How Valid Are Denaturant-Induced Unfolding Free Energy Measurements? Level of Conformance to Common Assumptions over an Extended Range of Ribonuclease A Stability†

Min Yao and D. W. Bolen*

Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch at Galveston, 5.154 Medical Research Building, Galveston, Texas 77555-1052

Received December 22, 1994⊗

ABSTRACT: A thermodynamic cycle is used to explore a host of assumptions, conditions, and criteria which must be met for evaluation of authentic unfolding free energy changes. The thermodynamic cycle involves measurement of the unfolding free energy change ($\Delta G°_{N-U}$) for RNase A at a reference pH, along with determination of the titration free energy changes for the native and unfolded species over an extended pH range. From these free energy changes, $\Delta G°_{N-U}$ at any pH in the range can be predicted and compared with $\Delta G°_{N-U}$ determined by use of the linear extrapolation method (LEM). Good agreement is found between predicted and determined free energy changes covering a broad range of protein stability changes (5 kcal/mol), pH (pH from 3 to 8.5), and lengths of linear extrapolation ($C_{1/2}$ values from 2.4 to 7.7 M urea). The agreement between predicted and LEM-determined $\Delta G°_{N-U}$ values demonstrates (1) that $\Delta G°_{N-U}$ determined by the LEM is a function of state; i.e., it has the properties of additivity and independence of pathway required of an authentic free energy quantity; (2) the ability to obtain $\Delta G°_{N-U}$ values which are in agreement with the free energy change predicted by the cycle is independent of the length of linear extrapolation; and (3) the two-state assumption holds over an extensive pH range. The fact that the pH titration curve of unfolded RNase A in 6 M GdnHCl could be used in accurately predicting urea-induced $\Delta G°_{N-U}$ values shows that the unfolded ensemble in 6 M GdnHCl is thermodynamically identical to urea-unfolded RNase A as far as the pH dependence of protein stability is concerned. Urea-induced and GdnHCl-induced RNase A $\Delta G°_{N-U}$ values at pH 3 were found not to agree with one another, but this appears to be due to the inability to control the salt effect of GdnHCl on the native state of RNase A. These results provide strong evidence that the LEM applied to urea-induced unfolding of RNase A results in reliable free energy changes that meet a number of essential criteria for authentic thermodynamic quantities. The titration method is important in its own right in providing a means for evaluating the pH dependence of two-state protein unfolding free energy changes which does not require analysis of denaturant-induced unfolding transitions.

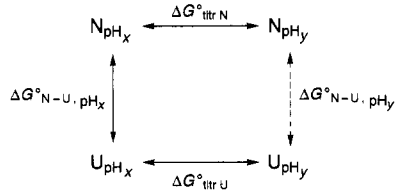
Except for kinetic measurements, much of what is known about protein folding centers around thermodynamic and structural considerations of protein stability. The primary index of stability is the unfolding free energy change ($\Delta G°_{N-U}$), a quantity frequently obtained from measurements of protein unfolding induced by the use of strong denaturants such as urea or guanidine hydrochloride (GdnHCl)[1] (Pace, 1975). The measurement of $\Delta G°_{N-U}$ is based upon several assumptions and conditions, the failure of any one of which could bring into question the validity of the quantity. These conditions and assumptions include such matters as the order of the transition, the number of states in the transition region, and the reversibility of the unfolding (Griko et al., 1994; Lumry et al., 1966). Closely associated with these matters are practical issues involving the evaluation of unfolding free energy changes, i.e., whether linear extrapolation is valid in evaluating $\Delta G°_{N-U}$, or whether a binding model or a transfer model is more appropriate for obtaining this quantity (Pace, 1975; Pace & Vanderburg, 1979; Santoro & Bolen, 1992). Once $\Delta G°_{N-U}$ is obtained from analysis of denaturant-induced unfolding measurements, the focus is often to interpret this quantity in terms of the stability of the protein or in terms of structural differences between the native states or unfolded states of wild-type and mutant proteins (Pace, 1975). However, due to the various assumptions and procedures necessary to evaluate $\Delta G°_{N-U}$, the question is whether the measured $\Delta G°_{N-U}$ is a property of the protein alone.

Surprisingly, little attention has been paid to the meaning of the $\Delta G°_{N-U}$ quantity obtained from denaturant-dependent analysis, what its properties are, and how it relates to the interpretation placed on the quantity (Bolen & Santoro, 1988; Santoro & Bolen, 1988). By way of illustration, it is not uncommon to find proteins which give different values of $\Delta G°_{N-U}$ for urea unfolding in comparison to GdnHCl-induced unfolding (Pace, 1975). For such systems, this means that the quantity, $\Delta G°_{N-U}$, is not solely a property of the protein, independent of the nature of the denaturant (Santoro & Bolen, 1988). Clearly, a dependence of $\Delta G°_{N-U}$ on the nature of denaturant would compromise the interpretation of this quantity as a measure only of a change in protein structure. In another issue, for any given set of

† Supported by U.S. Public Health Service Research Grant GM22300 and National Science Foundation Grant DMB 8904394.
⊗ Abstract published in *Advance ACS Abstracts,* March 1, 1995.
[1] Abbreviations: LEM, linear extrapolation method; GdnHCl, guanidine hydrochloride; RNase A, ribonuclease A; RCAM-RNase A, reduced and carboxamidated ribonuclease A.

0006-2960/95/0434-3771$09.00/0

Scheme 1



denaturant-induced unfolding data, $\Delta G°_{N-U}$ values obtained by use of the linear extrapolation method (LEM), the denaturant binding model, or the transfer model frequently differ from one another; i.e., the $\Delta G°_{N-U}$ is model-dependent (Pace, 1975). Regardless of which model is used, the $\Delta G°_{N-U}$ quantities obtained from application of the different models are generally interpreted to have the same meaning; i.e., the free energy change represents the difference in free energy between native and denatured states. Obviously, a given equilibrium can have only one free energy change, and the fact that different models give different values again brings into question the meaning of the $\Delta G°_{N-U}$ quantity used in analysis of protein unfolding.

The fact is, the foundation of the measurement of denaturant-induced $\Delta G°_{N-U}$ is weak, and there is more uncertainty in the interpretation of this quantity than is commonly acknowledged. It is useful, then, to investigate the processes and assumptions associated with $\Delta G°_{N-U}$ determination over an extensive range of protein stability, and evaluate how reliable and robust the methods and assumptions are in producing unfolding free energy changes that meet criteria expected of authentic thermodynamic quantities. We have previously cited criteria necessary for interpretation of $\Delta G°_{N-U}$ in terms of structural stability (Bolen & Santoro, 1988; Santoro & Bolen, 1988). In addition to meeting the criterion of reversibility for unfolding, $\Delta G°_{N-U}$ values derived from analysis of denaturant-induced protein unfolding should (1) be independent of the nature of the denaturant, (2) be analyzable in terms of a finite number of states of the protein, e.g., two-state analysis, and (3) have the properties (additivity and predictability) of being a thermodynamic function of state (Bolen & Santoro, 1988). These assumptions and criteria encompass most of the issues and uncertainties associated with $\Delta G°_{N-U}$ interpretation and measurement. The degree to which the assumptions are valid and criteria are met is essential in establishing a foundation on which unfolding free energy measurements can be evaluated and interpreted.

The work described here addresses assumptions and criteria associated with denaturant-induced $\Delta G°_{N-U}$ determined for ribonuclease A unfolding over an extensive range of stability and pH. Since the linear extrapolation model (LEM) is the most commonly used method for obtaining unfolding free energy changes, we have chosen to use it to obtain $\Delta G°_{N-U}$ at fixed pH values in the acidic to basic pH range. These free energy changes at different fixed pH values (x and y) are represented as the vertical equilibria in the thermodynamic cycle of Scheme 1 while the horizontal equilibria represent titration free energy changes associated with the native and unfolded states of the protein. The cycle sets the constraint that free energy changes of all four branches of the cycle, determined independently, must sum to zero if the quantities are valid free energy changes. This constraint results in eq 1a, from which it is shown that the

$$\Delta G°_{\text{titr U}} - \Delta G°_{\text{titr N}} = \Delta G°_{\text{N-U,pH}_y} - \Delta G°_{\text{N-U,pH}_x} \quad \text{(1a)}$$

difference in LEM determined unfolding free energy changes at pH_y and pH_x (right side of eq 1a) is equal to the difference in titration free energy changes of the native and denatured states (left side of eq 1a) between the two pH values. Since the titration free energies deal only with the proton binding polynomials of the two end states, the cycle shows that the unfolding free energy change at one pH (e.g., $\Delta G°_{N-U,pH_y}$) is related to the unfolding free energy change at another pH (e.g., $\Delta G°_{N-U,pH_x}$) only if the unfolding free energy changes represent the free energy difference between *two states*, native and unfolded protein. Though the thermodynamic cycle requires the free energies to be expressed in terms of only two states, it should be noted that titration free energy changes (left side of the equation) are not dependent on the shape of the denaturant-induced transition and therefore represent a means of evaluating pH-dependent changes in the unfolding free energy which is totallyindependent of the assumptions and approximations associated with the LEM model used in evaluating the right side of eq 1a. Except for the requirement of the two-state condition, the independence of the measurements for the left and right sides of eq 1a makes the cycle an important means of testing assumptions, conditions, and validity of $\Delta G°_{N-U}$ measurements which differ in pH. In this paper, we present results obtained by use of the linear extrapolation model as the means for evaluating $\Delta G°_{N-U}$. Work will be presented at a later date on application of the denaturant binding and transfer models to such data.

MATERIALS AND METHODS

Ribonuclease A from Sigma Chemical Co. was further purified by the method of Taborsky (Taborsky, 1959), and protein concentration was determined spectrophotometrically at 278 nm using a molar absorptivity of 9800 cm^{-1} M^{-1} (Tanford & Hauenstein, 1956). Ultrapure urea was purchased from Schwartz Mann Biotech ICN Co. Prior to its use, a solution of 10 M urea was further purified by treatment with a mixed-bed ion-exchange resin (AG501-X8) from Bio-Rad Laboratories for at least 1 h to get rid of ions formed from the decomposition of urea (Hagel et al., 1971). Solutions prepared from this purified urea were then filtered through 0.22 μm GV filter paper (Millipore Corp.). Ultrapure guanidine hydrochloride (GdnHCl) from Amresco was used without further purification, and concentrations of urea and GdnHCl were determined by refractometry, using data reported by Nozaki and Pace (Nozaki, 1972; Pace, 1986).

Buffers include the following: β-alanine, PIPES disodium salt, Bis-Tris, and HEPES sodium salt, all from Sigma Chemical Co.; glycine from Matheson, Coleman & Bell Mfg.; trisodium citrate from Fisher Scientific Co.; and taurine from Fluka Chemie AG.

Unfolding measurements were performed at 287 nm using matched 1 cm quartz cuvettes with screw caps. Mixing was brought about by a 2 min rapid back and forth rotation of the cuvette with the cuvette restrained in the vertical position. Experiments were performed using a Varian 2200 UV−Vis spectrophotometer thermostated at 25.0 ± 0.1 °C with a Perkin Elmer temperature controller. Unfolding data at pH 3 were also taken using circular dichroism at 222 nm to monitor the transition.

Unfolding data were analyzed by nonlinear least-squares fitting of the primary data to the linear extrapolation model

following the method of Santoro and Bolen (1988). The fittings were performed using the software Nonlin for Mac (Robelko Software, Murphysboro, IL).

Potentiometric titrations were carried out using a pHM85 Precision pH meter and calomel electrode, both made by Radiometer, along with a Metrohm E415 pH-stat titration vessel thermostated at 25.0 ± 0.1 °C and purged with purified nitrogen gas. The nitrogen gas was purified by passing prepurified nitrogen gas through three successive scrubbers, the first containing alkaline $BaCl_2$, the second containing 2 M phosphoric acid, and the third containing a solution identical in composition with that used in the titration vessel. The titration apparatus was standardized using certified standard buffer solutions from Analytical Products, Inc. The HCl titrant (0.015 M HCl) for native RNase A titration and $\Delta\nu$ experiments were prepared in 0.2 M NaCl while HCl titrant (0.015 M HCl) for unfolded RNase A was prepared in the presence of 6 M GdnHCl containing 0.2 M NaCl. Concentrations of the HCl titrants were determined from Gran plots using trizma base as a primary standard (Rossotti & Rossotti, 1965).

RNase A (about 10 mg/mL) used in $\Delta\nu$ experiments was first dialyzed at 4 °C overnight against 0.2 M NaCl at pH 3.5. A solution of 6.67 M GdnHCl in 0.2 M NaCl, or 8 M urea in 0.2 M KCl or NaCl, was adjusted to slightly above pH 3.5, and the dialyzed protein solution along with the 0.2 M NaCl solution it was dialyzed against were both adjusted to exactly pH 3.500. $\Delta\nu$ measurements were performed by first mixing a volume of 6.67 M GdnHCl solution with a sufficient volume of the pH 3.500, 0.2 M NaCl solution in the titration vessel to give a final volume of 5.00 mL. The resulting control solution was back-titrated to pH 3.500 and the volume of titrant used recorded. The titration vessel was cleaned and dried, and the 6.67 M GdnHCl stock was mixed with pH 3.500 RNase stock solution in the same volumes and proportions as the control experiment. The volume of titrant needed to return the mixed solution containing RNase A and GdnHCl to pH 3.500 was recorded, and the difference in moles of HCl between this solution and the control was divided by the total moles of RNase present in the mixture to evaluate $\Delta\nu$. In order to determine $\Delta\nu$ at the different final GdnHCl concentrations reported, the ratio of RNase A solution to GdnHCl solution was varied while keeping the total mixed volume at 5.00 mL.

Titrations of native RNase A in 0.2 M NaCl and of 6 M GdnHCl unfolded RNase A in 0.2 M NaCl were performed in a manner very similar to that described by Huang and Bolen (1993). RNase A at pH 3.0 in 0.2 M NaCl was dialyzed at 4 °C overnight against 0.2 M NaCl, pH 3.0, and the protein concentration was determined at 278 nm. Immediately prior to titration, a 0.2 M NaCl solution was prepared in a 50 mL volumetric flask in freshly degassed water, and titration was performed by mixing 4.5 mL of the freshly prepared 0.2 M NaCl solution with 0.5 mL of dialyzed RNase A in the titration vessel. For titration of unfolded RNase A, 0.5 mL of dialyzed RNase A was mixed with 4.5 mL of freshly prepared and degassed 6.67 M GdnHCl containing 0.2 M NaCl.

The titration was carried out immediately after adjusting the pH to around pH 8.5 using microliter amounts of 0.1 M (CO_2-free) NaOH (Aldrich Chemical Co.). The RNase A sample solution was continuously titrated from pH 8.5 to about pH 2.8 with 15 s intervals between each aliquot (10 μL) of HCl titrant, and the pH and volumes added were recorded. After reaching pH 2.8, the pH of the sample solution was again jumped to 8.5 using 0.1 M CO_2-free NaOH, and the sample was subjected to retitration from pH 8.5 to about 2.8 using the same procedure.

Several control experiments were performed by titrating 4.5 mL of the 0.2 M NaCl solution mixed with 0.5 mL of the 0.2 M NaCl solution used in the dialysis. Ratios were determined of the volume of HCl titrant used in titrating the blank solution from pH 8.50 to any particular pH within the range of interest, divided by the total volume of the blank solution at that pH. These ratios for each control were plotted as a function of the corresponding pH and the resulting data fitted to an eight-degree polynomial. This equation was used in the calculation of the degree of protonation of the protein.

The number of moles of H^+ bound per mole of protein was determined at each pH by subtracting the moles of titrant needed to titrate the protein to the particular pH, minus the moles of titrant used to achieve that pH in the blank, and this difference was divided by the total moles of protein present.

RESULTS

Previous studies have shown RNase A unfolding to be reversible and two-state in character (Salahuddin & Tanford, 1970). Our experience with both urea- and GdnHCl-induced unfolding of this protein under the experimental conditions reported here also finds that the unfolding of RNase A is reversible and two-state in character as judged by identical values of $\Delta G°_{N-U}$, m, and $C_{1/2}$ parameters regardless of which observable (difference extinction coefficient or ellipticity) is used to monitor unfolding (ellipticity data not shown).

Urea and guanidine hydrochloride (GdnHCl) were both used in determining $\Delta G°_{N-U}$ for unfolding of RNase A at pH 3.0. Figure 1 presents unfolding data for both denaturants monitored by extinction coefficient differences between native and unfolded forms of the protein. The solid lines represent the nonlinear least-squares best fits of the data to the linear extrapolation model giving $\Delta G°_{N-U}$ values of 5.07 ± 0.10 and 4.00 ± 0.19 kcal/mol for GdnHCl and urea, respectively. These free energy quantities are statistically different from one another, demonstrating a dependence of $\Delta G°_{N-U}$ on the nature of the denaturant and raising the disconcerting possibility that $\Delta G°_{N-U}$ is a quantity which is not a property of the protein alone. One possible source of the different values for urea and GdnHCl unfolding could be from ionic strength or salt effects on the native and/or unfolded species which accompany GdnHCl unfolding. To investigate that possibility, urea unfolding was performed as a function of NaCl concentration, and the results of those studies are presented in Figure 2. Clearly, there is a marked dependence of the unfolding transition on salt concentration, with all three fitting parameters ($\Delta G°_{N-U}$, m, $C_{1/2}$) changing as a function of salt (see Table 1). A plot of $\Delta G°_{N-U}$ vs [NaCl] is hyperbolic and appears to reach a maximum value of about 5.5 kcal/mol near 1 M NaCl. This unfolding free energy change in the limit of high NaCl concentration also does not agree with the $\Delta G°_{N-U}$ obtained from GdnHCl-induced unfolding. The lack of agreement between $\Delta G°_{N-U}$ values for urea (in high [NaCl]) and GdnHCl unfolding presents a serious problem in interpretation of this quantity. Due to the fact that the effects of neutral salts such as GdnHCl and NaCl are significant and present an additional

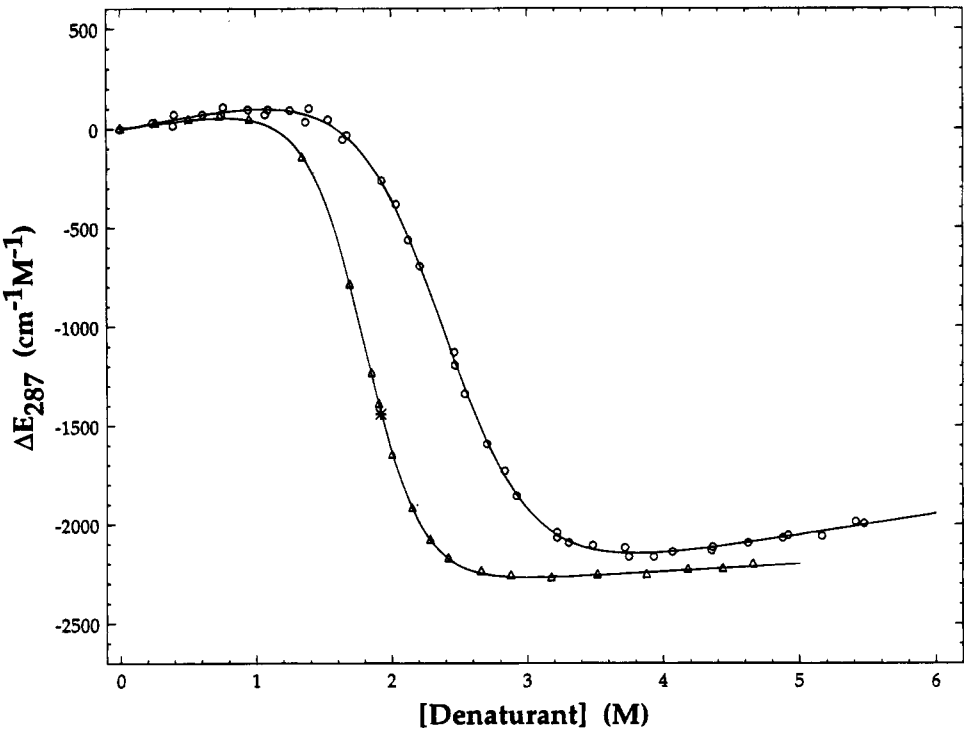


FIGURE 1: GdnHCl (△) and urea (○) induced unfolding of RNase A at pH 3.00 and 25 °C was monitored by changes in the molar extinction coefficient (ΔE_{287}). A reversible point is shown for GdnHCL-induced unfolding (*). The solid lines are nonlinear least-squares best fits of the data using the linear extrapolation method. Unfolding free energy parameters evaluated from the fittings for GdnHCl and urea, respectively, include 5.07 ± 0.10 and 4.00 ± 0.19 kcal/mol for $\Delta G°_{N-U}$ and 2.77 ± 0.07 and 1.84 ± 0.08 for m. The solutions were buffered by 0.1 M β-alanine.

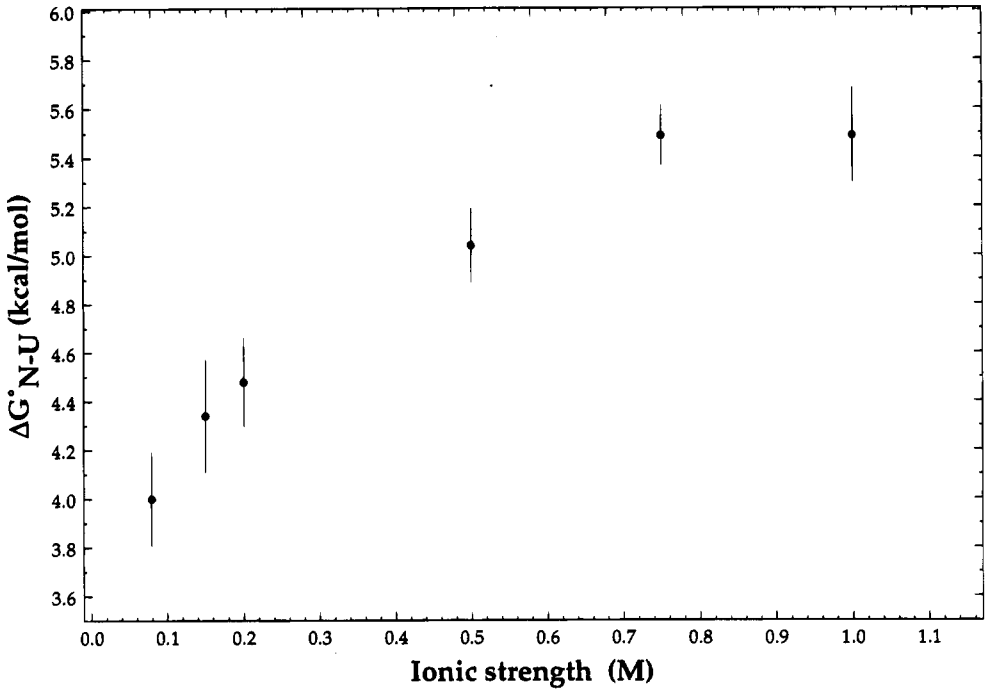


FIGURE 2: Salt and/or ionic strength effect on the unfolding free energy change ($\Delta G°_{N-U}$). Urea-induced unfoldings of RNase A were performed at pH 3.00, 25 °C, in 0.1 M β-alanine buffer with sufficient NaCl to give the ionic strengths indicated (see Table I for actual concentrations). Error bars shown are symmetrical ± values estimated from the nonsymmetrical 67% confidence intervals obtained from the NonLin program used.

parameter to control, all further unfolding experiments were performed using urea as the denaturant at a fixed NaCl concentration of 0.2 M.

Figure 3 presents urea-induced unfolding profiles for RNase A at fixed pH values over a range from pH 3 to 8.5, and the corresponding fitted thermodynamic parameters are provided in Table 2. Over this pH range, the stability of the protein increases by approximately 5 kcal/mol, showing that electrostatic effects over this pH range contribute a substantial fraction of the overall stability of the protein.

The objective of this paper is to determine whether or to what extent the free energy change evaluated by use of the linear extrapolation method ($\Delta G°_{N-U}$) can be used to predict the $\Delta G°_{N-U}$ at any other pH in accordance with the

Table 1: Effect of NaCl on RNase A Unfolding at pH 3.0[a]

[NaCl] (M)	ionic strength	$\Delta G°_{N-U}$ (kcal/mol)	$C_{1/2}$ (M)	m
0.0	0.079	4.00 ± 0.19	2.185 ± 0.14	1.84 ± 0.08
0.071	0.15	4.34 ± 0.23	2.38 ± 0.17	1.82 ± 0.09
0.121	0.2	4.48 ± 0.21	2.42 ± 0.15	1.85 ± 0.08
0.421	0.5	5.04 ± 0.15	3.02 ± 0.13	1.67 ± 0.05
0.671	0.75	5.49 ± 0.12	3.33 ± 0.11	1.65 ± 0.04
0.921	1.00	5.49 ± 0.19	3.59 ± 0.17	1.53 ± 0.05

[a] The 67% confidence intervals obtained from nonlinear least-squares fitting of the data described under Materials and Methods were nonsymmetrical. However, since they were not too far from being symmetrical, we have chosen to display and report them as symmetrical for the sake of space. Experiments were carried out in 0.1 M β-alanine buffer at pH 3.00 and 25 °C.

thermodynamic cycle of Scheme 1. With an arbitrary choice of RNase A unfolding at pH 3.5 as the reference pH ($\Delta G°_{N-U,pH_x}$), one should be able to predict $\Delta G°_{N-U}$ at other pH values ($\Delta G°_{N-U,pH_y}$) by determining the free energy of titration for the native ($\Delta G°_{titr\ N}$) and unfolded forms ($\Delta G°_{titr\ U}$) from pH 3.5 to the pH of interest (see eq 1b). In order to

$$\Delta G°_{N-U,pH_y} = \Delta G°_{N-U,pH_x} + (\Delta G°_{titr\ U} - \Delta G°_{titr\ N}) \quad (1b)$$

evaluate the titration free energy changes, the relative protonation of native and unfolded forms of RNase A at some fixed pH needs to be determined so that the native and unfolded titration curves can be scaled relative to one another. The difference in the degree of protonation of the native and unfolded forms of the protein at a fixed pH can be evaluated by direct measurement of the proton uptake or release ($\Delta\nu$) which accompanies denaturant-induced unfolding at the fixed pH. This experiment was performed as described under Materials and Methods and consists of mixing unbuffered protein solution (in 0.2 M NaCl at pH 3.500) with unbuffered GdnHCl (also in 0.2 M NaCl and at pH 3.500) and then back-titrating with HCl to pH 3.500. The equivalents of HCl used in this back-titration are subtracted from the equivalents of HCl used in a separate control experiment, and the result is divided by the number of moles of protein used to give $\Delta\nu_{pH\ 3.500}$, the proton uptake at the fixed pH of 3.500 accompanying the mixing of denaturant with protein. The control experiment consists of the identical mixing and titration protocol described above but without protein being in the solution. A plot of $\Delta\nu$ as a function of GdnHCl concentration in the mixture is given in Figure 4.

Substantive proton changes take place over two GdnHCl concentration ranges, namely, proton uptake occurs in the predenaturational range of about 1.3 M and below, and again in the transition range from 1.3 to 3 M GdnHCl. Urea as a denaturant could not be used in the $\Delta\nu$ determinations since the electrode response became increasingly sluggish with increased urea concentration. In particular, the electrode became highly unresponsive to the addition of hydrogen ion at urea concentrations greater than about 3−4 M. A limited amount of $\Delta\nu$ data are shown in Figure 4 at urea concentrations in which the electrode response was still reasonable. It is seen from these limited data that urea in the presence of 0.2 M NaCl has little effect on the proton binding behavior of native RNase A, in contrast to GdnHCl. Given that salt alone is known to alter the $\Delta\nu$ of native RNase A (Tanford & Hauenstein, 1956), these data might mean that GdnHCl is perturbing the proton binding behavior of native RNase A by means of an ionic strength effect on the highly positively charged protein. At pH 3.50, the overall change in $\Delta\nu$ between native protein with 0.2 M NaCl or KCl in the presence of urea, and unfolded protein (in 6 M GdnHCl containing 0.2 M NaCl), was found to be 2.24 ± 0.06.

With $\Delta\nu_{pH\ 3.500}$ determined, we are left with the problem of determining the free energy change for titration of the native and unfolded RNase A species over a defined pH range. The primary data for determining titration unfolding free energy changes for native and unfolded protein are the titration plots. A potentiometric titration curve for RNase A in the presence of 0.2 M NaCl in 6 M GdnHCl over the pH range from 2.8 to 8.6 is shown in Figure 5 along with titration data by Nozaki and Tanford over a more extensive pH range (Nozaki & Tanford, 1967c). The RNase A concentration used by Nozaki and Tanford (5−10 mg/mL) was significantly greater than ours (1 mg/mL), and the excellent agreement between the titration curves indicates no dependence of titration on protein concentration over this range (Nozaki & Tanford, 1967b). Figure 6 gives potentiometric titration data for RNase A in 0.2 M NaCl along with a replot of the titration of unfolded RNase A from Figure 5. The two titration curves have been scaled so that they are separated at pH 3.5 by 2.24 mol of proton per mole of protein as determined from $\Delta\nu$ measurements at pH 3.500. For convenience, the titration curves are presented with the ν value for unfolded protein at pH 8.5 arbitrarily set at zero. From this plot, it is possible to evaluate the pH dependence of the free energy change for RNase A unfolding from pH 3 to 8.5.

The titration free energy changes for native ($\Delta G°_{titr\ N}$) and unfolded ($\Delta G°_{titr\ U}$) RNase A can be obtained over the range from pH 3 to higher pH values by numerical integration of plots of ν versus pH for the two protein species (Huang & Bolen, 1993; Bolen & Santoro, 1988). Since, from eq 1b, we are most interested in the difference in the titration free energy changes of native and unfolded RNase A ($\Delta G°_{titr\ U} - \Delta G°_{titr\ N}$), this quantity can be obtained from the area between the two titration curves ($\Delta\nu$ vs pH) as given in Figure 6 and illustrated in eq 2. Here ν_N and ν_U represent

$$\Delta G°_{titr\ U} - \Delta G°_{titr\ N} = 2.303RTf(\nu_U - \nu_N)\ \mathrm{d\ pH} \quad (2)$$

the degree of protonation for native and unfolded protein, respectively, as a function of pH. Integration between the limits of the reference pH (pH 3.50) and any other pH will give the pH dependence of the unfolding free energy change over that pH range. Figure 7 provides a plot of the pH dependence of protein unfolding (solid curve) obtained from integration of the area between the titration curves in Figure 6 and using the unfolding free energy change at pH 3.5 as reference. Also plotted are the unfolding free energy changes determined independently at a variety of pH values from the data in Figure 3. The unfolding free energy changes predicted from use of the thermodynamic cycle (Scheme 1 and eq 1b) are in very good agreement with the unfolding free energy changes determined independently at various pH values using the linear extrapolation method.

DISCUSSION

The linear extrapolation method has long been used as a preferred method for evaluating denaturant-induced unfolding free energy changes of proteins (Greene & Pace, 1974; Pace, 1975, 1986). Its use, however has been a source of concern (Pace, 1975, 1986) since (1) the extrapolation is frequently

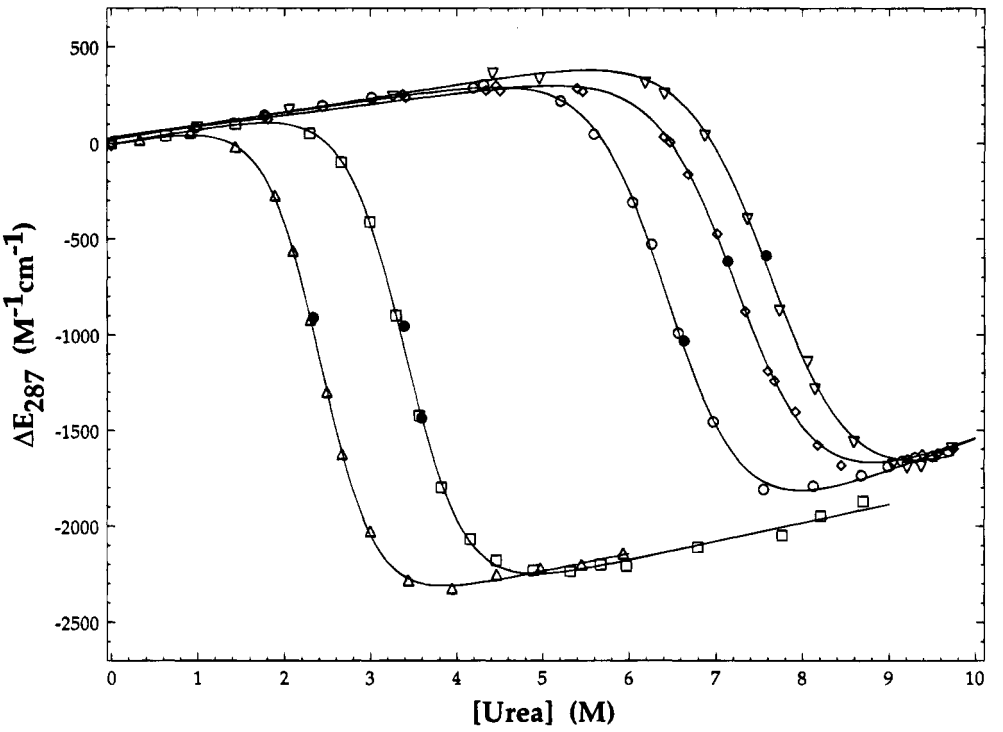


FIGURE 3: Urea-induced unfolding free energy changes as a function of pH. All unfolding experiments were conducted in the buffer concentrations indicated below with sufficient NaCl added to bring the ionic strength to 0.2 M. The buffers and pHs investigated include the following: 0.1 M β-alanine, pH 3.00 (△); 0.1 M β-alanine, pH 3.5 (□); 0.1 M Bis-Tris, pH 6.0 (○); 0.1 M HEPES, pH 7.0 (◇); and 0.1 M taurine, pH 8.5 (▽). A common slope and intercept for the unfolded base line was assumed in the fitting of the data at pH 6.0 and 7.0. The slope of the unfolded base line for data at pH 6.0 and 7.0 was also used in fitting the data at pH 8.5. Filled symbols indicate reversibility points obtained by fully unfolding RNase A at the pH indicated and then diluting the denaturant solution into the transition zone and monitoring ΔE_{287}.

Table 2: pH-Dependent Thermodynamic Parameters for RNase A Unfolding[a]

	$\Delta G°_{N-U}$ (kcal/mol)			
pH	experimental	predicted	$C_{1/2}$ (M)	m
3.0	4.48 ± 0.21	4.48 ± 0.01	2.42	1.85 ± 0.08
3.5	5.78 ± 0.37	5.77 ± 0.02	3.40	1.70 ± 0.10
6.0	8.82 ± 0.52	8.68 ± 0.09	6.49	1.36 ± 0.08
7.0	9.26 ± 0.31	9.38 ± 0.12	7.26	1.28 ± 0.05
8.5	9.30 ± 0.66	9.43 ± 0.16	7.69	1.21 ± 0.08

[a] The 67% confidence intervals obtained from nonlinear least-squares fitting of the data described under Materials and Methods were nonsymmetrical. However, since they were not too far from being symmetrical, we have chosen to display and report them as symmetrical for the sake of space. Buffers and conditions are given in the legend of Figure 3.

long and there are indications that the unfolding ΔG may not be a linear function of denaturant (Pace & Vanderburg, 1979; Santoro & Bolen, 1992), (2) there are instances in which denaturants do not give the same value of $\Delta G°_{N-U}$ from extrapolation (DeKoster et al., 1993; Pace et al., 1990; Ropson et al., 1990; Swint & Robertson, 1993), and (3) there has been no rigorous demonstration that the $\Delta G°_{N-U}$ obtained from linear extrapolation has the properties of an authentic thermodynamic quantity (Bolen & Santoro, 1988; Santoro & Bolen, 1988). The thermodynamic cycle illustrated by Scheme 1 provides a means to test some of the fundamental assumptions and questions concerning the linear extrapolation method, including whether the quantity obtained by linear extrapolation and referred to as $\Delta G°_{N-U}$ has the properties of a thermodynamic function of state. Furthermore, since prediction of the unfolding free energy change at some pH other than the reference pH involves the properties of only the two end states (N and U), the scheme also places demands on the extent to which two-state character must be exhibited over the range of pH and protein stability investigated.

Figure 7 provides strong evidence that the pH dependence of $\Delta G°_{N-U}$ obtained by means of linear extrapolation agrees with the pH dependence obtained independently from potentiometric titration of native and unfolded RNase A. The good agreement demonstrates that $\Delta G°_{N-U}$ obtained at one pH can be incorporated into Scheme 1 and $\Delta G°_{N-U}$ at any other pH can be successfully predicted. Thus, the quantity obtained from linear extrapolation ($\Delta G°_{N-U}$) is shown to be a state function with respect to Scheme 1 (i.e., independent of pathway), exhibiting the properties of additivity expected of an authentic free energy change. In evaluating the rigor with which these experiments demonstrate that $\Delta G°_{N-U}$ is a thermodynamic function of state, it is important to note that agreement between the independently determined unfolding free energy changes involves a broad range of protein stability changes (3400-fold in equilibrium constant) covering a wide pH range (from pH 3 to pH 8.5). In addition, the denaturant concentrations at the midpoint of the transitions ($C_{1/2}$) over this pH range extend from 2.4 to 7.7 M urea (Table 2). This large range in $C_{1/2}$ values covers lengths of linear extrapolation ranging from moderately short to very long. Clearly, the length of linear extrapolations does not affect the ability to obtain $\Delta G°_{N-U}$ values which are in good agreement with unfolding free energy changes predicted by use of Scheme 1. This is strong evidence that RNase unfolding free energy can be expressed legitimately as a linear function of urea concentration, with transitions which begin with as little as 1 M urea (beginning of the transition at pH 3 in Figure 3) and extend to transitions which near completion at 9 M urea (see pH 8.5 transition, Figure 3).

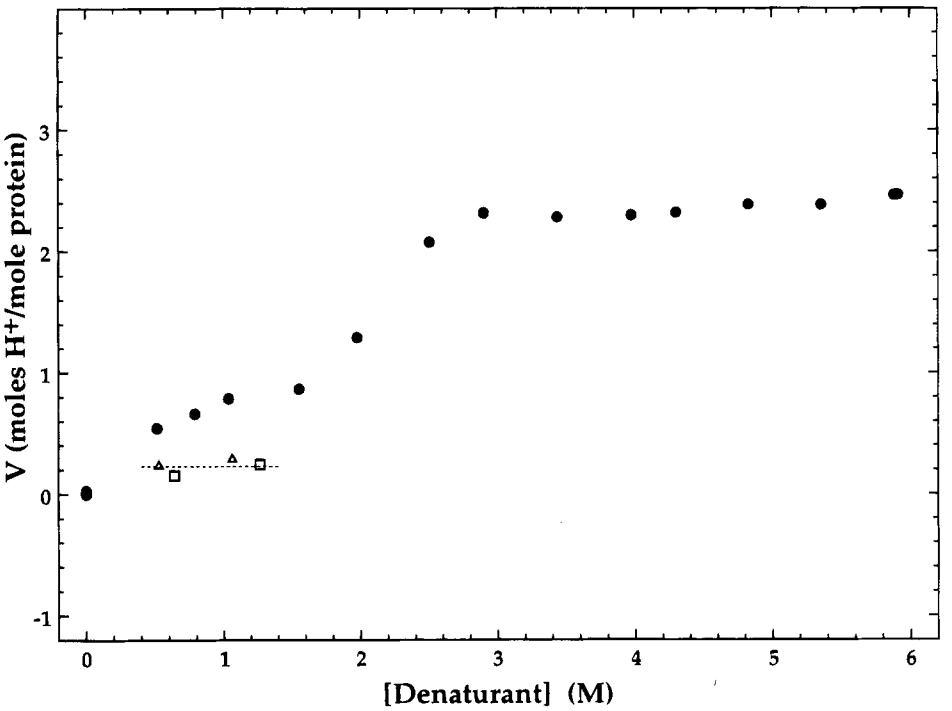


FIGURE 4: Determination of hydrogen ion uptake at pH 3.500 by RNase A as a function of [GdnHCl]. Data shown: GdnHCl at the concentrations indicated (●), 0.2 M NaCl in the presence of the urea concentrations indicated (△); 0.2 M KCl in the presence of the urea concentrations indicated (□). A $\Delta\nu$ value of 2.24 mol of H^+/mol of RNase A was determined from the difference between the average number of proton uptake in the presence of the predenaturational concentrations of urea shown (0.23, see dashed line) and the proton uptake in the presence of 6 M GdnHCl (2.47).

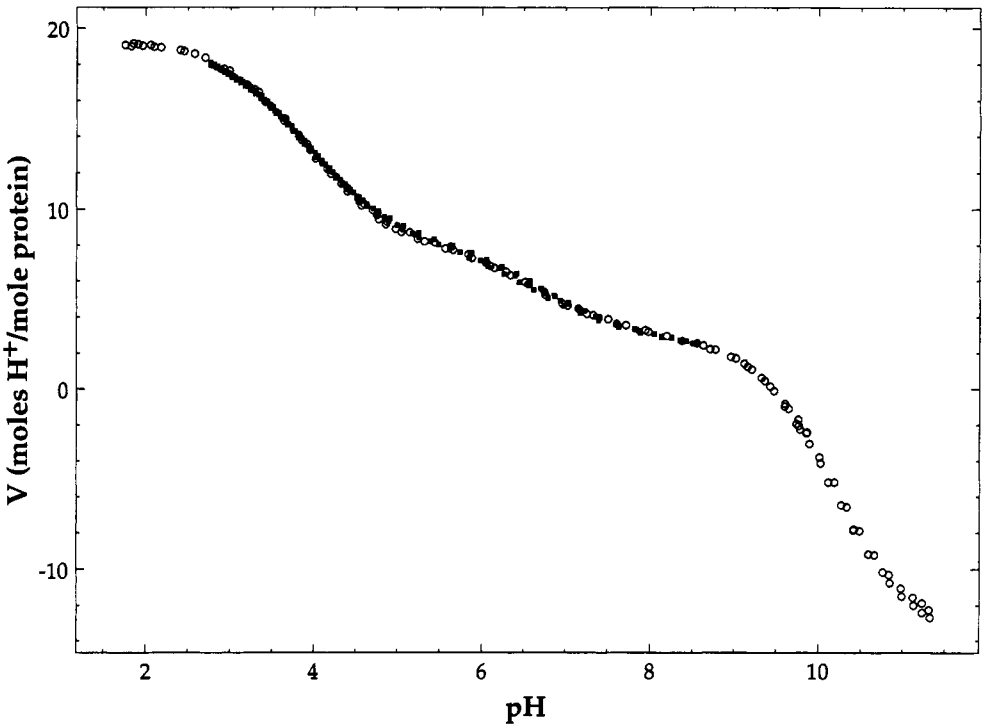


FIGURE 5: pH titration curve of RNase A in 6 M GdnHCl at 25 °C. The concentration of RNase A used in the titration was ca. 1 mg/mL, and the solution contained 0.2 M NaCl in 6 M GdnHCl. Open circles are digitized data taken from Figure 1 of Nozaki and Tanford (1967) involving titration of RNase A in 6 M GdnHCl.

One of the major difficulties with the linear extrapolation method is that it is largely empirical and, as such, the method requires authentication of both the assumptions and the putative thermodynamic quantities which are derived from it. The agreement between the two methods (unfolding measurements and potentiometric titrations) of determining unfolding free energy changes over such a broad range of conditions demonstrates that the linear extrapolation model using urea as a denaturant is an appropriate thermodynamic framework for describing free energy changes for RNase A. This, however, may not be true for all proteins.

Scheme 1 is based on the proposition that the proton binding polynomials of only the two end states (N and U) are important in predicting the pH dependence of unfolding. That is, two-state behavior is a requirement of Scheme 1, and how well RNase A unfolding is described by this

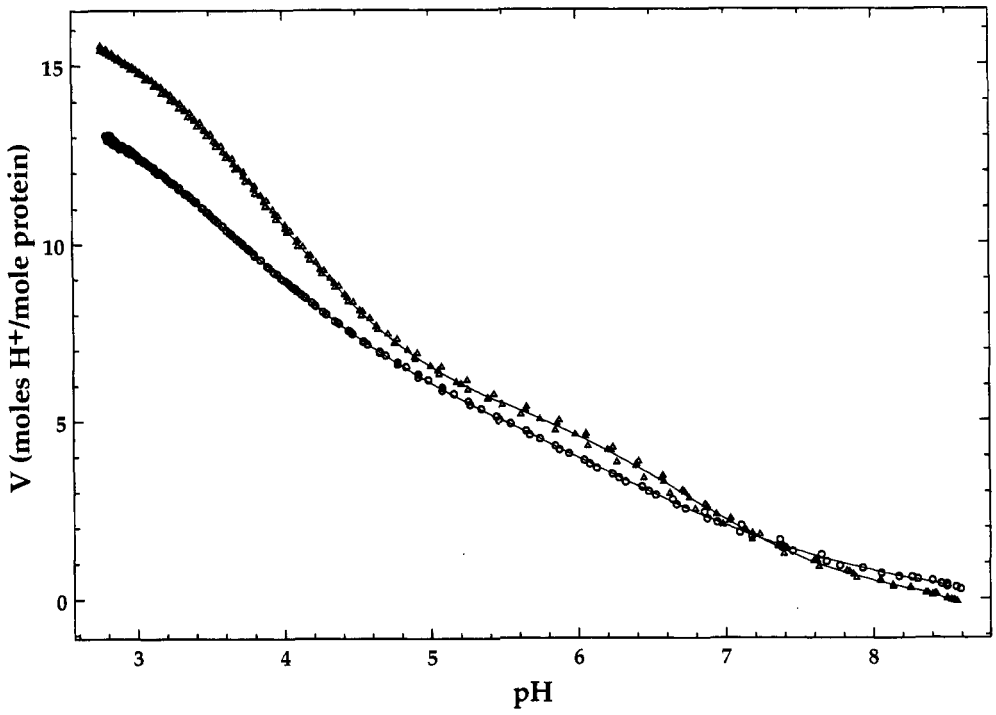


FIGURE 6: Reversible titration data for native (○) at 1.10 mg/mL and unfolded (△) RNase A at 1.12 mg/mL between pH 2.8 and 8.5. The unfolded RNase A data are an expanded replot of data reported in Figure 5 in 0.2 M NaCl plus 6 M GdnHCl. The unfolded titration curve was arbitrarily set to have a zero value of ν at pH 8.5. The titration curve for native RNase A is placed on the plot relative to the unfolded titration curve such that its ν value was 2.24 mol of H^+/mol of protein lower than that of unfolded RNase A at pH 3.50.

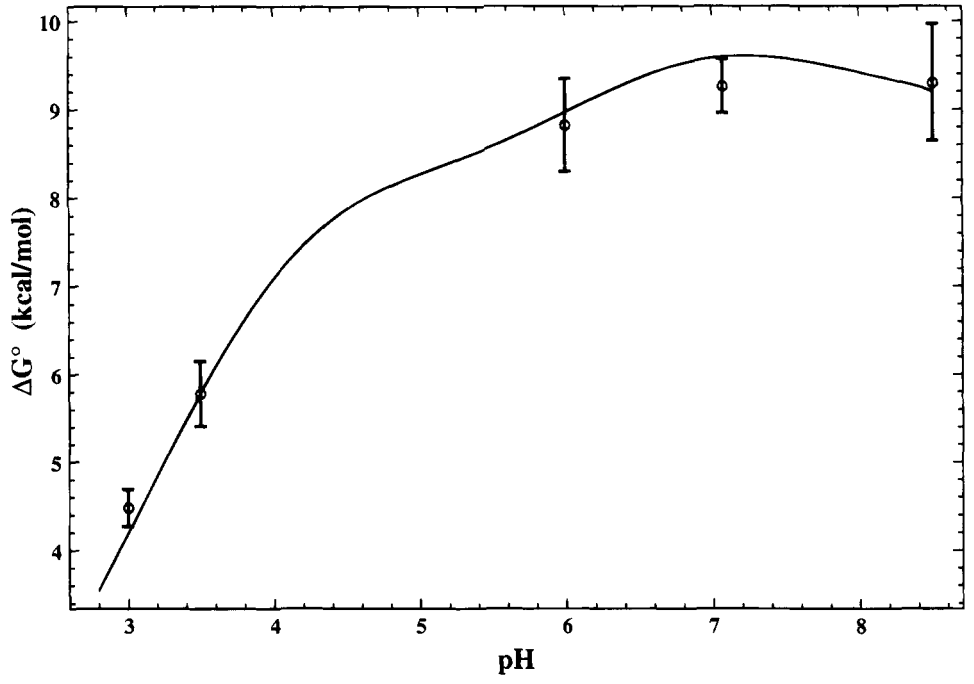


FIGURE 7: Unfolding free energy changes of RNase A as a function of pH. The solid line is the pH−stability profile obtained by use of Scheme 1, and its construction follows from eq 1b. With a $\Delta G°_{N-U}$ value of 5.78 kcal/mol at the reference pH of 3.50 as the starting point, the pH dependence of protein stability is obtained by integrating the area between the two curves in Figure 6 from the reference pH of 3.50 to other pH values in the range from pH 2.8 to 8.5. The area between the two titration curves represents the difference in free energy between native and unfolded RNase A ($\Delta G°_{titr\ U} - \Delta G°_{titr\ N}$). This combination of $\Delta G°_{N-U,pH_i} + (\Delta G°_{titr\ U} - \Delta G°_{titr\ N})$ is presented as the solid line in the plot. The open circle points and their associated errors are the unfolding free energy changes measured by means of the LEM (see Table 2).

approximation determines how good the agreement will be between the linear extrapolation method and application of Scheme 1. The accurate prediction of two-state unfolding free energy changes spanning a 3400-fold range in protein stability over a large pH range is offered as strong support for the two-state assumption. The presence of intermediate species at any pH which might affect linear extrapolation would result in disagreement between $\Delta G°_{N-U}$ derived from the linear extrapolation model and the unfolding free energy changes predicted from titration of native and unfolded RNase A.

Effect of Salt and/or Ionic Strength on $\Delta G°_{N-U}$. We have shown in Figure 1 that urea-induced unfolding in 0.2 M NaCl and GdnHCl-induced unfolding give different values of

$\Delta G°_{N-U}$. In principle, comparisons of $\Delta G°_{N-U}$ values for urea- and GdnHCl-induced protein unfolding should seldom agree if unfolding free energy changes depend on salt concentration. Figure 2 shows that salt affects $\Delta G°_{N-U}$ significantly in the case of urea-induced RNase A unfolding, but Thompson and Bigelow have shown that several neutral (nondenaturing) salts do not affect $\Delta G°_{N-U}$ derived from GdnHCl-induced unfolding of RNase A (Thomson & Bigelow, 1986). These observations may be understood if neutral salts like NaCl act by interacting with the highly positively charged native state of the protein to stabilize it by a general ionic strength effect in urea-induced unfolding, while in GdnHCl-induced unfolding the ionic strength in the predenaturation range of GdnHCl-induced unfolding of RNase A is already so high that addition of neutral salts contributes little or nothing to the effect. Whether this is the correct explanation for these observations is not clear without additional data. Mayr and Schmid have also suggested that sodium chloride in concentrations at 1 M and below, at pH 5, stabilizes RNase A against thermal unfolding, and the work of Matthews and Richards indicates that anion binding contributes to the stabilization of RNase A in the acid pH range (Matthew & Richards, 1982; Mayr & Schmid, 1993).

A question of interest is whether salt affects the native state, the unfolded state, or both states. Figure 4 provides evidence that the native state of RNase A is certainly affected by predenaturational concentrations of up to around 1 M GdnHCl. In this case, added GdnHCl is perturbing pK's in native RNase A, causing a $\Delta\nu$ with a maximum proton release of around 0.8 mol of proton per mole of protein. These changes occur prior to the onset of the unfolding transition, and such perturbations of native RNase A by GdnHCl are probably related to the GdnHCl-dependent amide exchange phenomena reported by Mayo and Baldwin (1993). GdnHCl is not the only salt that affects the state of protonation of native RNase A. It is known, for example, that addition of KCl to native RNase A also causes significant $\Delta\nu$ increases (Tanford & Hauenstein, 1956). This indicates that KCl and GdnHCl both affect the proton binding polynomial of the native state of RNase A in a significant manner. Since $\Delta\nu$ is responsible for the pH dependence of the unfolding free energy change (i.e., $\Delta G°_{\text{titr U}} - \Delta G°_{\text{titr N}}$ arises from the integration of $\Delta\nu$ vs pH; see Figure 6), any salt which affects the protonation state of native RNase A necessarily affects the pH dependence of the unfolding free energy change. Thus, some or all of the increase in $\Delta G°_{N-U}$ as a function of salt concentration arises from the effect of salt on native RNase A. In a later section, we will argue that salt affects the native state of RNase A but has little or no influence on unfolded RNase A.

Use of Titration Data in 6 M GdnHCl To Predict Urea-Induced $\Delta G°_{N-U}$ Values. The use of 6 M GdnHCl in carrying out potentiometric titration of unfolded RNase A and in predicting urea unfolding $\Delta G°_{N-U}$ values is a critical feature of this study. Titrations of unfolded protein and determinations of $\Delta\nu$ in the presence of GdnHCl have a long and successful history and are relatively easy to perform (Nozaki & Tanford, 1967b). From extensive titration studies, Tanford and associates made the surprising finding that the pK's of amino acids determined in 6 M GdnHCl are within 0.1−0.2 pK unit of the pK's of amino acids titrated in the presence of 0.1−0.2 M salt solutions (Nozaki & Tanford, 1967a,c). As a result of the pK's being essentially the same in 0.2 M salt and in 6 M GdnHCl, the hydrogen ion titration curves for lysozyme and RNase A in 6 M GdnHCl were found to be completely predictable on the basis of model compound pK's (determined in ca. 0.2 M NaCl) assuming full exposure of the titratable groups, subject to no interactions of any kind (Nozaki & Tanford, 1967c; Roxby & Tanford, 1971). This shows that titration of RNase A in 6 M GdnHCl gives pH-dependent behavior like that expected for completely unfolded protein in 0.2 M salt with noninteracting titratable groups. While 6 M GdnHCl is a good solvent for titrating unfolded RNase A, 8 M urea is not. Our inability to get good electrode response in high concentrations of urea prevented its use in titrations of unfolded protein and left titration in GdnHCl as the only means of getting the $\Delta G°_{\text{titr N}}$ data called for in Scheme 1.

The agreement between predicted and determined unfolding free energy data in Figure 7 raises the question: How is it possible that titration data *in 6 M GdnHCl* can be used to predict $\Delta G°_{N-U}$ values obtained from *urea-induced* unfolding data, especially since the linear extrapolation method gives different $\Delta G°_{N-U}$ values for urea- and GdnHCl-induced unfolding? The concurrence of the unfolding free energy values in Figure 7 would be understandable, and it would be legitimate to use GdnHCl-unfolded titration data to predict $\Delta G°_{N-U}$ values from urea-induced unfolding, if salt affected the native but not the unfolded state of the protein. From Figure 4, we see that the neutral salt, GdnHCl, significantly affects the protonation state of native RNase A, but little or no change in the protonation state of unfolded RNase A is observed once unfolded protein is present. It is not possible to investigate GdnHCl effects on protonation of unfolded RNase A at GdnHCl concentrations below 2.8 M because unfolded RNase is not highly populated under such conditions. In an attempt to look at effects of GdnHCl on at least one source of unfolded RNase A at lower GdnHCl concentration, titration experiments as a function of GdnHCl concentration were performed on reduced and carboxamidated RNase A (RCAM-RNase A), a species which is reported to be completely unfolded even in the absence of GdnHCl [see evidence cited in Tanford (1968)]. In the presence of from 1.0 to 4.8 M GdnHCl over the pH range from 3 to 9, we find the titration curves of RCAM-RNase A to be identical and independent of GdnHCl concentration (Silva and Bolen, unpublished results). This shows that the proton binding polynomial for this unfolded RNase is unaffected by GdnHCl from low to high concentration. Finally, we previously mentioned that the titration curve for RNase A in 6 M GdnHCl can be calculated from the pK's of model compounds obtained in salt concentrations around 0.2 M (Nozaki & Tanford, 1967b; Roxby & Tanford, 1971). It appears then that the titration data for RNase A in 6 M GdnHCl may be used to evaluate urea-induced unfolding free energy changes in 0.2 M NaCl because 6 M GdnHCl does not perturb the pK's of fully exposed titratable groups to any greater extent than does 0.2 M NaCl.

Thermodynamic Equivalence of Unfolded RNase A in Urea and in 6 M GdnHCl. The consequence of being able to use the titration curve of 6 M GdnHCl-unfolded RNase A to predict urea-induced unfolding free energy changes means that with respect to pH the unfolded ensemble in 6 M GdnHCl is thermodynamically identical to the unfolded ensemble in urea. It is seen in Figure 3 that, depending on pH, the unfolded ensemble becomes the predominant state at urea concentrations in the range of 4−9 M. The unfolded

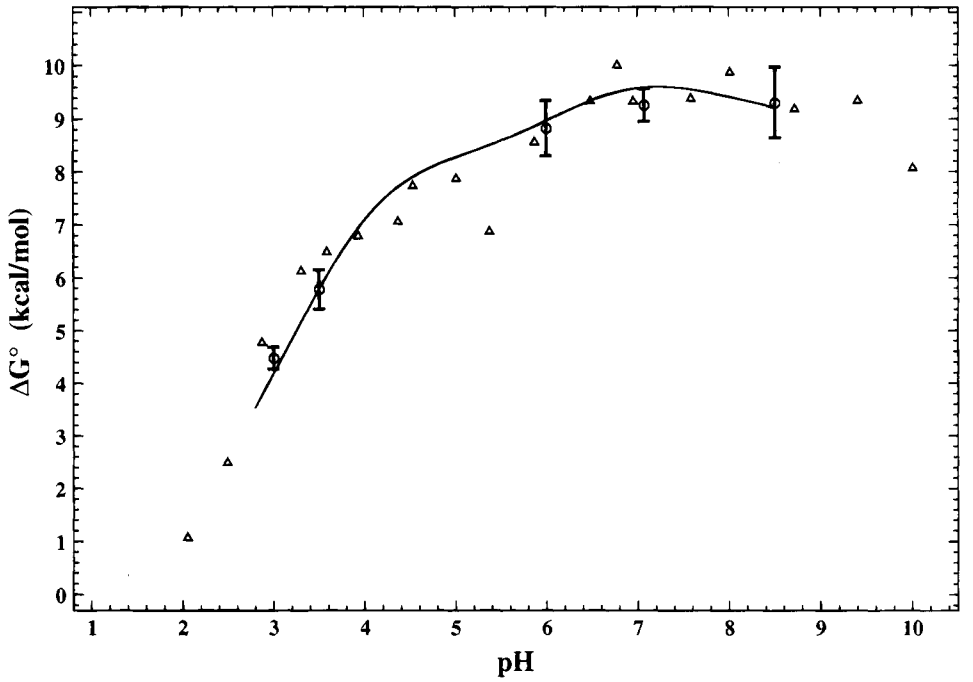


FIGURE 8: Unfolding free energy changes of RNase A as a function of pH from Figure 7 presented along with the data of Pace et al. (1990) (△) digitized from their Figure 6A.

ensembles in the various urea concentrations at the pHs indicated are fully represented by 6 M GdnHCl unfolded RNase A in terms of the state of protonation. The state of protonation of unfolded RNase A is the only property of importance in determining $\Delta G°_{titr\ U}$, and the amount of urea needed to bring about that state at a specific pH in the present set of experiments ranges from around 4 M at low pH to more than 9 M at higher pH. In principle, thermodynamic identity of the denatured states does not require the GdnHCl- and urea-unfolded ensembles to be identical in structural terms, but it does place severe constraints on how much of a structural difference would be possible without perturbing the proton binding polynomials. Privalov and Makhatadze have proposed that proteins denatured by different means may be thermodynamically identical as far as their heat capacities are concerned but could differ structurally (Privalov & Makhatadze, 1990). The extent to which different amounts of residual structure could exist in a protein denatured by different means and still be thermodynamically equivalent is likely to depend upon which thermodynamic parameter is being observed and the sensitivity of the parameter to the experimental condition imposed on the protein.

Is $\Delta G°_{N-U}$ a Property of both the Denaturant and Protein or the Protein Alone? The final characteristic desired of $\Delta G°_{N-U}$ is that it be a property of the protein alone, not of the denaturant used to induce the unfolding. We have shown that $\Delta G°_{N-U}$ values derived from urea- and GdnHCl-induced RNase A unfolding do not agree under the same conditions of pH and ionic strength, and this means that one or both of the $\Delta G°_{N-U}$ values reflect properties of both the protein and denaturant. Since urea-induced unfolding is dependent on NaCl concentration and there is no way of controlling salt concentration effects such as ionic strength in GdnHCl-induced unfolding, $\Delta G°_{N-U}$ from GdnHCl-induced RNase A unfolding is likely to contain contributions from both the denaturant and the protein through effects on the free energy of the native state. The question of whether the $\Delta G°_{N-U}$ obtained from urea-induced unfolding is only a property of the protein and not also of urea remains unanswerable from our data. The agreement of unfolding free energy changes between the two methods used here is a correlation of important consequence, but it does not adequately address the question of whether urea influences the magnitude of $\Delta G°_{N-U}$. However, the fact that we can substitute titration of unfolded RNase A in 6 M GdnHCl in place of titration of unfolded RNase A in urea and get excellent agreement between the urea-induced $\Delta G°_{N-U}$ and unfolding free energy changes from titration methods implies that the proton binding polynomial of the unfolded state is independent of which denaturant is used. Thus, if urea-induced $\Delta G°_{N-U}$ values are dependent on both urea and protein, the dependence will enter as an effect of urea on the free energy of the native state of the protein.

Comparisons of RNase A Unfolding Free Energy Changes with Other Reports. Figure 8 gives a comparison of the data of Pace et al. with the unfolding free energy data we report in Figure 7 (Pace et al., 1990). Overall, the agreement appears to be good. There is, however, one difference in quantitative aspects of the two studies. Unlike Pace et al., we find a quantitative difference between $\Delta G°_{N-U}$ values determined from urea- and GdnHCl-induced unfolding of RNase A at pH 3 (Pace et al., 1990). Urea-induced unfolding of RNase A is salt-dependent, and agreement between the $\Delta G°_{N-U}$ values for GdnHCl- and urea-induced unfolding is reached only when urea unfolding is conducted in the presence of about 0.5 M NaCl. We have no explanation for these differences between our results and those of Pace.

A pH Profile of Protein Unfolding without Need of Unfolding Transitions. The titration methods for native and 6 M GdnHCl-denatured protein coupled with $\Delta\nu$ measurements at a fixed pH provide sufficient data for determining the pH profile for two-state (native and 6 M GdnHCl-denatured) protein unfolding over the pH range of the titration measurements. The advantage of this method is that the pH dependence for two-state protein unfolding is model-

independent; i.e., it can be obtained without need for linear extrapolation, denaturant binding, and transfer models and all of their foibles. There are certain advantages to studying the pH-dependent part of protein unfolding on its own. Electrostatic effects account for some 5−10 kcal out of the estimated of 5−15 kcal/mol in total stabilities of globular proteins (Hu et al., 1992; Pace, 1975; Pace et al., 1990, 1992; Privalov, 1979). When it is considered that protein function is extremely dependent on pH, it is evident that proton linkage effects play primary roles in both the structure and function of proteins. The advantage of the titration−$\Delta\nu$ method is that it focuses on the pH-dependent free energy contribution to protein unfolding separate from the pH-independent contribution.

Summary. In order for application of Scheme 1 to successfully predict urea-induced RNase A unfolding free energy changes over such an extended range of pH, urea, and protein stability, a large number of assumptions and conditions have to either hold or compensate one another for their failures. These assumptions include the two-state assumption, the thermodynamic equivalence of 6 M GdnHCl-unfolded RNase A in comparison with urea-unfolded RNase A, and the legitimacy of the linear extrapolation method in terms of the scope, rigor, and validity of the thermodynamic parameter ($\Delta G°_{N-U}$) derived from use of this method. The good agreement between the unfolding free energy changes determined from titration and urea-induced unfolding along with the large range of urea, pH, and protein stability covered works against the likelihood of compensatory effects among the assumptions and approximations that would lead to agreement in the free energy changes. We have applied Scheme 1 to what is considered to be a well-behaved protein (RNase A), and the validity of the long-held assumptions appears to be borne out. We do not expect this type of test to extent to many proteins without some breakdown of one or more of the tenets upon which the thermodynamics of denaturant-induced unfolding are based.

The titration-$\Delta\nu$ method is important in its own right in providing a means to evaluate the pH dependence of two-state protein unfolding free energy changes which does not require analysis of denaturant-induced unfolding transitions.

ACKNOWLEDGMENT

We thank Dr. Yingwen Huang for helpful discussions throughout the course of this work and Dr. Andrew Robertson, Liskin Swint, and Greg DeKoster for reading an earlier draft of the manuscript.

REFERENCES

Bolen, D. W., & Santoro, M. M. (1988) *Biochemistry 27*, 8069−8074.
DeKoster, G. T., Robertson, A. D., Stock, S. M., & Stock, J. B. (1993) *Techniques in Protein Chemistry IV* (Angeletti, R. H., Ed.) pp 533−540, Academic Press, Inc., San Diego.
Greene, R. F., & Pace, C. N. (1974) *J. Biol. Chem. 249*, 5388−5393.
Griko, Y. V., Gittis, A., Lattman, E. E., & Privalov, P. L. (1994) *J. Mol. Biol. 243*, 93−99.
Hagel, P., Gerding, J. J. T., Fieggen, W., & Bloemendal, H. (1971) *Biochim. Biophys. Acta 243*, 366−373.
Hu, C.-Q., Sturtevant, J. M., Thomson, J. A., Erickson, R. E., & Pace, C. N. (1992) *Biochemistry 31*, 4876−4882.
Huang, Y., & Bolen, D. W. (1993) *Biochemistry 32*, 9329−9339.
Lumry, R., Biltonen, R., & Brandts, J. F. (1966) *Biopolymers 4*, 917−944.
Matthew, J. B., & Richards, F. M. (1982) *Biochemistry 21*, 4989−4999.
Mayo, S. L., & Baldwin, R. L. (1993) *Science 262*, 873−876.
Mayr, L. M., & Schmid, F. X. (1993) *Biochemistry 32*, 7994−7998.
Nozaki, Y. (1972) *Methods in Enzymology* (Hirs, C. H. W., & Timasheff, S. N., Eds.) pp 43−50, Academic Press, New York.
Nozaki, Y., & Tanford, C. (1967a) *J. Am. Chem. Soc. 89*, 736−742.
Nozaki, Y., & Tanford, C. (1967b) *Enzyme Structure* (Hirs, C. H. W., Ed.) pp 715−734, Academic Press, New York.
Nozaki, Y., & Tanford, C. (1967c) *J. Am. Chem. Soc. 89*, 742−749.
Pace, C. N. (1975) *CRC Crit. Rev. Biochem. 3*, 1−43.
Pace. C. N. (1986) *Enzyme Structure, Part L* (Hirs, C. H. W., & Timasheff, S. N., Eds.) pp 266−280, Academic Press, Inc., New York.
Pace, C. N., & Vanderburg, K. E. (1979) *Biochemistry 18*, 288−292.
Pace, C. N., Laurents, D. V., & Thomson, J. A. (1990) *Biochemistry 29*, 2564−2572.
Pace, C. N., Laurents, D. V., & Erickson, R. E. (1992) *Biochemistry 31*, 2728−2734.
Privalov, P. L. (1979) *Advances in Protein Chemistry* (Anfinsen, C. B., Edsall, J. T., & Richards, F. M., Eds.) pp 167−241, Academic Press, New York.
Privalov, P. L., & Makhatadze, G. I. (1990) *J. Mol. Biol. 213*, 385−391.
Ropson, I. J., Gordon, J. I., & Frieden, C. (1990) *Biochemistry 29*, 9591−9599.
Rossotti, F. J. C., & Rossotti, H. (1965) *J. Chem. Educ. 42*, 375−378.
Roxby, R., & Tanford, C. (1971) *Biochemistry 10*, 3348−3352.
Salahuddin, A., & Tanford, C. (1970) *Biochemistry 9*, 1342−1347.
Santoro, M. M., & Bolen, D. W. (1988) *Biochemistry 27*, 8063−8068.
Santoro, M. M., & Bolen, D. W. (1992) *Biochemistry 31*, 4901−4907.
Swint, L., & Robertson, A. D. (1993) *Protein Sci. 2*, 2037−2049.
Taborsky, G. (1959) *J. Biol. Chem. 234*, 2652−2656.
Tanford, C. (1968) *Advances in Protein Chemistry*, pp 121−217, Academic Press, New York.
Tanford, C., & Hauenstein, J. D. (1956) *J. Am. Chem. Soc. 78*, 5287−5291.
Thomson, J., & Bigelow, C (1986) *Biochem. Cell Biol. 64*, 993−998.

BI942963E