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Evidence for Residual Structure in Acid- and Heat-denatured Proteins*

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Kirk C. Aune, Ahmad Salahuddin, Mario H. Zarlengo, and Charles Tanford
From the Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27706

SUMMARY

Ribonuclease, lysozyme, and chymotrypsinogen undergo thermal transitions at low pH, which have been studied in considerable detail in several laboratories. This paper shows, by measurement of optical rotation, that the products of these transitions retain regions of ordered structure, susceptible to disruption by guanidine hydrochloride.

A number of very careful studies have been made in recent years of the thermal transitions which small globular proteins undergo at low pH. These transitions reflect the destruction of the ordered conformation of the native protein, and the products of the transition have the properties of highly disordered polypeptide chains. The principal objective of such studies has, however, been to determine the thermodynamic parameters of the transition, and relatively little effort has been made to characterize the products of the transitions precisely. Excellent examples of such studies are the studies of the thermal unfolding of ribonuclease, as reported by Hermans and Scheraga (1), Scott and Scheraga (2), and by Brandts (3); the thermal unfolding of lysozyme, reported by Sophianopoulos and Weiss (4); and the thermal unfolding of chymotrypsinogen, as reported by Brandts and Lumry (5) and by Brandts (6, 7).

Recent papers from this laboratory (8–11) on the other hand, have been primarily concerned with characterization of the product of denaturation. The studies have been confined, however, to proteins which have been denatured at room temperature by the addition of guanidine hydrochloride. The results have shown that this denaturation process leads to the formation of random coils, devoid of long-range, noncovalent structure. This conclusion appears to apply not only to proteins which have no disulfide cross-links, or in which disulfide bonds have been broken, but also to proteins which retain disulfide bonds. If disulfide bonds are present, the freedom of motion of the disordered polypeptide chains is naturally restricted, and the domain of the coiled chain in solution is reduced in volume. The experimental evidence indicated, however, that no structured regions, maintained by noncovalent bonds, are created (in concentrated guanidine HCl) as a result of these physical constraints.

These studies suggest the need for a comparison between the product obtained by guanidine HCl denaturation and by heat denaturation at low pH. Is the product of the thermal denaturation also a random coil, devoid of regions of cooperative structure, or does it retain portions of the native conformation or some similar ordered structure? Brandts (7) has already suggested that thermally denatured chymotrypsinogen may not be a completely disordered chain, because he found that the reduced viscosity of the denatured protein could be further increased by the addition of urea. He pointed out, however, that the evidence could not be considered conclusive, and a more direct attack on the problem seems warranted.

The objective of this paper is to provide more information on this subject. This is provided, not by a detailed comparison between the physical properties of thermally denatured and guanidine HCl-denatured proteins, but by a method designed specifically to detect the presence of elements of cooperative structure in a protein which is predominantly in a disordered state. Specifically, we have taken a protein which has been completely denatured by one denaturation procedure, and have attempted to induce an additional transition, in the direction of a random coil, by using a second denaturing agent. If the initially denatured protein possesses no structured regions, then the additional perturbation of the environment simply exerts a monotonic effect on the properties of the polypeptide chains. If structured regions do exist, then a cooperative transition to a truly disordered chain may be observed.

We have first used this method to reinforce the earlier conclusion that proteins possess no long-range structure in 6 M guanidine HCl. No cooperative transitions are observed when proteins dissolved in 6 M guanidine HCl are heated to high temperature. Application of the same criterion to proteins which have been thermally denatured by heating at acid pH leads to the contrary result. A small but unequivocal cooperative transition is observed upon the addition of guanidine HCl to thermally denatured proteins.

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EXPERIMENTAL PROCEDURE

The reagents used in this study, the preparation of solutions for measurement, and the measurements themselves were carried out as described previously (10), with only minor modifications. Optical rotation measurements were made in a thermostatted cell holder. Corrections were made for the drop in temperature between the bath, from which circulating water was fed, and the cell holder.

Optical rotation data are reported in terms of mean residue rotations,

$$[m'] = \frac{3M_0}{100(n^2 + 2)} [\alpha] \quad (1)$$

where $[\alpha]$ is the specific rotation at any wavelength, M_0 the mean residue weight of the protein, and n the refractive index of the solvent. Dispersion curves were measured over a wide range of wavelengths for several samples of each protein. The dispersion parameters proved not to be particularly useful, and are mentioned only briefly in the "Discussion." The dispersion curves were used to select a wavelength for each protein which would be convenient for following conformational transitions, and the results reported will be confined to data obtained at these particular wavelengths.

The refractive index of guanidine HCl solutions was measured, at the Na D line, at several concentrations and temperatures. The dispersion was estimated as previously described (12). No cooperative changes in refractive index were observed: $3/(n^2 + 2)$ is essentially a linear function of the concentration of guanidine HCl. The temperature coefficient is very small, essentially the same as has been observed for water alone.

RESULTS

The results of this paper are based entirely on measurements of optical rotation, at wavelengths well above the Cotton effect region. This choice of method for indicating structural change is a convenient one for the purpose of this study, because the expected influence of an increase in temperature or guanidine HCl concentration, in the absence of conformational change, is in the opposite direction from the change in rotation which ordinarily accompanies the disruption of protein structure. Thus, structural transitions, even though small in extent, are readily distinguished from simple environmental effects of temperature or of guanidine HCl.

Simple environmental effects on a disordered chain are governed by the rule of Kauzmann and Eyring (13), the application of which to proteins has been discussed by Schellman (14). According to this rule, the magnitude of optical rotation depends on the freedom of internal rotational motion of the molecule: greater flexibility diminishes the magnitude of the optical rotation. Since an increase in temperature facilitates internal molecular motion, it should be accompanied by a decrease in the magnitude of $[m']$. Moreover, all the data described in this paper were obtained at very low pH, where all three proteins carry a considerable net positive charge. Under these conditions a disordered chain will become stiffened by internal electrostatic repulsion, and this effect will be reversed by addition of an electrolyte such as guanidine HCl. Addition of guanidine HCl to a disordered chain will therefore also result in a decrease in the magnitude of the optical rotation. These conclusions are expected to apply not only to a completely disordered polypeptide chain, but also to a partially disordered one. In the absence of structural change, environmental effects on the optical rotation of native proteins are comparatively small, and structured regions on otherwise unfolded chains would thus presumably also make only a small contribution to the total effect of an increase in temperature or guanidine HCl concentration.

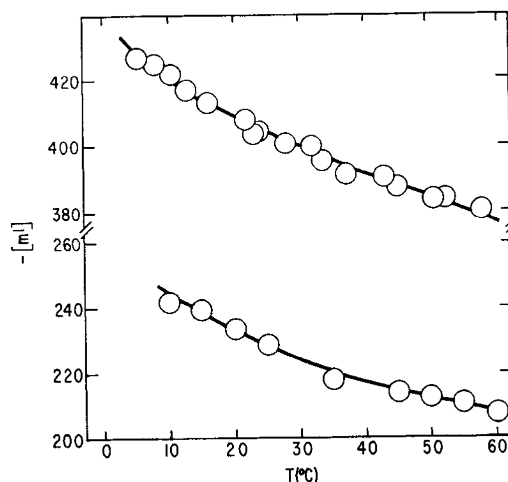

FIG. 1. The effect of temperature on the optical rotation of randomly coiled proteins in 6 M guanidine HCl (GuHCl). The *upper curve* is for lysozyme at pH 4.4: $[m']$ was measured at 320 mμ. The *lower curve* is for ribonuclease at pH 6.7: $[m']$ was measured at 400 mμ.

On the other hand, all known transitions of proteins from an ordered structure to a random coil or to a predominantly unfolded state are accompanied by an increase in the magnitude of rotation at the relatively high wavelengths which we have used. Presumably, the disruption of limited regions of ordered structure will also increase the magnitude of $[m']$. (Even if this generalization were not valid, an increase in the magnitude of $[m']$ under the conditions of our experiments could not be interpreted as a simple environmental effect, and would have to be construed as evidence for a conformational change.)

Effect of pH and Temperature on Proteins Unfolded by Action of Guanidine HCl—Fig. 1 shows the effect of temperature on the optical rotation of lysozyme and of ribonuclease in 6 M guanidine HCl. The monotonic decrease in levorotation which is predicted by the rule of Kauzmann and Eyring is observed. No indications of an additional conformational change exist.

Similar evidence indicates that pH changes have no further effect on guanidine HCl-denatured proteins. All experiments carried out so far indicate that guanidine HCl-denatured proteins are completely disordered, and incapable of undergoing additional unfolding.

Effect of Guanidine HCl on Proteins Unfolded by Combined Effects of Low pH and High Temperature—The thermal transitions at low pH lead to unique products. Transition temperatures are strongly dependent on pH, but the products of the transitions have properties which vary monotonically, and only to a small extent, with additional variations in temperature and pH. It is thus possible to identify a wide range of pH and temperature as a region in which (for each protein) a single "conformation" exists. The conformation is of course a flexible one, and its properties depend on environmental factors according to the

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Kauzmann-Eyring rule. An increase in temperature decreases the magnitude of $[m']$. A decrease in pH increases the charge, and, in dilute salt solutions, therefore increases the magnitude of $[m']$.

Fig. 2 shows the effect of adding guanidine HCl to thermally denatured ribonuclease, at pH 2.18 (measured at 25°). The thermal transition at this pH is complete at 50° or below (1, 3). The addition of guanidine HCl was carried out at 56°, *i.e.* well above the end of the thermal transition region.

The results show that there is an initial decrease in the magnitude of $[m']$, which is the expected effect of guanidine HCl in the absence of conformational change.[1] Between 2 and 4 M guanidine HCl, on the other hand, there is an increase in the magnitude of $[m']$ which indicates a new conformational transition. After the transition is complete, the monotonic decrease in the magnitude of $[m']$ is again observed.

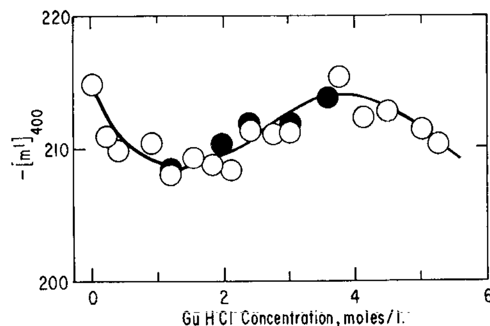

FIG. 2. Addition of guanidine HCl (*GuHCl*) to thermally denatured ribonuclease. Initial conditions: 56°, pH 2.18, ionic strength 0.1. ○, addition of guanidine HCl; ●, data obtained by dilution from high guanidine HCl concentrations.

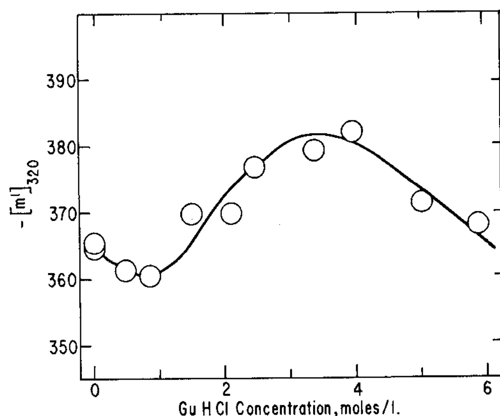

FIG. 3. Addition of guanidine HCl (*GuHCl*) to thermally denatured lysozyme. Initial conditions: 60.5°, pH 1.65, ionic strength 0.1.

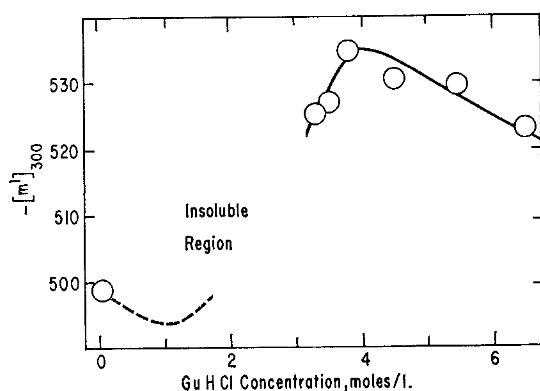

FIG. 4. Addition of guanidine HCl (*GuHCl*) to thermally-denatured chymotrypsinogen. Initial conditions: 60°, pH 2.0, ionic strength 0.01.

The data show that the conformational change between 2 and 4 M guanidine HCl is a reversible one. The transition curve is sigmoid, of the type normally observed for cooperative transitions (16). A measure of the degree of cooperativeness can be obtained from the dependence of the apparent equilibrium constant for the transition on the concentration of guanidine HCl (16). If X is used to designate the thermally-denatured protein, and U the completely unfolded protein, $[m']_X$ and $[m']_U$ can be determined as a function of guanidine HCl concentration by extension of the initial and final parts of Fig. 2 into the transition region. The apparent equilibrium constant (K_{app}) is then defined[2] as $([m']_{obs} - [m']_X)/([m']_U - [m']_{obs})$. This equilibrium constant obeys a relation of the kind

$$K_{app} = K_0 \text{ (guanidine HCl)}^n \tag{2}$$

over a limited range of guanidine HCl concentration near the midpoint of the transition. The exponent n of Equation 2 is a measure of the degree of cooperativeness.

The experimental data of Fig. 2 can of course yield only an approximate value of n, because the change in optical rotation which accompanies the transition from X to U is extremely small. The difference between $[m']_U$ and $[m']_X$ is in fact only of the same order of magnitude as the changes in either $[m']_U$ or $[m']_X$ which arise from solvent effects alone, and the values of K_{app} are necessarily not very precise. Nevertheless, the value of n can be determined to within about 25%. The result obtained is $n = 4 \pm 1$. This is significantly different from the value of n for the transition between native protein (N) and random coil by addition of guanidine HCl at 25°, for which $n = 12$.[3] The transition $X \rightarrow U$ is thus considerably less cooperative than the transition $N \rightarrow U$, a result compatible with the idea that the structured region which is disrupted in the transition $X \rightarrow U$ is only a fraction of the ordered structure of the native protein.

Fig. 3 shows similar results for lysozyme. The initial conditions before addition of guanidine HCl were pH 1.65 (measured at 25°) and 60.5°. Complete conversion of native protein to the heat-denatured form has taken place under these conditions (4). As with ribonuclease, addition of guanidine HCl first leads to a decrease in the magnitude of the optical rotation, representing the expected pure solvent effect. This is followed by a cooperative transition to a state with a more negative rotation, the magnitude of which again decreases after the transition is complete.

The data of Fig. 3 can again be expressed in terms of Equation

[1] It should be noted that the addition of GuHCl decreases the pH, because of the increase in the activity coefficient of H^+ ions (15). There is thus no possibility that the initial fall in the magnitude of $[m']$ reflects a partial reversal of the thermal transition. The transition is favored by a decrease in pH.

[2] We believe that all the transitions described in this paper are two-state transitions (7, 17), *i.e.* transitions in which only the initial and final states contribute significantly to the measured quantities. The apparent equilibrium constant becomes a true equilibrium constant, $K = [U]/[X]$, under these conditions. However, this is not a necessary part of the argument.

[3] A. Salahuddin, unpublished data.

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2 to obtain a measure of the degree of cooperativeness of the transition. The value of n is found to be about the same as for ribonuclease, $n = 4 \pm 1$. For complete unfolding of lysozyme by guanidine HCl at 25°, $n = 16$ (18).

The attempt to obtain similar data for the addition of guanidine HCl to heat-denatured chymotrypsinogen was not entirely successful. Initial conditions were pH 2.0 (measured at 25°) and a temperature of 60°. The thermal transition is complete under these conditions (6). Addition of guanidine HCl to the denatured protein unfortunately led to precipitation of the protein, so that no data on the initial effect of guanidine HCl could be obtained. The protein redissolved, however, when the concentration of guanidine HCl reached 3.2 M, and data above that concentration are shown in Fig. 4. It is seen that $[m']$ is larger in magnitude than it was for the initial heat-denatured protein. In view of the fact that the expected effect of guanidine HCl, in the absence of a conformational change, is in the opposite direction, and because this expectation was experimentally confirmed for ribonuclease and lysozyme, it can be concluded that heat-denatured chymotrypsinogen also undergoes additional unfolding upon addition of guanidine HCl, even though the transition itself could not be observed in this case.

DISCUSSION

Disordered polymer chains are flexible. The precise spatial arrangement of monomer units undergoes continuous change, and the properties which one observes represent averages over all accessible states. The relative contributions of microscopically distinct arrangements (*e.g.* relatively extended forms *versus* more compact ones) is, however, necessarily affected by environmental conditions. It is therefore characteristic of randomly coiled, or predominantly randomly coiled, polymers that observable average properties show a marked dependence on environmental factors. It thus becomes difficult to use physical measurements alone to distinguish between the conformations of proteins which have been denatured under quite different experimental conditions, and to determine whether one represents a more completely disordered molecule than another.

For example, lysozyme which has been denatured thermally at low pH has $[m']_{320} = -365°$, measured at pH 1.7 and 60°. Lysozyme denatured by guanidine HCl has $[m']_{320} = -399°$ (10), measured in 6 M guanidine HCl, at neutral pH and 25°. The corresponding parameters of the Moffitt-Yang equation (19) are $a_0 = -398$ and $b_0 = -85$ for the thermally-denatured protein, and $a_0 = -494$ and $b_0 = -22$ for the guanidine HCl-denatured protein (both based on $\lambda_0 = 212$ mμ). It is not possible to decide *a priori* whether these differences reflect a real difference in the extent to which the native structure has been disrupted, or whether the results simply represent the effects of differences in solvent composition and temperature, the protein being completely disordered under both conditions.

We have circumvented this problem by making use of the fact that ordered structures which are meaningful in protein chemistry are cooperative in nature, involving many amino acid residues. The disruption of structures of this kind is likewise cooperative: amino acid residues cannot be withdrawn from the ordered region one at a time. A transition which involves disruption of an ordered structure of this kind is therefore sigmoid in shape when plotted in the direct manner of Figs. 2 to 4, and the exponent n of Equation 2 is greater than unity (16).

By making use of this principle we have been able to show that heat-denatured ribonuclease, lysozyme, and probably also chymotrypsinogen contain ordered structures which can be disrupted by addition of guanidine HCl. We have also been able to strengthen the previous conclusion that proteins which are denatured by guanidine HCl at room temperature are random coils without any ordered regions (8–11): they certainly contain no ordered structures which can be disrupted by lowering the pH and heating to 60°.

It may be speculated that the coefficient n of Equation 2 is a measure of the length of polypeptide chain which is disrupted by the action of guanidine HCl. This interpretation would be approximately true under certain conditions: for example, if the transitions are two-state transitions, and if the driving force for the disruption of structure is the binding of guanidine HCl to peptide groups, as suggested by Robinson and Jencks (20). In any event, if the results are interpreted in this way, they suggest that the ordered regions which remain in heat-denatured proteins contain one-fourth to one-third as much ordered polypeptide chain as the native molecule. This would be a reasonable result.

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