Free Energy Changes in Denaturation of Ribonuclease A by Mixed Denaturants

EFFECTS OF COMBINATIONS OF GUANIDINE HYDROCHLORIDE AND ONE OF THE DENATURANTS LITHIUM BROMIDE, LITHIUM CHLORIDE, AND SODIUM BROMIDE*

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The denaturation of ribonuclease A by guanidine hydrochloride, lithium bromide, and lithium chloride and by mixed denaturants consisting of guanidine hydrochloride and one of the denaturants lithium chloride, lithium bromide, and sodium bromide was followed by difference spectral measurements at pH 4.8 and 25°C. Both components of mixed denaturant systems enhance each other’s effect in unfolding the protein. The effect of lithium bromide on the midpoint of guanidine hydrochloride denaturation transition is approximately the sum of the effects of the constituents ions. For all the mixed denaturants tested, the dependence of the free energy change on denaturation is linear. The conformational free energy associated with the guanidine hydrochloride denaturation transition in water is 7.5 ± 0.1 kcal mol⁻¹, and it is unchanged in the presence of low concentrations of lithium bromide, lithium chloride, and sodium bromide which by themselves are not concentrated enough to unfold the protein. The conformational free energy associated with the lithium bromide denaturation transition in water is 11.7 ± 0.3 kcal mol⁻¹, and it is not affected by the presence of low concentrations of guanidine hydrochloride which by themselves do not disrupt the structure of native ribonuclease A.

We have reported elsewhere (3, 4) the results of the effect of mixed denaturants (mixtures of urea and one of the denaturant salts LiCl, LiClO₄, and CaCl₂). These results lead to various important conclusions. If low concentrations of urea, which by themselves are not enough to denature RNase A, are added to the salt inducing a transition, we found that urea causes the transition to occur at lower denaturant salt concentration, but that the product of denaturation is still in the salt-denatured conformation. If low concentrations of one of the denaturant salts, which by themselves do not disrupt the structure of native RNase A, are added to urea which is inducing a transition, unexpected results were obtained. We found that the low concentrations of each salt actually stabilize the protein against urea denaturation, but do not change the conformation of the urea-denatured molecule. At somewhat higher concentrations of denaturant salts, we found that the added denaturant cooperates with urea in unfolding, again with no change in the nature of the end product of denaturation. As we have suggested elsewhere (3), a possible explanation for these unexpected observations lies in the ability of the carbonyl oxygen of the urea molecule to form strong complexes with lithium and calcium ions (8). However, the observed stabilization could also be explained if there is a unique binding site for lithium and calcium salts on native RNase A (9). The latter possibility, not considered earlier (3), will be discussed here.

In this paper we would like to report measurements of the denaturation transitions of RNase A by GdnHCl,¹ by LiBr, and by mixed denaturants consisting of GdnHCl and one of the denaturants LiBr, LiCl, and NaBr. The rationale for using GdnHCl is that it gives the same denaturation transition as urea does (5), but it should not interact with lithium salts owing to the lack of a carbonyl oxygen in the molecule. It is therefore expected that both components of the mixed denaturants should enhance each other’s effect in denaturing RNase A; no competition, as found in the case of the urea/lithium mixture (3, 4), should be observed. It is interesting to note for each denaturant-denaturant system we found, as we had expected, that when both components of the mixture are used on RNase A at the same time, they cooperate.

We also report here our measurements of the free energy change on denaturation (ΔG₀) of RNase A by mixed denaturants. The values of ΔG₀ are determined from denaturation transitions of the protein induced by a denaturant in the presence of fixed concentrations of a second denaturant which by themselves are not enough to denature the protein. From the dependence of ΔG₀ on GdnHCl concentration we were able to estimate the stability of the native protein (i.e. the

¹The abbreviation used is GdnHCl, guanidine hydrochloride.

Measurements of the physical properties of ribonuclease A under various denaturing conditions using different physical methods have suggested that different denaturants could produce different denatured states, i.e. conformations with different amounts of secondary and tertiary structures (1–5). Only urea, guanidine hydrochloride, and GdnHCNS give the most extensively unfolded state which is devoid of all the elements of native structure and cause the molecule to behave as a cross-linked random coil (6, 7). The denatured states obtained in other denaturants are “intermediate” states between native and randomly coiled states. Supporting evidence that intermediate states contain residual structure comes from the observation that another cooperative transition occurs when urea is added to denatured states obtained in salts such as LiCl, LiClO₄, and CaCl₂ (3, 4). It should, however, be noted that the denatured states obtained in lithium and calcium salts are different from one another.

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value of $\Delta G_D$ at zero concentration of GdnHCl in the presence of low concentrations of lithium and sodium salt denaturants. Similarly, stability of the native protein was estimated from LiBr denaturation of RNase A in the presence of low concentrations of GdnHCl. It has been found that the stability of the native protein is unchanged in the presence of a denaturant at concentrations which do not disrupt the native structure of RNase A. This finding seems to suggest that there is no "specific" binding site on the native RNase A for the denaturants used in this study.

MATERIALS AND METHODS

RNase A (bovine pancreas) was obtained from Sigma. Ultrapure GdnHCl was purchased from Schwarz/Mann. LiCl and NaBr were from J. T. Baker Chemical Co., while LiBr was from Matheson, Coleman & Bell. These and other analytical grade chemicals were used without further purification.

Difference spectral measurements were made in a Cary 219 spectrophotometer using tandem cells whose temperature was maintained at 25.0 ± 0.1 °C. The sample cell contained buffer in one compartment and protein in denaturant in the other compartment. One half of the reference cell contained native protein in buffer, and the other contained denaturant solution. Protein and denaturant concentrations were identical in both cells. All difference spectral results were converted to difference molar extinction coefficient, $\Delta \varepsilon$, defined as the difference between molar extinction coefficients of the protein in denaturant and in buffer solutions. Protein concentration was determined using a value of 9800 for the molar extinction coefficient of RNase A at 277.5 nm (1).

Protein solutions for denaturation and renaturation experiments were prepared as follows. For denaturation experiments, known amounts of stock protein solution, buffer, and denaturant (single or mixed) solutions, all in 0.1 M sodium acetate buffer (pH 4.8), were mixed and incubated overnight, which was sufficient for completion of the reaction. A similar procedure was used in preparing protein solutions for renaturation experiments with the only exception that RNase A was first denatured in a concentrated denaturant solution containing a fixed concentration of the second denaturant of interest, and then diluted with buffer having the same amount of the second denaturant.

Measurements of pH were made with a Radiometer type TTT1C pH meter.

RESULTS

The GdnHCl denaturation transition curves in the presence of low concentrations of LiBr, LiCl, and NaBr are shown in Figs. 1, 2, and 3, respectively. For RNase A, GdnHCl denaturation is reversible and closely approaches a two-state mechanism (6, 7). We have found that all the transitions shown in Figs. 1–3 are reversible. It will be assumed that all the transitions shown here are essentially a two-state process in the presence of low concentrations of added denaturant which by themselves do not disrupt the native conformation of the enzyme.

For a two-state denaturation, the free energy change on denaturation, $\Delta G_D$, can be calculated from the results of Figs. 1–3, using

$$\Delta G_D = -RT \ln \frac{\Delta \varepsilon_{\text{obs}} - \Delta \varepsilon_N}{\Delta \varepsilon_D - \Delta \varepsilon_{\text{obs}}}$$

(1)

where subscripts $N$, $D$, and obs denote, respectively, the value of $\Delta \varepsilon_{\text{obs}}$ for the native state, the value for the denatured state, and the value observed in the presence of the denaturant of interest. Since the spectral properties for the native state (the linear portion of the pretransition regions) and denatured state (the linear portion of the post-transition region) showed dependence on denaturant concentration (see Figs. 1–3), allowances have therefore been made for the dependence of both $\Delta \varepsilon_N$ and $\Delta \varepsilon_D$ on the denaturing medium in calculating the values of $\Delta G_D$. It should be noted that the dependence of

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FIG. 1. GdnHCl denaturation of RNase A in the presence of different concentrations of LiBr at 25 °C. Curve 1, 0 M; curve 2, 0.69 M; curve 3, 1.38 M; curve 4, 2.08 M. All measurements were made in 0.1 M sodium acetate buffer (pH 4.8). At least two points in the transition region and one point in the pretransition region represent both denaturation and renaturation experiments. All the points in pre- and post-transition regions are not shown to maintain brevity. Lines in the pre- and post-transition regions are least squares best fit to the data.

FIG. 2. GdnHCl denaturation of RNase A in the presence of different concentrations of LiCl. Curve 1, 0 M; curve 2, 1.12 M; curve 3, 1.89 M; curve 4, 2.94 M; curve 5, 4.20 M. In order to maintain brevity, points are not shown in the figure. Experimental conditions are the same as those described in the legend to Fig. 1.

FIG. 3. GdnHCl denaturation of RNase A in the presence of different concentrations of NaBr. Curve 1, 0 M; curve 2, 1.09 M; curve 3, 1.64 M; curve 4, 2.59 M. Experimental conditions are same as those described in the legend to Fig. 1.
\[ \Delta G_0 = \Delta G'_0 - m[\text{denaturant}] \]  

where \( \Delta G'_0 \) is the value of \( \Delta G_0 \) when the GdnHCl concentration is zero, [denaturant] represents the concentration of the denaturant, and \( m \) is the slope of the straight line. Results of these analyses are presented in Table I.

Results of GdnHCl denaturation of RNase A in the presence of the various concentrations of LiCl (Fig. 2) and NaBr (Fig. 3) were also analyzed according to Equations 1 and 2. From the linear plots of \( \Delta G_0 \) against [denaturant] (not shown), values of \( \Delta G'_0 \), \( m \), and \( C_m \), the midpoint of the transition, were determined and are given in Table I.

We have also studied the LiBr denaturation of RNase A in the presence of low concentrations of GdnHCl which by themselves do not disrupt the state \( N \) of the protein. These changes are reversible and are shown in Fig. 5. All the plots of \( \Delta G_0 \) versus LiBr concentration are good straight lines (not shown). The results of least squares analysis are given in Table II.

All we know about LiBr denaturation of RNase A tells us that the LiBr-denatured protein is less unfolded than the GdnHCl-denatured protein (1). It is therefore expected that \( \Delta G'_0 \) LiBr denaturation ought to be less than that for GdnHCl denaturation (11, 12). The results of Tables I and II suggest the contrary. We have presented earlier (13) a possible explanation for this discrepancy and we will not discuss it here.

**Discussion**

We have studied the denaturation of RNase A by GdnHCl, LiBr, and by mixed denaturants consisting of GdnHCl and one of the denaturants LiBr, LiCl, and NaBr. Measurements of difference spectra at 287 nm were used to follow the transition. It is known that changes in \( \Delta T_m \) reflect alterations in the environment of tyrosyl residues on exposure to solvent. It has been shown elsewhere (4) that difference spectral transitions are identical to those obtained by circular dichroism measurements.

The results of Fig. 5 suggest that the value of \( \Delta T_D \) depends on LiBr concentration, but is independent of the presence of GdnHCl. A value of -1700 for \( \Delta T_D \) at [LiBr] = 0 suggests that 2 of the 3 buried tyrosyl residues in native RNase A are normalized on LiBr denaturation (1). In contrast, \( \Delta T_D \) for GdnHCl denaturation depends on concentrations of both GdnHCl and the second denaturant (Figs. 1–3). We believe that the product of GdnHCl denaturation in the presence of various concentrations of lithium and sodium salts is always a cross-linked random coil. The reason for this belief is our earlier observations that the end product of RNase A denaturation in the presence of lithium salt denaturants is a random coil, constrained by four intact disulfide bonds (3, 4).

The mechanism of GdnHCl denaturation is very important in the analysis of results such as shown in Figs. 1–3 (10). The kinetics of GdnHCl denaturation of RNase A suggests the unfolding of the protein is a two-state process (14). On the other hand, fast kinetic studies reveal that the denaturation of RNase A by GdnHCl is a sequential process (15); but the high cooperativity of the equilibrium transition curves suggests that intermediates are unstable in the transition region and therefore do not contribute significantly to \( \Delta G_D \) (15). In light of the steepness of each transition of Figs. 1–3, we have assumed that GdnHCl denaturation in the presence of various concentrations of LiBr, LiCl, and NaBr is a two-state process.

A least squares analysis was applied to the data such as shown in Fig. 4 according to Equation 2 (10) which was shown to be a more general thermodynamic model for analyzing the denaturation of proteins by chemical denaturants (16). The

### Table I

**Parameters characterizing the GdnHCl denaturation of RNase A in the presence of various concentrations of LiBr, LiCl, and NaBr**

The \( m \) values, \( d(\Delta G_0)/d[\text{denaturant}] \), are slopes of plots such as those shown in Fig. 4; \( C_m \) is the molar concentration of GdnHCl at which \( \Delta G'_0 = 0 \); and \( \Delta G'_0 \) is the extrapolated value of \( \Delta G_0 \) at [denaturant] = 0 and is obtained from the plots such as shown in Fig. 4.

<table>
<thead>
<tr>
<th>LiBr</th>
<th>LiCl</th>
<th>NaBr</th>
</tr>
</thead>
<tbody>
<tr>
<td>[LiBr]</td>
<td>( \Delta G'_0 )</td>
<td>( -m )</td>
</tr>
<tr>
<td>M</td>
<td>kcal mol(^{-1})</td>
<td>kcal mol(^{-1}) M(^{-1})</td>
</tr>
<tr>
<td>0</td>
<td>7.6</td>
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</tr>
<tr>
<td>0.69</td>
<td>7.4</td>
<td>3.44</td>
</tr>
<tr>
<td>1.38</td>
<td>7.5</td>
<td>4.46</td>
</tr>
<tr>
<td>2.08</td>
<td>7.6</td>
<td>6.33</td>
</tr>
</tbody>
</table>

* \( \Delta G'_0 \) values are apparent due to the fact that they are measured by a pseudothermodynamic method (7).
The effect of the addition of a denaturant is to reduce the energy of solvation of the peptide group is very large (18, 19).

The final conclusion from the results of Table I is that the $\Delta G_{\text{D}}$ value for the $N \rightarrow D$ transition does not depend upon the presence of low concentrations of lithium and sodium salts. This is an expected result if there is no specific binding site for lithium and bromide ions on native RNase A, because Pace and McGrath (9) and Pace and Marshall (20) presented evidence to show that the binding of a small molecule to a unique site on the native conformation of protein results in a readily observable change in protein stability.

In order to verify the above conclusions, led by the results in Table I, we reversed the denaturants. That is, the LiBr denaturation of RNase was studied in the presence of low concentrations of GdnHCl, which by themselves are not enough to denature the enzyme (see Fig. 5). It is interesting to note that the data in Table II lead to the same conclusions as those obtained from the results in Table I: (i) that both components of the mixed denaturants when used on the protein at the same time cooperate; (ii) that the $m$ value increases as GdnHCl concentration is increased; and (iii) that the conformational stability of the native protein ($\Delta G_{\text{D}}$) is unaffected by the presence of low concentrations of GdnHCl, and hence there is no specific site for the added denaturant on native RNase A.

We would like to conclude that there is no unique binding site on the native RNase molecule for the denaturants used in this study, and that denaturant-denaturant interaction offers the best explanation for the results on the effect of stabilization of RNase A against urea denaturation by lithium salts (3).

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Free energy changes in denaturation of ribonuclease A by mixed denaturants. Effects of combinations of guanidine hydrochloride and one of the denaturants lithium bromide, lithium chloride, and sodium bromide.

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