Effects of Guanidine Hydrochloride on the Proton Inventory of Proteins: Implications on Interpretations of Protein Stability†

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ABSTRACT: The ΔG°_{N-D} value obtained from extrapolation to zero denaturant concentration by the linear extrapolation method (LEM) is commonly interpreted to represent the Gibbs energy difference between native (N) and denatured (D) ensembles at the limit of zero denaturant concentration. For ΔG°_{N-D} to be interpreted solely in terms of N and D, as is common practice, it must be shown to be independent of denaturant concentration. Because ΔG°_{N-D} is often observed to be dependent on the nature of the denaturant, it is necessary to determine the circumstances under which ΔG°_{N-D} can be interpreted as a property solely of the protein. Here, we use proton inventory, a thermodynamic property of both the native and denatured ensembles, to monitor the thermodynamic character of denaturant-dependent aspects of N and D ensembles and the N ⇄ D transition. Use of a thermodynamic rather than a spectral parameter to monitor denaturation provides insight into the manner in which denaturant affects the meaning of ΔG°_{N-D} and the nature of the N ⇄ D transition. Three classes of proteins are defined in terms of the thermodynamic behaviors of their N ⇄ D transition and N and D ensembles. With guanidine hydrochloride as a denaturant, the classification of protein denaturations by these procedures determines when the LEM gives readily interpretable ΔG°_{N-D} values with this denaturant and when it does not.

The Gibbs energy change for protein denaturation (ΔG°_{N-D}), as determined from denaturant-induced unfolding of proteins, is a thermodynamic parameter often used to define the stability of a protein (*1*−*3*). As a result, ΔG°_{N-D} values have served as the basis for a wide range of studies on the energetic differences between wild-type and mutant proteins. The validity of the conclusions drawn from these studies is, of course, completely dependent on the validity of the ΔG°_{N-D} values. Given that ΔG°_{N-D} values are often determined from a method that is empirical in origin (the linear extrapolation method, LEM) (*4*−*6*), it is doubtful that the quantity we call ΔG°_{N-D} deserves the complete credibility it is often given.

The LEM is a procedure for evaluating ΔG°_{N-D} from the cooperative denaturant-induced transition of the protein from native to denatured states (*1*−*3*). Operationally, the method involves obtaining a plot of some spectral observable as a function of denaturant concentration, in which the spectral observable differs for the native and denatured states. The resulting plot is sigmoid-shaped, signifying a denaturant concentration-induced cooperative transition from the native state of the protein to the denatured state. Assuming that the transition can be described by the simple equilibrium N ⇄ D involving just the two states, N and D, linear extension of the pre- and postdenaturation baselines into the transition zone provides a scale from which it is possible to evaluate equilibrium constants [D]/[N] as a function of denaturant concentration (*1*−*3*). Converting these equilibrium constants into observed Gibbs energy changes [$\Delta G_{obs} = -RT \ln ([D]/[N])$] and plotting ΔG_{obs} vs denaturant concentration gives a linear plot with a negative slope that is extrapolated to the limit of zero denaturant concentration. The intercept of this extrapolation to zero denaturant concentration is given the name ΔG°_{N-D} and is said to represent the Gibbs energy difference between native and denatured protein at the limit of zero denaturant concentration (*7*).

It is clear from the degree of empiricism used in evaluating ΔG°_{N-D} that much is needed to establish the thermodynamic validity of values obtained from the LEM and to understand what ΔG°_{N-D} actually represents. Because ΔG°_{N-D} is nearly always interpreted as a property solely of the N and D states of the protein, an obvious test to exclude solvent contributions would be to show that ΔG°_{N-D} is independent of the nature of the denaturant. Experiments showing that urea- and GdnHCl-induced denaturation extrapolate to the same ΔG°_{N-D} value for a protein are frequently not performed, but when they are, urea denaturation is often found to give a different ΔG°_{N-D} from that of GdnHCl (*2*, *8*). Obviously, either one or both of these ΔG°_{N-D} values depend on the denaturant and/or denaturant concentration, and when ΔG°_{N-D} is dependent on the denaturant or denaturant concentration, the highly desirable interpretation of ΔG°_{N-D} as a property of the protein alone is invalid.

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[1] Abbreviations: LEM, linear extrapolation method; PMS-Ct, phenylmethanesulfonyl-α-chymotrypsin; RNase A, ribonuclease A; wtSN, wild-type staphylococcal nuclease; GdnHCl, guanidine hydrochloride; RCAM, reduced and carboxyamidated ribonuclease A; F-Ct, furoyl-chymotrypsin; Ct, α-chymotrypsin.

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What is needed is a concise and experimentally accessible way of dealing with the empirical nature of the LEM, a way of assessing the validity of the LEM for any particular protein. There are two issues of importance, one of which is to determine whether ΔG°_{N-D} depends on the *nature of the denaturant*. The second issue is less obvious, but more far-reaching, and it deals with the basic requirement that ΔG°_{N-D} be devoid of *dependence on denaturant concentration*. In principle, instead of using a spectral parameter to monitor the N $\rightleftarrows$ D transition, it should be possible to use a thermodynamic quantity, one that is strongly linked to protein structure and differs in its magnitude for the N and D states.

Let us consider a case of two-state denaturation in which the characteristic thermodynamic quantity being monitored is observed to be independent of denaturant concentration in both the pre- and postdenaturation concentration ranges. In this case, the *thermodynamic characters* of the N and D states are said to be denaturant concentration-independent, and under such circumstances we should see a sigmoidal denaturation profile with flat baselines. Because changes only occur in the transition zone and not in the baselines, only the ratio [D]/[N] is dependent on denaturant concentration. If the LEM is the correct way to analyze protein stability in this system, its use will result in a denaturant concentration-independent ΔG°_{N-D}. However, ΔG°_{N-D} will not be denaturant concentration-independent if either the native or denatured baseline has a slope, for then the thermodynamic character of the native or denatured ensemble will be changing in the transition zone along with the [D]/[N] ratio. Application of the LEM in this case will result in a denaturant concentration-dependent ΔG°_{N-D}.

For ΔG°_{N-D} to be denaturant concentration-independent, the requirement that denaturant have no effect on the thermodynamic character of N or D states not only applies to the transition zone but also extends to the pre- and postdenaturation concentration ranges. As will be shown, a denaturant that alters the thermodynamic character of the native or denatured ensembles in the pre- or postdenaturation concentration range can lead to ΔG°_{N-D} having a different numerical value for urea-induced compared with GdnHCl-induced denaturation.

Here, our goal is to establish a parameter that will monitor the individual thermodynamic characters of the native and denatured ensembles. The proton binding sites on the N and D ensembles are numerous and the proton occupancies (i.e., proton inventory) at the various sites are sensitive to the local structural and chemical environments (*7*). Because of this strong linkage between protein structure and the thermodynamic property of proton occupancy, any change in the proton inventory of the (N or D) ensemble as a function of denaturant concentration at fixed pH (*7*) will be a measure of how the thermodynamic character of the species is altered by denaturant concentration.

The experimental parameter used in monitoring whether or how the thermodynamic character of the N and/or D states changes as a function of denaturant concentration is $\Delta\nu$, a quantity that measures the change in proton inventory (*7*). Such measurements involve mixing unbuffered protein in 0.2 M NaCl at a specified fixed pH with unbuffered guanidine hydrochloride (GdnHCl) in 0.2 M NaCl at the same fixed pH (*5, 6, 9, 10*). A pH change will occur if protons are taken up or given off on mixing, and with appropriate controls, the number of moles of proton change per mole of protein ($\Delta\nu$) is determined by back-titrating the solution to the original fixed pH value (*5, 6, 9, 10*). The results of such measurements define the thermodynamic character of the particular ensemble as a function of denaturant concentration and provide insight into the origin of denaturant-dependent ΔG°_{N-D} values.

MATERIALS AND METHODS

Descriptions of the batch method for evaluating $\Delta\nu$ have been published previously, and the apparatus used in these experiments, the means of quantifying the 0.01 M HCl (in 0.1 M NaCl) titrant concentration, and the procedures for evaluating $\Delta\nu$ have been amply described (*5, 6, 9, 10*).

Due to different choices of reference conditions, the $\Delta\nu$ data reported in the literature for PMS-Ct and α-chymotrypsin (α-Ct) do not begin with a zero value (*5, 9*). To compare results from different sources, a common reference state was selected so that all $\Delta\nu$ data begin with $\Delta\nu = 0$ at zero concentration of denaturant.

While the batch method for evaluating $\Delta\nu$ gives the most accurate results, it uses around 4−5 mg of protein per mixing experiment, with a final protein concentration of 1−1.5 mg/mL (*5, 6, 9*). To reduce the amount of protein used, we have developed a continuous titration method for evaluating $\Delta\nu$ as a function of GdnHCl concentration (*11*). The advantages of the continuous titration method are that it uses much less protein while giving many more points to define the $\Delta\nu$ vs GdnHCl concentration plot. The disadvantage is that systematic errors can occur in serial additions, so the batch method ultimately is more accurate. A description of the continuous titration method is provided below, which makes use of the titration apparatus previously described (*6, 9, 10*).

Wild-type SN (4 to 5 mg/mL) in 0.1 M NaCl was dialyzed overnight against freshly degassed unbuffered 0.1 M NaCl at neutral pH, 4 °C, and the protein and dialyzing solutions respectively were kept as sample and control solutions. The sample solution was adjusted to pH 4.5 with very small amounts of HCl (2−0.1 M HCl stocks) in 0.1 M NaCl, and solutions of 0.1 M NaCl (adjusted to pH 4.5) and 7.5 M GdnHCl in 0.1 M NaCl (adjusted to pH 8) were prepared and degassed in a MicroCal vacuum control box with attached diaphragm pump. The continuous titration procedure of $\Delta\nu$ measurements involves proton uptake/release measurements for protein sample and two controls (GdnHCl blank and protein dilution blank). In this procedure, serial additions of concentrated GdnHCl solution to the protein solution are made, and the number of protons taken up or released by the protein upon each GdnHCl addition is evaluated. To cover the 0−6 M GdnHCl concentration range, $\Delta\nu$ experiments were conducted in two stages, one from 0 to 3 M and the other from 3 to 6 M GdnHCl. For the 0−3 M range, exactly 2.00 mL of the wtSN sample solution at pH 4.5 and 2.00 mL of 0.1 M NaCl solution (pH 4.5) were individually delivered into the titration vessel, giving an initial protein concentration of 2−2.5 mg/mL. After mixing, the pH of the mixed solution was adjusted to exactly pH 4.500 with 0.1−2.0 M HCl or 0.1−2.0 M NaOH (CO_2-free) containing 0.1 M NaCl. The volumes of HCl or NaOH solutions added to the titration vessel to adjust pH were recorded.

In the first titration step, by use of a Hamilton dispenser (Micro Lab 500) equipped with two 100-μL Hamilton syringes, an aliquot of 25 μL of solution in the titration vessel was removed, and an aliquot of 25 μL of 7.5 M GdnHCl containing 0.1 M NaCl solution (pH 8) was added to the titration vessel with the same Hamilton dispenser. Since dilution of concentrated GdnHCl solution significantly decreases the pH of unbuffered solution, a pH of 8 for the 7.5 M GdnHCl stock solution was needed to obtain a pH of slightly higher than pH 4.500 on addition of 7.5 M GdnHCl stock to the titration vessel. This avoids the need for base titrant. After mixing, the pH of the solution in the titration vessel became slightly higher than pH 4.500 and was titrated back to exactly pH 4.500 with 0.01 M HCl titrant containing 0.1 M NaCl, and the volume of titrant was recorded [V_{HCli}(gs) where $i = 1$] along with the total volume at this step (Vol$i$). Sufficient time was allowed (3−30 min) to attain stable pH readings after each addition of HCl titrant to the titration vessel.

In the second titration step, another aliquot of 25 μL of the solution in the titration vessel was removed and 25 μL of 7.5 M GdnHCl solution was added. Again, the solution was back-titrated to pH 4.500 with HCl titrant and the titrant volume was recorded [V_{HCli}(gs) where $i = 2$]. Further additions of GdnHCl were repeated until [GdnHCl] in the titration vessel reached about 3 M, with V_{HCli}(gs) recorded after each step i.

GdnHCl blank experiments were conducted in the same manner as for protein sample except that the control solution (0.1 M NaCl at pH 4.5) was used in place of the protein sample solution. At each serial GdnHCl addition step (i = 1, 2, 3, ...), the volume of HCl titrant added, V_{HCli}(gb), was recorded.

Protein dilution blank experiments include two parts: protein dilution and control dilution. Protein dilution was conducted in the same manner as for protein sample except that at each serial addition step, 0.1 M NaCl solution at pH 8 was used in place of 7.5 M GdnHCl containing 0.1 M NaCl. After each serial addition of 0.1 M NaCl (dilution) (i = 1, 2, 3, ...), the volume of HCl titrant added, V_{HCli}(ds), was recorded along with the total volume in the titration vessel (Vol$i$). Dilution of the control was conducted in the same manner as for protein dilution except that the control solution was used instead of the protein sample solution. At each titration (dilution) (i = 1, 2, 3, ...), the volume of HCl titrant added for dilution of control, V_{HCli}(db), was recorded.

At each step i of GdnHCl addition (i = 1, 2, 3, ...), the volume of HCl titrant used in restoring protein sample solution to pH 4.500 was corrected by use of the GdnHCl blank. V_{HCli}(gb) for the GdnHCl blank was subtracted from V_{HCli}(gs) for protein sample, giving the net volume (see eq 1) of HCl titrant used, dV_{HCli}(g):

$$dV_{HCli}(g) = V_{HCli}(gs) - V_{HCli}(gb) \quad (1)$$

The protein concentration at one GdnHCl addition step (P_i) is different for each step due to different dilution factors. So at titration step i, protein concentration (P_i) is normalized to the initial protein concentration (P_0) by dividing dV_{HCli}(s) by the dilution factor of protein concentration (P_i/P_0). The normalized net volume of HCl titrant added to the protein sample, d$V_{HCli°}$(s), is given by

$$dV_{HCli°}(s) = dV_{HCli}(s)/(P_i/P_0) \quad (2)$$

$\delta\nu_\iota$, which represents the number of moles of protons taken up or released per mole of the protein at the ith titration step, is calculated from

$$\delta\nu_\iota = dV_{HCli°}(s)M_{HCl}/(P_0V_0) \quad (3)$$

Here, M_{HCl} represents the concentration of HCl titrant, P_0 represents the initial concentration of the protein (about 2−2.5 mg/mL), and V_0 represents the initial volume of the sample solution in the titration vessel. $\delta\nu_\iota$ value is the amount of hydrogen ion bound or released by the protein on addition of an aliquot of stock 7.5 M GdnHCl to the protein in going from step $i - 1$ to i.

The sum of $\delta\nu_\iota$ from titration step 1 to i gives $\Delta\nu$, representing the moles of protons taken up or released per mole of protein in going from zero to the total concentration of GdnHCl at step i.

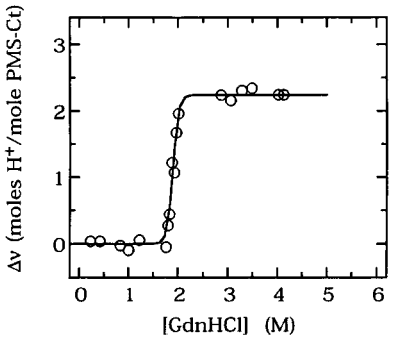


FIGURE 1: Moles of hydrogen ion per mole of protein taken up by PMS-Ct in 0.2 M KCl on denaturation induced by GdnHCl at pH 4.000, 25 °C. These data were obtained by the batch method of measuring $\Delta\nu$ as described by Bolen and Santoro (*5*). The final concentrations of GdnHCl (in 0.2 M KCl) after mixing are presented on the abscissa, and the final concentration of PMS-Ct for each point is in the range of 1−1.5 mg/mL. The data shown are replotted from Figure 3 of ref *5* after being referenced to zero denaturant as described under Materials and Methods herein. An uptake of 2.26 ± 0.08 mol of H^+/mol of PMS-Ct is shown to occur in the transition zone.

RESULTS

The $\Delta\nu$ data to be presented here are from a number of different workers spanning more than three decades in time (*5*, *6*, *9*, *10*, *12*). The denaturant used in all the experiments is GdnHCl. Nozaki and Tanford found long ago that aqueous solutions of GdnHCl are quite suitable for acid−base titrations of proteins and that pKs of titratable amino acid groups in 6 M GdnHCl differ by no more than 0.2 pK unit from their pKs in 0.1 M KCl (*13*−*17*). By contrast, urea solutions cause slow response in calomel and silver−silver chloride pH electrodes, and because of this poor response GdnHCl has been the preferred denaturant for $\Delta\nu$ and titration experiments.

With proper controls, Figure 1 presents changes in proton uptake/release on mixing unbuffered phenylmethanesulfonyl-chymotrypsin (PMS-Ct) in 0.2 M KCl (pH 4.000) with unbuffered GdnHCl containing 0.2 M KCl (pH 4.000) and then back-titrating to pH 4.000 to evaluate the proton uptake

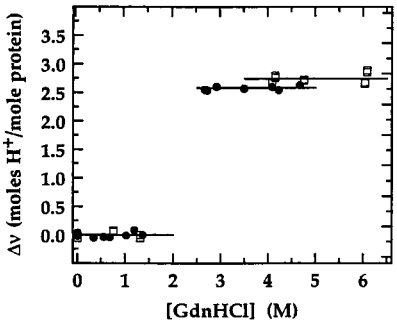


FIGURE 2: Proton uptake by α-chymotrypsin (Ct) (●) and furoyl-chymotrypsin (F-Ct) (□) on GdnHCl-induced denaturation at pH 3.500 and 25 °C in the presence of 0.2 M KCl. These data were obtained by the batch method of measuring $\Delta\nu$ as described by Huang and Bolen (*9, 10*). The final concentrations of GdnHCl (in 0.2 M KCl) after mixing are presented on the abscissa, and the final concentration of Ct or F-Ct for each point is in the range of 1−1.5 mg/mL. The data shown are replotted from Figure 3 of Huang and Bolen (*10*) after being referenced to zero denaturant as described under Materials and Methods herein.

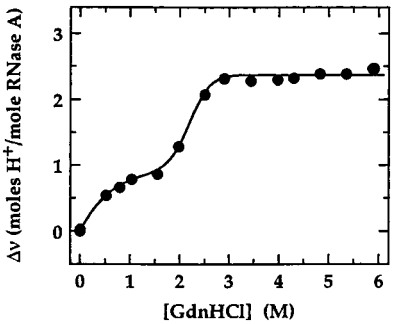


FIGURE 3: Proton uptake by RNase A on GdnHCl-induced denaturation at pH 3.500 and 25 °C in the presence of 0.2 M NaCl. These data were obtained by the batch method of measuring $\Delta\nu$ as described by Yao and Bolen (*6*). The final concentrations of GdnHCl (in 0.2 M NaCl) after mixing are presented on the abscissa, and the final concentration of RNase A for each point is in the range of 1−1.2 mg/mL. The data shown are replotted from Figure 4 of ref *6*.

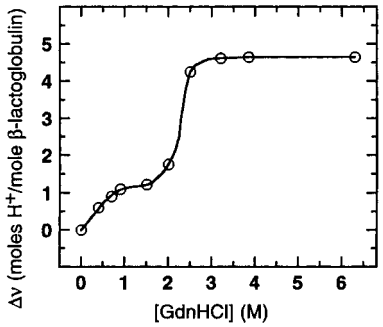


FIGURE 4: Proton uptake by β-lactoglobulin on GdnHCl-induced denaturation at pH 6.500 and 25 °C in the presence of 1 M KCl. These data were obtained by the direct difference titration method as described by Nozaki and Tanford (*12*). The solution at zero GdnHCl contained 1 M KCl, and final concentrations of GdnHCl containing 1 M KCl after mixing are presented on the abscissa. The data shown are replotted from Figure 6 of Nozaki and Tanford (*12*).

($\Delta\nu$) (*5*). By use of $\Delta\nu$ as an observable, these data show the GdnHCl-induced transition between native and denatured states that occurs in the GdnHCl concentration range from 1.5 to 2.5 M GdnHCl. It is clear from this figure that proton uptake only occurs in the transition range, where the ratio of [N]/[D] changes. Significantly, the $\Delta\nu$ values in the pre- and postdenaturation GdnHCl concentration ranges do not change with GdnHCl concentration, showing that no GdnHCl-induced perturbation occurs for the proton inventories of either the native or the denatured ensembles of PMS-Ct. Similarly, Figure 2 gives the results of $\Delta\nu$ experiments taken from the literature on α-chymotrypsin and furoyl-chymotrypsin (*9, 10*). Unfortunately, no $\Delta\nu$ measurements were taken in the transition zone, but it is evident that no proton uptake or release occurs in the pre- and postdenaturation concentration ranges, signifying that no GdnHCl-induced changes occur in the proton inventories of the native and denatured species. These data provide examples of GdnHCl-induced denaturations of a class of proteins in which proton uptake only occurs as a result of conversion of native to denatured protein; the proton inventories of native and denatured ensembles themselves are unaffected by the presence of GdnHCl.

Figures3 and 4 present examples of a separate class of proteins with a more complex pattern of $\Delta\nu$ dependence on GdnHCl concentration. As shown previously for ribonuclease A (RNase A) denaturation by GdnHCl at pH 3.50 (Figure 3), the transition from native to denatured protein occurs within the sigmoidal part of the data between 1.5 and 3 M GdnHCl (*6*). While the $\Delta\nu$ in the postdenaturational concentration range beyond 3 M GdnHCl is independent of denaturant concentration, $\Delta\nu$ is dependent on GdnHCl concentration in the predenaturation range in which the native-state ensemble is believed to exist. Clearly, GdnHCl is affecting the proton inventory of the native-state ensemble, changing $\Delta\nu$ by 0.8 mol of proton/mol of protein in the predenaturation range from zero to 1.5 M GdnHCl. A similar pattern of $\Delta\nu$ vs [GdnHCl] is also exhibited in the pH 6.50 GdnHCl-induced denaturation of β-lactoglobulin as shown in Figure 4 (*12*). As with RNase A, the proton inventory of β-lactoglobulin changes most in the N ⇄ D transition range, it is invariant with GdnHCl concentration in the posttransition range, and it varies with denaturant concentration in the predenaturation zone.

Of the proteins studied thus far, wild-type staphylococcal nuclease (wtSN) presents the most complex dependence of $\Delta\nu$ on GdnHCl concentration. At pH 4.50, Figure 5A shows that the $\Delta\nu$ versus [GdnHCl] profile for wtSN bears no resemblance to $\Delta\nu$ versus [GdnHCl] plots of either of the other two classes of proteins. In this figure, we have also included results of GdnHCl-induced N ⇄ D denaturation of wtSN at pH 4.5 (solid line) as detected by fluorescence emission and expressed in terms of fraction denatured. To provide greater clarity, the results in the transition zone are expanded in Figure 5B. It can be seen that $\Delta\nu$ is changing in the transition zone, but unlike the cases involving RNase A, β-lactoglobulin, chymotrypsin, and PMS-Ct, $\Delta\nu$ does not track with the fraction denatured. It is evident that $\Delta\nu$ increases dramatically over the same GdnHCl concentration range (0−0.2 M) in which the native-state population changes little. At GdnHCl concentrations (≥0.9 M) where the fraction of denatured protein is nearly 100%, $\Delta\nu$ continues to change with increasing denaturant concentration. The data show that the thermodynamic characters of the N and D states are changing both within and outside the transition zone and that the fluorescence and proton inventory results do not correspond with one another.

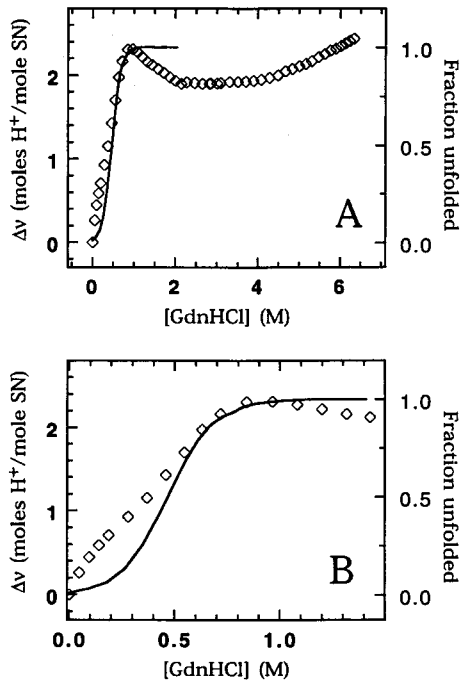


FIGURE 5: Proton uptake by wtSN on GdnHCl-induced denaturation at pH 4.500 and 25 °C in the presence of 0.1 M NaCl. These data were obtained by the continuous titration method of measuring $\Delta\nu$ as described under Materials and Methods. The final concentrations of GdnHCl (in 0.1 M NaCl) after mixing are presented on the abscissa. The initial wtSN concentration at the beginning of the titration procedure was 2−2.5 mg/mL. The solid line is the fraction denatured wtSN as evaluated from fluorescence-detected denaturation of the protein performed at pH 4.50 and 25 °C in the presence of 0.1 M NaCl. Panel A gives the full range of GdnHCl concentrations; panel B gives an expanded view of just the transition zone.

DISCUSSION

The puzzle of why some proteins give the same LEM-determined $\Delta G°_{N-D}$ values from urea and GdnHCl denaturation and some do not has served as a serious challenge to the use of $\Delta G°_{N-D}$ values as measures of protein stability (*6*, *18*). Lack of agreement between urea- and GdnHCl-derived $\Delta G°_{N-D}$ values emphasizes a gap in understanding what these quantities mean and how they are to be interpreted. At least part of the problem lies in the ensemble nature of the native and denatured states and how these ensembles depend on denaturant and denaturant concentration. That is, in monitoring the N ⇄ D transition it is essential to know how denaturant affects the thermodynamic properties of the N and D ensembles as well as the effect of denaturant on the transition. No spectral parameter provides direct measurements on the thermodynamics of the individual N and D species nor the thermodynamics of the transition. To monitor all these aspects of the protein, we require a thermodynamic property as an observable, one that is sensitive to the N ⇄ D transition and the structural thermodynamics of the individual N and D ensembles. A parameter that reports on a thermodynamic property of an ensemble will be monitoring an aspect of what we shall call the *thermodynamic character* of the ensemble.

A thermodynamic property that provides the sensitivity for monitoring the thermodynamic character of native and denatured ensembles is the proton occupancy or proton inventory of the ensemble. The reason proton inventory is a good choice for reporting on thermodynamic character is that protein stability and protein structure are inextricably linked to the proton inventories of the respective native and denatured ensembles, and this linkage accounts for the full pH-stability profile of the protein (*6*, *7*). The numbers of protons associated with the native and denatured ensembles are determined by the ensemble-averaged pK_as of the ionizable groups on these protein species (*7*). Because native and denatured species of a protein are structurally quite different, one can generally expect that at any given pH the proton inventories of the native and denatured ensembles will be measurably different from one another. The exact relationship between the Gibbs energy of the protein species and the proton inventory is expressed in the Wyman relationship (*19*):

$$(\partial \ln \Sigma/\partial \mathrm{pH}) = -\nu \qquad (4)$$

Here, Σ is the proton binding polynomial of the ensemble, a quantity that directly involves the Gibbs energy of the ensemble, and ν is the degree of proton binding or proton inventory (*20*). If a denaturant such as GdnHCl is added to the protein solution and alters the proton inventory, eq 5 is obtained:

$$(\partial \ln K/\partial \mathrm{pH}) = \Delta\nu \qquad (5)$$

in which $K = (\Sigma_{GdnHCl}/\Sigma_{zeroGdnHCl})$ is the ratio of proton binding polynomials of the ensembles with and without GdnHCl and $\Delta\nu$ is the difference in proton inventory between the two GdnHCl conditions the protein is in at the given pH (*7*). Thus, $\Delta\nu$ can be used to discover whether (and by how much) the denaturant is altering the thermodynamic character of an ensemble as well as to monitor the transition between ensembles. Experiments involving $\Delta\nu$ as a function of GdnHCl have been reported for several proteins (*5*, *6*, *9*, *10*, *12*), and the patterns that occur provide insight into the thermodynamic effects of denaturing solutes both within and outside the transition zone. Three classes of proteins that differ in the manner in which $\Delta\nu$ values depend on denaturant concentration are presented below, along with the thermodynamic consequences of these dependencies.

PMS-Ct: A Case with "Fixed" Thermodynamic Character of Native and Denatured Ensembles

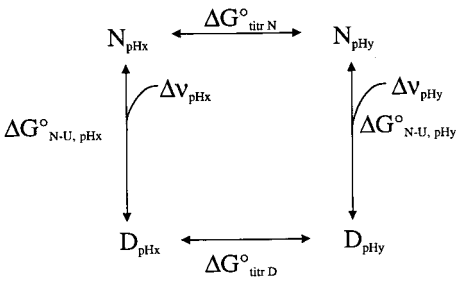
The $\Delta\nu$ vs GdnHCl profile for PMS-Ct (Figure 1) exhibits the simplest behavior of the proteins studied. The baselines in the pre- and postdenaturation ranges are independent of denaturant concentration, meaning that the thermodynamic characters of the native and denatured ensembles with respect to denaturant concentration are invariant or "fixed" (*21*−*23*). It is clear from Figure 1 that all of the $\Delta\nu$ occurs in the transition zone, demonstrating that the change in Gibbs energy between native and denatured ensembles is associated exclusively with the ratio of the populations of N and D. Under these circumstances, application of the LEM will provide the desired result of a denaturant concentration-independent $\Delta G°_{N-D}$. If this $\Delta G°_{N-D}$ is truly a property of the protein, independent of denaturant concentration, it will also likely be independent of the nature of the denaturant. Table 1 shows that within error urea, GdnHCl, and dimethylurea all give the same $\Delta G°_{N-D}$ for PMS-Ct (*5*). The

Table 1: LEM Determined ΔG°_{N-D} Values at 25 °C

protein	denaturant	pH	ΔG°_{N-D}	*m*
PMS-Ct	urea	4.0	9.20 ± 0.45[a]	2.22
PMS-Ct	GdnHCl	4.0	8.69 ± 0.44[a]	4.19
PMS-Ct	1,3-di methylurea	4.0	8.83 ± 0.56[a]	3.0
RNase A	urea	3.0	4.48 ± 0.21[b]	1.85
RNase A	GdnHCl	3.0	5.07 ± 0.10[b]	2.77
β-lacto globulin	urea	3.2	10.5[c]	2.10
β-lacto globulin	GdnHCl	3.2	12.5[c]	3.89
wtSN	urea	4.50	3.59 ± 0.08[d]	1.80
wtSN	GdnHCl	4.50	2.45 ± 0.07[d]	2.40

[a] Reference *4*; 0.2 M ionic strength from 50 mM acetate buffer in 0.14 M NaCl. [b] Reference *6*; 0.2 M ionic strength from 0.1 M β-alanine buffer containing 0.07 M NaCl. [c] Reference *1*. [d] Errors reported are multiple measurements of GdnHCl and urea denaturation. Errors for footnotes *a* and *b* are fitting errors.

Scheme 1



LEM-determined ΔG°_{N-D} meets essential criteria to qualify it as a property of the protein alone (*5*, *6*, *24*).

A most important consequence of "fixed" thermodynamic character of the native and denatured ensembles and the resulting denaturant-independent ΔG°_{N-D} is that it is possible to determine the entire pH-dependent stability profile for the protein in a model-independent manner (*6*, *9*, *10*). Scheme 1 defines a relationship

$$\Delta G^\circ_{N-D,pHy} - \Delta G^\circ_{N-D,pHx} = \Delta G^\circ_{titrD} - \Delta G^\circ_{titrN} \quad (6)$$

that combines two types of measurements: $\Delta G^\circ_{N-D,pHy}$ and $\Delta G^\circ_{N-D,pHx}$ are LEM-determined denaturation Gibbs energy changes at pH *x* and *y*, and ΔG°_{titrD} and ΔG°_{titrN} represent potentiometrically determined titration Gibbs energy differences between D and N states over the pH range from *x* to *y*. The terms on the left side of eq 6 can be determined from LEM analysis, and those on the right can be determined from potentiometric titration data. In evaluating the right side of eq 6, it can be shown that

$$\Delta G^\circ_{titrD} - \Delta G^\circ_{titrN} = 2.303RT \int_{pH_x}^{pH_y} \Delta\nu \, d\mathrm{pH} \quad (7)$$

where $\Delta\nu$ is the difference in proton inventory between the N and D states and can be determined as a function of pH directly from the potentiometric titration curves for native and denatured protein over the pH range from *x* to *y* (*9*). Numerical integration of $\Delta\nu$ as a function of pH as specified by eq 7 will give an evaluation of the pH-stability profile for the protein without the need for analyzing transitions involving a two-state model (*5*, *6*, *9*, *10*). This evaluation is said to be model-independent.

From the perspective of the LEM, the $N_{pHx/y}$ and $D_{pHx/y}$ species represented in Scheme 1 are at fixed pH at 0 M denaturant. From the perspective of potentiometric titration, ΔG°_{titrN} can be obtained readily by potentiometric titration of the native state from pH*x* to pH*y* in the absence of denaturant. However, the denatured state is not populated at 0 M denaturant and this causes a practical problem in how to obtain ΔG°_{titrD} experimentally. That practical issue is solved by performing the potentiometric titration of the protein in the presence of 6 M GdnHCl.

At first glance, it may not seem legitimate to use the titration of 6 M GdnHCl-denatured protein to represent the same denatured state define in $\Delta G^\circ_{N-D,pHy}$ or $\Delta G^\circ_{N-D,pHx}$ through LEM analysis. Justification for using 6 M denatured protein comes from the fact that, in the LEM extrapolation, the properties of the N $\rightleftarrows$ D equilibrium in the transition zone are being projected to 0 M denaturant. This means that the ΔG°_{N-D} obtained at 0 M denaturant retains the attributes of the N and D states that occur in the transition zone; these states do not have the attributes (e.g., degree of compactness of the D state or certain thermodynamic characteristics) of the N and D ensembles that actually occur in 0 M denaturant (*5*, *25*−*28*). In a case like PMS-Ct, the proton inventories of both N and D are invariant (fixed) in the transition zone, and when these species are extrapolated via the LEM to 0 M denaturant, they retain the same proton inventories they had in the transition zone. This means that, with regard to proton inventory, 6 M GdnHCl-denatured PMS-Ct is a proper model for the denatured species represented in ΔG°_{N-D}, and potentiometric titration of 6 M GdnHCl-denatured PMS-Ct will give the same pH-titration profile as the denatured species represented in ΔG°_{N-D} (*5*, *6*).

An important facet of $\Delta\nu$ as a thermodynamic parameter, then, is that all information about the pH dependence of protein stability difference between the native and denatured ensembles of the protein is manifested in the pH dependence of $\Delta\nu$ (*5*−*7*, *9*, *10*). This condition holds as long as the thermodynamic characters of the native and denatured ensembles are "fixed". Scheme 1 provides a means to test whether the model-dependent LEM-determined ΔG°_{N-D} values can be verified by the model-independent potentiometric titrations of native and denatured protein. PMS-Ct has been shown to obey the constraints of the thermodynamic cycle (Scheme 1) and because $\Delta G^\circ_{N-D,pHy} = (\Delta G^\circ_{titrD} - \Delta G^\circ_{titrN} + \Delta G^\circ_{N-D,pHx})$, $\Delta G^\circ_{N-D,pHy}$ is path-independent (*5*). A path-independent ΔG°_{N-D} is a direct demonstration that this ΔG°_{N-D} is a thermodynamic function of state. The ability to show that a LEM-determined ΔG°_{N-D} is a thermodynamic function of state provides strong independent evidence that ΔG°_{N-D} for PMS-Ct is a property of the protein alone (*5*, *6*).

Cases Involving "Variable" Thermodynamic Character in the Native-State Ensemble but with "Fixed" Thermodynamic Character in the Denatured Ensemble

RNase A. The $\Delta\nu$ data for RNase A as a function of GdnHCl concentration exhibit a significant $\Delta\nu$ in the transition zone (1.5−3 M GdnHCl), reflective of the changing ratio of N $\rightleftarrows$ D within this limited concentration range. The independence of $\Delta\nu$ on GdnHCl in the postdenaturation range defines the thermodynamic character of the denatured

ensemble as "*fixed*" (*21*−*23*). This means that 6 M GdnHCl-denatured RNase A can be used to represent the proton inventory of the LEM-determined denatured ensemble at the limit of zero denaturant concentration and that titration of the protein in the presence of 6 M GdnHCl can be used to obtain $\Delta G°_{titrD}$ (Scheme 1). What distinguishes RNase A from PMS-Ct is that GdnHCl causes $\Delta\nu$ to change in the predenaturational zone of RNase A, and this defines the thermodynamic character of the native-state ensemble as being "*variable*"or denaturant concentration-dependent. The significance of the "variable" thermodynamic character of the ensemble in the predenaturation range is that $\Delta G°_{N-D}$ determined by the LEM for GdnHCl-induced denaturation will be denaturant-dependent, i.e., $\Delta G°_{N-D}$ will not be solely a property of the protein (*22*, *23*). The denaturant concentration dependence of $\Delta G°_{N-D}$ makes it much less valuable as a thermodynamic quantity for two reasons: first, it is not legitimate to interpret it simply as the Gibbs energy difference between N and D states, as is the common practice; and second, the LEM-determined $\Delta G°_{N-D}$ values from GdnHCl cannot be shown to be a denaturant concentration-independent function of state through application of Scheme 1.

In earlier work on RNase A we have shown that (unlike GdnHCl) urea does not alter $\Delta\nu$ in the predenaturational zone, so the native ensemble in the presence of urea exhibits "fixed" thermodynamic character (*6*). The fact that the native ensemble in the presence of urea exhibits "fixed" thermodynamic character while with GdnHCl it is "variable" means that LEM analysis of urea denaturation will necessarily extrapolate to a different $\Delta G°_{N-D}$ from that given by LEM analysis of GdnHCl denaturation of RNase A. Such results are shown in Table 1. Nonagreement of $\Delta G°_{N-D}$ values from two denaturants calls into question one or both of the quantities, so to provide more insight into the meaning of these $\Delta G°_{N-D}$ values we determine whether either of the values fits the criterion of being a thermodynamic function of state. By application of Scheme 1, we have shown previously with RNase A that the $\Delta G°_{N-D}$ obtained from urea denaturation exhibits the property of being a thermodynamic function of state, while the LEM-determined $\Delta G°_{N-D}$ from GdnHCl does not (*6*). This important test shows that the *urea-determined* $\Delta G°_{N-D}$ values obtained through application of the LEM are solely properties of this protein and that the thermodynamic character of the native and denatured ensembles as a function of urea concentration are "fixed". It is also significant that while the native ensembles of RNase A in urea and GdnHCl are "fixed" and "variable" (respectively) and are not thermodynamically equivalent to one another, the RNase A denatured ensembles in high urea and 6 M GdnHCl are "fixed" and are thermodynamically identical to one another (*6*).

In the case of RNase A, it is useful to ask why GdnHCl affects the thermodynamic character of the native RNase A while urea does not. When urea denaturation of RNase A was conducted in the presence of various NaCl concentrations, the urea-derived $\Delta G°_{N-D}$ increased to values much closer to the GdnHCl-derived $\Delta G°_{N-D}$ (*6*). Undoubtedly, the effects have to do with the fact that GdnHCl is a salt and perturbs the proton inventory of the native-state ensemble. While earlier work made some progress in understanding the importance of salt in comparing urea- and GdnHCl-induced denaturation (*24*), Monera et al. (*29*) were first to show the strong electrostatic screening effects GdnHCl has on protein stability and the native state. More recently, Ibarra-Molero et al. (*30*) have also given a striking example of this effect. There is also the possibility that specific site binding by GdnHCl to the native state can have effects on protein stability (*1*, *4*, *6*, *25*, *31*), and it is likely that specific site binding would also alter proton inventory of the native-state ensemble.

Though electrostatic screening and/or specific site binding of GdnHCl to the native state are the most likely causes of the changes in proton inventory with the native-state ensemble, they may not be the source of all proton inventory effects with the denatured ensemble. As the compact denatured ensemble at low denaturant concentration expands with increasing concentration of denaturant, the resulting changes in distance between ionizable residues should cause changes in the proton inventory of the ensemble. Expansion of the denatured ensemble with increasing denaturant concentration will occur regardless of the denaturant, so in principle, "variable" thermodynamic behavior in the denatured ensemble can arise with either urea or GdnHCl.

From the perspective of proton inventory, urea- and GdnHCl-induced denaturations of RNase A give different $\Delta G°_{N-D}$ values because GdnHCl concentration causes the native ensemble to exhibit "variable" thermodynamic character while urea concentration results in "fixed" thermodynamic character of N. Scheme 1 and $\Delta\nu$ are key experiments in providing a means to diagnose and distinguish denaturant concentration-dependent and independent $\Delta G°_{N-D}$ values.

β-Lactoglobulin. The $\Delta\nu$ vs GdnHCl profile reported for β-lactoglobulin is identical in form to that of RNase A, with the major change in $\Delta\nu$ occurring in the transition zone (1.5−3 M GdnHCl) (*12*). Like RNase A, the native-state ensemble of β-lactoglobulin exhibits "variable" thermodynamic character in the predenaturation concentration range but "fixed" thermodynamic character of the denatured ensemble. The prediction is that GdnHCl-derived $\Delta G°_{N-D}$ values will be denaturant-dependent, that they cannot be successfully accommodated in the thermodynamic cycle of Scheme 1, and that it will not be legitimate to interpret the quantity as a property solely in terms of the protein components of the N ⇄ D equilibrium. If urea gives "fixed" thermodynamic behavior in the native ensemble of β-lactoglobulin, we would then expect urea-induced denaturation of β-lactoglobulin to give denaturant concentration-independent $\Delta G°_{N-D}$ values.

Nearly all the $\Delta\nu$ data presented here were taken at low pH. The data for β-lactoglobulin, however, were taken in the neutral pH range (pH 6.5) and show that significant proton inventory changes can occur at pH ranges well removed from the acidic and basic ranges where most ionizations take place.

A Case of "Variable" Thermodynamic Character in both the Native and Denatured Ensembles

Staphylococcal Nuclease. Unlike the other proteins studied, the denatured ensemble of wtSN exhibits "variable" two-state thermodynamic behavior (*21*−*23*). Even in 6 M GdnHCl the thermodynamic character of the denatured ensemble continues to change with GdnHCl concentration

(see Figure 5A). This means that 6 M GdnHCl is insufficient either to expand the dimensions of the ensemble to the point that ionizable groups are no longer perturbed or to screen all electrostatic effects. The fixed thermodynamic characters of the denatured ensembles of PMS-Ct, RNase A, and β-lactoglobulin clearly distinguish these relatively well-behaved proteins from this third class of proteins.

Within the transition zone, the inability of $\Delta\nu$ to track with the fluorescence-detected denaturation of wtSN in Figure 5B means that the $\Delta\nu$ associated with the changing ratio of the N and D states is not the only proton uptake/release process accompanying the transition. The changing behavior of $\Delta\nu$ in the pre- and postdenaturation concentration zones immediately adjacent to the transition zone strongly suggests that the thermodynamic characters of the N and D states are changing at the same time the ratio of [D]/[N] is changing (*23*, *32*, *33*). The thermodynamic consequence of the changing thermodynamic character of N and D in the transition zone is that the species of N and D at the beginning of the transition are thermodynamically distinct from the N and D species at the end of the transition. That is, the transition is composed not of one equilibrium, $N \rightleftarrows D$, but of a series of native to denatured equilibria represented by $N_A \rightleftarrows D_\alpha$ at the beginning of the transition and ending with $N_Z \rightleftarrows D_\zeta$ at the end of the transition (*22*, *23*):

$$N_A \rightleftarrows D_\alpha$$
$$N_B \rightleftarrows D_\beta$$
$$\vdots$$
$$N_Z \rightleftarrows D_\zeta$$

The thermodynamic character of the native-state ensemble is changing through a continuum of stages from A to Z as GdnHCl is increased, while the denatured ensemble proceeds through stages α to ζ. Thus, when the LEM is applied to the fluorescence vs GdnHCl data, the apparent ΔG°_{N-D} obtained is not representative of a single $N \rightleftarrows D$ equilibrium; rather, it is a complex quantity representing a continuum of reactions.

For proteins that exhibit "variable" thermodynamic character in their native and denatured ensembles either within or outside the transition zone, the *apparent* ΔG°_{N-D} values cannot have the meaning currently ascribed to them in the field of protein folding. The quantities are highly ambiguous in terms of the degree to which they reflect the Gibbs energy differences between the denaturant concentration-dependent native and denatured states, and the additional ambiguity of the multitude of thermodynamically changing N and D species within the transition also renders any clean interpretation impossible.

Several additional studies support this view of the variable behavior of the native and denatured ensembles of wtSN and the thermodynamic consequences of this behavior on the LEM. Yang et al. (*23*) have used calorimetric heats of mixing measurements of GdnHCl with wtSN to describe the enthalpy changes as a function of GdnHCl within the $N \rightleftarrows D$ transition. The sharpness of the transition defined by the calorimetric measurements deviates from that defined by fluorescence measurements in a manner suggestive of variable thermodynamic behavior. In a more recent report, we have studied the effect of GdnHCl on both $\Delta\nu$ and the Stokes radius of reduced and carboxyamidated ribonuclease A (RCAM) (*33*), a member of a class of disulfide-free RNase A derivatives considered to be random coils in aqueous solution (*34*−*37*). The measurements show that $\Delta\nu$ changes measurably for RCAM over the 0−1.5 M GdnHCl range and marginally, if at all, beyond 1.5 M denaturant. RCAM is also observed to expand as a function of increasing [GdnHCl], with the greatest increase in Stokes radius occurring in the 0−1.5 M GdnHCl range. These results show that even for a denatured protein already known to be highly expanded in aqueous solution, a measurable $\Delta\nu$ is observed with increasing [GdnHCl] that is correlated with expansion of the ensemble. Thus, it is highly likely that the denatured ensembles of proteins that denature with a C_m in the range of 0−1.5 M GdnHCl will exhibit "variable" thermodynamic character, and analysis of the denaturation data by LEM should give denaturant-dependent ΔG°_{N-D} values (*33*). With wtSN, the C_ms of 0.8 and 2.5 M for GdnHCl and urea denaturations, respectively, are quite low (*22*), and the respective denatured states are significantly more compact at the beginning of the transition than at the end. By way of example, the Stokes radius of wtSN is 29.2 Å at the beginning of the urea-induced denaturation transition and 32.8 Å at the end [evaluated from the data in Baskakov and Bolen (*22*)]. For the denatured ensemble represented by the Stokes radius, this amounts to an increase of 40% in volume and 25% in surface area taking place within the transition zone. Naturally, the denatured ensemble is not an impenetrable sphere, but it is clear that the expansion of the ensemble is significant and the average distance between ionizable side chains will be increased, enhancing the potential for p*K* changes. Given the strong linkage between proton inventory and protein structure, it would be surprising if the changes in dimensions of the denatured ensemble within the transition zone would not lead to accompanying changes in proton inventory.

It is important to note that with PMS-Ct, RNase A, β-lactoglobulin, and chymotrypsin, the C_m values for GdnHCl are all above 1.5 M GdnHCl and all of their denatured ensembles exhibit "fixed" thermodynamic character (*1*, *4*, *6*, *31*). Apparently, at these higher GdnHCl concentrations the denatured ensembles of these proteins are sufficiently expanded and electrostatic screening effects are sufficiently complete that the proton inventory becomes invariant with GdnHCl concentration in the transition zone and the post-denaturation range.

CONCLUSIONS

Currently in the field of protein folding, LEM-determined ΔG°_{N-D} values are tacitly assumed to be a property of the protein alone, and these quantities are invariably interpreted as being the Gibbs energy difference solely between the N and D states at the limit of zero denaturant concentration (*7*). However, only for those proteins that exhibit "fixed" thermodynamic character in their native and denatured states is such an interpretation valid. Denaturants that cause "variable" thermodynamic character either in the native ensemble and/or in the denatured ensemble will always give a LEM-determined ΔG°_{N-D} that is denaturant or denaturant concentration-dependent in one way or another. For these proteins, the LEM-determined ΔG°_{N-D} cannot be success-

fully accommodated in the thermodynamic cycle given in Scheme 1, because the $\Delta G°_{N-D}$ values for these proteins fail the criterion of being a (denaturant concentration-independent) function of state. It is also not correct to compare or contrast the $\Delta G°_{N-D}$ values of wild-type and mutant species of proteins that exhibit "variable" native and/or denatured ensembles within the transition zone. The reason is that the m and/or C_m values will generally be different for the proteins being compared, and these differences involve different dependencies on denaturant concentration in the transition zone. In short, $\Delta\Delta G°_{N-D}$ quantities for such proteins have no stronger basis for interpretation than do the individual denaturant concentration-dependent values of $\Delta G°_{N-D}$.

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