A Test of the Linear Extrapolation of Unfolding Free Energy Changes over an Extended Denaturant Concentration Range†

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ABSTRACT: Guanidine hydrochloride (GdnHCl) and thermally induced unfolding measurements on the oxidized form of *Escherichia coli* thioredoxin at pH 7 were combined for the purpose of assessing the functional dependence of unfolding free energy changes on denaturant concentration over an extended GdnHCl concentration range. Conventional analysis of GdnHCl unfolding exhibits a linear plot of unfolding ΔG vs [GdnHCl] in the transition zone. In order to extend unfolding ΔG measurements outside of that narrow concentration range, thermal unfolding measurements were performed using differential scanning calorimetry (DSC) in the presence of low to moderate concentrations of GdnHCl. The unfolding ΔG values from the DSC measurements were corrected to 25 °C using the Gibbs–Helmholtz equation and mapped onto the ΔG vs [GdnHCl] plot. The dependence of unfolding ΔG on [GdnHCl] was found to be linear over the full denaturant concentration range, provided that the chloride ion concentration was kept at a threshold of $\geq$1.5 M. In the DSC experiments performed in the presence of GdnHCl, chloride concentrations were maintained at 1.5 M by addition of appropriate amounts of NaCl. The linear extrapolation method (LEM) gives an unfolding free energy change in the absence of denaturant ($\Delta G°_{N-U}$) in excellent agreement with the ΔG determined by DSC measurement in 1.5 M NaCl. The various methods give a consensus unfolding ΔG value of 8.0 kcal/mol at 25 °C in the absence of denaturant. Within error, $\Delta G°_{N-U}$ values obtained from urea- and GdnHCl-induced unfolding are in agreement, suggesting that urea, GdnHCl, and thermal unfolding involve the same N $\rightleftarrows$ U equilibrium in this system.

Free energy is the single most important parameter for quantitating protein stability and comparing stabilities of closely related proteins. Nearly all theoretical and experimental aspects of protein folding relate in some way to unfolding free energy changes, and much of the current work involving comparisons of mutant proteins is highly dependent on accurate evaluation of unfolding free energy measurements. The linear extrapolation method (LEM) is the most frequently used method of determining protein unfolding free energy changes induced by urea or guanidine hydrochloride (GdnHCl) (Pace, 1986; Schellman, 1987). It is based upon the premise that (two-state) unfolding free energy changes are linearly dependent on denaturant concentration and that extrapolation to zero denaturant concentration gives $\Delta G°_{N-U}$, the unfolding free energy change in the absence of denaturant (Greene & Pace, 1974). Despite its widespread use, the linear extrapolation method does not rest on the strongest of foundations. Experimentally, little is known about the functional dependence of unfolding free energy changes on denaturant concentration outside of the transition zone, and the validity of $\Delta G°_{N-U}$ as a true thermodynamic quantity is not well established experimentally (Bolen & Santoro, 1988; Santoro & Bolen, 1988).

The popularity of the LEM in determining $\Delta G°_{N-U}$ stems from observations that the method generally appears to give equivalent values for $\Delta G°_{N-U}$ regardless of which denaturant (urea or GdnHCl) is used to induce unfolding. By contrast, analyses of the same unfolding data using the transfer or denaturant binding models give $\Delta G°_{N-U}$ values which often vary with the denaturant used (Pace, 1975, 1986). Clearly, $\Delta G°_{N-U}$ should be a property only of the protein and since the LEM is the method which most often gives denaturant-independent values of $\Delta G°_{N-U}$ it has become the method of choice. This preference over the transfer and denaturant binding models should, however, be viewed with caution since there may be legitimate reasons why these other two models are not as uniform in giving denaturant-independent values of $\Delta G°_{N-U}$. Since all three methods often result in different values for $\Delta G°_{N-U}$ for the same unfolding data, the dilemma which arises is which $\Delta G°_{N-U}$ should be used and what does the quantity mean?

The LEM began as a strictly empirical method of obtaining $\Delta G°_{N-U}$ with no attempt to rationalize the assumption of linearity in terms of molecular properties (Greene & Pace, 1974). Since that time, Schellman has shown from thermodynamic derivations that a linear dependence of ΔG on denaturant concentration could be achieved by retaining appropriate terms of the excess free energy for protein unfolding (Schellman, 1987). However, the derivation in no way excludes the possibility of nonlinear dependencies. Dill has made use of transfer free energy data in developing a theory for denaturant-induced unfolding and has concluded that urea-induced unfolding should result in a linear dependence of ΔG on urea concentration but that guanidine hydrochloride induced unfolding should be somewhat curvilinear (Alonso & Dill, 1991; Dill, 1985). These results provide some theoretical rationale for linearity but with considerable ambiguity as to the generality of such a dependence.

In terms of the three experimental methods for evaluating $\Delta G°_{N-U}$, the issue of linearity takes on model-dependent importance, especially in the range of low denaturant concentration (Alonso & Dill, 1991; Pace, 1986; Pace & Vanderburg, 1979). The denaturant binding model predicts a nearly linear dependence of ΔG versus denaturant concentration at high

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denaturant concentrations, but in the low concentration range, ΔG is predicted to deviate from linearity to give a more positive value of $\Delta G°_{N-U}$ than that obtained from the LEM (Pace, 1986). In the only experimental study of which we are aware which reports ΔG values outside of the transition zone, Pace and Vanderburg show deviations of ΔG vs [denaturant] for myoglobin unfolding consistent with the denaturant binding model (Pace & Vanderburg, 1979). In addition, the prediction by Alsonso and Dill of curvilinear behavior for GdnHCl is in a direction that would also result in a value of $\Delta G°_{N-U}$ more positive than one would obtain by applying the LEM (Alonso & Dill, 1991).

The status of the measurement of $\Delta G°_{N-U}$ is quite ambiguous both in terms of the meaning of this parameter and in terms of the validity of the method used in determining it. The question of the functional dependence of ΔG on denaturant over an extended concentration range lies at the heart of some of these issues, but there are few unambiguous ways of determining the dependence outside of the denaturation transition zone. In the work described here, we combine reversible thermal unfolding data (ΔG, ΔH, and ΔC_p) obtained for *Escherichia coli* thioredoxin in varying concentrations of GdnHCl, along with GdnHCl-induced unfolding data, to provide a ΔG versus denaturant concentration plot for thioredoxin at 25 °C over the range from 0 to 2.75 M GdnHCl. The GdnHCl-induced unfolding transition zone at this temperature covers the range from about 2.0 to 2.75 M, and data outside this concentration range were obtained from differential scanning calorimetric (DSC) experiments in the presence of from 0.25 to 1.5 M GdnHCl. The DSC data were corrected to 25 °C by use of the Gibbs–Helmholtz equation and then mapped onto the ΔG versus GdnHCl concentration plot to illustrate the dependence over the extended GdnHCl concentration range.

Materials and Methods

Wild-type thioredoxin (TRX) was isolated from *E. coli* strain JF521. This strain contains the plasmid pCJF4, encoding the wild-type thioredoxin gene (Lim et al., 1985). Purified wild-type thioredoxin was kindly provided by Drs. Clare Woodward and Knut Langsetmo from the University of Minnesota, and the protein purification procedure used has been described (Langsetmo et al., 1989; Laurent et al., 1964). Thioredoxin concentrations were determined spectrophotometrically, using a molar extinction coefficient of 13 700 M^{-1} cm^{-1} at 280 nm and molecular weight of 11 700. GdnHCl (from Amresco) and urea (from Schwarz/Mann Biotech) were recrystallized before use while other reagents were used without further purification.

Differential scanning calorimetry was performed with a MicroCal MC2 scanning calorimeter. All solutions used in these experiments were in 0.01 M phosphate buffer, adjusted to pH 7.0 at 25 °C. Thioredoxin solutions containing the buffered salt and/or GdnHCl concentrations indicated were dialyzed against the buffer salt and/or GdnHCl overnight, and dialyzed protein and dialyzing buffer solutions were used to load the sample and reference cells, respectively. Baseline runs were made by scanning the appropriate buffered dialysate against itself. In those few instances in which thioredoxin was dissolved in buffer and used in DSC runs without previous dialysis, no significant differences in the thermodynamic parameters were noticed. The DSC cells were operated under about 30 psi of nitrogen during the scans, and in most experiments, relatively high scanning rates of 60 or 90 °C/h were used to minimize irreversible effects which may accompany protein unfolding. At least two consecutive scans were performed on the same sample to check for reversibility. All DSC experiments were performed under computer control using an IBM PC interfaced to the calorimeter. The computer interface, data collection, and data analysis software were supplied by MicroCal. Occasionally at very high temperature, software supplied by MicroCal was used to evaluate ΔH and T_m for two-state unfolding without the necessity of a postunfolding baseline.

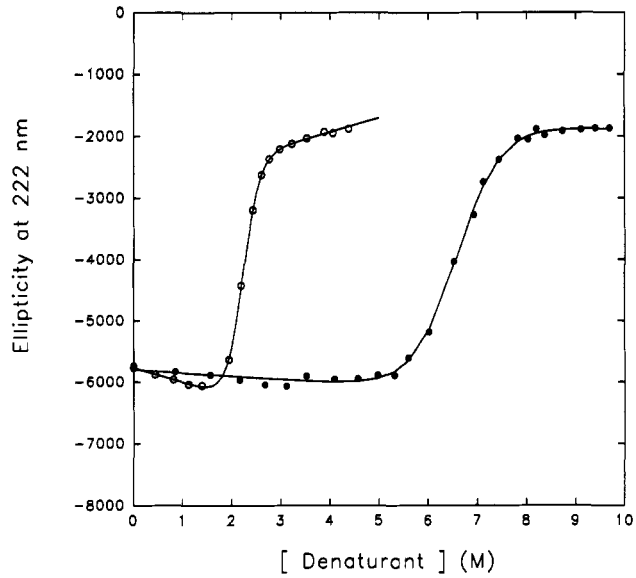

FIGURE 1: Urea- and GdnHCl-induced unfolding of oxidized thioredoxin in 0.05 M MOPS, pH 7.0, buffer at 25 °C. θ_{222} values were obtained for thioredoxin (0.5–0.15 mg/mL) as a function of guanidine hydrochloride (○) and urea (●) concentration. The urea data were obtained in the presence of 0.5 M NaCl. The solid lines are the results of nonlinear least-squares best fits of the data to the equation given under Materials and Methods.

Denaturant-induced unfolding of thioredoxin was performed at 25 °C in the presence of 0.05 M MOPS, pH 7.00, buffer. Guanidine hydrochloride and urea were used as denaturants, and the urea unfolding data were collected in the presence of 0.5 M NaCl. Changes in ellipticity at 222 nm were monitored using an AVIV Model 62 DS circular dichroism spectropolarimeter, and temperature was controlled to 25.0 ± 0.1 °C with a Lauda RC 6 bath circulator. CD data were collected by computer and analyzed using software provided by AVIV Associates. The data were fitted by nonlinear least-squares analysis using the linear extrapolation model as presented by Santoro and Bolen (1988). This model results in an expression relating θ_{222} to denaturant concentration ([D]), with $\Delta\theta_N$, $\Delta\theta_U$, m_N, m_U, m_G, and $\Delta G°_{N-U}$ as fitting parameters. Here, $\Delta\theta_N$ and $\Delta\theta_U$ represent the intercepts and m_N and m_U the slopes of the pre- and postunfolding baselines, respectively, while $\Delta G°_{N-U}$ and m_G are the intercept and slope, respectively, of the linear extrapolation of unfolding free energy changes versus denaturant concentration. The solid lines in Figure 1 represent the nonlinear least-squares best fits of the data to the equation: $\theta_{222} = \{(\Delta\theta_N + m_N[D]) + (\Delta\theta_U + m_U[D]) \exp[-(\Delta G°_{N-U}/RT + m_G[D]/RT)]\}/\{1 + \exp[-(\Delta G°_{N-U}/RT + m_G[D]/RT)]\}$.

Results

E. coli thioredoxin (oxidized form) is a small (11 700 Da) relatively hydrophobic protein resistant to both denaturant-induced and thermally induced unfolding. Figure 1 shows GdnHCl- and urea-induced unfolding profiles obtained by observing the increase in ellipticity at 222 nm. The solid lines represent the nonlinear least-squares best fits of the data to the linear extrapolation model using the analysis previously

Table I: Unfolding Free Energy Changes for *E. coli* Thioredoxin

mode of unfolding	[NaCl] (M)	T_m (°C)	ΔH (kcal/mol)[a]	$\Delta G°_{unf}$ or $\Delta G°_{N-U}$ (kcal/mol)	$C_{1/2}$ (M)[g]	m[h]
thermal	0.1	87.0	106.9 ± 1.1	8.9 ± 0.3[b]		
thermal	0.5	87.0	103.5 ± 1.1	8.4 ● 0.3[b]		
thermal	1.0	87.1	98.1 ± 0.2	7.5 ● 0.3[b]		
thermal	1.5	88.0	99.8 ± 0.6	7.7 ± 0.3[b]		
GdnHCl[e]				7.8 ± 0.2[e]	2.22	3.51
urea[e,f]	0.5			8.6 ± 0.9[c]	6.57	1.32
thermal/GdnHCl	≥1.5			8.1 ± 0.1[d]	2.25	3.62

[a] Errors determined from the fit of the endotherm to the two-state model. [b] $\Delta G°_{unf}$ calculated from DSC data and ΔC_p = 1.66 ± 0.05 kcal/(mol·deg) as described in the text. Error was calculated by propagation of errors in ΔH and ΔC_p as applied to the Gibbs–Helmholtz equation. [c] $\Delta G°_{N-U}$ evaluated by use of the LEM as described in the text. [d] ΔG intercept (±SD) from regression analysis of the combined data for GdnHCl-induced unfolding data and thermal unfolding (DSC) data. The DSC data were obtained in 1.5 M total chloride. [e] Data obtained in 0.05 M MOPS, pH 7.0, buffer at 25 °C. [f] Data obtained in the presence of 0.5 M NaCl. [g] $C_{1/2}$ represents the concentration of denaturant at the midpoint of the transition. [h] m represents the slope of the linear dependence of ΔG on denaturant concentration.

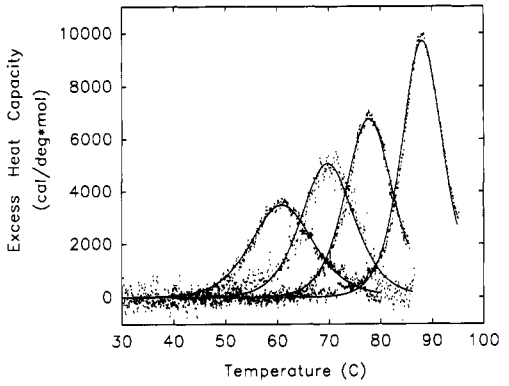

FIGURE 2: Representative DSC scans of thioredoxin in 0.01 M pH 7.0 phosphate buffer containing different concentrations of GdnHCl. Endotherms from left to right contain 1.5, 1.0, 0.5, and 0 M GdnHCl. The run shown at 0.0 M GdnHCl was performed in the presence of 1.5 M NaCl. The solid lines represent fits of the data to the two-state model ($\Delta H_{cal} = \Delta H_{van't\ Hoff}$). The scan rate was 60 °C/h with protein concentrations of about 1.0 mg/mL.

described (Santoro & Bolen, 1988). Figure 1 also illustrates that the effective ranges over which unfolding free energy data can be collected are from about 2 to 2.75 M GdnHCl and from about 5.75 to 7.5 M urea.

Figure 2 presents representative DSC profiles for thioredoxin at pH 7.0 in the presence of varying concentrations of GdnHCl. The solid lines are two-state fits to the data, and the degree of reversibility upon rescanning is found to be dependent upon the magnitude of the temperature in the unfolding range in conjunction with how long the protein is left in the unfolded state at high temperature. Use of fast scan rates (90 °C/h) and rapid cooling after proceeding through about 75% of the endotherm permit recovery of 90% or more of the endotherm upon rescanning. The DSC endotherms for thioredoxin were found to be independent of scan rate, indicating that the native and unfolded forms equilibrate rapidly in comparison with the time constant of the instrument.

The scans in Figure 2 show that though GdnHCl destabilizes thioredoxin, reversible two-state character is retained. ΔH_{cal} for each of these scans was found to be a linear function of T_m (see Figure 3), and from such data, an apparent ΔC_p can be determined from the slope of the plot. The points which deviate most from the linear dependence are those low in ionic strength. An investigation of the ionic strength effect showed that the thermal unfolding enthalpy change decreased with increasing NaCl concentration, reaching a constant ΔH value in the limit of 1–1.5 M NaCl (see Table I). On the basis of the salt dependence, the data used to construct the line in Figure 3 were restricted to experiments performed in ≥1 M chloride, contributed by NaCl and/or GdnHCl. The apparent

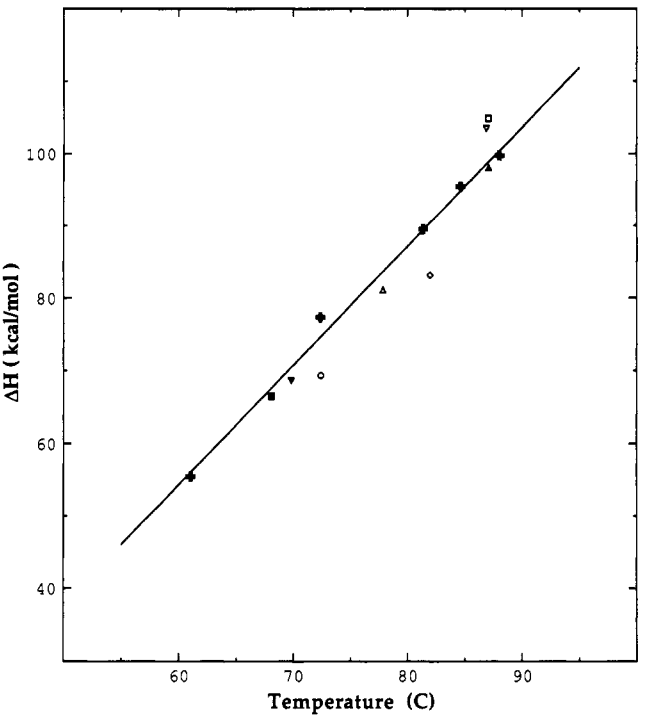

FIGURE 3: Dependence of ΔH_{cal} on T_m for thioredoxin unfolding in the presence of varying NaCl and GdnHCl concentrations. Experiments were performed in the presence of 0.01 M phosphate buffer at pH 7.0 containing no added salt (□, T_m = 87.1 °C), 0.5 M NaCl (▽, T_m = 86.9 °C), 0.25 M GdnHCl (◇, T_m = 82 °C), 0.5 M GdnHCl (△, T_m = 77.8 °C), 0.75 M GdnHCl (○, T_m = 72.4 °C), 1.0 M GdnHCl (▼, T_m = 69.84 °C), 1.1 M GdnHCl (■, T_m = 68.1 °C), and 1.0 M NaCl (▲, T_m = 87.1 °C). Solutions at constant chloride ion concentration of 1.5 M are all given the symbol (+). From left to right in the figure, these solutions are 1.5 M GdnHCl (T_m = 61.1 °C), 1.0 M GdnHCl/0.5 M NaCl (T_m = 72.4 °C), 0.5 M GdnHCl/1.0 M NaCl (T_m = 81.4 °C), 0.25 M GdnHCl/1.25 M NaCl (T_m = 84.7 °C), and 1.5 M NaCl (T_m = 88.0 °C). A ΔC_p of 1.66 ± 0.05 kcal/(mol·deg) is obtained from the line formed by all closed symbols.

ΔC_p obtained from the slope of the line is 1.66 ± 0.05 kcal/(mol·deg).

The ΔH, ΔG, and T_m values obtained by DSC in varying concentrations of GdnHCl were used in conjunction with the Gibbs–Helmholtz equation (eq 1) to evaluate what the ΔG

$$\Delta G°_{unf} = \Delta H_m(1 - 298/T_m) - \Delta C_p[(T_m - 298) + 298 \ln (298/T_m)]$$

values for unfolding would be at 25 °C ($\Delta G°_{unf}$). These data are mapped onto the ΔG versus GdnHCl concentration plot presented in Figure 4. Also presented in Figure 4 are the 25 °C unfolding free energy data obtained in the transition region of Figure 1 along with the linear extrapolation (solid line)

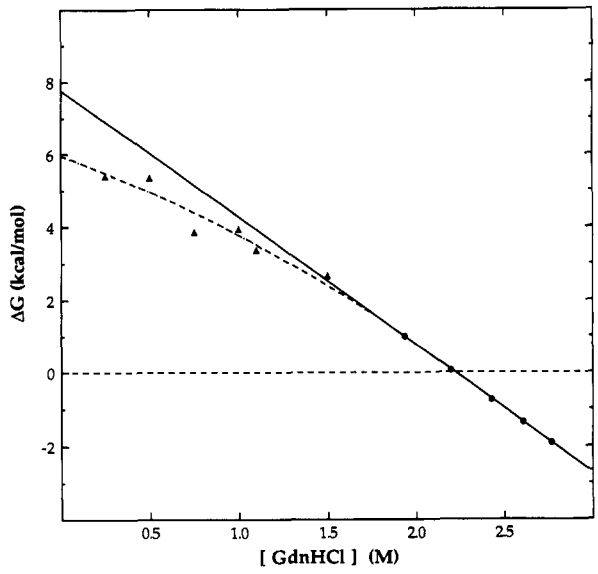

FIGURE 4: Unfolding free energy changes as a function of [GdnHCl] at 25 °C obtained without controlling ionic strength. GdnHCl unfolding free energy changes in Figure 1 were evaluated using nonlinear least-squares analysis, and the data from the transition zone (●) were mapped onto this plot. Data shown at 1.5 M GdnHCl and below (▲) were provided by first performing DSC experiments in the presence of the indicated concentrations of GdnHCl and then correcting the DSC data to 25 °C by use of the Gibbs–Helmholtz equation and mapping them onto the plot. The solid line represents the result of the application of the linear extrapolation model to the GdnHCl-induced unfolding data presented in Figure 1. The dashed line is an arbitrary line drawn through the data to illustrate deviation from linearity.

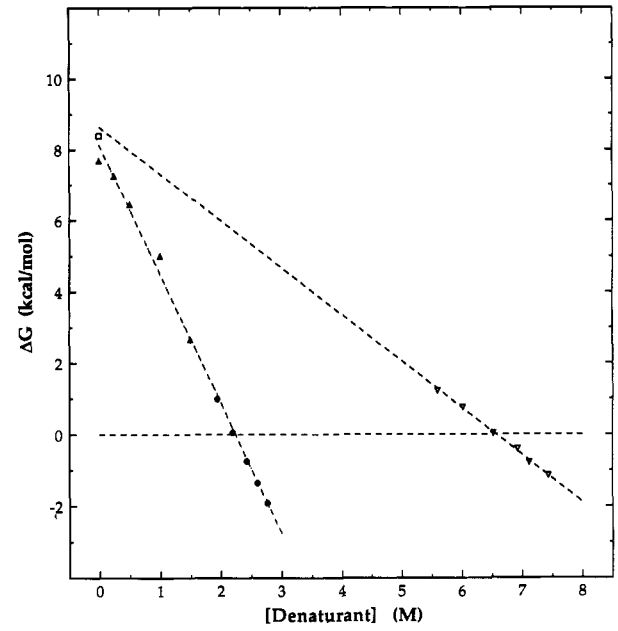

FIGURE 5: Unfolding free energy changes as a function of GdnHCl concentration obtained in the presence of chloride concentrations ≥1.5 M. ΔG data represented by (●) are the result of application of the linear extrapolation model to the GdnHCl-induced unfolding data in Figure 1. Free energy data at 1.5 M GdnHCl and below (▲) were obtained from DSC measurements of thioredoxin in solutions containing the concentration of GdnHCl indicated plus enough NaCl to bring the total chloride concentration to 1.5 M. The two ΔG values at zero GdnHCl (▲) and (□) are derived from DSC scans in the presence of 1.5 and 0.5 M NaCl, respectively. All of the DSC data obtained were corrected to 25 °C using the ΔH and T_m evaluated for the particular scan, plus the ΔC_p of 1.66 ± 0.05 kcal/(mol-deg). Also shown are urea unfolding data at 25 °C (▽) derived from the application of the linear extrapolation model to data given in Figure 1.

evaluated from the nonlinear least-squares analysis. From the highest GdnHCl concentrations down to around 1.5 M, linear dependence appears to hold rather well. However, below 1.5 M, ΔG deviates in a manner that would result in a more negative value for the intercept [$\Delta G°_{N-U}$] than would be predicted using the LEM.

One uncontrolled variable inherent in GdnHCl-induced unfolding experiments is the ionic strength. It is clear from Figure 3 that salt concentration below around 1 M deviates from the linear dependence found between ΔH and T_m. Also Figure 4 shows deviation from the linear extrapolation model at concentrations somewhat below 1.5 M GdnHCl. In an attempt to control the apparent salt and ionic strength effects, we performed DSC scans at differing GdnHCl concentrations while maintaining the total chloride ion concentration at 1.5 M by the use of added NaCl. Figure 5 shows these ΔG data mapped onto the LEM plot. By keeping the chloride ion concentration at 1.5 M or higher, linear dependence is observed all along the concentration range, from 0.25 through 2.75 M GdnHCl. The $\Delta G°_{unf}$ data point at zero GdnHCl concentration comes from DSC of thioredoxin in 1.5 M NaCl, and the linear dependence shown takes into account free energy changes derived from DSC data as well as from the GdnHCl-induced unfolding measurements at 25 °C.

To summarize, there are three evaluations of interest in determining the free energy change for N ⇄ U at 25 °C: the DSC data in 1.5 M NaCl corrected to 25 °; the GdnHCl-induced unfolding data obtained at 25 °C by use of the LEM; and the thermal unfolding data in the presence of varying [GdnHCl] but constant (1.5 M) [chloride] shown in Figure 5. All three quantities converge to the same free energy change within narrow error limits (see Table I). Though we were unable to obtain urea unfolding free energy measurements at 1.5 M NaCl because of noise in the CD signal in the presence of high urea and high salt, we do show urea unfolding data (in 0.5 M NaCl) analyzed in Figure 1 and mapped onto Figure 5. The $\Delta G°_{N-U}$ obtained from the urea data agrees very well with that obtained from GdnHCl unfolding; however, the error is large and encompasses the more precise $\Delta G°_{unf}$ and $\Delta G°_{N-U}$ data obtained using DSC and GdnHCl. Attempts at performing thermal unfolding experiments on thioredoxin in urea solutions were abandoned after aberrations in baselines were observed, making analysis difficult. While the sources of these aberrations were not investigated, we assume they are related to thermal decomposition of urea.

Discussion

The functional dependence of ΔG vs denaturant concentration is important for a variety of reasons. First, nonlinearity in the low denaturant concentration range has important implications with respect to both the denaturant binding model and Dill's model for protein unfolding, a model based in part on changes in amino acid side chain transfer free energies (Alonso & Dill, 1991; Dill, 1985; Pace, 1986). Second, the LEM is the method used overwhelmingly in the field, so the question arises as to what nonlinear dependence might mean to interpretation of $\Delta G°_{N-U}$ obtained by this method. Third, these issues are further compounded by the notion that while unfolding may be represented by a simple equation (N ⇄ U), the N and U species take on denaturant-dependent character such that the very nature of the reaction itself changes as a function of denaturant, particularly at low denaturant concentration (Dill & Shortle, 1991). There are few ways of answering these question without knowledge of the dependence of unfolding ΔG on denaturant concentration. The problem is to devise a way of getting such data.

The most cited study in the literature which attempts to determine unfolding ΔG as a function of denaturant is that by Pace and Vanderburg (1979). Horse myoglobin at pH 7 is quite stable, giving a ΔG°_{N-U} value of 7.5 kcal/mol obtained from linear extrapolation of GdnHCl unfolding data. Pace and Vanderburg found that myoglobin stability is highly pH-dependent and determined GdnHCl unfolding data at several pH values. At the lowest pH investigated (pH 4.6), the protein is so unstable that it is estimated to already be 25% unfolded even in the absence of GdnHCl. Conversion to a 50/50 mixture of U/N required less than 0.1 M GdnHCl, and the transition appeared to be complete in the presence of 0.3 M. By application of pH corrections to all of their data, a plot of ΔG vs GdnHCl concentration normalized to pH 7 was obtained. This plot showed deviations from linearity such that the unfolding ΔG at very low [GdnHCl] was found to be more positive than the ΔG°_{N-U} obtained from linear extrapolation (Pace & Vanderburg, 1979). This direction of curvature is consistent with that expected for both the denaturant binding model and Dill's model of protein unfolding (Alonso & Dill, 1991; Dill, 1985; Pace, 1986).

We have gone about determining the dependence of unfolding ΔG on denaturant concentration in a very different manner, and the results we have obtained are quite different from those of Pace and Vanderburg. We believe there are good reasons why our results differ from those of Pace and Vanderburg and that the differences provide some insight into the meaning of ΔG°_{N-U}.

The experimental procedures we have used combine results from thermal unfolding measurements with denaturant-induced data for oxidized *E. coli* thioredoxin. The rationale behind combined use of thermal- and denaturant-induced unfolding data stems from our observation that thermal unfolding of thioredoxin corrected to 25 °C gives an unfolding free energy change, ΔG°_{unf}, quite comparable to the ΔG°_{N-U} obtained by the use of the LEM. This result suggested that thermal- and denaturant-induced unfolding of thioredoxin might be related to one another in this system. We reasoned that the relationship might be tested, thermodynamically, by conducting thermal unfolding experiments in the presence of varying concentrations of GdnHCl, followed by correction of the unfolding free energy changes to 25 °C by means of the Gibbs–Helmholtz equation. The values of these temperature-corrected unfolding free energy changes in the presence of varying [GdnHCl] could provide information on the destabilization of the protein brought about by predenaturational concentrations of GdnHCl at 25 °C. Such data may then be related to GdnHCl-induced unfolding free energy measurements of thioredoxin also carried out at this temperature.

Of the data collected, we are asking most of the DSC experiments since they give the ΔH, ΔG, T_m, and ΔC_p data necessary for evaluating ΔG values at 25 °C. In order to get reasonably accurate ΔG_{unf} data, the errors in ΔH, T_m, and ΔC_p must be kept low. Also, the DSC data must be very well behaved if the corrections to 25 °C are to be meaningful, and to this end, we show in Figure 2 that the thermal unfolding endotherms in the presence of GdnHCl are quite reversible and exhibit two-state character. Such endotherms give ΔH, ΔG, and T_m values for use in the Gibbs–Helmholtz equation, but ΔC_p is harder to obtain. A common practice in evaluating ΔC_p for thermal unfolding is to plot the ΔH of unfolding as a function of T_m from DSC experiments conducted at different pH values, and the slope of such a plot gives the ΔC_p for unfolding. An alternative way of getting ΔC_p would be to plot ΔH vs T_m for DSC scans of thioredoxin unfolding carried out at constant pH but in the presence of varying GdnHCl concentrations. This method gave a ΔC_p [1.66 ± 0.05 kcal/(mol·deg)] with narrow error limits so long as the ionic strength of the data used to make the plot was at least 1 M or higher. Since Table I shows a clear dependence of ΔH on NaCl concentration that levels off at 1 M NaCl and higher, it is likely that GdnHCl affects the unfolding of thioredoxin both by its denaturing ability and by its ionic strength.

Figure 4 shows unfolding free energy changes at 25 °C in varying concentrations of GdnHCl. The ΔG data at 1.5 M GdnHCl and below were obtained from DSC data collected in the presence of varying concentrations of GdnHCl and corrected to 25 °C using the Gibbs–Helmholtz equation. Also shown are the ΔG data obtained using the LEM. It is evident that below 1.5 M GdnHCl, nonlinear dependence becomes apparent. The deviation at low GdnHCl concentration leads to a more negative value of ΔG at zero denaturant concentration than that obtained using the LEM. Since application of the denaturant binding model or Dill's model of GdnHCl-induced unfolding predicts a deviation from linearity leading to a more positive value for unfolding ΔG at zero denaturant, the experimental results are in total disagreement with the linear extrapolation model, the denaturant binding model, and Dill's model for protein unfolding. It must be pointed out, however, that the experimental conditions for the DSC-derived unfolding free energy data in low concentrations of GdnHCl are very different from the GdnHCl-dependent unfolding free energy changes predicted using the denaturant binding model and the model of Dill and co-workers. Our results involve the effect of low GdnHCl concentrations on unfolding free energy changes at high temperature which are then corrected to 25 °C using the Gibbs–Helmholtz equation. By contrast, the denaturant binding model and the model of Dill and co-workers deal with the binding behavior of GdnHCl or transfer free energy data, respectively, in GdnHCl at the set temperature of 25 °C. These experimental differences and the differences in the apparent unfolding free energies they give at low [GdnHCl] provide a basis for proposing that the N ⇄ U equilibrium at low [GdnHCl] and high temperature that is projected to 25 °C is different from the N ⇄ U unfolding equilibria carried out at 25 °C in the presence of low denaturant concentration.

Having identified salt concentration as a factor of importance, we recognized the need to eliminate or at least control the effect of salt (or ionic strength) in experiments involving low GdnHCl concentration. Noting in Figure 4 that deviations from linearity occur at GdnHCl concentrations below 1.5 M, we carried out DSC experiments in the presence of varying ratios of NaCl and GdnHCl, keeping the total chloride concentration constant at 1.5 M. Under these high ionic strength conditions, the unfolding free energy changes at 25 °C calculated from the DSC data were found to be linearly dependent upon GdnHCl concentration and congruent with the GdnHCl-induced unfolding data obtained at 25 °C (see Figure 5). The line presented in Figure 5 represents the linear least-squares best fit of all of the GdnHCl data in the figure along with the ΔG°_{unf} determined in the presence of 1.5 M NaCl shown at zero GdnHCl concentrtaion. The linear dependence of the ΔG data at ≥1.5 M total chloride concentration holds over an extensive GdnHCl concentration range, consistent with the linear extrapolation model. Also shown in Figure 5 are the LEM results of urea unfolding data carried out in the presence of 0.5 M NaCl at 25 °C (cf. Figure 1). Higher chloride concentrations could not be used in the urea unfolding experiments due to the high noise level in the CD

signal in the presence of high urea plus NaCl concentrations. The extrapolated value of ΔG°_{N-U} from the urea data in 0.5 M NaCl agrees very well with the unfolding ΔG determined from DSC in 0.5 M NaCl (Table I). On the basis of the ionic strength differences, we would expect to get slightly different values of ΔG°_{N-U} for urea (in 0.5 M NaCl) in comparison with GdnHCl. However, the error in the ΔG°_{N-U} value from urea is too large to distinguish between the two values. With the quality of data at hand, we conclude that urea and GdnHCl give the same value of ΔG°_{N-U} within the errors of the measurements.

In contrast with the urea unfolding data, the DSC-derived measurements and GdnHCl unfolding measurements give relatively precise values for the unfolding free energy change at 25 °C. The error limits for the ΔG°_{N-U} value from GdnHCl, the ΔG_{unf} derived from DSC measurement in 1.5 M NaCl, and the intercept obtained from linear extrapolation of the combined thermal/GdnHCl data are all within rather tight unfolding free energy confidence intervals (see Table I). This agreement among ΔG values at 25 °C demonstrates the strong correlation between thermal unfolding and GdnHCl-induced unfolding in this system.

The agreement of ΔG°_{N-U} values for urea and GdnHCl unfolding in conjunction with ΔG°_{unf} from thermal measurements suggests that denaturant-induced and thermally induced unfolding are dealing with the same basic phenomenon and either that the distribution of species which comprises the unfolded state must be the same in all three modes of unfolding or that the differences that exist in the unfolding ensembles in high [urea], high [GdnHCl], or high temperature must not contribute much to ΔG°_{N-U} at 25 °C. The convergence of ΔG°_{N-U} and ΔG°_{unf} data from all three means of unfolding provides some assurance that in the case of thioredoxin under these experimental conditions, ΔG°_{N-U} is a property of the protein, independent of the means used to induce unfolding.

The LEM and the Gibbs–Helmholtz equation take the unfolding equilibrium that exists in the denaturant-induced or thermally induced unfolding transition zone and project that equilibrium to other conditions, such as zero denaturant or some other temperature, respective. Thus, the ΔG°_{N-U} and ΔG°_{unf} values obtained by these methods represent hypothetical reactions of N $\rightleftarrows$ U in which the properties of both N and U are the properties derived at high denaturant concentration or at high temperature. In the case of GdnHCl-induced unfolding, the LEM reflects the effects of high salt concentration on the properties of both N and U as well as the effects of the unfolding ability of GdnHCl. Similarly, thermal unfolding in the presence of salt will reflect the effects of both salt (or ionic strength) and heat on the properties of N and U, and the Gibbs–Helmholtz equation projects that equilibrium to 25 °C. Fixing the salt concentration at a constant and high level (1.5 M), near that in GdnHCl-induced unfolding, provides some assurance that salt effects on the properties of N and U will be similar to that found in GdnHCl-induced unfolding. The fact that ΔG values obtained from DSC measurements in varying [GdnHCl] but at a constant ionic strength of 1.5 M fall on the linear extrapolation line indicates that thermal unfolding is monitoring the same N $\rightleftarrows$ U equilibrium that the LEM is monitoring.

If we accept the hypothesis that the linear extrapolation method projects the N $\rightleftarrows$ U equilibrium that occurs in the transition zone to the limit of zero denaturant and that ΔG°_{N-U} retains the attributes of the N and U states in high [GdnHCl], we may understand the source of differences between the linear ΔG vs [denaturant] dependence which we observe and the nonlinear result obtained by Pace and Vanderburg (1979). All of the data we used to obtain ΔG°_{N-U} or ΔG°_{unf} require either high concentrations of GdnHCl, urea, or heat or a combination of GdnHCl and high heat. The resulting N $\rightleftarrows$ U equilibria observed are under strongly unfolding conditions, and only the ratios of U/N change as a function of [GdnHCl] and/or temperature; the character of the microdistributions of states making up N and U is presumed to be independent of [GdnHCl] or, at most, very weakly dependent on [GdnHCl]. The strong unfolding conditions used in collecting the data on thioredoxin differ markedly from the conditions Pace and Vanderburg had to use in their approach. In order to get data on myoglobin unfolding at low [GdnHCl], Pace and Vanderburg used pH conditions in which the protein was marginally stable, requiring very little denaturant in the transition zone. The N $\rightleftarrows$ U equilibrium in the transition zone under these very mild and nonsaturating denaturant conditions is not comparable to the equilibrium at high [denaturant]; the two extremes of conditions give rise to very different N $\rightleftarrows$ U reactions. Low [GdnHCl] is insufficient to disrupt hydrophobic interactions, so the extensively unfolded distribution of microstates comprising the U state at high denaturant concentrations is largely replaced by a distribution of unfolded microstates with a more compact character in low [GdnHCl] (Dill & Shortle, 1991).

A highly stable protein is more likely to require enough denaturant such that the N $\rightleftarrows$ U equilibrium has the same character throughout the transition zone and additional denaturant beyond the transition zone does not further alter the distributions of species which comprise the N and U states. In such cases (e.g., thioredoxin), the LEM appears to project the character of that N $\rightleftarrows$ U equilibrium to zero denaturant and the ΔG°_{N-U} value is a property of the protein and not of denaturant. That is, the LEM is more likely to reflect uniform (denaturant-independent) properties of N and U when it is applied to proteins which are considered stable or highly stable. The problem in applying the LEM arises when it is applied to marginally stable proteins since the nature of the unfolded state changes with denaturant concentration even in the transition zone. Thus, application of the LEM to marginally stable proteins will project an apparent ΔG°_{N-U} at zero denaturant which will be denaturant-dependent. Comparing LEM-derived ΔG°_{N-U} values for a relatively stable wild-type protein with its marginally stable mutant species can be a questionable practice since the ΔG°_{N-U} values for the two proteins may not represent the same N $\rightleftarrows$ U reaction. Also, for marginally stable proteins, the apparent ΔG°_{N-U} will very likely be a property of both the protein and the denaturant, and this will compromise interpretation of the quantity.

The ultimate importance of ΔG°_{N-U} derived from application of the LEM to urea- or GdnHCl-induced protein unfolding data depends upon our understanding of what it represents. The results presented here give empirical evidence that the unfolding free energy change for thioredoxin depends linearly on GdnHCl concentration over an extended concentration range, provided that total salt concentrations are maintained at high and constant levels and the N $\rightleftarrows$ U equilibrium is projected from strongly unfolding conditions. Agreement of ΔG°_{N-U} with ΔG°_{unf} calculated from DSC data implies that the same N $\rightleftarrows$ U equilibrium described by ΔG°_{N-U} and ΔG°_{unf} is operative in the thermal- and denaturant-induced transition regions. Furthermore, since two-state behavior is exhibited by the DSC experiments and the two-state hypothesis was used in obtaining all of the consensus ΔG values, getting the same value for ΔG°_{N-U} and ΔG°_{unf} re-

gardless of the means of inducing unfolding provides strong support for representing the N ⇄ U equilibrium by the two-state model. The connection between thermal- and denaturant-induced unfolding is rarely ever made because the thermally unfolded state is not generally considered to be as extensively unfolded as it would be in GdnHCl or urea. In the presence of very high and constant chloride ion concentration, the connection between GdnHCl and thermal unfolding of thioredoxin appears to be remarkably simple in this particular system. Only further studies with other proteins will tell whether the linear relationship is of a general nature.

Registry No. GdnHCl, 50-01-1; urea, 57-13-6.

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Pentraxin Family of Proteins Interact Specifically with Phosphorylcholine and/or Phosphorylethanolamine†

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ABSTRACT: Pentraxins are a family of serum proteins characterized by five identical subunits that are noncovalently linked. The two major types of pentraxins are C-reactive protein (CRP) and serum amyloid P component (SAP). CRP proteins are identified by their calcium-dependent interaction with phosphorylcholine. This study showed that SAP also bound to phosphorylated compounds but had a high specificity for phosphorylethanolamine. Thus, human CRP and SAP show high specificity that is complementary for the related compounds, phosphorylcholine and phosphorylethanolamine, respectively. This relationship suggests a complementary and/or related function for the pentraxins. Pentraxins from other species were also examined. Mouse SAP showed binding interactions and specificity similar to human SAP. Female protein (FP) from hamster and rat CRP showed a hybrid specificity and bound to both phosphorylethanolamine and phosphorylcholine. All of the proteins that bound phosphorylethanolamine also associated with human C4b-binding protein (C4BP). With the exception of human and rat CRP, all the proteins also bound to vesicles containing acidic phospholipids. All of these binding interactions were calcium-dependent and mutually exclusive, suggesting that they involved the same site on the protein. These findings suggest possible ways to examine the function of the pentraxins.

The pentraxin family of proteins have been highly conserved throughout evolution in vertebrate species (Pepys et al., 1978; Baltz et al., 1982). A common structural feature is five identical subunits noncovalently bound in a cyclic manner (Gotschlich & Edelman, 1965; Kushner & Sommerville, 1970; Bach et al., 1977; Oliveria et al., 1980; Pepys et al., 1978, 1982; Pontet et al., 1981; de Beer et al., 1982; Baltz et al., 1982; Pepys & Baltz, 1983). In fact, molecular structure is a vital criterion for classification as a pentraxin (Osmand et al., 1977). The members of this family include C-reactive protein (CRP),[1] serum amyloid P component (SAP), and female protein (FP) from hamster (Skinner & Cohen, 1988). CRPs and SAPs are distinguished by specific binding properties and whether or not the pentraxin is associated with amyloid deposits (Pepys

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[1] Abbreviations: PE, phosphorylethanolamine; PC, phosphatidylcholine; CRP, C-reactive protein; SAP, serum amyloid P component; FP, female protein from Syrian hamster; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; dansyl-PtdEtn, dansyl phosphatidylethanolamine; CPS, pneumococcal C-polysaccharide; EDTA, ethylenediaminetetraacetate.

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