Free Energy Changes in Ribonuclease A Denaturation

EFFECT OF UREA, GUANIDINE HYDROCHLORIDE, AND LITHIUM SALTS*

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The unfolding of ribonuclease A by urea, guanidine hydrochloride, lithium perchlorate, lithium chloride, and lithium bromide has been followed by circular dichroic and difference spectral measurements. All three abnormal tyrosyl residues are normalized in urea and guanidine hydrochloride ($\Delta \epsilon_{287}^\text{urea} = -2700$), only two are normalized in lithium bromide and lithium perchlorate ($\Delta \epsilon_{287}^\text{LiClO}_4 = -1700$), and only one is exposed in lithium chloride solutions ($\Delta \epsilon_{287}^\text{LiCl} = -700$). The Gibbs energies are $4.7 \pm 0.1$ kcal mol$^{-1}$ for urea- and guanidine hydrochloride-denaturation, $3.8 \pm 0.2$ kcal mol$^{-1}$ for lithium perchlorate-denaturation, and $12.7 \pm 0.2$ kcal mol$^{-1}$ for lithium chloride- and lithium bromide-denaturation of ribonuclease A. The latter results suggest that the mechanism of the unfolding process in urea and guanidine hydrochloride is quite different from that in lithium salts.

One of the interesting results from a study of the denaturation of folded proteins is an estimation of the Gibbs energy (conformational free energy change) involved in converting the globular conformation to an unfolded state in water. This is done by measuring the free energy change $\Delta G_{\text{sep}}$ as a function of denaturant concentration in the transition region and extrapolating to zero concentration, $\Delta G_{\text{sep}}^0$ (7). However, we do not know whether or not the values of $\Delta G_{\text{sep}}^0$ for all denatured states are internally consistent, in the sense that $\Delta G_{\text{sep}}^0$ is larger when more extensive unfolding takes place and is smaller when less extensive unfolding occurs. For this reason and others, we have been carrying out systematic studies of denaturation of proteins by urea and by various salt denaturants, namely, GdnHCl, LiClO$_4$, LiBr, and LiCl. It is felt that it would be worthwhile to present in detail here some rather unexpected results of $\Delta G_{\text{sep}}^0$ measurements for the denaturation of one protein, namely, RNase A by salt denaturants giving intermediate states. It has been observed that although LiCl and LiBr cause partial unfolding, the value of $\Delta G_{\text{sep}}^0$ is about three times larger than that obtained for a randomly coiled RNase A. A possible explanation for this discrepancy is proposed.

MATERIALS AND METHODS

RNase A (bovine pancreas) was obtained from Calbiochem. Ultra-pure urea and GdnHCl were purchased from Schwarz/Mann, LiCl was from Baker, while LiClO$_4$ and LiBr were from Matheson, Coleman & Bell. These and other analytical grade chemicals were used without further purification.

Absorption spectra were measured in a Cary 118 or a Cary 219 spectrophotometer using tandem thermostatted cells whose temperature was maintained within $\pm 0.01 ^\circ$C. The protein concentration was determined using a value of 9800 for the molar extinction coefficient of native RNase A at 277.5 nm (1).

CD measurements were made in a Jasco J-20 automatic recording spectropolarimeter. Thermostatted cells of 0.1- and 1.0-cm path lengths were used. Base-line corrections for solvents were made routinely. CD data were reduced to the concentration-independent parameter [$\theta$], the mean residue ellipticity, defined as:

$$[\theta] = \frac{M \theta_x}{10^1}$$

where $\theta_x$ is the observed ellipticity in millidegrees at wavelength $\lambda$, $M_x$ the mean residue weight of RNase A ($M_x = 110$), $c$ the protein concentration (milligrams/cm$^3$), and $l$ the path length (centimeters). All CD results are presented in units of degrees cm$^2$ dmol$^{-1}$

RNase A solutions were prepared as follows. For unfolding experiments, known amounts of stock protein solution, buffer, and denaturant solutions, all in 0.1 M glycine-HCl buffer (pH 3.0) were mixed and incubated overnight which was sufficient for completion of the reaction. A similar procedure was employed in preparing the protein solution for refolding experiments with the only exception that RNase A was first denatured in concentrated denaturant solution and then diluted with buffer. Solutions for all measurements were routinely filtered.

Measurements for pH were made with a Radiometer type TTTIC pH meter.

RESULTS

The unfolding transitions in urea and in salt denaturants, followed by observing changes in $[\theta]_{222}$, are shown in Fig. 1. A decrease in the magnitude of $-\Delta [\theta]_{222}$ is taken to reflect the loss of secondary structure. All transitions were found reversible; the experimental points obtained from denaturation and renaturation experiments lie on the same transition curve.

In Fig. 2 the difference molar absorption changes at 287 nm, $\Delta \varepsilon_{287}$, are plotted as a function of denaturant concentration. At this wavelength, it is expected that changes would reflect alteration in the environment of tyrosine residues on
The subscripts $N$, $U$, and obs denote, respectively, the value of $(\theta)_{220}$ or $\Delta \varepsilon_{287}$ for the native state, the value for unfolded state, and the value observed in the presence of denaturant at a temperature $T$°K. Unlike the CD results (Fig. 1), since the difference spectral properties of the native and unfolded protein molecules showed dependence on the denaturant concentration (Fig. 2), allowance has been made for the dependence of both $Y_N$ and $Y_U$ on the denaturant concentration in calculating $K_{app}$.

The values of $\Delta G_{app}$ were plotted against each denaturant concentration (Fig. 3). A linear least squares analysis was applied to the data according to empirical Equation 3 (8), which was later shown to be a more general thermodynamic model for analysing the solvent denaturation of proteins (9):

$$\Delta G_{app} = \Delta G_{app}^{\text{iso}} - mC$$

where $C$ is the molar concentration of the denaturant and $m$ the slope, i.e. $(\Delta \varepsilon_{287}/\partial C)_{T,p}$. The choice of Equation 3 is also based on our recent study in which we have tested the linear model (Equation 3) by studying the denaturation of several proteins by urea, guanidine hydrochloride, and guanidine thiocyanate (10).

A least squares method was used to analyse the reversible equilibrium results of Figs. 1 and 2. The resulting values of $\Delta G_{app}^{\text{iso}}$, $m$, and $C_{\text{eq}}$ the denaturant concentration at which $K_{app}$

exposure to solvent. The intercepts at zero denaturant concentration, obtained from the linear extrapolation of results in the post-transition region are assumed to be the value of $\Delta \varepsilon_{287}$ which would be observed for the unfolded state, if it could somehow be studied in the absence of the denaturant (1). These effects of denaturation are also reversible.

The apparent equilibrium constant $K_{app}$ and free energy change for a two-state process can be obtained from the results shown in Figs. 1 and 2 using the following relation:

$$K_{app} = \frac{Y_U - Y_N}{Y_U - Y_{obs}} = e^{-\Delta G_{app}/RT}$$

$$\Delta G_{app} = \Delta G_{app}^{\text{iso}} - mC$$

TABLE I

| Denaturant | $\Delta G_{app}^{\text{iso}}$ kcal mol$^{-1}$ | $-m$ kcal mol$^{-1}$ M$^{-1}$ | $C_{eq}$ M |
|------------|-------------------------------------------|-----------------------------|----------|
| GdnHCl     | (4.8)                                    | (2.0)                       | (2.0)    |
| Urea       | (4.7)                                    | (1.52)                      | (3.3)    |
| LiCl       | 12.9 (12.9)                              | 2.19 (2.19)                 | 5.9 (5.9) |
| LiBr       | 12.5 (12.5)                              | (3.20)                      | (3.9)    |
| LiClO$_4$  | 3.5 (4.0)                                | 1.55 (1.82)                 | 2.2 (2.2) |
expected that AG$ associated with the unfolding by a lithium salt is likely to be less than that for unfolding to a random coil (11–13). On the other hand, lithium salts lead to an incomplete unfolding of RNase A (see Figs. 1 and 2), i.e. in our terminology a state "intermediate" between the native and randomly coiled state (1). Indeed such intermediate states have been shown to contain some residual structures that can be removed in a co-operative manner by the addition of urea to the partially denatured states (3, 4).

The observation that two independent physical methods used to follow a denaturation transition gave identical values of $\Delta G_{\text{app}}^{\text{obs}}$ and $C_m$, within the experimental error, suggests that each transition shown in Figs. 1 and 2 is a two-state process. It should, however, be noted that this is a necessary but not a sufficient condition for an all-or-none process. Additional evidence for the absence of any stable intermediate(s) in the course of denaturation of RNase A by urea (14) and GdnHCl (15) comes from kinetic studies on unfolding and refolding of this protein. However, fast kinetic studies of the denaturation of RNase A by GdnHCl and urea suggested that the conformational transition of the enzyme is a sequential process (16). But the high cooperativity of unfolding measured by equilibrium experiments inside the transition region suggests that intermediates are unstable relative to native and denatured protein (16) and therefore do not contribute significantly to $K_{\text{app}}$.

An attempt was made to study the kinetics of denaturation and renaturation of RNase A by lithium salts. It was concluded that meaningful kinetic data for the refolding of RNase A cannot be obtained due to a large heat of dilution of these salt solutions. For example, the rise in temperature of a 10 M LiBr solution at 25 °C was more than 10 °C on 50% dilution with water at 25 °C. Nevertheless, we assumed a two-state behavior of lithium salts denaturation in the light of the coincidence of transition curves measured by two independent physical properties (see Table I).

RNase A in concentrated solutions of urea and GdnHCl is devoid of all the elements of its native conformation and behaves as a cross-linked random coil (11–13). It is therefore possible to estimate the Gibbs energy change for the process, folded conformation $\rightleftharpoons$ random conformation, assuming that water in the absence of the denaturant. A value of 4.7 ± 0.1 kcal mol$^{-1}$ for $\Delta G_{\text{app}}^{\text{obs}}$ was obtained (see Table I). This value for the two denaturants is the same, which indicates that the extent of unfolding in the two denaturants cannot differ appreciably.

In most cases the products of acid and thermal denaturation of several proteins are less completely unfolded than those of urea and GdnHCl denaturation (11). In his review Pace (8) compares the values of $\Delta G_{\text{app}}^{\text{obs}}$ for GdnHCl and acid-thermal denaturation of several proteins. These results suggest that for a protein $\Delta G_{\text{app}}^{\text{obs}}$ for unfolding to a random coil is greater than that for unfolding to a denatured state produced by acid-thermal denaturation. All available data on the effect of lithium salts suggest that these denaturants unfold RNase A to a lesser extent than GdnHCl and urea (1–4). It is therefore expected that $\Delta G_{\text{app}}^{\text{obs}}$ associated with the unfolding by a lithium salt is likely to be less than 4.7 ± 0.1 kcal mol$^{-1}$. As can be seen in Table II, the analysis of equilibrium results of denaturation by LiCl and LiBr gave unexpected results; a value of 12.7 ± 0.2 kcal mol$^{-1}$ for $\Delta G_{\text{app}}^{\text{obs}}$ was estimated from these denaturation results in the absence of lithium salts.

The discrepancy between the observed and expected values of $\Delta G_{\text{app}}^{\text{obs}}$ prompted us to study the denaturation by LiCl of two other proteins, namely lysozyme and $\alpha$-lactalbumin; LiCl is known to give rise to a conformation intermediate between the native and denatured states of these proteins (5, 6). The results of the LiCl denaturation, along with those for denaturation by urea and GdnHCl (10) are listed in Table II. As can be seen in Table II, $\Delta G_{\text{app}}^{\text{obs}}$ values from LiCl denaturation are larger than those associated with the process, native $\rightleftharpoons$ random coil conformation.

The discrepancy between the expected and observed values of $\Delta G_{\text{app}}^{\text{obs}}$ is confusing unless it means that we have to reconsider the two-state behavior of protein denaturation by lithium salts, as assumed in the present analysis. The mechanism of denaturation is very important in analysing transition curves such as shown in Figs. 1 and 2. As we shall see below, one can explain the denaturation results by assuming a mechanism in which native protein (N) goes to a "definite" state A, obtained in a lithium salt, through another denatured state (X) which is unfolded more than A and is not on the direct path between N and A:

$$N \rightleftharpoons X \rightleftharpoons A$$

On the other hand, as shown by Tanford (11) and Pace (8), if X is on the direct path between N and A, i.e. if X is less unfolded than A, then the estimate of $\Delta G_{\text{app}}^{\text{obs}}$ derived from a two-state analysis will be lower than the true value of this parameter.

A mathematical treatment for the equilibrium constant for a process such as shown in Equation 4 is as follows. If all the states are characterized by a property Y and fractional concentration $f$, then $Y_{\text{obs}}$ at any point on the salt-induced denaturation curve between N and A is given by

$$Y_{\text{obs}} = f_N Y_N + f_X Y_X + f_A Y_A$$

and

$$f_N + f_X + f_A = 1$$

The observed fraction of the denatured protein, $f_{\text{obs}}$, for the process between N and A (N $\rightleftharpoons$ A) is given by

$$f_{\text{obs}} = \frac{Y_{\text{obs}} - Y_N}{Y_A - Y_N} = f_A + \alpha f_X$$

where $\alpha = (Y_X - Y_N)/(Y_A - Y_N)$. The value of $\alpha = 1$, if states A and X are the same. But $\alpha > 1$ for all values of $Y_A$ that lie between $Y_X$ and $Y_N$. The apparent equilibrium constant, $K_{\text{app}} = f_{\text{obs}}/(1 - f_{\text{obs}})$ can be written as

$$K_{\text{app}} = K_A \left[ \frac{1 + \alpha(K_X/K_A)}{1 + (1 - \alpha)K_X} \right]$$

where $K_A = f_A/f_N$ is the equilibrium constant for the process N $\rightleftharpoons$ A, and $K_X = f_X/f_N$ is the equilibrium constant for the process N $\rightleftharpoons$ X. Due to the fact that $K_{\text{app}}$ is positive, it must

| Denaturant | Lysozyme | $\Delta G_{\text{app}}^{\text{obs}}$ | $C_m$ | $\Delta G_{\text{app}}^{\text{obs}}$ | $C_m$ |
|------------|----------|----------------|------|----------------|------|
| GdnHCl$^+$ | 9.0      | 4.2            | 4.2  | 3.3            | 5.1  |
| Urea       | 8.8      | 6.8            | 4.4  | 5.0            | 5.1  |
| LiCl       | 18.5     | 7.1            | 5.8  | 5.1            | 5.1  |

$^*$ Data taken from Ref. 10.
therefore be kept in mind that the term \((1 - \alpha)K_X\) of the denominator of Equation 7 is less than one.

It is obvious from Equation 7 that for \(\alpha > 1\), the square bracketed term of this equation is always greater than 1. This means that \(K_{\text{app}} > K_A\), the true thermodynamic equilibrium constant for the process \(N \rightleftharpoons A\). That is, \(\Delta G_{\text{app}}^{\circ} > \Delta G_A^{\circ}\). We would therefore like to suggest that a cause for the apparent discrepancy among the values of \(\Delta G_{\text{app}}^{\circ}\) for various denaturation processes seems to be due to a problem in analysing the transition curve obtained in lithium salt solutions.

It has recently been reported that the mechanism of denaturation of RNase A by LiClO₄ (17) is more complex than that proposed here (see Equation 4). In the folding of the denatured protein from state \(A\) to the state \(N\), Denton et al. (17) observed three intermediates. Thus, there is kinetic evidence that the lithium denaturation of RNase A is not a two-state process.

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