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Calorimetrically-derived parameters for protein interactions with urea and guanidine–HCl are not consistent with denaturant *m* values

Gregory T. DeKoster, Andrew D. Robertson [1]

Department of Biochemistry, The University of Iowa, Iowa City, IA 52242, USA

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Abstract

A recent study used calorimetric data and a stoichiometric binding model to derive binding constants, enthalpies, and stoichiometries describing the interaction between proteins and the chemical denaturants, urea and guanidine–HCl (Makhatadze and Privalov, J. Mol. Biol., 226 (1992) 491). In the present study, these parameters have been used to calculate the excess free energy, ΔG^{ex}, associated with interactions between chemical denaturants and the three proteins examined in the calorimetric study: ribonuclease A, cytochrome c, and lysozyme. This free energy and its dependence on denaturant concentration, the denaturant *m* value, have then been compared to experimental results from chemical denaturation experiments. The magnitudes of *m* values calculated from the calorimetric studies are significantly greater, 20 to 100%, than the observed values in urea. Calculated *m* values for guanidine–HCl range from about 10% greater than observed values for cytochrome c to over 100% greater for lysozyme. Discrepancies between calculated and observed *m* values are probably attributable to incomplete binding isotherms in the calorimetric studies. An additional issue raised in this study concerns the correlation of *m* values with changes in accessible surface areas upon unfolding. For proteins that undergo a two-state unfolding reaction, experimental *m* values can vary by more than a factor of two for a given protein, depending on the solution conditions. This observation suggests that factors beyond changes in accessible surface areas play a major role in determining *m* values.

Keywords: Ribonuclease A; Protein folding; Chemical denaturation; Denaturant binding; Denaturant *m* values

1. Introduction

Weak interactions between proteins and small-molecule solutes are of considerable practical and theoretical interest. The biological milieu includes a large number of small molecule solutes that can affect protein stability and function [1]. Experimentalists routinely use any of a number of solutes, such as glycerol and sucrose, at high concentrations to stabilize proteins [2]. Chemical denaturants such as urea and guanidinium chloride (GuHCl) are widely used tools for the recovery of proteins from precipitates [3] and for study of the thermodynamics [4–6] and kinetics [4,7–11] of protein folding. In general, the effects of chemical denaturants on protein stabil-

[1] telephone: (319) 335-6515 FAX: (319) 335-9570 e-mail: andy-robertson@iowa.edu

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ity are more readily assessed experimentally than are effects of other types of solutes. Consequently, denaturants have served as prototypes for theoretical and experimental investigation of weak interactions between proteins and solutes.

Four methods or models have been proposed for analysis and interpretation of chemical denaturation data. All four approaches use the following general relationship to describe the observed free energy of unfolding, $\Delta G'_u$, in the presence of chemical denaturant

$$\Delta G'_u = \Delta G^o_u + \Delta G^{ex} \quad (1)$$

where ΔG^o_u is the free energy of unfolding in the absence of denaturant and ΔG^{ex} is the difference in excess free energy of denaturant interaction with native and denatured protein. One approach is the purely phenomenological linear extrapolation method (LEM) summarized in Eq. (2), in which ΔG^o_u is obtained by extrapolation of $\Delta G'_u$ values observed in the denaturation transition zone back to zero denaturant concentration, C [5]

$$\Delta G'_u = \Delta G^o_u - mC \quad (2)$$

where the denaturant m value is the slope describing the dependence of $\Delta G'_u$ on the molar denaturant concentration. A second model relies on data for the free energy of transferring amino acids and peptides from water into aqueous denaturant solutions [4,12,13]. These data are used in conjunction with knowledge of a protein's amino acid composition to derive a measure of changes in solvent-accessible surface area upon denaturation [4].

A third model views chemical denaturation as the result of denaturant binding by the native and denatured protein [4]. According to the most common form of this model, each binding site on native and denatured protein is assumed to have the same binding constant, k, but the number of binding sites on denatured protein exceeds that for native protein by Δn [4,5]

$$\Delta G'_u = \Delta G^o_u - \Delta nRT\ln(1 + ka) \quad (3)$$

where R is the gas constant, 1.987 cal mol^{-1} K^{-1}, and T is the absolute temperature. Denaturation is thus driven by mass action as the activity of denaturant, a, is increased. The fourth model is similar to the third model, in that a binding formalism is used in the mathematical development of the theory, but treats denaturant action as more akin to solvation than binding [14–20]. A key distinction between the solvent exchange model and the denaturant binding model is the formal inclusion of solvent in the reaction between protein and denaturant. This model leads to improved agreement between theory and experiment with respect to studies of preferential interactions. More detailed examination of the possible molecular mechanisms of interaction between proteins and denaturants is being pursued in Monte Carlo statistical mechanics calculations [21].

A variety of experimental approaches have been taken to investigate the mechanism of interaction between proteins and denaturants, particularly urea and GuHCl. The solubility of amino acids and peptides in aqueous denaturant solutions has been studied to derive thermodynamic parameters describing the transfer of protein groups from water to denaturant solutions [22–29]. Similar parameters have been obtained from isopiestic vapor pressure measurements [30]. The interaction of urea with helical peptides has been examined using circular dichroism (CD) spectropolarimetry and modified Zimm–Bragg theory [31].

Proteins in aqueous denaturant solutions have been examined using densitometric methods [32–34], isopiestic methods [35,36], and equilibrium dialysis [37]. The general picture from these and the model compound studies is that urea and GuHCl interact favorably with the peptide group as well the amino acid side chains. Molecular details of these interactions cannot be assessed directly in the thermodynamic studies, but interesting data in this regard are beginning to appear from both crystallographic [38,39] and NMR studies [40–42].

Privalov and coworkers have provided a large body of valuable calorimetric data concerning interactions of proteins with denaturants [43,44]. The most recent study included use of the simple homogeneous binding model, Eq. (3), to interpret calorimetric data for three proteins [44]. The results of this analysis are binding constants, stoichiometries, enthalpies, and entropies describing the interaction of proteins and denaturants.

Data in the literature suggest that denaturant interactions with protein groups may be heterogeneous

(see Table 2 in [13] for compilation). Schellman has pointed out that, in the likely event that these weak interactions are indeed heterogeneous, there are at least two significant concerns with the use of a simple homogeneous binding model: first, the resulting binding parameters will not be the average over all sites and, second, the stoichiometry and enthalpy of interaction cannot be separated on the basis of calorimetric data alone [19,20]. An additional concern in applying the binding model to the calorimetric data stems from the paucity of data describing an approach to saturation [44], which may lead to an underestimate for the total number of denaturant binding sites [45]. In light of these concerns and the use of the calorimetrically-derived parameters by a number of laboratories, the present study is designed to test the accuracy of these parameters through comparison with an independent measure of the energetics of interactions between denaturants and the three proteins used in the calorimetric study.

Comparison of Eq. (2) and Eq. (3) suggests that denaturant binding parameters can be used to calculate m values. More specifically, the dependence of $\Delta G'_u$, the experimentally observed free energy of unfolding in the transition zone, on denaturant concentration resides entirely in ΔG^{ex} for both models. In this study the quantitative relationship between m values and denaturant binding parameters is developed. This relationship is then used to compare the calorimetrically derived binding parameters for denaturants and proteins with experimental m values published previously [46–48] and with additional values for ribonuclease A (RNase A) determined in the present study.

2. Materials and methods

2.1. Thermal denaturation of RNase A

RNase A (Type XII-A) was used as purchased from Sigma Chemical Co. (St. Louis, MO) and stored at −20°C. GuHCl (Heico Chemical Co., Delaware Water Gap, PA) and urea (Boehringer Mannheim, Indianapolis, IN) were used without further purification. Concentrations of stock solutions of aqueous GuHCl and urea were determined by refractometry [5].

Thermal denaturation of RNase A was monitored at 222 nm by CD spectropolarimetry on an Aviv 62 DS spectropolarimeter equipped with thermoelectric temperature control [49]. The concentration of RNase A was 0.05 mg ml^{-1} in 10 mM potassium acetate and varying concentrations of either GuHCl or urea. The pH was measured with an Orion model 611 pH meter equipped with a 3 mm Ingold glass electrode (Wilmad Glass Co., Buena, NJ) calibrated at two points with standards from VWR Scientific (West Chester, PA). Samples were stirred continuously in a 1-cm pathlength cuvette and the scan rate was 1°C min^{-1}. Temperature was monitored with a thermocouple inserted in the cuvette.

2.2. Data analysis

Raw data from thermal denaturation experiments, θ_{obs}, were fit to Eq. (4), which describes a two-state unfolding process, to obtain the enthalpy of unfolding, ΔH_m, the midpoint of unfolding, T_m, and parameters describing the pre- and post-transition baselines [49]

$$\theta_{obs} = \frac{(\theta_n + m_n T) + (\theta_d + m_d T)\{\exp[(\Delta H_m/R)(1/T_m - 1/T)]\}}{\{1 + \exp[(\Delta H_m/R)(1/T_m - 1/T)]\}} \quad (4)$$

The parameters θ_n and θ_d are the y-intercepts of the pre- and post-transitional baselines, respectively, and m_n and m_d are the slopes of these baselines. The fitted parameters were used to generate curves describing the fraction of native RNase A versus temperature. Conversion of the thermal denaturation data at varying denaturant concentrations to chemical denaturation at varying temperatures has been described previously [49] and is outlined briefly here. Isothermal slices were taken through the fraction native curves to transpose the data, which then represented chemical denaturation at a variety of temperatures. The isothermal slices were taken at temperatures where at least 4 data points fell between frac-

tion native values of 0.9 and 0.1, corresponding to a range in $\Delta G'_u$ of about ± 1.5 kcal mol^{-1}. Fraction native values were converted to $\Delta G'_u$ and replotted versus denaturant concentration. These data were then fit to Eq. (2) to obtain ΔG°_u and m values.

3. Results

3.1. Calculation of m values from binding parameters

The relationship between denaturant m values and denaturant binding parameters is a bit more complicated than indicated by a comparison of Eq. (2) and Eq. (3). The m value of Eq. (2) assumes a linear dependence of $\Delta G'_u$ on denaturant concentration while the binding parameters of Eq. (3) may lead to a nonlinear relationship. The result is that the two equations extrapolate to different values for ΔG°_u when used to fit the same data set [50]: Eq. (3) typically results in larger ΔG°_u values than does Eq. (2). However, within about ± 1.5 kcal mol^{-1} of the midpoint for chemical denaturation, experimentally determined values of $\Delta G'_u$ are the same regardless of whether Eq. (2) or Eq. (3) is being used to *interpret* the data. The resulting equality is

$$\Delta G^\circ_u(\mathrm{LEM}) - m[\mathrm{D}] = \Delta G^\circ_u(\mathrm{bind}) - \Delta nRT\ln(1 + ka) \qquad (5)$$

where ΔG°_u(LEM) and ΔG°_u(bind) are the extrapolated ΔG°_u values of Eq. (2) and Eq. (3), respectively. These two terms are constants, so while ΔG^{ex} may be different depending on whether Eq. (2) or Eq. (3) is used, all variation in $\Delta G'_u$ with denaturant concentration is in the excess free energy terms. Thus, one should be able to use the calorimetrically derived binding parameters to predict the dependence of $\Delta G'_u$ on denaturant concentration near the midpoint of chemical denaturation.

Substituting the denaturant activity, a, with $C\gamma$, the product of denaturant concentration, C, and the activity coefficient, γ, and taking the derivative of both sides of Eq. (5) with respect to denaturant concentration gives

$$-m = \frac{-\Delta nRTk\left[\gamma + C\left(\frac{d\gamma}{dC}\right)\right]}{(1 + kC\gamma)} \qquad (6)$$

All of the parameters necessary for calculating m with Eq. (6) are reported by Makhatadze and Privalov [44]. In determining m values, however, experimentalists actually measure $\Delta G'_u$ over a finite interval of denaturant concentration, typically ± 0.5 to 1.0 M, near the midpoint for unfolding. The experimental m value is thus a mean m value, $\langle m \rangle$, over the experimental range of denaturant concentration. How is $\langle m \rangle$ to be calculated from binding parameters?

In principle, $\langle m \rangle$ can be calculated by determining the weighted average of m over the experimental interval of denaturant concentration. In practice, $\langle m \rangle$ is going to be very close to the m value at the midpoint of denaturation. This follows from the relatively small intervals in denaturant concentration over which $\Delta G'_u$ is measured experimentally, which leads to the commonly observed linear concentration dependence of $\Delta G'_u$. This is illustrated in Fig. 1, where the calorimetrically derived binding parameters and Eq. (7) have been used to calculate ΔG^{ex} for the unfolding reaction of RNase A at 25°C

$$\Delta G^{ex} = -\Delta nRT\ln(1 + ka) \qquad (7)$$

The variation in free energy with denaturant is going to appear linear over all 1 M and 2 M intervals in GuHCl and urea, respectively, which are typical experimental intervals for these denaturants. The m

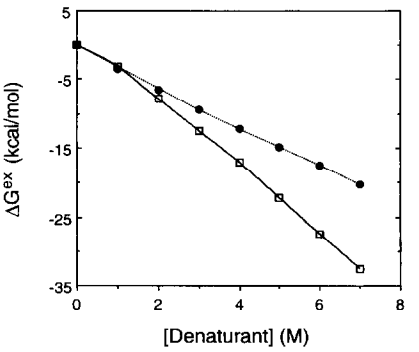

Fig. 1. The net excess free energy of interaction, ΔG^{ex}, between RNase A and GuHCl (□) and urea (●) at 25°C. ΔG^{ex} was calculated using Eq. (7). Denaturant activities, on a molar scale, and binding parameters for RNase A are as reported in Tables 1, 5 and 6 of Makhatadze and Privalov [44]. For GuHCl, the binding parameters are $\Delta n = 31$ and $k = 0.60$ M^{-1}. For urea, the parameters are $\Delta n = 118$ and $k = 0.052$ M^{-1}. The lines are meant to aid inspection.

values for RNase A calculated using Eq. (6) are plotted in Fig. 2 (▽), while calculated m values for cytochrome c and lysozyme are reported in Table 1.

To mimic the experimental determination of m values, additional m values for RNase A were calculated in a two-step process. First, the binding parameters of Makhatadze and Privalov [44] were used to calculate ΔG^{ex} for RNase A, cytochrome c and lysozyme over a range of denaturant concentrations centered at midpoints determined experimentally. The concentration range was limited to an interval of ± 1.5 kcal mol^{-1} in ΔG^{ex} about the midpoint. Each set of ΔG^{ex} values was then fit to a line to obtain an m value. The resulting m values for RNase A are plotted in Fig. 2 (▲).

3.2. Predicted versus observed m values

RNase A was one of the proteins used in the calorimetric study and is one of the best characterized proteins with regard to chemical denaturation. Chemical denaturation of RNase A has been examined over a wide range of pH values by the Pace and Bolen laboratories using tyrosine fluorescence and tyrosine absorbance, respectively [46–48]. Chemical denaturation of RNase A is a two-state process [48].

Fig. 2 summarizes the comparison of experimental and calculated m values for RNase A at 25°C. The two sets of experimental values agree very well for GuHCl and for urea concentrations greater than 3 M. Experimental values at low denaturant concentrations have larger uncertainties than those at higher concentrations because the pre-transition baseline tends to be short. For example, when the denaturation midpoint is less than about 2 M urea, the fitting error in urea m values from the Pace laboratory (●) averages about ± 200 cal mol^{-1} M^{-1}). Uncertainties in urea m values at concentrations above 2 M urea tend to be ≤ 100 cal mol^{-1} M^{-1} for both laboratories [47,48]. The two sets of calculated m values (▲, ▽) are in good agreement, which is consistent with the arguments presented above concerning the linearity of free energy in denaturant concentration. However, calculated m values for GuHCl are about 50% greater in magnitude than the experimental values while calculated m values for urea range

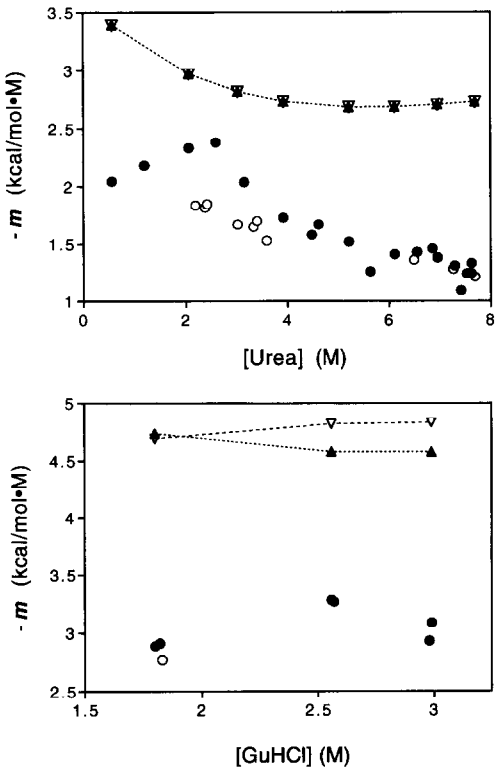

Fig. 2. Calculated and experimental m values for RNase A versus denaturant concentration at 25°C. The experimental values are those of Pace and coworkers (●) [47] and Yao and Bolen (○) [48]. One set of calculated m values was determined by linear fits of ΔG^{ex}, obtained with Eq. (7), versus denaturant concentration (▲), as described in the text. The concentration dependences for activities at 25°C, as reported in Table 1 of Makhatadze and Privalov [44], were fit to fourth- and fifth-order polynomials for urea and GuHCl, respectively. For urea, the equation for activity, a, as a function of molar denaturant concentration, C, is: $a = (6.915\times10^{-5}) + 0.9961C - (3.685\times10^{-2})C^2 + (4.651\times10^{-3})C^3 - (1.230\times10^{-4})C^4$. For GuHCl, the equation is: $a = (-2.844\times10^{-4}) + (5.556\times10^{-2})C + (3.400\times10^{-1})C^2 - (1.062\times10^{-1})C^3 + (1.843\times10^{-2})C^4 - (9.939\times10^{-4})C^5$. The concentration dependence of the denaturant activity coefficient, γ, is required for calculating m values using Eq. (6) (▽). This concentration dependence was obtained for both denaturants at 25°C from ratios of activities and denaturant concentrations reported in Table 1 of Makhatadze and Privalov [44]. These were fit to third-order polynomials to facilitate interpolation of activity coefficients at denaturant concentrations where activities were not measured experimentally. The derivatives of these polynomials with respect to denaturant concentration, C, provided the other parameter, $d\gamma/dC$, needed for calculations with Eq. (6). The lines are to aid inspection.

Table 1
Experimental and calculated m values for equine cytochrome c and hen egg white lysozyme [a]

	C_m /M	m^{expt}	m^{calc}	Ref.
Cyt c				
GuHCl	2.62	3270	2700	[51]
	2.5	2950	2700	[52]
	2.42	3010	2700	[53]
Urea	7.3	1200	2700	[51]
Lysozyme				
GuHCl	4.24	2330	4100	[54]
	4.2	1880	4100	[55]
	3.07	1880	4200	[56]
Urea	6.8	1290	2800	[55]
	5.21	1120	2800	[56]

[a] C_m is the experimental midpoint of unfolding, m^{expt} is the experimentally observed m value and m^{calc} is the m value calculated at C_m using Eq. (6) and the binding parameters for cytochrome c and lysozyme reported by Makhatadze and Privalov [44]. All experimental m values were determined at 25°C except those of Hagihara et al. [51], which were determined at 20°C.

from about 20 to 100% greater in magnitude than experimental values. The largest differences in urea are observed at concentrations where uncertainties in experimental m values are low.

The observed differences in GuHCl may not be statistically significant. The reported relative uncertainty in the binding constant at 25°C is ±15% while no uncertainty in Δn is reported. We estimate this to be about 30% from the reported stoichiometry for denatured RNase A, 74 ± 8, and assuming a similar relative error for the native state value of 43. Similar calculations for urea suggest that the differences in calculated and oberved m values are significant.

Results for cytochrome c are similar to those for RNase A: calculated and observed m values in GuHCl are similar while a large difference is observed in urea (Table 1). In contrast, calculated m values for lysozyme are about half the experimental values with both denaturants.

3.3. Temperature dependence of m

The binding parameters can also be used to predict temperature dependences for m. In the binding model, the temperature dependence of ΔG^{ex} at a given molar concentration of denaturant is a consequence of two competing factors: the negative enthalpy of denaturant binding, Δh, which leads to a decrease in the magnitude of ΔG^{ex} with increasing temperature, and the temperature dependence of the denaturant activity, $a(T)$, which leads to increasing ΔG^{ex} with increasing temperature [44]. Substituting into Eq. (7) leads to the following expression for the temperature dependence of ΔG^{ex}

$$\Delta G^{ex}(T) = -\Delta nRT\ln \times \left[1 + k_{ref}\exp\left[\frac{\Delta h}{R}\left(\frac{1}{T_{ref}} - \frac{1}{T}\right)\right]a(T)\right] \quad (9)$$

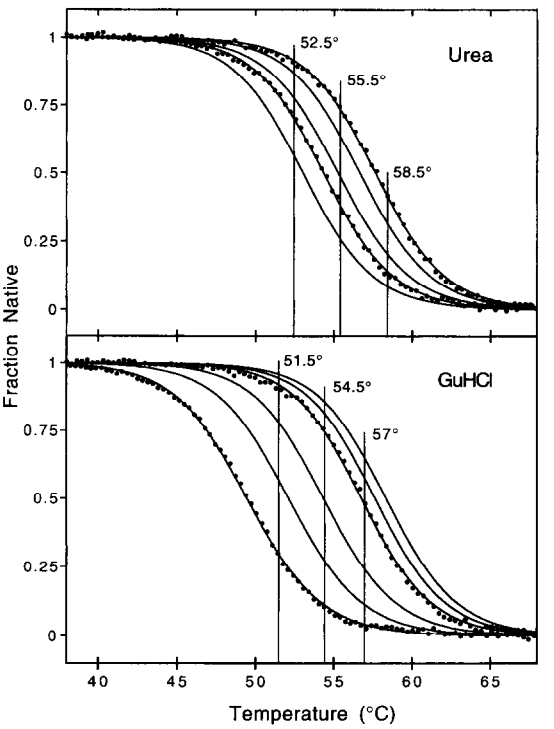

Fig. 3. Fraction native RNase A versus temperature in varying concentrations of urea (top) and GuHCl (bottom) at pH 5 and in 10 mM potassium acetate. The raw data were acquired as described in Section 2 and fit to Eq. (4). The fitted parameters reported in Table 2 were used to convert the data to fraction native versus temperature, as described in Section 2. The continuous lines are generated from the fitted parameters of Table 2. Representative raw data, normalized to the fraction native scale, are shown as small circles. The concentrations of urea (top panel) are, from right to left, 0, 0.25, 0.50, 0.75, and 1.0 M. The concentrations of GuHCl are, from right to left, 0.1, 0, 0.25, 0.50, 0.75, and 1.0 M. The vertical lines indicate the temperatures at which isothermal slices have been taken through the data to generate the plots in Fig. 4.

Table 2
Thermodynamic parameters of RNase A unfolding at pH 5.0, 10 mM potassium acetate

Denaturant/M	ΔH_m /kcal mol^{-1}	T_m /°C
0.00	96.0 (2.1)	57.9 (0.1)
0.00	98.1 (2.3)	57.7 (0.1)
Urea		
0.25	95.8 (2.0)	56.7 (0.1)
0.50	93.9 (4.6)	55.3 (0.1)
0.75	95.7 (1.9)	54.3 (0.1)
1.00	94.2 (4.7)	53.0 (0.1)
GuHCl		
0.10	97.9 (2.0)	58.4 (0.1)
0.25	98.1 (2.0)	56.8 (0.1)
0.25	102.4 (2.2)	57.1 (0.1)
0.50	93.4 (1.8)	54.4 (0.1)
0.75	90.7 (2.0)	52.1 (0.1)
1.00	88.2 (1.8)	49.5 (0.1)

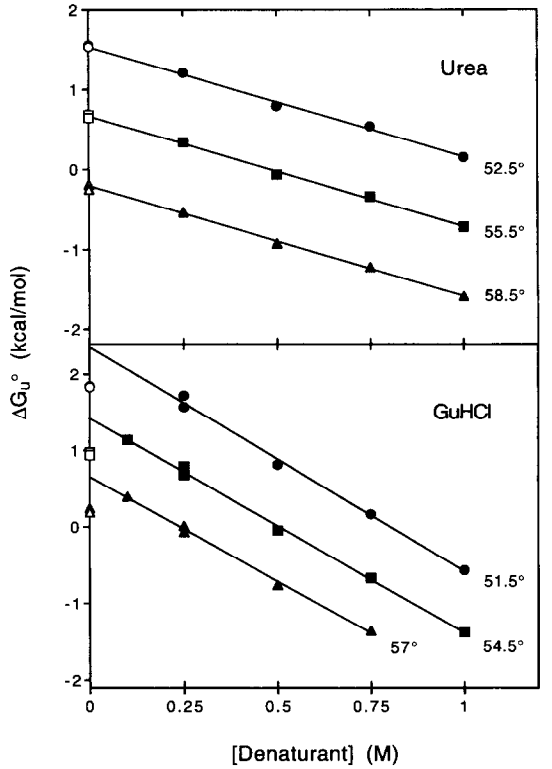

Fig. 4. The free energy of unfolding for RNase A at pH 5.0 versus concentration of urea (top) or GuHCl (bottom) at the temperatures indicated in Fig. 3. Data are indicated by the symbols and were obtained as described in the text and in the legend to Fig. 3. The lines are fits to Eq. (2) using data indicated by filled symbols. The ΔG_u° values at zero denaturant concentration were obtained directly from the thermal transition in the absence of denaturant.

The binding constant, k_{ref}, is at the reference temperature, T_{ref}. The contribution of $a(T)$ is $\leq 15\%$ of the enthalpy effect on k for both urea and GuHCl, so the predicted trend for both urea and GuHCl is decreasing ΔG^{ex} and m, with increasing temperature at fixed denaturant concentration. More specifically, if one assumes a constant Δn with varying temperature, then a 10 deg increase in temperature should lead to approximately 5% and 10% declines in m values in 1 M urea and 1 M GuHCl, respectively.

To test if the binding parameters can be used to predict the temperature dependence of m, RNase A was denatured with urea and GuHCl at a variety of temperatures (Fig. 3). After fitting to Eq. (4), the

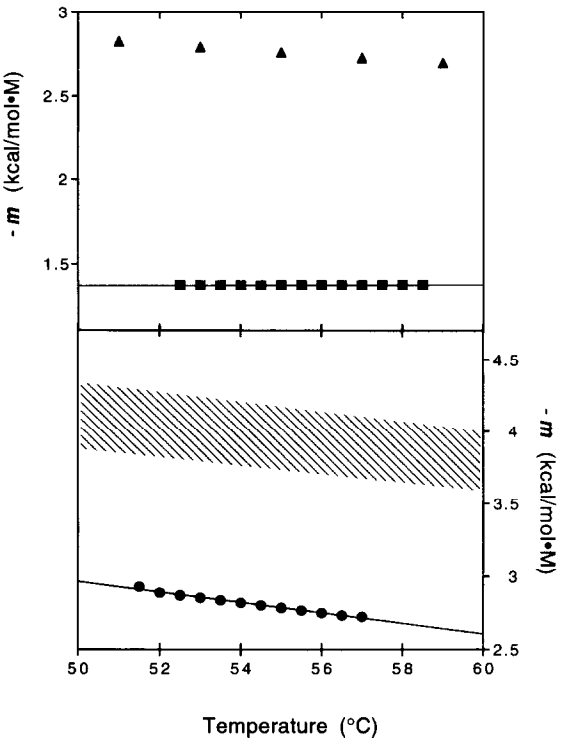

Fig. 5. Temperature dependence of experimental and calculated m values for RNase A in urea (top) and GuHCl (bottom) at pH 5.0. Experimental m values in urea and GuHCl are indicated by filled squares (■) and filled circles (●), respectively. Calculated urea m values (▲) were obtained by linear fits of ΔG^{ex}, obtained with Eq. (7), versus denaturant concentration. Similar calculations to obtain calculated m values for GuHCl showed that ΔG^{ex} was distinctly nonlinear at low denaturant concentrations. The hatched region in the lower panel shows the range of GuHCl m values obtained by linear extrapolation from 0.1 to 0.75 M GuHCl and 0.25 to 1.0 M GuHCl.

fitted parameters (Table 2) were used to transpose the data to yield chemical denaturation at a variety of temperatures (Fig. 4). Performing experiments at temperatures near the thermal transition permits a direct comparison of extrapolated values of ΔG_u^o with those determined directly [49]. Knowledge of ΔG_u^o also permits direct evaluation of experimental versus predicted values of ΔG^{ex} derived from Eq. (2) and Eq. (3).

Urea denaturation of RNase A at concentrations ranging from 0 to 1 M and temperatures ranging from 52 to 58°C yields m values that are nearly independent of temperature, as expected (Fig. 5). In contrast, GuHCl denaturation over a similar range of concentrations and temperatures shows the predicted 10 to 15% decline in m values (Fig. 5). The overall modest temperature dependence for m values has been observed with other proteins [57–60].

The disagreements between experimental and calculated m values observed at 25°C are also seen at the elevated temperatures with both denaturants. An interesting result with GuHCl is the disagreement between the extrapolated and directly determined values of ΔG_u^o (Fig. 5). The apparent stabilization of RNase A at low concentrations of GuHCl is consistent with previous observations from other laboratories [48,61].

4. Discussion

Experimentally determined m values for RNase A, cytochrome c, and lysozyme generally differ from those predicted using calorimetrically derived binding parameters for urea and GuHCl. Given the large uncertainties in binding stoichiometries for GuHCl, the differences in GuHCl for RNase A and cytochrome c may not be statistically significant. Nevertheless, the present study suggests that some or all of the average binding parameters for proteins in both denaturants are significantly greater than the true values. Some possible origins for the differences are: 1) inaccuracies in the experimental m values, 2) inaccuracies in the binding parameters, and 3) different solution conditions for the spectroscopic and calorimetric studies. The latter is unlikely given that, with the exception of m values at low concentrations of urea, the absorbance and fluorescence studies show good agreement in spite of the different buffers, ionic strengths, and, presumably, order of magnitude differences in protein concentrations [62]. Similar arguments tend to rule out inaccuracies in the experimental m values with, again, the possible exception of values obtained at less than 2 M urea. The most likely explanation for the difference between experimental and calculated m values is inaccuracies in the binding parameters. An additional argument concerning the accuracy of the binding parameters is found in Fig. 1: values for ΔG^{ex} calculated using the calorimetrically-derived binding parameters suggest that urea and GuHCl should be equally effective as denaturants up to about 2 M concentrations when, in fact, GuHCl is typically about twice as effective as urea at all concentrations (Figs. 2 and 5).

The simplest explanation for inaccurate binding parameters is that the binding isotherms described by the calorimetric data are incomplete. The fitted parameters reported in Table 5 of Makhatadze and Privalov [44] probably reflect the bias introduced in using incomplete isotherms, where both the binding constant and stoichiometry appear to fall with increasing temperature. For denatured RNase A, the apparent stoichiometry of urea binding varies from 258 at 10°C to 208 at 40°C. Equivalent arguments for GuHCl binding to denatured RNase A lead to decreases in stoichiometry from 79 to 67. An additional complication is the likely heterogeneity in the interactions between proteins and denaturants. In principle, fitted parameters from the homogeneous binding model of Eq. (3) can provide an accurate phenomenological description of heterogeneous binding [19,20], but the data must be complete. Finally, separation of the enthalpies and stoichiometries of binding may be problematic using calorimetric data alone [19,20]. The analysis presented here bears only on the *interpretation* of the calorimetric data of Makhatadze and Privalov. The data themselves are in accord with and greatly augment results from other studies [11,43].

The denaturant m values that have been tabulated for this study raise additional questions about their relationship to the molecular details of protein denaturation. Myers and coworkers recently identified significant correlations between m values, calculated surface area changes upon unfolding, and the heat capacity change upon unfolding, ΔC_p [63]. Uversky

and Ptitsyn made similar observations with regard to m values and molecular weights for proteins [64]. One problem with comparing m values with surface area changes, ΔC_p values, or molecular weight is that m values can vary considerably for the same protein depending on the solution conditions (Fig. 2; Fig. 5) [47,48,65]. Pace and coworkers have attributed variations in m for RNase T1, RNase A, and barnase to electrostatic repulsion at extremes of pH, where high net charges lead to expansion of the denatured state [47,65]. Alternatively, m depends on the denaturant concentration, which would be consistent with a binding mechanism for denaturation. In either event, no one m value appears to be characteristic of a given protein: urea m values for a single protein can vary by nearly two-fold (Fig. 2) [47,65].

The molecular basis for the energetics of interactions between proteins and denaturants is not well understood. A crude outline of factors governing these interactions may be provided by correlation of the calorimetric data and denaturant m values with specific features of protein structure, but a more detailed molecular and energetic picture is more likely to come from studies of model compounds [19,20,31].

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