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FEATURE ARTICLE

Thermodynamics of Protein Interactions with Urea and Guanidinium Hydrochloride

George I. Makhatadze*

Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061

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Urea and guanidinium hydrochloride (GdmCl) are used as chemical denaturing agents in the study of protein folding and stability. It has been known for a long time that the free energy of unfolding estimated using the linear extrapolation model (LEM) for the GdmCl-induced unfolding is often higher than the corresponding values obtained using LEM for the urea-induced unfolding. It was noted by Nick Pace that "This disagreement is puzzling and deserves further studies" (Pace, C. N. *Methods Enzymol.* **1986**, *131*, 266). This paper summarizes thermodynamic information on the denaturing action of urea and GdmCl that may explain why LEM analysis of GdmCl-induced unfolding in many cases does not provide a "true" stability of the protein. The answer to the puzzle seems to be the nonapplicability of LEM for the data analysis obtained using GdmCl-induced denaturation.

Understanding the mechanisms of protein folding and protein stability requires the knowledge of the thermodynamic parameters (Gibbs energy, enthalpy, entropy, heat capacity, partial volume) characterizing the difference between the unique native state of a protein and its unfolded state.[1] One way to measure these thermodynamic parameters is to induce the unfolding reaction using some type of perturbant and monitor the changes in the populations of states upon transition. There are a number of properties that can provide information about the population of different states: spectral properties (UV or VIS−absorption, fluorescence, circular dichroism, infrared, NMR), heat capacity, viscosity, or enzyme activity. The changes in the population of states is then used to determine the equilibrium constant and other thermodynamic parameters. When chemical denaturants such as urea or guanidinium chloride (GdmCl) are used as a perturbant, the equilibrium constant, $K_{eq}(C)$, for a two-state transition

$$\mathrm{N} \leftrightarrow \mathrm{U} \tag{1}$$

is defined as

$$K_{eq}(C) = \frac{F_U(C)}{F_N(C)} \tag{2}$$

where $F_N(C)$ and $F_U(C)$ are the fractions of the protein in the native and unfolded states, respectively, at a given concentration of denaturant, C. $F_N(C)$ and $F_U(C)$ are related to the experimental observable, $y_X(C)$, as:

$$F_N(C) = 1 - F_U(C) = \frac{y_X(C) - y_U(C)}{y_N(C) - y_U(C)} \tag{3}$$

where $y_N(C)$ and $y_U(C)$ are the dependencies of the observable on denaturant concentrations for the native and unfolded states, respectively (Figure 1). Using eq 3, the expression for the equilibrium constant can be written as

$$K_{eq}(C) = \frac{F_U(C)}{F_N(C)} = \frac{F_U(C)}{1 - F_U(C)} = \frac{y_N(C) - y_X(C)}{y_X(C) - y_U(C)} \tag{4}$$

The Gibbs energy at a given concentration of denaturant, $\Delta G(C)$, is related to the equilibrium constant as

* Phone: (806) 742-3062. Fax: (806) 742-1289. E-mail: GIA@TTU.EDU.

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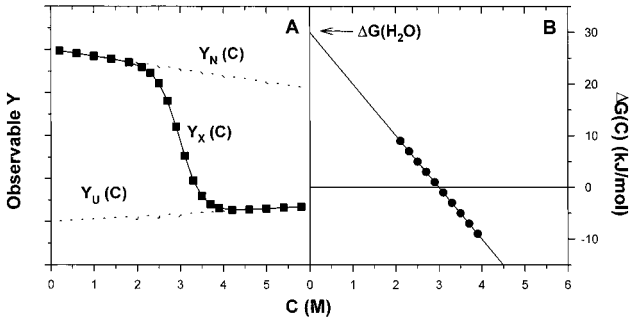

Figure 1. Simulated unfolding experiment. Panel A: Changes in the experimental observable, *Y*, as a function of the concentration of denaturant, *C*. The concentration dependences of the observable signals for the native and unfolded states are shown as dotted lines. Panel B: Linear extrapolation of the $\Delta G(C) = -RT \ln K_{eq}(C)$ term to zero concentrations of denaturant. See text for details.

$$\Delta G(C) = -RT \ln K_{eq}(C) \tag{5}$$

and can be obtained experimentally only in the denaturant concentration range where the transition is observed experimentally. For different proteins, the range of concentrations where the transition occurs is different, and the Gibbs energy at zero concentration of denaturant, $\Delta G(H_2O)$, is of exceptional importance. Thus, to obtain the values $\Delta G(H_2O)$, a reliable procedure for the extrapolation of $\Delta G(C)$ to zero denaturant concentration, i.e.,

$$\Delta G(H_2O) = \lim_{C \to 0} \Delta G(C) \tag{6}$$

is required. Numerous observations on a large number of different proteins showed that in the transition range, the Gibbs energy of unfolding is a linear function of denaturant concentration in a relatively narrow range of the denaturant concentrations (see, e.g., ref 2 and references therein). Thus, following the Ockham's Razor principle (why use a complicated explanation when the simple one will suffice), these observations led to the so-called linear extrapolation method (LEM) for determination of the Gibbs energy of unfolding in the absence of denaturant:

$$\Delta G(C) = \Delta G(H_2O) - mC \tag{7}$$

where the so-called denaturant *m*-value is the dependence of the Gibbs energy of unfolding on denaturant concentration[3] and the *mC* term thus represents the difference in transfer Gibbs energy between the unfolded and native states.

Analysis of the protein stability using LEM is widespread due to the simplicity of both the experimental setup for denaturant-induced unfolding and corresponding data analysis. However, the invariable applicability of LEM analysis has been debated and its applicability has been questioned.[4−15] Two compounds, urea and guanidinium chloride (GdmCl), are particularly popular protein denaturants. They have very similar chemical structure but differ in their "efficiency" to denature proteins. One indication of the inadequacy of the LEM for the analysis of protein stability is the difference in the $\Delta G(H_2O)$ values obtained using urea or GdmCl as denaturant.[4,5] The difference "*...in the values of* $\Delta G(H_2O)$ *derived from GdmCl data are consistently 20−50% higher than the values derived from urea. This disagreement is puzzling and deserves further studies*".[5] Here I will consider three different sets of experimental evidence showing that the LEM is *applicable* for the urea-induced unfolding and is *not applicable* for the GdmCl-induced unfolding reactions.

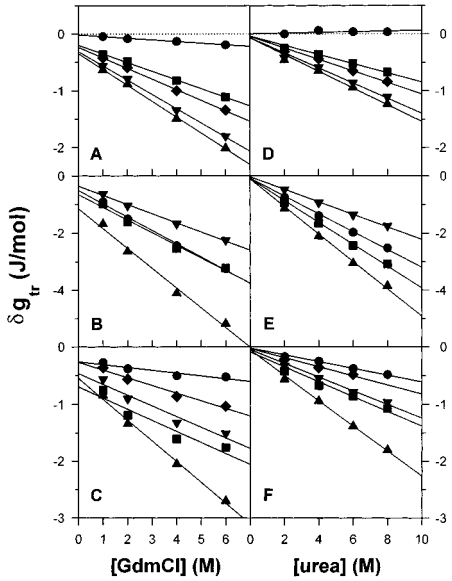

Figure 2. Gibbs energy of transfer of amino acid residues from water into aqueous solutions of different concentrations of GdmCl (panels A, B, C) or urea (panels D, E, F).[5,16−18] Panels A and D: side chains of alanine (●), valine (■), leucine (▲), isoleucine (▼), and proline (◆). Panels B and E: side chains of phenylalanine (●), tyrosine (■), tryptophane (▲), and methionine (▼). Panels C and F: side chains of threonine (●), histidine (■), asparagine (▲), glutamine (▼) and the peptide unit (◆). The $\delta g_{tr,i}(C)$ values for side chains were obtained as a difference in the Gibbs energy of transfer between a given amino acid residue and that of glycine. Solid lines represent the linear fit of the experimental data.

Transfer Model

In the transfer model, the Gibbs energy of unfolding of a protein in aqueous solution in the absence of denaturant, $\Delta G(H_2O)$, is related to the Gibbs energy at a given concentration of denaturant, $\Delta G(C)$, corrected for the sum of Gibbs energies of transfer of individual components:[16]

$$\Delta G(C) = \Delta G(H_2O) + \sum_i \alpha_i n_i \delta g_{tr,i}(C) \tag{8}$$

where n_i is the number of the group type i, α_i is fractional exposure of this group, and $\delta g_{tr,i}(C)$ is the Gibbs energy of transfer of this group from water to a given concentration of denaturant. Comparison of the transfer model (eq 8) with the LEM (eq 7) shows that the *mC* term is analogous to $-\sum_i \alpha_i n_i \delta g_{tr,i}(C)$. The native state is unique. The unfolded state ensemble does depend on denaturant concentration, although this dependence in many cases is not significant.[6−8] Thus for a given protein, the $n_i\alpha_i$ term can be assumed to be independent of the denaturant concentrations. With this in mind, $\delta g_{tr,i}(C)$ is the only term that will depend on the denaturant concentration. Thus, the validity of LEM can be assessed from the dependence of $\delta g_{tr,i}(C)$ on the denaturant concentrations. For LEM to be applicable, the plot of $\delta g_{tr,i}(C)$ vs *C* must satisfy two conditions. First, the dependence of $\delta g_{tr,i}(C)$, on denaturant concentration must be a linear function. Second, the dependence of $\delta g_{tr,i}(C)$ on denaturant concentration must extrapolate to the origin, i.e., at zero denaturant concentration the transfer energy must be zero.

Figure 2 presents the dependence of the Gibbs energies of transfer of different amino acid residues from water into aqueous solutions of GdmCl or urea as a function of concentration of these denaturants at 25 °C. The experimental data reported by the Tanford group[5,16−18] cover broad concentrations of GdmCl

(1−6 M) and urea (1−8 M) and cover the range where most of the protein unfolding experiments are performed. All experiments are performed at 25 °C and include more than a dozen amino acids and short peptides. Independent of the nature of the solute, two important features of the Gibbs energy for the transfer into denaturant solutions can be observed. First, the Gibbs energy of transfer is a linear function of denaturant concentrations for both GdmCl and urea. Second, the Gibbs energy of transfer into urea solution linearly extrapolates to the origin, i.e., at 0 M urea the energy of transfer is 0 J/mol. However, in the case of GdmCl linear extrapolation of the Gibbs energy of transfer to zero concentration of denaturant in all cases leads to large negative values.

Thus experimental data on the Gibbs energy of transfer of different model compounds into an aqueous urea solution satisfies the criteria for LEM, i.e., the dependence of $\delta g_{tr,i}(C)$ on the urea concentration is *both* a linear function and it extrapolates to the origin. This can be considered as an important indication for the *validity* of LEM for the urea-induced unfolding.

In the case of GdmCl, however, the experimental data on the Gibbs energy of transfer of model compounds into aqueous GdmCl solutions satisfies *only one* of two criteria for LEM. The dependence of $\delta g_{tr,i}(C)$ on GdmCl concentration is linear, but it does not extrapolate to the origin. Independent of the nature of model compounds values of $\delta g_{tr,i}(C)$, extrapolated to zero concentration of GdmCl, are significantly negative. This predicts that the $\Delta G(H_2O)$ value for protein unfolding calculated using LEM will be overestimated, in agreement with the observations of Pace.[4,5]

Therefore the thermodynamics of transfer of model compounds from water into an aqueous solution of denaturants shows that LEM is applicable to the urea-induced unfolding but is not applicable to the GdmCl-induced protein unfolding reactions.

Binding Model

In the binding model of the effects of urea and GdmCl on proteins, it is assumed that urea and GdmCl interact with the solvent-exposed groups on a protein via direct binding. The Gibbs energy of unfolding of a protein in aqueous solution in the absence of denaturant, $\Delta G(H_2O)$, in this model is related to the Gibbs energy at a given concentration of denaturant, $\Delta G(C)$, corrected for the Gibbs energy of denaturant binding, $\Delta G_b(C)$, as

$$\Delta G(C) = \Delta G(H_2O) + \Delta G_b(C) \qquad (9)$$

$\Delta G_b(C)$ is defined as

$$\Delta G_b(C) = -\sum_i \Delta n_i RT \ln(1 + K_{b,i}C) \qquad (10)$$

where $K_{b,i}$ is the binding constant for the site type i and $\Delta n_i = n_U - n_N$ is the difference in the number of binding sites of type i between the unfolded, n_U, and the native states, n_N. Assuming that the denaturant binding sites are identical and independent, eq 10 can be written as

$$\Delta G(C) = \Delta G(H_2O) - \Delta nRT \ln(1 + K_bC) \qquad (11)$$

where Δn is the difference in the number of denaturant binding sites between the unfolded and the native states and K_b is the binding constant.

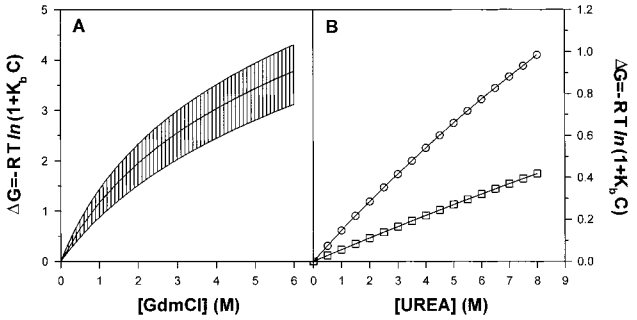

Figure 3. Gibbs energy of binding per site calculated as $-RT \ln(1 + K_bC)$. Panel **A**. Binding per site for GdmCl. Thick solid line $K_b = 0.60$ M^{-1}, shaded area shows the Gibbs energy calculated with 30% higher and lower values of K_b. Panel **B**. binding per site for urea: (open circles) Gibbs energy calculated using maximal reported values for K_b 0.06 M^{-1}; (open squares) Gibbs energy calculated using minimal reported values for K_b 0.023 M^{-1}. See text for details.

Comparison of the binding model (equation 11) and LEM (equation 7) shows that since ΔnRT is independent of denaturant concentration, for the validity of LEM from the point of view of the binding model, will rely on the dependence of $\ln(1 + K_bC)$ on denaturant concentrations. The functional dependence of $\ln(1 + K_bC)$ must be closely approximated by a linear function in the experimental concentration range of denaturants, i.e., 0−6 M for GdmCl and 0−8 M for urea (see also Scholtz et al.[19]).

The dependence of the $-RT \ln(1 + K_bC)$ term on C can be easily calculated using values of K_b. Estimation of K_b for urea was the subject of a number of studies using different systems. Schönert and Stroth[20] reported a value of 0.038 M^{-1} for the urea−peptide group interactions. Calorimetric studies of dissolution of cyclic diglycyl peptide by Sijpkes et al.[21] led the authors to conclude that K_b for urea is 0.045 M^{-1}. Using a homologous set of cyclic dipeptides, Zou et al.[22] reported the value of 0.023 M^{-1} for the binding constant of urea to the peptide backbone amide. Calorimetric studies of several proteins[14] gave an estimate for K_b of urea to be (0.06 ± 0.01) M^{-1}. The value of K_b for GdmCl $(0.60 \pm 0.09$ $M^{-1})$ was determined by the calorimetric studies of several proteins[14] and is in agreement with the value of 0.60 M^{-1} for GdmCl−peptide group interactions reported by Pace.[5]

Figure 3 shows the dependence of $-RT \ln(1 + K_bC)$ on concentrations of GdmCl (panel A) or urea (panel B). The expression $-RT \ln(1 + K_bC)$ for urea was calculated for two different values of K_b, 0.023 and 0.061 M^{-1}, representing minimal and maximal reported values. In the case of GdmCl, the K_b value of 0.6 M^{-1} is shown, with the results of calculations with 30% higher and lower K_b values indicated as vertical error bars. The difference in $-RT \ln(1 + K_bC)$ vs C dependencies for urea and for GdmCl are dramatic. Due to the low K_b values for urea, the $-RT \ln(1 + K_bC)$ dependence on the urea concentration is very close to a straight line in the 0−8 M urea concentration range. The linearity does not depend on whether maximal or minimal reported values of K_b were used and is a simple consequence that when $K_b \ll 1$ the expression $-RT \ln(1 + K_bC)$ can be approximated by $-RTK_bC$. In contrast, the dependence of the $-RT \ln(1 + K_bC)$ term for GdmCl on the concentration of this denaturant is far from linear (Figure 3). The nonlinear behavior of the $-RT \ln(1 + K_bC)$ vs C plot persists at 30% higher and lower than 0.60 M^{-1} values of K_b for GdmCl. Linear extrapolation of the data obtained at the moderately low concentrations of GdmCl (1−3 M) to the zero denaturant concentrations would lead to a nonzero value for the term $-RT \ln(1 + K_bC)$ and thus predicts an overestimate

of the $\Delta G(H_2O)$ using LEM, which is in agreement with experimental observations.[5]

The binding model of interactions of denaturants with proteins leads to the same conclusion as the transfer model: LEM can be used for the analysis of the urea-induced protein unfolding but will lead to a significantly overestimated value of $\Delta G(H_2O)$ if used for the analysis of GdmCl induced protein unfolding reactions.

Electrostatic Effects

The electrostatic nature of GdmCl has been recognized for a long time. Indeed, the guanidinium group has a pK_a value of ~12.4; thus at neutral pH in aqueous solution GdmCl will exist in a fully dissociated state as Gdm^+ and Cl^-. The effect of electrolytes on the stability of proteins has been studied intensively by von Hippel's group.[23] They show that different ions affect protein stability in different ways. Particularly important for this discussion are the observed effects of different guanidinium salts and urea on the transition temperature (T_m) of ribonuclease A (RNase). It was found that the effect of the guanidinium ion on the T_m of RNase A depends on the nature of the anion.[23] Thiocyanate (SCN^-) and chloride (Cl^-) salts of guanidinium act as destabilizers, with GdmSCN being about twice as potent as GdmCl. The acetate salt of guanidinium (GdmAc) is also destabilizing, but its effect on the transition temperature is very similar, on the molar bases, to that of urea. Guanidinium sulfate (Gdm_2SO_4) actually increases the transition temperature of RNase A. Interestingly, the effects of different cations and anions on the stability of RNase A were found in a first approximation to be additive. This observation provided an explanation for the observed difference in the effects of guanidinium salts on the RNase A transition temperature. The effect of guanidinium ions (Gdm^+) is to decrease the transition temperature; however, the overall effect will depend on the nature of the anion. A strongly "salting out" anion such as sulfate (SO_4^{2-}) will overcompensate the destabilizing effect of guanidinium ions, whereas the destabilizing effect of guanidinium will be enhanced by "salting in" anions such as thiocyanate and chloride.

The first attempt to dissect electrostatic effects of GdmCl on protein stability was made by Santoro and Bolen[7] and further elaborated by Yao and Bolen.[8] Using oxidized thioredoxin as a model protein, Santoro and Bolen[7] showed that in order to obtain the Gibbs energy of stabilization in agreement between temperature- or urea-induced denaturation and GdmCl-induced denaturation using LEM, it is necessary to maintain a constant ionic strength of the solution. The ionic strength required to obtain similar values of $\Delta G(H_2O)$ using different perturbants is quite high, >1.5 M.[7] These experiments provided a first direct indication that the effect of GdmCl on protein stability cannot be as simple as LEM. In 1993 Goto's group[24] reported that proteins unfolded at low pH can be refolded by increasing concentrations of electrolytes in general and by GdmCl in particular. Later the same year, Mayr and Schmid[25] reported that RNase T1 can actually be stabilized by 100 mM GdmCl. The most dramatic effects of GdmCl on protein stability have been observed in the case of the ubiquitin molecule.[26] This small globular protein (76 amino acid residues, molecular mass 7.4 kDa) can be thermostabilized by neutral salts, such as 500 mM NaCl, by as much as 25 deg. GdmCl also thermostabilizes the ubiquitin molecule: addition of 500 mM GdmCl increases the transition temperature for this protein by 16 deg, whereas addition of 2 M GdmCl increases the transition temperature by 13 deg. This is a remarkable stabilization since many proteins are already unfolded at these concentrations of GdmCl.

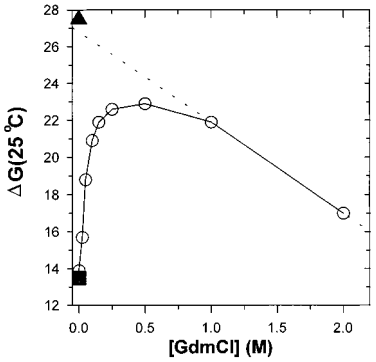

Figure 4. Gibbs energy of unfolding of ubiquitin as a function of GdmCl concentration at pH 2.0 (10 mM glycine−HCl buffer): (open circles) Gibbs energy values calculated from the differential scanning calorimetry data;[25] (dashed line) linear extrapolation to zero GdmCl concentration using the data points at the two highest concentrations of GdmCl; (solid square) Gibbs energy obtained from LEM analysis of the urea-induced unfolding shown in Figure 5; (solid triangle) Gibbs energy obtained from LEM analysis of the GdmCl-induced unfolding shown in Figure 5.

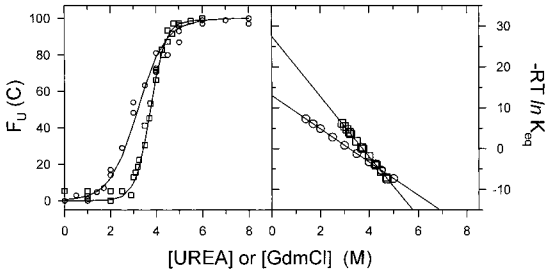

Figure 5. Denaturant-induced unfolding of ubiquitin at pH 2.0. Panel A: Fraction of ubiquitin unfolded, $F_U(C)$, as a function of concentrations of urea (○) or GdmCl (□). The $F_U(C)$ was calculated from the experimental data on the changes in the ellipticity at 222 nm or fluorescence intensity as described in Makhatadze et al.[26] Panel B: LEM analysis (eq 7) of the experimental data for the urea-(○) or GdmCl-induced (□) unfolding of ubiquitin shown in panel A. The following parameters from the analysis have been obtained: urea $\Delta G(H_2O)$ = 13 kJ/mol and m = 4070 J/mol^2; GdmCl $\Delta G(H_2O)$ = 27.5 kJ/mol and m = 7350 J/mol^2. The m-value for GdmCl is in excellent agreement with the value reported in the literature.[2,27a]

Using our reported experimental data on ubiquitin,[26] one can calculate the dependence of the Gibbs energy at a given concentration of GdmCl, $\Delta G(C)$, on the concentration of this denaturant (Figure 4). It must be specified that the Gibbs energy values, including $\Delta G(H_2O)$, obtained from the calorimetric experiments are model independent. Linear extrapolation to the zero GdmCl concentration using two points obtained at the highest concentrations of GdmCl gives the value for $\Delta G(H_2O)$ of 26.8 kJ/mol, almost 100% higher than 14 kJ/mol, the value obtained from the calorimetric experiments in the absence of GdmCl (Figure 4). Thus, linear extrapolation overestimates the Gibbs energy of unfolding of ubiquitin. To further corroborate this result, we performed the analysis of urea- and GdmCl-induced unfolding of ubiquitin using LEM (Figure 5). The profiles for the fraction of ubiquitin unfolded as a function of denaturant (urea or GdmCl) concentrations were obtained by monitoring either the changes in fluorescence intensity or the changes in ellipticity at 222 nm (for details see Figure 5 in ref 26). The profiles were fitted according to the LEM (eq 7) to estimate $\Delta G(H_2O)$ values. The LEM analysis of the urea-induced unfolding gave 13.5 kJ/mol of the Gibbs energy of stabilization of ubiquitin in the absence of this denaturant, in agreement with the calorimetric data given above. The Gibbs energy of stabilization of ubiquitin in the absence of denaturant,

obtained from the LEM analysis of GdmCl-induced unfolding was found to be 27.5 kJ/mol (Figure 5), comparable with the estimates of 26.8 kJ/mol from the linear extrapolation of the calorimetric data (Figure 4), but clearly higher than the true Gibbs energy of stabilization (14 kJ/mol at pH 2.0) (Figures 4 and 5).

Concluding Remarks

Using three different approaches this paper shows that there is ample amount of evidence that LEM analysis of the urea-induced denaturation provides very reasonable if not excellent estimates for the Gibbs energy of stabilization, comparable with the stability estimates using temperature-induced unfolding. This is not true in the case of GdmCl-induced unfolding, for which LEM analysis gives stability values significantly different from that estimated from the analysis of the urea- or temperature-induced unfolding transitions. This, possible deviation from LEM for GdmCl-induced unfolding might be the consequence of the electrolyte nature of GdmCl, which affects the electrostatic interactions of the native state of the protein molecule. Due to the uniqueness of the charge distribution for different proteins and different contributions of electrostatic interactions to protein stabilization (see, e.g., refs 13 and 27), the deviation from LEM for individual proteins will be different. This will preclude the development of a simple yet universal extrapolation procedure for the analysis of the GdmCl-induced denaturational transition in proteins, rendering GdmCl-induced unfolding reactions unsuitable for the study of protein stability.

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