role of urea-protein interaction in the mechanism by which urea denatures proteins.

The data reported in this paper show that urea interacts with lysozyme in a fashion and extent similar to that found with bovine serum albumin. In the absence of added NaCl, spectropolarimetry and viscometry failed to reveal additional alteration in the conformation of lysozyme following interaction with urea. Nevertheless, the native stability of intermolecular disulfide bonds to mercaptans was lost upon such interaction. In the presence of 0.2 M NaCl a conformational change upon urea interaction was clearly detected by viscometry. Thus, as with serum albumin, there exists a positive correlation between the degree of urea-lysozyme interaction and the magnitude of protein perturbation.

A preliminary report has been published (5).

MATERIALS AND METHODS

Materials—Lysozyme (crystalline (Worthington), Lots LY8HA, LY8HB, LY8HC), purified by extensive dialysis against distilled water, was stored over Drierite under a vacuum at 4° until used. Residual water as determined by exposure of the protein to dry heat (105° for 12 hours) never exceeded 3% (w/w).
Organic and inorganic reagents were the best available commercial products and were used without further purification.

**Ultrafiltration**—Ultrafiltration experiments were performed as detailed previously (1), utilizing a Diaflo model 50 ultrafiltration cell (Amicon). Briefly, pressure in the cell was maintained by nitrogen gas at 46-pound pressure. Amicon UM-2 membranes were used exclusively and previously equilibrated with solvent. Desiccated lysozyme was next dissolved in a 20-ml aliquot of the solvent, the solution then being placed into the ultrafiltration cell. Six to eight ultrafiltrates were obtained and analyzed over a 43- to 60-min period. The refractive index at 20°C of each ultrafiltrate was determined to an accuracy of ±0.00002 on a Bellingham and Stanley Abbey 60 high accuracy refractometer. Changes in concentration were then ascertained by comparison of the average refractive index of the ultrafiltrates with the refractive index of the retentate determined before the addition of protein. The difference in refractive index was translated into change of turbidity by comparison with standard turbidity-refractive index curves from 0 to 8 M for aqueous urea and acetamide (6). Variation between ultrafiltrates in a series obtained from an individual experiment was less than ±0.005 M. Protein impermeability of the UM-2 membrane in each experiment was previously tested as before (1) and reconfirmed by absence of protein absorption at 292 mp for the pooled ultrafiltrates.

**Spectropolarimetry**—Relatively concentrated stock solutions of salt-free protein were prepared with deionized and once distilled water. Solutions for polarimetry were obtained by volumetric dilution of the stock solutions with precision microburets in the appropriate solvents. Lysozyme concentrations were determined by spectrophotometry with ε282 = 26.41. The pH was determined by a Radiometer model TTTlc meter. Results are reported as the mean residue rotation at 578 mp where

\[ [\alpha]_\text{res}^\text{obs} = \frac{3M_p}{100(\text{n}^2 + 2)} \]

in which \([\alpha]_\text{res}^\text{obs}\) is the specific rotation at 20°C, \(M_p\) is the mean residue weight of the protein (\(M_p\) lysozyme, 111), and \(n\) is the refractive index of the solvent. The refractive index of water appropriate to each wave length was used in all calculations. No adjustment was made for the contribution of urea or acetamide to the refraction of the solvent (1). The mean residue rotation for separate preparations of each solution was reproducible within ±2.0° at 578 mp. The dispersion of rotation with wave length was analyzed by the Moffitt-Yang equation

\[ [\alpha]_\text{res}^\text{obs} = \frac{a_0\lambda^2}{\lambda^2 - \lambda_0^2} + \frac{b_0\lambda^4}{(\lambda^2 - \lambda_0^2)^2} \]

with \(\lambda_0\) set at 212 mp. The values of \(b_0\) reported in this paper were derived from the slope of linear plots of \([\alpha]_\text{res}^\text{obs}(\lambda^2 - \lambda_0^2)^{-2}\) against \((\lambda^2 - \lambda_0^2)^{-2}\). ORD measurements of lysozyme in water and 8.1 M urea were also made from 230 to 340 mp in a 2.0-mm water-jacketed cell with a Cary model 60 automatic recording spectropolarimeter. Measurements below 212 mp in 8.1 M acetamide were not possible because of the large absorbance of the incident beam by the solvent.

The abbreviation used is: ORD, optical rotatory dispersion.

**Viscometry**—Viscosities were determined in Cannon-Manning semimicro viscometers having water flow times of around 250 sec at 20°C. Flow times in the presence of mercaptan were measured 48 hours following the preparation of the solution to permit reduction and unfolding of the protein, if occurring, to proceed to completion. Results are given as the intrinsic viscosity which is calculated by extrapolating the reduced viscosity determined at three separate protein concentrations ranging from 0.7% to 2.5% lysozyme in each solvent studied to zero protein concentration. The reduced viscosity is given as

\[ \eta_\text{red} = \frac{t - t_0}{t_\infty} + \frac{t - t_0}{t_\infty} \]

where \(t\) and \(t_0\) are the flow times of protein-containing and protein-free solvent respectively, \(c\) is protein concentration (grams per cc), \(t_0\) is the density of protein-free solvent, and \(\bar{v}\) is the partial molar volume of lysozyme taken to be 71. Variation in the reduced viscosity as determined on separate aliquots of the same solution was less than 1%.

**Conductance**—All conductivity measurements were reproducible to ±1.0% on 3.0-ml aliquots of solution utilizing a YSI model 31 conductivity bridge (Yellow Springs Instrument Company) with a 3403 conductivity cell in the upright position.

**Sulfhydryl Determination**—The method of Zahler and Cleland (7) was modified as follows. A 0.05-ml aliquot of 1.0% lysozyme in the appropriate solvent (dithiothreitol in water or 8.1 M urea) was added to 0.55 ml of 5.7 × 10⁻⁴ M sodium arsenite made up in a 0.2 M Tris buffer. After 5 min, 7.00 ml of 3.7 × 10⁻⁴ M 5,5'-dithiobis(2-nitrobenzoic acid) were added to the reaction mixture, giving a final pH of 8.1. The change in optical density at 412 mp was then followed for 60 sec on a Beckman DU spectrophotometer against a solvent blank prepared identically but without lysozyme. Sulfhydryl content was calculated from the observed absorbance by reference to standard curves prepared with aqueous 0.14 to 1.00 × 10⁻² M 2-mercaptoethanol solutions.

**Enzymatic Assay**—A stock solution of extensively dialyzed, salt-free 0.5% lysozyme was prepared. The substrate consisted of aqueous suspensions of Micrococcus lysodeikticus cells (Worthington) extensively dialyzed against distilled water to a final conductance of 50 μhos and optical density at 450 mp near 1.3 units. Enzyme activity was determined (8) by adding 0.1 ml of stock solution to 3.0 ml of substrate solution and following the change of absorbance at 450 mp on a Cary model 15 automatic recording spectrophotometer. The presence of 0.1 M NaCl in the reaction mixture increased conductance from 50 μhos to 5500 μhos. Final enzyme concentrations were higher than the usual assay conditions in order to observe the reaction under conditions of no salt.

**RESULTS**

To measure urea-lysozyme interaction accurately, ultrafiltration was initially performed utilizing solvents containing only two dialyzable components (e.g. urea and water). Under such conditions a decrease of urea concentration from the initial urea concentration occurred in ultrafilters obtained from aqueous solutions following the addition of anhydrous lysozyme (Table I). A ratio of interaction of 1 urea molecule to 3 amino acid residues in each lysozyme molecule was observed in the most concentrated urea solution studied. In contrast to the dependence of urea-protein interaction upon urea concentration, we
TABLE I

Interaction of denaturants with lysozyme in absence of reducing agents

<table>
<thead>
<tr>
<th>Denaturant</th>
<th>Initial concentration (AM riz 0.005)</th>
<th>Change in absorbance (ΔM ± 0.005)</th>
<th>Calculated ratios of interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Denaturant to protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>9</td>
<td>-0.118</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>-0.103c</td>
<td>37c</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-0.094</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-0.066c</td>
<td>24c</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-0.059</td>
<td>21</td>
</tr>
<tr>
<td>Acetamide</td>
<td>8</td>
<td>-0.077</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>-0.054</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>-0.057</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-0.052</td>
<td>11</td>
</tr>
</tbody>
</table>

* Anhydrous lysozyme added to 4.0% (grams per volume).
* Values are the average of two or more separate ultrafiltration experiments and are adjusted for the hydration of lysozyme, assumed to be 2070, as described previously (1).
* Same values obtained upon the addition of 0.20 M NaCl to the indicated urea solvents (see text).

found that the optical rotatory dispersion of lysozyme was independent of urea concentration (Lines 1, 3, 5 and 8, Table III; Fig. 1). In addition, the intrinsic viscosity of lysozyme with and without urea was nearly identical (Lines 1 and 2, Table II) and significantly higher than that reported for native globular proteins (9).

Since the viscosity of lysozyme in pure water suggests an unusually swollen conformation, viscometric determinations were repeated in the presence of 0.2 M NaCl. The intrinsic viscosity of lysozyme decreased by about 40% with increase in ionic strength to 0.2 (Lines 1 and 3, Table II). The enzymatic activity of the protein was also significantly increased upon transfer of lysozyme from water to 0.1 M NaCl (Fig. 2). When this more compact lysozyme molecule was then exposed to 4.0 M urea at the same ionic strength, intrinsic viscosity of the protein increased by 21% (Lines 3 and 4, Table II), when exposed to 8.1 M urea by 64% (Lines 3 and 5, Table II). These changes in intrinsic viscosity for lysozyme in water, 0.2 M NaCl, or 8.1 M urea-0.2 M NaCl were unaccompanied by change in [m] 233 or b 0 of the protein. Ultrafiltration experiments were repeated at 4 M and 8 M urea in the concomitant presence of 0.2 M NaCl. Change of salt concentration in the filtrates was detected by conductivity measurements and contributed less than 10% to the total refractive index change. The extent of urea interaction was found to be identical in the presence or absence of NaCl (Table I).

A nonreactive group in native lysozyme toward mercaptoethanol, the intramolecular disulfide bonds (10, 11), was next studied in salt-free urea solvents to interpret the significance of the solute-protein interaction seen in such solvents. Following the addition of lysozyme to 3.6, 6.3, 8.1, or 9.0 M urea solutions containing

* Upon the addition of 8.1 M urea to lysozyme in water or 0.2 M NaCl, a small but reproducible change in the Moffitt-Yang parameter a0 from -280 to -255 occurred. Although indicative of some disruption in the nonhelical portion of the lysozyme molecule, the magnitude of the increase in a0 was too small for a study of the effect of varying urea concentration.

0.09 M 2-mercaptoethanol, the levorotation of the protein became significantly more negative (Fig. 3, A and B) and b 0 less negative (Lines 4, 6, and 9, Table III). The intrinsic viscosity of lysozyme in 8.1 M urea-0.09 M 2-mercaptoethanol was also found to be more than 3 times greater than that observed for the protein in 8.1 M urea alone (Lines 2 and 7, Table II). Alteration of the rate and extent of change in optical rotation by the possible carbamylation of ε-amino or free sulfhydryl groups by cyanate (12) was excluded by our observation that preliminary incubation of the protein in 8.1 M urea for 4000 min at room tempera

![Fig. 1. Optical rotatory dispersion of lysozyme in the near ultraviolet region. The reduced rotation of lysozyme at 27° in water (O-O), 8.1 M urea (O-O), and 8.1 M acetamide (△-△) is plotted against wave length.](image-url)
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FIG. 2. Kinetic analysis for the turbidimetric assay of lysozyme activity in water (△) and 0.1 M NaCl (○) for which \( f_{UL} \) (fraction of unlysed cell membranes) =

\[
\frac{\text{OD}_{\text{final}} - \text{OD}_{\text{time}}}{\text{OD}_{\text{final}} - \text{OD}_{\text{initial}}}
\]

with OD being optical density at 450 nm, and \( k \) = rate of cell lysis \( \times 10^4 \).

TABLE III

Optical rotatory parameters of lysozyme in various solvents

Lysozyme was added to 1% (grams per volume). Reported parameters represent final, unchanging values in each solvent (see text).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>2-Mercaptoethanol ( \times 10^{-6} )</th>
<th>Apparent pH</th>
<th>[( \psi )] ( \times 10^4 )</th>
<th>( \delta )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HOH</td>
<td>0</td>
<td>6.0</td>
<td>-55.2</td>
<td>-144.0</td>
</tr>
<tr>
<td>2. HOH</td>
<td>+</td>
<td>6.1</td>
<td>-52.3</td>
<td>-141.7</td>
</tr>
<tr>
<td>3. 3.6 M urea</td>
<td>0</td>
<td>6.5</td>
<td>-49.4</td>
<td>-142.8</td>
</tr>
<tr>
<td>4. 7.6 M urea</td>
<td>+</td>
<td>7.0</td>
<td>-86.9</td>
<td>-34.8</td>
</tr>
<tr>
<td>5. 8.1 M urea</td>
<td>0</td>
<td>6.9</td>
<td>-82.7</td>
<td>-144.7</td>
</tr>
<tr>
<td>6. 8.1 M urea</td>
<td>+</td>
<td>7.5</td>
<td>-95.6</td>
<td>-16.5</td>
</tr>
<tr>
<td>7. 8.1 M urea</td>
<td>+</td>
<td>6.2</td>
<td>-92.3</td>
<td>-33.6</td>
</tr>
<tr>
<td>8. 9.0 M urea</td>
<td>0</td>
<td>7.1</td>
<td>-49.7</td>
<td>-139.5</td>
</tr>
<tr>
<td>9. 9.0 M urea</td>
<td>+</td>
<td>7.5</td>
<td>-95.7</td>
<td>-22.6</td>
</tr>
<tr>
<td>10. 8.1 M acetamide</td>
<td>0</td>
<td>5.8</td>
<td>-47.2</td>
<td>-141.2</td>
</tr>
<tr>
<td>11. 8.1 M acetamide</td>
<td>+</td>
<td>5.9</td>
<td>-47.5</td>
<td>-143.8</td>
</tr>
</tbody>
</table>

* When present, added to a concentration of 0.09 M.

* Experimental pH of protein-containing solutions to an accuracy of \( \pm 0.05 \) unit.

* Solution made up with \( \times 15 \) Sørensen's phosphate buffer.

Substitution of 0.02 M dithiothreitol for mercaptoethanol as a reducing agent induced similar changes in the ORD parameters of lysozyme in 8.1 M urea. Following complete unfolding of lysozyme in 0.02 M dithiothreitol-8.1 M urea, the number of free sulfhydryl groups per lysozyme molecule was found to be 7.5 ± 2.0. In contrast, 0.5 ± 1.5 sulfhydryl groups per lysozyme were observed for the protein in aqueous 0.02 M dithiothreitol without urea.

To clarify the mechanism by which urea facilitates the reduc-

Fig. 3. Optical rotatory parameters of lysozyme in the presence and absence of mercaptoethanol. A, reduced rotation at 578 m\( \mu \) and 90° in 8.0 m (△), 8.1 m (○), 6.8 m (⊙), and 3.6 m (△) urea containing 0.09 m 2-mercaptoethanol and 8.0 m urea lacking thiol (○) is plotted as a function of time. B, Moffitt-Yang parameter \( k_2 \) as a function of time.
TABLE IV
Forward rate of unfolding for lysozyme in various mercaptoethanol-containing solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Apparent pH</th>
<th>min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. HOH</td>
<td>6.1</td>
<td>No unfolding</td>
</tr>
<tr>
<td>2. 3.6 M urea</td>
<td>7.0</td>
<td>0.03</td>
</tr>
<tr>
<td>3. 6.3 M urea</td>
<td>7.5</td>
<td>0.23</td>
</tr>
<tr>
<td>4. 8.1 M urea</td>
<td>7.5</td>
<td>0.98</td>
</tr>
<tr>
<td>5. 8.1 M urea</td>
<td>6.2</td>
<td>0.43</td>
</tr>
<tr>
<td>6. 8.1 M urea-0.2 M NaCl</td>
<td>7.5</td>
<td>0.81</td>
</tr>
<tr>
<td>7. 9.0 M urea</td>
<td>7.5</td>
<td>1.06</td>
</tr>
</tbody>
</table>

a Solution made up with m/15 Sørensen’s phosphate buffer.

TABLE V
Interaction of urea with lysozyme in presence of reducing agent

<table>
<thead>
<tr>
<th>Reducing agent</th>
<th>Protein conformation</th>
<th>Change in molarity (Δm = 0.005)</th>
<th>Calculated ratios of interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td>Folded</td>
<td>-0.099</td>
<td>35/100</td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>Unfolded</td>
<td>-0.128</td>
<td>18/20</td>
</tr>
<tr>
<td></td>
<td>Folded</td>
<td>-0.106</td>
<td>38/20</td>
</tr>
<tr>
<td></td>
<td>Unfolded</td>
<td>-0.131</td>
<td>47/20</td>
</tr>
</tbody>
</table>

a Anhydrous lysozyme added to 4.0% (grams per volume) to aqueous 8 M urea containing the indicated mercaptan.

b At 0.09 M.

c Ultrafiltration performed within 45 min of exposure of lysozyme to thiol. Folded conformation confirmed by ORD.

d Ultrafiltration performed 2160 to 3600 min after exposure of lysozyme to thiol. Unfolded conformation confirmed by ORD.

e At 0.02 M.

The unfolding of lysozyme, the following experiments were performed. First, there is evidence that at an alkaline pH the action of sulphydryl group-reducing agents is potentiated by hydrogen bond-breaking solvents (13). However, we observed that at pH values closer to neutrality the large decrease in the levorotation of the model compound l-cystine upon exposure to 0.09 M 2-mercaptoethanol was very similar in rate and extent for the amino acid either in water or 8.1 M urea (Table VI). Since this loss in optical activity of cystine is dependent upon cleavage of its disulfide bond (14), aqueous urea solutions near neutrality appear not to facilitate the action of mercaptoethanol. Second, the rate of unfolding of lysozyme in 8.1 M urea buffered to pH 6.2 was diminished by about 60% (Line 5, Table IV). However, the extent of unfolding in buffered and unbuffered 8.1 M urea was the same (Lines 6 and 7, Table III). Third, lysozyme was seen to unfold partially under very mild oxidizing conditions, but only in the presence of 8.1 M urea. Thus, in aqueous 0.33% perchloric acid or 0.2501, performic acid the protein retained native values for both $[\theta]_227$ and $b_0$. Upon the addition of 8.1 M urea to each solution, $[\theta]_227$ decreased to around $-80^\circ$ and $b_0$ increased to $-74^\circ$. Since the action of oxidizing acids shows a strong preference for disulfide bonds independent of the oxidation-reduction potential of these bonds (15), it is reasonable to attribute the 50% unfolding observed to scission of the protein’s disulfides. Therefore, the reduction and unfolding of lysozyme by mercaptans in aqueous urea are independent of possible nonspecific solvent, pH, or oxidation-reduction potential effects.

The unfolding of lysozyme in urea-mercaptoethanol solutions can be represented by a modified two-state transition $N' \rightarrow U$ where $N'$ is folded lysozyme and $U$ is the completely unfolded lysozyme molecule. The fraction of folded lysozyme at any given time, $f_{N'}$, in each of the urea-mercaptoethanol solvents studied is expressed as

$$f_{N'} = \frac{[\theta]_227 - [\theta]_227_{100}}{[\theta]_227_{100} - [\theta]_227}$$

where $[\theta]_227_{100}$ is the reduced mean residue rotation of fully unfolded lysozyme, $[\theta]_227$ is the rotation of the folded protein, and
Optical rotatory power of L-cysteine in different solvents

<table>
<thead>
<tr>
<th>Solvent</th>
<th>2-Mercaptoethanol</th>
<th>Apparent pH</th>
<th>$\Delta [m']_{365}^{460}$</th>
<th>Loss of optical activity$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOH</td>
<td>0</td>
<td>5.5</td>
<td>None</td>
<td>0%</td>
</tr>
<tr>
<td>HOH M urea</td>
<td>+</td>
<td>5.6</td>
<td>+715$^c$</td>
<td>82%</td>
</tr>
<tr>
<td>8.1 M urea</td>
<td>+</td>
<td>6.1</td>
<td>+700$^c$</td>
<td>80%</td>
</tr>
<tr>
<td>8.1 M acetamide</td>
<td>+</td>
<td>5.5</td>
<td>+754$^c$</td>
<td>87%</td>
</tr>
</tbody>
</table>

$^a$ All solutions made up with 0.2 M Sørensen's phosphate buffer (pH 5.5); L-cystine was added to a concentration of 0.013% (grams per volume).

$^c$ Constant value obtained approximately 120 min after exposure of cystine to 0.09 M mercaptoethanol.

DISCUSSION

At least four distinct forms of lysozyme can be defined under the conditions of our study (Fig. 6). Lysozyme Form I represents the very compact form of the protein observed in aqueous 0.2 M NaCl. Since the protein revealed a low intrinsic viscosity comparable to most native globular proteins (9) and greatest enzymic activity at this ionic strength, Conformation I can be taken to represent native lysozyme. Upon the complete removal and fully unfolded lysozyme molecule. To eliminate the covalent interaction of mercaptetoethanol with free sulphydryl of lysozyme and the resultant concentration change in ultratfiltrates, the free mercaptoethanol was used in large excess (0:1) to protein thioles (16). Upon complete unfolding of lysozyme in the presence of mercaptoethanol the number of urea molecules reacting with each lysozyme increased from that seen in the absence of thiol (Table V). A similar increase in urea interaction upon unfolding of lysozyme was seen with 0.02 M dithiothreitol (Table V), a thiol with an oxidation-reduction potential (17) and molar refractive increment much different from mercaptoethanol. It appears, therefore, that the number of molecules of urea interacting with each lysozyme molecule in 8 M urea increased from 1 urea per 3.5 amino acid residues for the unreduced protein to 1 urea per 2.8 residues for the reduced, unfolded protein.

Acetamide was also seen to interact with lysozyme, although to a lesser extent than urea (Table I). A final value of 1 acetamide molecule interacting per 4.5 amino acid residues in each lysozyme molecule in 8 M acetamide was observed. Unlike urea, however, the optical rotatory parameters of lysozyme in 8 M acetamide remained unchanged and similar to those for the protein in water (Lines 10 and 11, Table III). The observed loss in optical activity of L-cystine in 8 M acetamide upon exposure to 2-mercaptopethanol excludes any inhibitory effect of the acetamide solvent itself on the reduction of disulfide bonds by mercaptoethanol (Table VI). A "destabilization" of the native conformation of lysozyme in acetamide solutions was expressed by the increased sensitivity of the protein to heat in acetamide compared to water (Fig. 5). With van't Hoff plots, the enthalpy of unfolding of lysozyme in 8 M urea was +59.2 kcal mole$^{-1}$, and in 8 M acetamide was +90.1 kcal mole$^{-1}$. Thus the extent of urea and acetamide interaction with lysozyme paralleled the thermal stability of the protein.
of salt from lysozyme an increase in intrinsic viscosity occurred, giving Form II. The interaction of urea with lysozyme in 8 M urea-0.2 M NaCl induced considerable swelling or increased asymmetry of the protein molecule (or both), the resulting conformation being referred to as lysozyme Form III. Lysozyme Form III, in marked contrast to Forms I and II, showed instability of its intramolecular disulfide bonds toward mercaptans. The reduction of the disulfide bonds of Form III resulted in a completely unfolded, randomly coiled protein, lysozyme Form IV (18). Each form defined by us for lysoyme may actually represent a composite of closely related yet separate conformers. However, it is useful to discuss the question of urea-lysozyme interaction in terms of these four forms, each operationally defined by ORD parameters, viscosity, and disulfide bond reactivity.

Transformation of lysozyme from Form I to III seen upon interaction with urea was a conformational change easily detected by measurement of intrinsic viscosity. Thus, interaction of urea with lysozyme giving a protein molecule less compact than the native form is compatible with our earlier observations on serum albumin (1). The transition from Form I to II can be interpreted as a conformational shift dependent upon a decrease in the ionic strength of the solvent. A similar viscosity change has been reported by Josefsson at a more acid pH (19). It is unlikely that protein aggregation could account for such a large change in viscosity (20). Since lysozyme is a very basic protein (21), swelling with diminution of enzymatic activity upon transition to lysozyme Form II could result from increased electrostatic repulsion between charged groups on the protein molecule. However, a change in protein conformation following the removal of NaCl is not unequivocally indicated since electroviscous effects (22) or increased protein hydration alone could explain the increased viscosity. Likewise, distortion of the substrate or alteration in the mode of substrate-enzyme binding would diminish enzymatic activity. If future work provides evidence that Form II is indeed not separate from Form I, the basic conclusion that urea-lysozyme interaction is accompanied by perturbation of the protein's conformation is strengthened.

Since the interaction of urea with lysozyme in the absence of supporting electrolyte did not result in conformational change of the protein clearly detectable by viscometry or ORD, the possibility still remained that urea can interact with a quasi-native or partially unfolded form of lysozyme without further alteration of conformation. However, transition from Form II to III did involve drastic change in the disulfide bond chemistry of the protein. The complete resistance of the intramolecular disulfide bonds of lysozyme in pure water to thiols (indicated by native values for ORD parameters with absence of the increased dextro-rotation seen upon disulfide reduction (14), complete absence of the protein precipitate observed in aqueous solutions of reduced lysozyme (23), and failure to detect a significant number of free sulfhydryl groups in such solutions) is similar to that observed for the native conformation of most other globular proteins (11). Thus, even though lysozyme Form II is possibly swollen with less enzymatic activity than the native Conformation I, both forms share resistance of intramolecular disulfides to reduction. The decreased stability of lysozyme's disulfide bonds upon exposure to concentrated urea as seen by us and others (2, 10, 24) is independent of pH, nonspecific solvent effects, or changes in the oxidation-reduction potential of the disulfide bonds, as documented in our investigation. Therefore, two reasonable explanations could account for the loss of native disulfide bond stability in urea. First, if those regions of the lysozyme molecule containing disulfide bonds are not stabilized by the disulfide bonds themselves, urea could act by decreasing the stability of the noncovalent forces in the disulfide-containing regions following reduction. However, the importance of intact intramolecular disulfide bonds (25, 26) in lysozyme's thermal and chemical stability (27-31) contradicts such a mechanism. A more likely explanation of the diminished disulfide bond resistance to mercaptans would be increased accessibility of the intramolecular disulfides of lysozyme to solvent following urea interaction. Gorin, Fulford, and Deonier (32) have recently demonstrated the probable importance of accessibility or non-accessibility in determining the reactivity of lysozyme's intramolecular disulfides toward different thiols. Nagy and Straub observed a direct relationship between resistance to electrolytic reduction and the number of nonpolar amino acid residues neighboring the disulfide bonds in globular proteins, electrolytic reduction of lysozyme occurring in aqueous 30% ethanol but not in water (33). Finally, conversion of other proteins from their native to denatured conformation increases the reactivity of their disulfides (16, 34, 35), most easily explained by increased accessibility of their disulfides to thiols. Thus, in the absence of salt, we conclude that urea interaction with the lysozyme molecule induces further rearrangement of structure in the vicinity of the disulfide bonds. Direct evidence of such a conformational rearrangement has been obtained by preliminary investigations in this laboratory showing an enhanced 260 μm circular dichroic (disulfide) band in Lysozyme III.

The values for both the intrinsic viscosity of lysozyme in the presence of salt and the rate constant for unfolding of reduced lysozyme were directly proportional to the added urea concentration. The effect of urea upon either the extent of swelling and disulfide bond accessibility of each lysozyme molecule or upon the magnitude of the equilibrium constant for the I = III transition could explain this urea dependence. Also, reduction of those disulfide bonds exposed in each lysozyme molecule or the irreversible conversion of each lysozyme molecule to completely unfolded Form IV through the pathway I = III → IV accounts for the reduction and unfolding of all lysozyme molecules even in dilute urea solutions.

Two fundamental issues remain unsettled in this report. First, identification of the nature of specific sites of interaction of urea and acetamide with lysozyme cannot be unequivocally revealed by the techniques of the present investigation. Structurally, urea could function as a strong bifunctional hydrogen bond donor. It is conceivable, therefore, that urea could interact with lysozyme through hydrogen bond formation with two peptide groups (30, 37). The lesser acetamide interaction would thus be explained by the inability of this compound to participate as a bifunctional hydrogen bond donor. The near identity of the final interacting ratios between urea and completely reduced and unfolded lysozyme (Form IV), urea and completely denatured albumin (1), or urea and other denatured proteins does suggest sites on the unfolded protein molecule independent of amino acid composition or sequence, perhaps peptide groups. Second, the intrinsic viscosity of unreduced lysozyme in the presence of urea is very much less than that seen for the randomly coiled conformation assumed by most proteins in concentrated urea.

a J. R. Warren and J. A. Gordon, unpublished results.
Thus, we would be remiss in not pointing out that this report does raise some doubt as to the decisive role of the "binding" of urea (and perhaps other amides or guanidine hydrochloride (38)) for the complete unfolding of lysozyme. The complete unfolding of proteins which possess unusually stable regions of structure maintained by strong hydrophobic forces or intramolecular disulfide bond (or both), such as lysozyme (26, 39), might depend upon disruption of the structural "cores" by some property (or properties) of very concentrated urea separate from that (or those) involved in direct urea-protein interaction. A possible example of such a separate property is the ability of urea to diminish hydrophobic forces (40), perhaps through disorganization of solvent structure (41). Studies similar to the one reported here, but utilizing synthetic polypeptides, currently are underway hopefully to clarify both of these points.

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REFERENCES