Thermodynamics of the Denaturation of Ribonuclease by Guanidine Hydrochloride*

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ABSTRACT: Thermodynamic parameters for the transition of native ribonuclease to the cross-linked random coil, induced by addition of guanidine hydrochloride, have been determined. The results are quite similar to those reported earlier for lysozyme.

The effect of temperature can be described by assigning $\Delta H = 35 \pm 5$ kcal/mole at 25°, and $\Delta C_p = 2200 \pm 500$ cal/deg per mole, independent of temperature. The major part of the pH dependence of the equilibrium can be interpreted as arising from the presence of two carboxyl groups with abnormally low pK in the native state. Ribonuclease is more susceptible than lysozyme to denaturation by guanidine hydrochloride (and, presumably, the same facts apply to urea denaturation) for two reasons. (1) The intrinsic free energy of denaturation in the absence of denaturant is about 1 kcal/mole *smaller* for ribonuclease. (2) The number of new binding sites for denaturant that arise during the transition is about 10% *larger* for ribonuclease.

Previous papers from this laboratory (Aune and Tanford, 1969a,b, 1970) have reported the results of a study of the thermodynamics of the denaturation of lysozyme by guanidine hydrochloride (Gu·HCl).[1] It was found that the reaction involves only two states at 25°, the native state (N) and the cross-linked random coil (D), which is the end product of denaturation by Gu·HCl. At higher temperatures a third discrete state, the heat-denatured state (X), was also found to contribute to the equilibrium properties. Below about 35° the results were interpreted entirely in terms of a single equilibrium constant K_D, for the process N $\rightleftharpoons$ D. It was found that K_D could be written as a product of three independent functions

$$K_D = (D)/(N) = K_D^0 F(a_H) f(a_{Gu \cdot HCl}) \quad (1)$$

The function $F(a_H)$ describes the dependence of K_D on pH, and it was shown that it could be taken to be independent of the concentration of Gu·HCl and of temperature, within the range over which these parameters were varied in the investigation. The function $f(a_{Gu \cdot HCl})$ describes the dependence of K_D on the activity of Gu·HCl, and it was shown that it could be taken to be independent of pH and temperature. The parameter K_D^0 is a function of temperature alone. It *formally* represents the value of K_D when $F(a_H) = f(a_{Gu \cdot HCl}) = 1$, which, with the forms used for $F(a_H)$ and $f(a_{Gu \cdot HCl})$ corresponds to zero activity of Gu·HCl, and to the lower limit of the pH range encompassed by the data (pH $\simeq$ 1).

Above 35°, where the state X becomes important, the experimental data yield an apparent equilibrium constant, K_{app}, which is a function of both K_D and of the equilibrium constant, K_{NX}, for the reaction N $\rightleftharpoons$ X. K_{NX} in turn can be described by an equation analogous to eq 1.

The present paper describes a similar, though somewhat less detailed, study of the denaturation of ribonuclease by Gu·HCl. The results are quite similar to those obtained for lysozyme. Subsequent papers will show, however, that this similarity does not apply to all proteins: substantial differences are found in the Gu·HCl denaturation of cytochrome *c* and β-lactoglobulin, for example.

Experimental Procedure

Materials. Bovine pancreatic ribonuclease A was purchased from Sigma Chemical Co. Both preparations labeled type IIA and type IIIA were used: no significant differences between them were found. Stock solutions were deionized by the method of Dintzis (1952),[2] and the protein content was determined by drying to constant weight in air at 107°. Gu·HCl was purchased from J. T. Baker Co., and purified according to the method of Nozaki and Tanford (1967b).

Methods. The methods used were essentially the same as were used to study the denaturation of lysozyme (Aune and Tanford, 1969a). Optical rotation was measured at 400 mμ, and difference spectral measurements were made at 287 mμ. These wavelengths were chosen so as to maximize the relative change in the measured parameters for the transition from native to denatured states. All measurements were carried out at protein concentrations between 0.1 and 0.5 g per 100 ml.

Results

Figure 1 shows the transition from state N to state D as measured by optical rotation at pH 6 and 25°. The transition is seen to be thermodynamically reversible: experimental

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[1] The abbreviation Gu·HCl is used for guanidine hydrochloride.

[2] A description of the apparatus used by us has been given by Nozaki and Tanford (1967a).

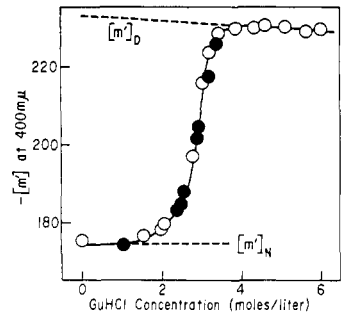

FIGURE 1: Transition curve measured by optical rotation at pH 6, 25°. Open circles represent denaturation, filled circles represent renaturation after exposure to 7 M Gu·HCl. The dashed lines represent the values of $[m']$ at 400 mμ for native and fully denatured protein.

results obtained after exposure to 7 M Gu·HCl are seen to fall on the same curve as data obtained by addition of Gu·HCl to aqueous protein solutions. The line representing $[m']_D$ as a function of Gu·HCl concentration was determined by the method of least squares from the experimental points above 4 M Gu·HCl. The value of $[m']_N$ was assumed to be independent of Gu·HCl concentration within experimental error. Both $[m']_D$ and $[m']_N$ are in good agreement with previous determinations in this laboratory and elsewhere.

Figure 2 shows similar data obtained by difference spectroscopy. Because of the greater effect of Gu·HCl concentration on absorption intensity, allowance had to be made for the dependence of both ΔE_N and ΔE_D on denaturant concentration. The two lines representing these parameters were determined by the method of least squares from experimental points outside the transition zone.

Absence of Stable Intermediate States. When the results of Figures 1 and 2 are replotted as per cent change *vs.* Gu·HCl concentration, the data from optical rotation and difference spectroscopy fall on the same curve. This is a necessary, but not a sufficient criterion for characterizing the reaction as a two-state transition, in which only the initial and final states make a significant contribution to the measured properties, molecules in intermediate states of unfolding being present in insignificant amounts at all stages of the transition.

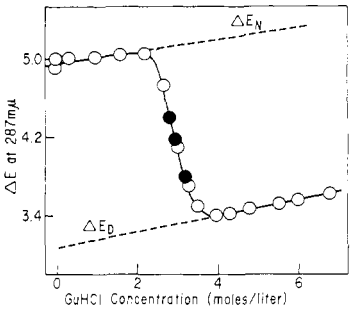

FIGURE 2: Transition curve measured by difference spectroscopy at 287 mμ, at pH 6, 25°. The ordinate ΔE represents absorption intensity for a protein concentration of 1 g/100 ml and a 1-cm light path, relative to an arbitrary reference. Open circles represent denaturation, filled circles represent renaturation after exposure to 7 M Gu·HCl. The dashed lines represent the values of ΔE for native and fully denatured protein.

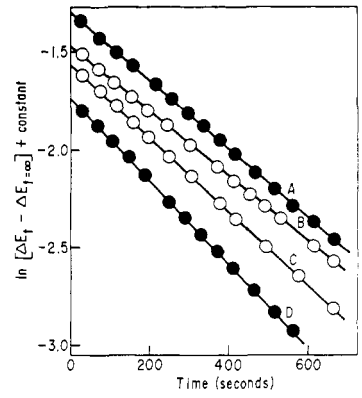

FIGURE 3: First-order kinetic plots for denaturation and renaturation in the transition zone, at pH 5.7, 25°. Open circles represent results from denaturation experiments at 2.69 M Gu·HCl (B) and 2.88 M Gu·HCl (C). Filled circles represent results from renaturation experiments at 2.50 M Gu·HCl (A) and 2.9 M Gu·HCl (D).

A decisive test for the absence of stable intermediates is to analyze the kinetics of the conversion of both N and D into the equilibrium state, as described earlier (Aune and Tanford, 1969a; Tanford, 1968). As shown by the data of Figure 3, and similar data at other concentrations of Gu·HCl, first-order kinetics were observed in both directions. Moreover, the extrapolated values of the ordinate $(\Delta E_{t=0} - \Delta E_{t=\infty})$ agree with the values expected on the basis of Figure 2. This result establishes the transition unequivocally as two state within the range of conditions of Figures 1 and 2.

With the two-state nature of the transition established (at pH 6 and 25°), the results of Figures 1 and 2 can be unequivocally converted into experimental values of the equilibrium constant for the reaction $N \rightleftharpoons D$. Figure 4 shows the resulting K_D values as a function of Gu·HCl concentration. As previously indicated, results from optical rotation and difference spectroscopy fall on the same curve.

Validity of Equation 1. The foregoing proof of the two-state nature of the transition being studied here applies only to the conditions under which the measurements were carried out, *i.e.*, pH 6 and 25°. We have *assumed* that the reaction can be treated as two state at other pH values and temperatures also, except under conditions where the heat-denatured

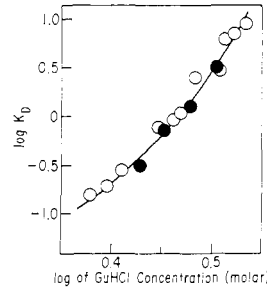

FIGURE 4: The equilibrium constant for the transition as a function of Gu·HCl concentration at pH 6, 25°. Open circles represent data from optical rotation (Figure 1), filled circles represent data from difference spectra (Figure 2).

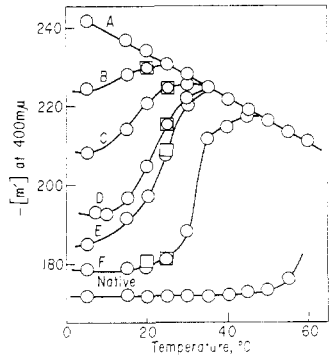

FIGURE 5: The effect of temperature, as measured by optical rotation at pH 6. Curve A was measured at 4.31 M Gu·HCl and is taken to represent the dependence of $[m']$ for the fully denatured protein on temperature. Curves B to F represent Gu·HCl concentrations of 3.44, 3.24, 3.10, 2.79, and 2.29 M, respectively. The squares demonstrate reversibility: they represent data obtained on cooling from 60°.

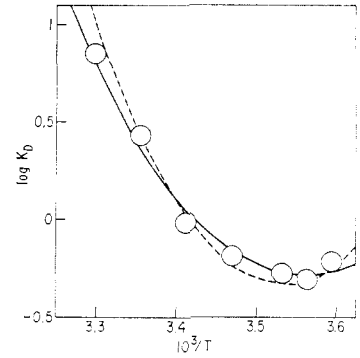

FIGURE 6: Log K_D at 3.1 M Gu·HCl, plotted as a function of reciprocal temperature. The curves are theoretical. The solid line is based on ΔH = 31 kcal/mole at 25° and ΔC_p = 1800 cal/deg per mole. The dashed line is based on ΔH = 40 kcal/mole at 25° and ΔC_p = 2650 cal/deg per mole.

state may be expected to make a contribution to the equilibrium mixture. With this assumption, K_D values can be estimated from measurements of optical properties at other pH values and temperatures. Before presenting these data and their analysis it should be noted that they support the validity of eq 1, with independent functions to represent the effects of pH, temperature, and Gu·HCl concentration. Specifically, the results will indicate that $\partial \ln K_D/\partial T$ is independent of Gu·HCl concentration at pH 6, and that $\partial \ln K_D/\partial$pH is independent of Gu·HCl concentration between pH 3 and pH 7 at 25°.

No measurements were made at Gu·HCl concentrations below 2 M. Because Gu·HCl is a strong electrolyte, this means that long-range electrostatic interactions do not influence any of our results significantly. Such an influence, particularly an influence on $F(a_H)$, is anticipated at low ionic strength. For this reason eq 1 is not expected to remain valid at Gu·HCl concentrations much below those covered by the experimental data, unless provision for the effect of ionic strength on $F(a_H)$ is added to the equation for $F(a_H)$ based on the results of this paper alone. The same problem was encountered in the analysis of the denaturation of lysozyme (Aune and Tanford, 1969a,b).

Dependence on Gu·HCl Concentration. As was true for the corresponding data for lysozyme, the results of Figure 4 can be fitted by a variety of expressions for $f(a_{Gu\cdot HCl})$. Perhaps the most plausible of the equations applied to lysozyme is eq 15 of Aune and Tanford (1969b)

$$f(a_{Gu\cdot HCl}) = (1 + k\, a_{\pm})^{\Delta n} \qquad (2)$$

in which $a_{\pm}$ is the mean ion activity of Gu·HCl. The model underlying this equation has binding sites for the ions of Gu·HCl (presumably $Gu\cdot H^+$ plays the major role) on both native and denatured molecules, but the number of sites is larger on the denatured molecule: Δn in eq 2 represents the difference between the two states in the number of sites per molecule. Though sites with different affinity undoubtedly exist, it is not possible to extract more than two parameters from the experimental data, and the same binding constant k was accordingly assigned to all sites on the lysozyme molecule, for both native and denatured protein. With this assumption, the best value of k was found to be 1.20 at 25°, with activity referred to molar concentration units. In applying eq 2 to the data of Figure 4 we have used this *same* single value of k and have determined the best value of Δn by least squares. The value obtained is Δn = 24.4, and the corresponding value of log K in the absence of Gu·HCl, *i.e.*, $\log K_D^0 + \log F(a_H)$ at pH 6, is −9.5. The curve in Figure 4 is drawn according to eq 2 with these values for the parameters.

If eq 11 of the preceding paper (Aune and Tanford, 1969b) is used

$$f(a_{Gu\cdot HCl}) = (1 + k a_{Gu\cdot HCl})^{\Delta n} \qquad (3)$$

corresponding to a model visualizing molecular binding of Gu·HCl, we obtain, with the same binding constant as was used for lysozyme (k = 3.00), Δn = 8.9, and $\log K_D^0 + \log F(a_H)$ at pH 6 = −6.54. Within the limits of Gu·HCl concentration covered by Figure 4, eq 3 with these parameters is indistinguishable from the curve drawn according to eq 2.

Effect of Temperature. Figure 5 shows the effect of temperature on the optical rotation of ribonuclease at various Gu·HCl concentrations. All measurements were made near pH 6. Curve A represents $[m']_D$ at 4.3 M Gu·HCl as a function of temperature. Values of $[m']_D$ at other Gu·HCl concentrations were obtained by assuming that the change in $[m']_D$ with Gu·HCl concentration, as given for 25° by Figure 1, is independent of temperature. The value of $[m']_N$ is seen to be independent of temperature to about 40°. The change above that temperature is not a change in $[m']_N$ *per se*, but reflects the onset of thermal denaturation (Hermans and Scheraga, 1961; Brandts and Hunt, 1967).

The results of Figure 5 prove to be insufficient to determine unequivocal values for ΔH and ΔC_p for the reaction. Figure 6, for example, shows the values of log K_D determined from the experimental data at 3.1 M Gu·HCl, plotted as a function of $1/T$, and two theoretical curves, one based on ΔH (25°) = 31 kcal/mole and ΔC_p = 1800 cal/deg per mole, the other on ΔH (25°) = 40 kcal/mole and ΔC_p = 2650 cal/deg per mole (ΔC_p was taken as independent of temperature for both

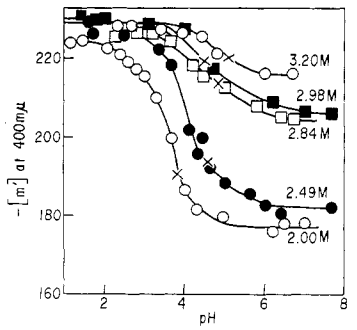

FIGURE 7: The effect of pH, as measured by optical rotation at 25°, at the Gu·HCl concentrations given in the figure. The crosses represent one measurement at each Gu·HCl concentration made after exposure to pH 1.5.

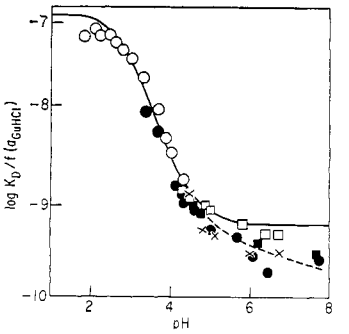

FIGURE 8: Equilibrium constants calculated from the data of Figure 7, plotted as log $[K_D/f(a_{Gu\cdot HCl})]$ *vs.* pH, with $f(a_{Gu\cdot HCl})$ given by eq 2. The symbols used correspond to those of Figure 7, except that crosses are used to represent the results at 3.2 M Gu·HCl. The solid curve is a theoretical curve according to eq 6.

equations). The experimental results clearly do not permit a choice between the two curves. The same conclusion applies to the results at other Gu·HCl concentrations. These results are somewhat more scattered than those at 3.1 M Gu·HCl and are readily represented by either of the two theoretical curves of Figure 6. One observes no systematic trend in the fitness of the theoretical curves with Gu·HCl concentration between 5 and 30°, *i.e.*, the temperature dependence of log K_D is independent of Gu·HCl concentration within the experimental error of the measurements.

Above 30°, equilibrium constants can be obtained only from the results at 2.29 M Gu·HCl. These data fit much better to an extension of the solid line of Figure 6 (*i.e.*, that based on ΔC_p = 1800 cal/deg per mole) than on the line based on the higher value of ΔC_p. However, deviations from log K values based on a two-state transition are expected to occur at higher temperatures, because of the expected contribution of the heat-denatured state (X) to the equilibrium properties. Under these conditions the equilibrium constant determined in the usual way from the data of Figure 5 is an *apparent* equilibrium constant, K_{app}, which is a function of the equilibrium constant K_{NX} for the reaction N $\rightleftarrows$ X, as well as of the equilibrium constant K_D, *i.e.* (Tanford, 1968)

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

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

The value for α, based on the data of Aune *et al.* (1967), is about 0.7 for ribonuclease.

Equation 4 can be used to estimate values of K_{NX} if K_D is known, as was done for lysozyme (Aune and Tanford, 1970). With values of K_D based on the thermal parameters ΔH = 40 kcal/mole, ΔC_p = 2650 cal/deg per mole we obtain log K_{NX} = 1.8 at 45° and 2.29 M Gu·HCl. This is a reasonable value. At pH 6, 45°, in the absence of Gu·HCl, Brandts and coworkers[3] have determined the value of log K_{NX} to be −2.75. This result applies to very low ionic strength. An increase in ionic strength decreases K_{NX}, as demonstrated by the effect of ionic strength on the transition temperature of the reaction N $\rightleftarrows$ X by Hermans and Scheraga (1961). A rough calculation based on their data near pH 6 indicates that log K_{NX} is about 0.9 less at ionic strength 2.0 than at low ionic strength, *i.e.*, leads to log K_{NX} = −3.6 at 45°, pH 6, and high ionic strength in the absence of Gu·HCl. The presence of Gu·HCl will greatly increase log K_{NX}. Assuming that this effect is about two-thirds of the effect that Gu·HCl has on log K_D (this being the result obtained for lysozyme), and using eq 2 and 3, we estimate the value of log K_{NX} in 2.29 M Gu·HCl to be increased by 5 ± 1, *i.e.*, the expected value becomes log K_{NX} = 1.4 ± 1.0, which is to be compared with the observed result of log K_{NX} = 1.8.

The overall conclusion is that the experimental data lead to ΔH = 35 ± 5 kcal/mole at 25°, and ΔC_p = 2200 ± 500 cal/deg per mole, and that comparison with the results of heat-denaturation studies favors values toward the upper end of the indicated uncertainty range.

Effect of pH. Figure 7 shows the effect of pH on the optical rotation of ribonuclease at a series of Gu·HCl concentrations at 25°. In the analysis of these results, the same value of $[m']_N$ was used as before. A single value of $[m']_D$ of −229° was determined from the limiting values of $[m']$ at low pH from the results at the three highest Gu·HCl concentrations. (The value at 3 M Gu·HCl based on the $[m']_D$ line of Figure 1 is −230.8°: the difference of about 2° is within the experimental error of the measurement.) The limiting value of $[m']$ at low pH at 2.0 M Gu·HCl is significantly *less* negative than the value obtained from the results at higher Gu·HCl concentrations. Since $[m']_D$ should become slightly *more* negative as the Gu·HCl concentration is reduced, this difference presumably arises from inability to achieve completeness of the N → D transition at 2.0 M Gu·HCl by a decrease in pH alone. Equilibrium constants were based on an $[m']_D$ value of −229°, the same as was used for the higher Gu·HCl concentrations: K_D values below pH 2.5 are subject to considerable error because the measured $[m']$ lies so close to $[m']_D$.

The equilibrium constants calculated from these data were divided by $f(a_{Gu\cdot HCl})$, as determined from the results at pH 6, using eq 2. A plot of log $[K_D/f(a_{Gu\cdot HCl})]$ *vs.* pH is shown in Figure 8, and it is seen that the results at all Gu·HCl concentrations can be reasonably described by a single curve.

[3] Personal communication from Dr. J. F. Brandts. These data represent an extension of the results of Brandts and Hunt (1967).

TABLE I: Thermodynamic Data in the Absence of Denaturant at 25°, pH 6.

	Ribonuclease	Lysozyme
Extrapolation by eq 2		
log K_D	−9.5	−10.4
$\Delta G_D°$, kcal/mole	+13.0	+14.2
$\Delta S_D°$, cal/deg per mole	+74 ± 16	+28
Extrapolation by eq 3		
log K_D	−6.5	−7.8
$\Delta G_D°$, kcal/mole	+8.9	+10.6
$\Delta S_D°$, cal/deg per mole	+88 ± 16	+40
Parameters independent of extrapolation		
ΔH, kcal/mole	35 ± 5[a]	22.4
ΔC_p, cal/deg per mole	2200 ± 500[a]	1375
Dependence on Gu·HCl concentration		
Δn, eq 2	24.4	21.5
Δn, eq 3	8.9	7.8
Principal groups responsible for effect of pH in acid region		
Number of groups	2	2
Identity	COOH	COOH
pK in native state	2.8–3.0	1.9

[a] Making allowance for the presence of heat-denatured ribonuclease at temperatures above 30° favors values toward the high end of the indicated range.

This indicates that the function $f(a_{Gu\cdot HCl})$ determined at pH 6 applies at lower pH also, and thus demonstrates the mutual independence of $f(a_{Gu\cdot HCl})$ and $F(a_H)$, at least to pH 3.5. (All the points below pH 3.5 refer to a single concentration of Gu·HCl.)

The maximum value of $-\text{d log } K_D/\text{d pH}$ in Figure 8 is about 1.25. This means that at least two titratable groups must have different pK values in states N and D. The fact that the maximum slope does not reach a value of 2.0 indicates that the difference between the acid dissociation constants for these groups in the two states is not as large as in lysozyme. The pH dependence of K_D for the latter protein in the same pH range also depends on the existence of two groups with anomalous pK's in the native state, but the maximum value of $-\text{d log } K_D/\text{d pH}$ is larger.

The interpretation of the results of Figure 8 is based on the principles used to analyze the pH dependence of the denaturation of lysozyme. The solid line in the figure represents eq 8 of Aune and Tanford (1969a),

$$F(a_H) = \frac{(1 + K_{1,D}/a_H)(1 + K_{2,D}/a_H)}{(1 + K_{1,N}/a_H)(1 + K_{2,N}/a_H)} \qquad (6)$$

with $pK_{1,N} = pK_{2,N} = 3.0$, $pK_{1,D} = 3.9$, and $pK_{2,D} = 4.4$. This is the theoretical equation for the influence of one aspartyl and one glutamyl group, with anomalous pK's of 3.0 in the native state, and normal pK's in the denatured state.[4] The equation fits the results below pH 5 reasonably well: no attempt was made to obtain a better fit to the experimental data below pH 2.5 because these data were all obtained at 2.0 M Gu·HCl and are subject to considerable uncertainty, as was noted above.

It is also likely that the heat-denatured state makes some contribution to the results at the lowest pH. Brandts and Hunt (1967) give a value of K_{NX} slightly above unity at pH 1.1, 25°, low ionic strength. An increase in ionic strength greatly decreases K_{NX} at this pH (Hermans and Scheraga, 1961), but nevertheless K_{NX} at 2.0 M Gu·HCl can be expected to be somewhat larger than at low ionic strength in the absence of Gu·HCl, though presumably $K_D > K_{NX}$. In that case the points of Figure 8 at the lowest pH values represent K_{app}, as given by eq 4, instead of K_D, and it is to be noted that $K_{app} < K_D$ if $K_D > K_{NX}$ when $K_{NX} > 1$. True K_D values at very low pH may thus be larger than the values shown in the figure. The influence on the analysis of the results by eq 6 would, however, be slight: only a slight reduction of $pK_{1,N}$ and/or $pK_{2,N}$ (say, to 2.8 rather than 3.0) would be required.

Equation 6 predicts that log $[K_D/f(a_{Gu\cdot HCl})]$ should become independent of pH above pH 5, whereas a small continued decrease is observed experimentally. To account for this it is necessary that there is at least one additional titratable group (with pK near 6) which has an anomalously low pK in the native state.[5] This very likely reflects the presence in ribonuclease of two histidyl residues with somewhat less-than-normal pK in the native state (Donovan, 1965; Bradbury and Scheraga, 1966; Meadows *et al.*, 1967). The experimental points of Figure 8 in the pertinent pH region are too few and too scattered to warrant an attempt to fit them theoretically by incorporating the presence of these groups in an extension of eq 16.

Discussion

A summary of all results of this paper, and a comparison with corresponding data for lysozyme, is given in Table I. Although the results for ribonuclease are less exact than those for lysozyme, they suffice to show the striking similarity between the two proteins with respect to their denaturation by Gu·HCl. This similarity is perhaps surprising in view of the well-known greater stability of lysozyme toward denaturation by Gu·HCl and by urea. Table I shows that this greater stability arises from two factors. Native lysozyme is *intrinsically* somewhat more stable than native ribonuclease ($\Delta G_D°$ is greater), and at the same time the N ⇌ D equilibrium is somewhat less influenced by Gu·HCl (Δn of eq 2 or 3 is smaller).

It is seen from Table I that the value of ΔC_p for the N → D reaction is considerably larger for ribonuclease than for lysozyme. It is likely that a major contribution to ΔC_p is made by the exposure of hydrophobic groups to the aqueous solvent medium, and the difference between lysozyme and

[4] The two groups can be identified as carboxyl groups by the same reasoning as was applied to lysozyme.

[5] In lysozyme experimental K_D values *increase* near pH 6 and a group with an anomalously *high* pK in the native state was required to account for this.

ribonuclease may thus be ascribable to the unusual degree of exposure of the aromatic groups of *native* lysozyme to the solvent (Williams *et al.*, 1965), which would increase C_p in the native state and reduce ΔC_p for denaturation. It is possible that the larger Δn value for ribonuclease, noted in the preceding paragraph, arises from the same cause.

Another interesting aspect of the value of ΔC_p for ribonuclease is that it is of the same order of magnitude as ΔC_p for the N → X reaction of that protein in the absence of Gu·HCl. The latter has been established, both from the temperature dependence of K_{NX} (Brandts and Hunt, 1967) and from direct calorimetric measurements (Danforth *et al.*, 1965), to be 2300 cal/mole per degree at 45°.[6] Because of the greater degree of unfolding of state D, compared with X, one might expect ΔC_p for the reaction N → D to be larger than ΔC_p for the reaction N → X, but this difference could be compensated by a lesser intrinsic ΔC_p per residue exposed when the solvent is a concentrated Gu·HCl solution than when it is pure water. (The detection of an effect of Gu·HCl concentration on ΔC_p is beyond the limits of precision of our data.)

The value of ΔH for the reaction N → X at 25° is about 50 kcal/mole, according to the results of Brandts and Hunt (1967). This value of ΔH is *larger* than ΔH for the reaction N → D. Thus, at low temperature, the additional unfolding that occurs in the reaction X → D is *exothermic*, with a ΔH of -15 ± 5 kcal/mole. A similar result was obtained with lysozyme.

The pH dependency of K_D provides another point of similarity between ribonuclease and lysozyme. For both proteins the major effect is a considerable increase in K_D as the pH is lowered from near-neutral pH to acid pH. In both proteins the data are compatible with the explanation that two carboxyl groups with anomalously low pK in the native state are responsible for the major part of this effect. The effect is greater, however, in lysozyme and the pK's of the anomalous groups are correspondingly lower (1.9 *vs.* 2.8–3.0 when both groups are assigned the same pK_N). In both proteins the effect of pH on the N ⇌ X transition is similar to that on the N ⇌ D transition when allowance is made for the difference in ionic strength between studies of the two processes. For the N ⇌ X transition in ribonuclease Hermans and Scheraga (1961) invoke one or two carboxyl groups with $pK_N = 2.5$, and for the same reaction in lysozyme, Sophianopoulos and Weiss (1964) find two abnormal groups with $pK_N = 1.46$ and 1.72, respectively. The same difference of about one pK unit between the abnormal groups in the two proteins is observed.

[6] ΔC_p emerges as temperature dependent from the studies of Brandts and Hunt (1967), but this feature of their results is probably within their experimental uncertainty.

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