Thermodynamics of the Denaturation of Lysozyme by Guanidine Hydrochloride. III. Dependence on Temperature*

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ABSTRACT: It has been previously shown that lysozyme may be denatured by raising the temperature in the absence of guanidine hydrochloride, and that the product (X) differs from the product (D) of denaturation by guanidine hydrochloride at room temperature. Investigation of the effect of temperature on denaturation by guanidine hydrochloride is therefore complicated by the fact that two different products may be obtained. The results obtained in this study, in the range of pH 1–4, indicate that the product X makes no significant contribution to the equilibrium below 35°, at any concentration of guanidine hydrochloride. Thermodynamic parameters for the reaction N $\rightleftharpoons$ D can thus be evaluated from the results below 35°. Results obtained above 35° deviate from expectations based on the equilibrium N $\rightleftharpoons$ D alone. These deviations are ascribed to the existence of form X, and thermodynamic parameters for the reaction N $\rightleftharpoons$ X are calculated on this basis. The validity of this procedure is demonstrated by showing that the results of this calculation are consistent with the direct measurements of the equilibrium N $\rightleftharpoons$ X by Sophianopoulos and Weiss, and with our own previous estimate that state X has a degree of unfolding of about 70% compared with the fully denatured state D.

In this paper we seek to analyze and interpret the temperature dependence of the equilibrium between native lysozyme (N) and the denatured product (D) obtained by the action of guanidine hydrochloride (GuHCl). The equilibrium constant, K_D, for this reaction, at 25°, has been shown to be the product of three terms

$$K_D = K_D^0 F(a_H) f(a_{GuHCl}) \quad (1)$$

where $F(a_H)$ is a function of pH alone, represented at 25° by eq 10 of the first paper of this series (Aune and Tanford, 1969a), and $f(a_{GuHCl})$ is a function of the activity of GuHCl alone, several alternative representations of which were considered in the second paper of this series (Aune and Tanford, 1969b). The most plausible of these is perhaps eq 15 of that paper

$$f(a_{GuHCl}) = (1 + 1.2a_{\pm})^{21.5} \quad (2)$$

where $a_{\pm}$ is the mean ion activity of GuHCl, numerically equal to the square root of a_{GuHCl}. We shall use eq 2 in this paper to describe the dependence of K_D on GuHCl concentration. Except insofar as extrapolation to GuHCl concentrations below 1 M is concerned, essentially identical results would be obtained if any of the other representations of $f(a_{GuHCl})$ were used.

In a previous study of the effect of temperature on the denaturation of β-lactoglobulin by urea (Pace and Tanford, 1968), it was found that the thermodynamic parameters for this reaction that reflect the effect of temperature, ΔH, and ΔC_p were independent of the pH or urea concentration at which the temperature dependence was determined. Identical thermodynamic parameters were also obtained in one experiment in which GuHCl was used as denaturant for the protein. These results imply that the functions describing the dependence of K_D on pH and the concentration of denaturant are independent of temperature for β-lactoglobulin,[1] and suggest that the corresponding functions of eq 1 are also temperature independent within the limits of experimental accuracy. We shall see that this expectation is confirmed by the results of this paper, *i.e.*, the measurements will reflect the thermodynamic parameters for the unfolding process *per se*, and will be only minimally influenced by the secondary processes (H^+ dissociation, GuHCl binding) that stabilize the unfolded form at high GuHCl concentration and at low pH.

Another complication does, however, affect the results of this study, this being the existence of a denatured state (X), different from state D, which is formed by heating lysozyme at low pH in the absence of denaturing agent (Sophianopoulos and Weiss, 1964). This state is converted into state D, in a cooperative transition, by the addition of GuHCl to heat-denatured lysozyme at 60° (Aune *et al.*, 1967). It is likely that state X will have significant stability under conditions that one would like to use for the study of the N $\rightleftharpoons$ D equilibrium in moderately concentrated GuHCl at elevated temperatures, so that the N $\rightleftharpoons$ D reaction, which at 25° is a true two-state process, is likely to become a three-state process, involving the equilibria N $\rightleftharpoons$ X $\rightleftharpoons$ D. The results obtained in a study of the process thus depend not only on the equilibrium constant K_D for the reaction N $\rightleftharpoons$ D, but also on the equilibrium constant K_{NX} for the reaction N $\rightleftharpoons$ X. In terms of optical rotation, which has been used to follow the denaturation in this study,

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[1] If ΔH and ΔC_p are independent of pH and GuHCl concentration, $[\partial^2 \ln K_D/\partial T \partial a_H]$ and $[\partial^2 \ln K_D/\partial T \partial a_{GuHCl}]$ must both be equal to zero. These derivatives are independent of the order of differentiation.

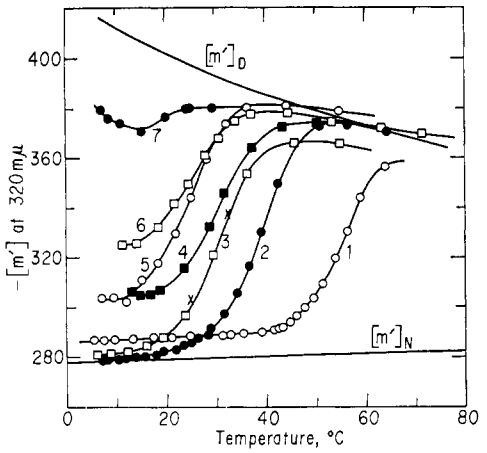

FIGURE 1: Optical rotation as a function of temperature under various conditions: (1) 0.85 M GuHCl, pH 2.82; (2) 1.92 M GuHCl, pH 1.97; (3) 2.23 M GuHCl, pH 1.85; (4) 2.79 M GuHCl, pH 1.76; (5) 3.08 M GuHCl, pH 2.85; (6) 3.14 M GuHCl, pH 3.64; and (7) 3.50 M GuHCl, pH 3.42. The two crosses on curve 3 represent reversals from high temperature. The line for $[m']_D$ is for 6 M GuHCl: at the higher temperatures $-[m']_D$ *increases* as the concentration of GuHCl is reduced. The line for $[m']_N$ is for 2.5 M GuHCl.

the state of equilibrium is expressed in terms of what may be called an apparent equilibrium constant

$$K_{app} = \frac{[m'] - [m']_N}{[m']_D - [m']} \quad (3)$$

where $[m']$ is the measured rotation at a particular pH, denaturant concentration, and temperature, while $[m']_N$ and $[m']_D$ are the rotations of the protein in the native and fully denatured states, respectively, under the same conditions. For a three-state process (Tanford, 1968)

$$K_{app} = \frac{K_D + \alpha K_{NX}}{1 + (1 - \alpha)K_{NX}} \quad (4)$$

where α is the fraction of the total change in rotation that occurs in going from N to X, *i.e.*,

$$\alpha = \frac{[m']_X - [m']_N}{[m']_D - [m']_N} \quad (5)$$

It is seen that K_{app} reduces to K_D when K_{NX} becomes insignificantly small, *i.e.*, when the equilibrium concentration of X is negligible. When K_{NX} is not negligible, it leads to $K_{app} > K_D$ when K_D itself is small (denaturation to state X occurs when D itself is formed to only a small extent), and it leads to $K_{app} < K_D$ when K_D itself is large (molecules in state X having undergone a lesser change in rotation than those in state D).

Experimental Section

All results of this paper are based on measurements of optical rotation at 320 mμ. Except for the variation in temperature, the procedures used are the same as previously described (Aune and Tanford, 1969a).

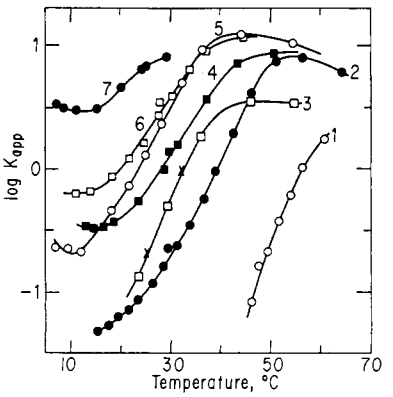

FIGURE 2: The results of Figure 1, replotted as log K_{app} as a function of temperature. Conditions are the same as in Figure 1.

Results

Figure 1 shows experimental heating curves obtained on heating lysozyme solutions at several pH values and GuHCl concentrations. These curves were shown to represent reversible equilibrium data: on cooling from a higher temperature, the path of the heating curves was retraced. Two experimental points obtained in this way are shown on curve 3 of the figure.

In order to convert these data into values of K_{app} by eq 3 it is necessary to know $[m']_N$ and $[m']_D$ as a function of the composition variables. The requisite data were obtained from measurements on the native and denatured proteins outside the transition region (*e.g.*, Figure 1 of Aune *et al.*, 1967), and were fitted to the following equations, which were assumed to apply within the transition region as well

$$[m']_N = -295.4 + 7.097C - 0.0534t \quad (6)$$

$$[m']_D = -396 - 9.538C + 0.7285C^2 + (0.574C - 0.0606C^2)t - (0.0059C - 0.000778C^2)t^2 \quad (7)$$

where C represents GuHCl concentration, and t the temperature in degrees centigrades. The rotations were found to be independent of pH within the pH range covered by the data. Representative curves of $[m']_N$ and $[m']_D$ as a function of temperature are shown in Figure 1.

It should be observed that the values of $[m']_N$ and $[m']_D$, as given by eq 6 and 7, are subject to far greater uncertainty than the values one would obtain for a single transition curve at constant temperature and pH. The equilibrium constants determined in this study are therefore less precise than those reported in the earlier papers. This is true especially of the values of K_{NX}, which often depend on relatively small differences between measured and calculated rotations.

Values of log K_{app} determined from the results of Figure 1, by use of eq 3, 6, and 7, are shown in Figure 2.

Figure 1 shows the expected formation of form X at low GuHCl concentrations in a rather obvious way: the heating curves, especially at the lowest GuHCl concentrations, asymptotically approach $[m']$ values at high temperature that are considerably less negative than the values of $[m']_D$ under these conditions. Curve 1 (0.85 M GuHCl, pH 2.82) resembles heating curves for lysozyme at low pH in the complete ab-

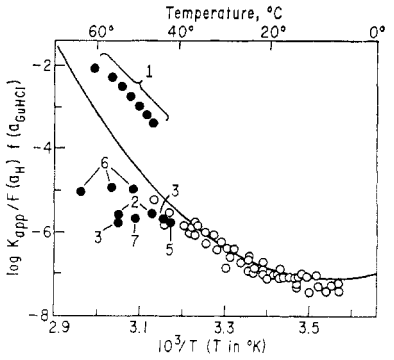

FIGURE 3: Log $[K_{app}/F(a_H)f(a_{GuHCl})]$ as a function of $1/T$. $F(a_H)$ and $f(a_{GuHCl})$ were taken as independent of temperature. Experimental points used for the calculation of K_{NX} are shown as filled circles, and are labeled with a number to identify the curves of Figures 1 and 2 from which they are taken. The curve is a theoretical curve for log K_D^0, based on the parameters of Table I. An explicit analytical expression is given by eq 9.

sence of GuHCl, as determined by Sophianopoulos and Weiss (1964).

Both Figures 1 and 2 display a feature that has been previously observed both for the thermal denaturation of ribonuclease (Brandts and Hunt, 1967), which is a reaction of the type $N \rightleftarrows X$, and for the urea or GuHCl-induced denaturation of β-lactoglobulin (Pace and Tanford, 1968). It is a feature that is probably characteristic of all denaturation equilibria in which the end product is largely disordered. It is seen in Figure 1 as a reversal of the approach of $[m']$ toward $[m']_N$ at low temperatures, and in Figure 2 as an increase in log K_{app} with cooling below a temperature of about 10°, at which the native form appears to have maximum stability. This reversal is a reflection of the large change in heat capacity that accompanies protein unfolding, which leads to a negative ΔH for denaturation at low temperature and a positive ΔH at high temperature (Tanford, 1968).

As stated earlier, the functions $F(a_H)$ and $f(a_{GuHCl})$ of eq 1 may be expected to be independent of temperature.[1] This makes it possible to identify those parts of Figure 2 in which K_{app} is essentially equal to K_D, and those in which K_{NX} contributes significantly. If $K_{app} \simeq K_D$, then $K_{app}/F(a_H)f(a_{GuHCl})$ should be essentially a unique function of temperature, regardless of the pH or GuHCl concentration at which measurements were made. Since significantly large values of K_{NX} will sometimes make $K_{app} > K_D$ and sometimes make $K_{app} < K_D$, values of $K_{app}/F(a_H)f(a_{GuHCl})$ will become divergent when K_{NX} becomes appreciably large.

The corrected equilibrium constants are shown as a function of $1/T$ in Figure 3. It is seen that all values of $K_{app}/F(a_H) \cdot f(a_{GwHCl},)$ cluster about a single curve below about 35°, but that they diverge above that temperature. We have therefore assumed that K_{app} may be equated with K_D below 35°, and that the ordinate of Figure 3 in this temperature range therefore represents the constant K_D^0 of eq 1. We have drawn a reasonable curve through these data, as shown in the figure, and have extended it to higher temperature to indicate the values of K_D^0 above 35°. The curve shown is determined by the parameters log K_D^0 at 25° = 6.68, ΔH at 25° = 20.7 kcal/mole, and ΔC_p = 1375 cal/deg per mole, independent of temperature.

It is possible that some of the scatter of points about this curve, below 35°, represents an incorrectness in the assumption that $F(a_H)$ and $f(a_{GuHCl})$ are independent of temperature. However, if separate values of ΔH and ΔC_p are determined from each of the sets of data from Figure 1, they do not vary in a systematic way with pH and GuHCl concentration. It is concluded that the scatter of points is a measure of experimental error, at least part of which is ascribable to error in the values of $[m']_N$ and $[m']_D$ at the higher temperatures. It may be noted that the value of log K_D^0 at 25°, determined by the curve of Figure 3, is essentially the same as the value of −6.65 determined from isothermal data in the preceding paper when eq 15 was used to represent the dependence of K_D on GuHCl concentration.[2]

It may be noted that the single set of results with the largest deviation from the curve of Figure 3 is the set of results obtained from the heating curve at pH 2.85, 3.08 M GuHCl. If these results are used as basis for an independent calculation of the thermodynamic parameters, one obtains ΔH = 21.6 kcal/mole at 25°, and ΔC_p = 1320 cal/deg per mole.

A decisive test for the validity of these thermodynamic parameters is to use them to determine values of K_D for the result above 35°, to introduce these values into eq 4, and then to use the observed values of K_{app}, both those greater and less than K_D, as a basis for calculation of the equilibrium constant K_{NX}. If reasonable values for the latter are obtained, this would indicate that the thermodynamic parameters for the temperature dependence of K_D are substantially correct. In making the calculations it was assumed that the parameter α (eq 5) is independent of temperature. A value of 0.68 was determined from comparison of the extrapolated value of $[m']_D$ at 60°, in the absence of GuHCl, with the known value of $[m']_N$ under these conditions (Aune *et al.*, 1967).

Figure 3 shows the experimental points used for this calculation, and Figure 4 shows values of log K_{NX} as a function of temperature for the four heating curves that yielded experimental points at more than one temperature. The average values of ΔH determined from these results are shown in the figure, and are seen to be in excellent agreement with the average value of ΔH of 66 kcal/mole, determined from the direct measurement of K_{NX} in the absence of GuHCl by Sophianopoulos and Weiss (1964). The results indicate that ΔH for the reaction $N \rightleftarrows X$, as for the reaction $N \rightleftarrows D$, is within experimental error independent of pH or the concentration of GuHCl.

When ΔH itself is large, the range of temperature over which log K can be determined experimentally is necessarily very narrow. As a result, it is not possible to evaluate a meaningful value for ΔC_p. Sophianopoulos and Weiss (1964), in their study of the thermal denaturation of lysozyme, did not consider the possibility that ΔH may be temperature dependent at all. Our results suggest that a temperature dependence does exist: curvature is clearly indicated by the bottom curve of Figure 4. The curve drawn through the points of this set of results is based on an assumed ΔC_p of 950 cal/deg per mole, the basis for which will be given below.

As was indicated in the first paper of this series, the pH dependence for the reaction $N \rightleftarrows X$ is probably the same as for

[2] The values of log K_D cited in Table I of the preceding paper apply to neutral pH. The value given here is log K_D^0, which applies to pH = $-\infty$.

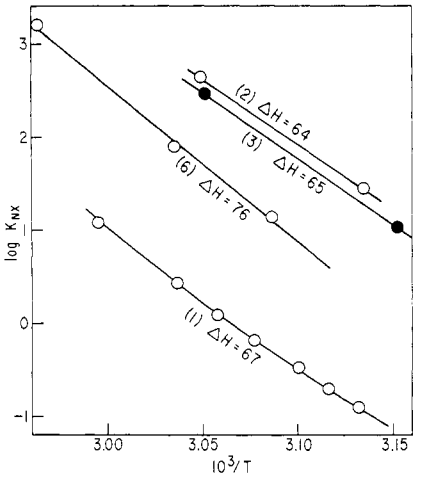

FIGURE 4: Plot of log K_{NX} as a function of temperature. Each line is numbered to indicate the origin of the results in the previous figures. The ΔH value is the average ΔH for each set of results, in kilocalories per mole, and is to be compared with the value of 66 kcal/mole reported by Sophianopoulos and Weiss (1964) for the reaction N $\rightleftharpoons$ X in the absence of GuHCl. The *curve* through the data of line 1 is a theoretical curve, allowing for temperature dependence of ΔH, with ΔC_p assumed equal to 950 cal/deg per mole.

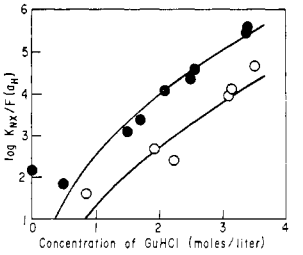

FIGURE 5: The dependence of K_{NX} on the concentration of GuHCl. The filled circles represent the addition of GuHCl to heat-denatured lysozyme, at a constant temperature of 60° (pH 1.7–1.3). The open circles represent interpolation or extrapolation of the results of Figures 2–4 to a single temperature of 50°. Each curve of Figure 2 (except curve 4) is represented by one experimental point. The two curves are theoretical curves, according to eq 8, with $\Delta n = 14$.

the reaction N $\rightleftharpoons$ D at the same ionic strength. Thus the same function $F(a_H)$ can be applied to correct all experimental values of K_{NX} to the same pH as was used for K_D. Figure 5 shows the results of this procedure. The data are based on one point from each of the lines of Figure 4, interpolated or extrapolated to a temperature of 50°. Two additional points are obtained from the two data sets of Figure 3 from which only a single value of K_{NX} could be obtained. The results are plotted as a function of the concentration of GuHCl, and fall reasonably close to a single curve, as they should, since $F(a_H)$ must be independent of GuHCl concentration. (It should be recalled that K_{NX} is determined from the *deviation* between K_{app}, itself relatively imprecise at these temperatures, and the calculated curve for K_D in Figure 3. The self-consistency of the results, and their agreement with the upper curve of Figure 5, are actually remarkably good.)

The upper curve of Figure 5 represents an alternative procedure for calculating K_{NX} and testing the validity of the thermodynamic parameters for K_D represented by the curve of Figure 3. They are the results of an experiment essentially the same as that given by Figure 3 of the paper by Aune *et al.* (1967). GuHCl was added to protein initially at 60° (GuHCl absent), under which conditions the protein exists largely in state X. The transition to state D is observed as GuHCl is added, and values of K_{NX} are determined as before. The values are plotted in the upper curve of Figure 5, and the dependence on GuHCl concentration obtained in this way is clearly in reasonably good agreement with the results based on the heating curves. The difference between the two curves is essentially that expected on the basis of the difference in temperature.

Figure 5 shows that log K_{NX} (at constant pH) increases with increasing concentration of GuHCl, as is to be expected for a reaction involving substantial unfolding of the molecule and consequent exposure of the same kind of binding sites for GuHCl as are exposed in unfolding to the fully denatured product. The magnitude of this dependence is, however, smaller than for the reaction N $\rightleftharpoons$ D, as given in the preceding paper, consistent with the fact that state X is less completely unfolded than state D.

To obtain comparable information, we have described the dependence of K_{NX} on GuHCl concentration by the same type of equation as was used for K_D, *i.e.*, eq 2. Thus

$$K_{NX}/F(a_H) = K_{NX}^0 (1 + 1.2a_\pm)^{\Delta n} \quad (8)$$

where Δn is the change in the number of binding sites (for GuH^+) that accompanies the conversion of N into X. The binding constant of 1.2 for the reaction between GuH^+ and sites on the protein, already assumed to be the same for all sites on the native protein and form D, must of course also be assigned the same value for state X. The constant has to be taken as independent of temperature because $f(a_{GuHCl})$ itself is independent of temperature.

From a plot of log $[K_{NX}/F(a_H)]$ *vs.* log $(1 + 1.2a_\pm)$ the best value of Δn was determined to be about 14 and theoretical curves according to eq 8 with that value of Δn are shown in Figure 5. It is seen that they provide a good fit for the data at GuHCl concentrations above 1 M, but that K_{NX} values are higher than predicted at lower concentrations of denaturant. As was noted in the preceding papers, this is the expected result. The function $F(a_H)$ used here is applicable to high ionic strength only, and will fail at low ionic strength, especially at low pH where short-range electrostatic interactions are magnified. It should be recalled that the function $K_{NX}/F(a_H)$ formally represents the value of K_{NX} at pH = $-\infty$.[3]

The value $\Delta n = 14$ required to fit the data of Figure 5 is the most significant aspect of the figure, and provides the best

[3] The actual discrepancy between the theoretical and experimental equilibrium constants depends very much on which of the alternative models of the preceding paper are used to generate the theoretical curves. The various equations used there are essentially identical above 1 M GuHCl, but differ greatly at lower concentrations of denaturant. We have used eq 15 of the preceding paper, because it represents what is perhaps the most plausible model. With it we obtain log $K_{NX}^0 = -0.54$ at 60°, compared with the experimental value of +2.15. (Essentially the same value is obtained from this study as from the previous work by Sophianopoulos and Weiss.) If eq 11 of the preceding paper had been used, the value of log K_{NX}^0 required for the theoretical curve would have been +1.16, which is closer to the experimental value.

TABLE I: Thermodynamic Parameters in 1 M GuHCl, 25°, pH 7.[a]

Reaction	Log K	$\Delta G°$ (kcal/mole)	ΔH (kcal/mole)	$\Delta S°$ (cal/deg per mole)	ΔC_p (cal/deg per mole)
N ⇌ D	−5.8	7.9	22.4	49	1375
N ⇌ X	−5.7	7.8	41.2	112	950
X ⇌ D	−0.1	0.1	−18.8	−63	425

[a] Thermodynamic parameters are given in 1 M GuHCl, rather than in the absence of denaturant, in order to avoid the problems inherent in the extrapolation from 1 to 0 M GuHCl. ΔH and ΔC_p are of course not affected by the concentration of GuHCl, and the negative ΔH for the reaction X → D would therefore apply to 0 M GuHCl also. Actual thermodynamic parameters for the reaction N ⇌ X, at 0 M GuHCl, are probably not substantially different from the values given in the table, because the effects of electrostatic repulsion, in the absence of a high salt concentration, would be expected to counteract in part the stabilization of the native state by removal of GuHCl.

indication of the correctness of our treatment of the data. Comparing it with the value of $\Delta n = 21.5$, required (eq 2) for the reaction N → D, it indicates that the extent of unfolding that accompanies the reaction N → X is 65% of the extent of unfolding that accompanies the reaction N → D. This estimate is in gratifying agreement with the estimate of 68% based on the relative change in optical rotation, as given by the parameter α of eq 5.

As was discussed elsewhere (Tanford, 1968), the change in heat capacity that accompanies protein denaturation arises at least in part from contacts between exposed hydrophobic groups and the aqueous environment, and the magnitude of ΔC_p should therefore also be a measure of the relative extent of unfolding, *i.e.*, one might expect ΔC_p for the reaction N → X to be about 70% of the value for the reaction N → D, or 950 cal/deg per mole. It is not possible to determine ΔC_p from the data of Figure 3, and only average ΔH values for the temperature range 50–60° were given there. The results obtained at 0.854 M GuHCl actually do show curvature, and, as the figure shows, are consistent with a ΔC_p value of the correct order of magnitude.

Using the value $\Delta C_p = 950$ cal/deg per mole for the reaction N → X, and assuming that it, like ΔC_p for the reaction N → D, is independent of temperature, all thermodynamic parameters for the reaction N ⇌ X can be evaluated.

Thermodynamic parameters for all three possible interconversions between the states N, X, and D, at 1 M GuHCl, pH 7, 25°, are shown in Table I. Values appropriate for other conditions (except at GuHCl concentration <1 M) are readily computed as follows.

(1) The pH dependence of the equilibrium constant for both N ⇌ D and N ⇌ X is given by eq 10 of the first paper of this series. The equilibrium constant for the reaction X ⇌ D is independent of pH.

(2) The dependence of the equilibrium constants on the activity of GuHCl is determined by equations of the form of eq 8, with $\Delta n = 21.5$ for N ⇌ D, 14 for N ⇌ X, and 7.5 for X ⇌ D. The parameter K^0 at 25° is $\log K_D^0 = -6.65$, $\log K_{NX}^0 = -4.9$, $\log K_{XD}^0 = -1.75$.

(3) The dependence of the equilibrium constants on temperature occurs entirely through the effect of temperature on K^0. It may be evaluated by the relations

$$\log K_D^0 = -2001.57 + 691.485 \log T + 84.6326 \times 10^3/T \quad (9)$$

$$\log K_{NX}^0 = -1365.2 + 478.060 \log T + 52.8881 \times 10^3/T \quad (10)$$

$$\log K_{XD}^0 = -636.4 + 213.425 \log T + 31.7445 \times 10^3/T \quad (11)$$

(4) ΔH and ΔC_p are independent of pH and GuHCl activity, and ΔC_p is independent of temperature. ΔH at any temperature is therefore obtained directly from ΔH at 25°, given in Table I, and the corresponding value of ΔC_p.

(5) $\Delta S°$ is most simply evaluated from the difference between $\Delta G°$ and ΔH.

Discussion

Although the denaturation of lysozyme under the conditions used in this study involves two distinct denatured states, it has been possible to determine thermodynamic parameters for the formation of the fully denatured state D (cross-linked random coil) by confining the analysis to relatively low temperatures. The validity of these parameters for higher temperatures was demonstrated by showing that the discrepancy between observed results at higher temperatures, and those calculated on the basis of K_D alone, leads to thermodynamic parameters for formation of the heat-denatured state X that are self-consistent, show reasonable dependence on GuHCl concentration, and extrapolate to values in the absence of GuHCl that are consistent with the results of the thermal denaturation study of Sophianopoulos and Weiss (1964). All thermodynamic data for both reactions, and for the transition X → D, have been summarized in Table I.

It is interesting to note that the value of ΔH for the reaction X → D is negative at 25°. This suggests that the structural elements that exist in form X of the protein may depend largely on hydrophobic contacts, as ΔH for exposure of hydrophobic groups to an aqueous medium is generally negative at low temperatures (Kauzmann, 1959). Because of the positive ΔC_p associated with the reaction X → D, ΔH for the reaction increases progressively with increasing temperature. At 50°, $\Delta H = -8.2$ kcal/mole; at 75°, $\Delta H = +2.5$ kcal/mole.

The parameters for the reactions N → D and N → X listed in Table I are comparable with similar parameters for other transitions to randomly coiled or predominantly randomly coiled states (Tanford, 1968). The values of ΔC_p are smaller than have been observed for other small proteins (*e.g.*, ΔC_p = 2000 cal/deg per mole for the thermal denaturation of ribonuclease). This may be a reflection of the fact that the aromatic side chains of lysozyme are exposed to an unusually high de-

gree in the native state (Williams *et al.*, 1965), so that the change in exposure of these groups that accompanies unfolding is less than for most other proteins. The increased exposure of hydrophobic groups in predominantly unfolded states, and the resultant increase in the ordering of water molecules, are believed to be the principal factors that lead to large values of ΔC_p for denaturation processes.

It should also be noted that the denatured lysozyme molecule occupies an exceptionally small volume in solution when disulfide bonds are intact. The intrinsic viscosity of state D is only about 45% as large as that of the true random coil (disulfide bonds broken). The corresponding figure for ribonuclease is 60%, and for β-lactoglobulin it is 85%. A consequence of the small volume would be that contacts between side chains, though presumably always transient, must on the average be more numerous than for other denatured proteins, and that the amount of water ordered by contacts between hydrophobic side chains and the solvent must be less than for other denaturated proteins.

Values of ΔH for unfolding of different proteins, unlike the values of ΔC_p, show considerable variation. Presumably they depend on details of the *native* conformation and the number and strength of internal bonds that must be broken. At 25°, ΔH values ranging from −21 kcal/mole for the reaction N → D of β-lactoglobulin to +47 kcal/mole for the reaction N → X of ribonuclease have been reported. The ΔH values for lysozyme are seen to lie toward the high end of this range.

References

Aune, K. C., and Tanford, C. (1969a), *Biochemistry 8*, 4579.

Aune, K. C., and Tanford, C. (1969b), *Biochemistry 8*, 4586.

Aune, K. C., Salahuddin, A., Zarlengo, M. H., and Tanford, C. (1967), *J. Biol. Chem. 242*, 4486.

Brandts, J. F., and Hunt, L. (1967), *J. Am. Chem. Soc. 89*, 4826.

Kauzmann, W. (1959), *Advan. Protein Chem. 14*, 1.

Pace, N. C., and Tanford, C. (1968), *Biochemistry 7*, 198.

Sophianopoulos, A. J., and Weiss, B. J. (1964), *Biochemistry 3*, 1920.

Tanford, C. (1968), *Advan. Protein Chem. 23*, 121.

Williams, E. J., Herskovits, T. T., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, 3574.

Small Angle X-Ray Scattering of a Homogeneous γG1 Immunoglobulin*

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ABSTRACT: A homogeneous human γG1 myeloma protein (Eu) of known primary structure has been studied by means of small angle X-ray scattering. Similar studies were also carried out on the Fab(t) and Fc(t) fragments and on the $F(ab')_2$ fragment of the molecule. Molecular weights obtained from scattering curves were in reasonable agreement with those determined by sedimentation equilibrium and those calculated from the primary structure. The hydration was calculated to range from 0.27 to 0.37 g of H_2O/g of protein for the fragments and whole molecule. The radii of gyration of the Fab(t) and Fc(t) fragments were similar (32.0 and 33.1 Å, respectively), whereas the radius of gyration of the whole protein was 75.8 Å. The data on the radii of gyration and the behavior of the scattering curves suggest that the Fab and Fc regions of the γG immunoglobulin molecule are relatively compact but that the whole molecule has an extended structure in solution.

A number of problems related to the size, shape, and flexibility of γG immunoglobulin[1] have emerged from electron microscopic and hydrodynamic analyses. Several studies (Feinstein and Rowe, 1965; Noelken *et al.*, 1965; Valentine and Green, 1967) indicate that the Fab and Fc regions of the γG immunoglobulin molecule are compact structures linked *via* a "hinge" region. The electron microscopic experiments of Valentine and Green (1967) have suggested that the two Fab regions are tightly but flexibly linked to the Fc region and that the molecule is a Y-shaped structure, the most extended dimension of which does not exceed 120 Å. Although a complete analysis of the three-dimensional structure will depend upon X-ray crystallography (Terry *et al.*, 1968; Goldstein *et al.*, 1968; Avey *et al.*, 1968), studies of the molecule in solution are necessary to give additional information on its length, degree of flexibility (Weltman and Edelman, 1967; Wahl and Weber, 1967; Wahl, 1969; Fayet and Wahl, 1969), and the manner in which its regions are connected.

We have measured the small angle X-ray scattering be-

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[1] The nomenclature used is that suggested by the World Health Organization (1964). Fab(t), Fc(t), tryptic fragments corresponding to papain fragments Fab and Fc; $F(ab')_2$, 5S fragment obtained by pepsin digestion.