Protein Stability: Functional Dependence of Denaturational Gibbs Energy on Urea Concentration†

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Received August 26, 1998; Revised Manuscript Received November 10, 1998

ABSTRACT: Determination of protein stability (ΔG_D^0) from the conformational transition curve induced by a chemical denaturant is problematic; for different values of ΔG_D^0, the value of the Gibbs energy change on denaturation (ΔG_D) in the absence of the denaturant are obtained when different extrapolation methods are used to analyze the same set of (ΔG_D, denaturant concentration) data [Pace, C. N. (1986) *Methods Enzymol. 131,* 266−280]. We propose a practical solution to this problem and use it to test the dependence of ΔG_D of lysozyme, ribonuclease-A, and cytochrome-c on [urea], the molar urea concentration. This method employs (i) measurements of the urea-induced denaturation in the presence of different guanidine hydrochloride (GdnHCl) concentrations which by themselves disrupt the native state of the protein at the same temperature and pH at which denaturations by urea and GdnHCl have been measured; (ii) estimation of ΔG_D^{cor}, the value of ΔG_D corrected for the effect of GdnHCl on the urea-induced denaturation using the relation ($\Delta G_D^{cor} = \Delta G_D + m_g$ [GdnHCl] $= \Delta G_D^0 - m_u$ [urea], where m_g and m_u are the dependencies of ΔG_D on [GdnHCl] and [urea], respectively) whose parameters are all determined from experimental denaturation data; and (iii) mapping of ΔG_D^{cor} onto the ΔG_D versus [urea] plot obtained in the absence of GdnHCl. Our results convincingly show that (i) [urea] dependence of ΔG_D of each protein is linear over the full concentration range; (ii) the effect of urea and GdnHCl on protein denaturation is additive; and (iii) KCl affects the urea-induced denaturation if the native protein contains charge−charge interaction and/or anion binding site, in a manner which is consistent with the crystal structure data.

We have been interested for some time in the determination of protein stability, defined as the decrease in Gibbs energy of the structureless protein polypeptide chain when it folds to give a native protein molecule in water (or dilute buffer), from the conformational transition curves induced by chemical denaturants at constant pH and temperature (*1*−*8*). This method of estimation involves the determination of values of ΔG_D,[1] the Gibbs energy change of denaturation, and their extrapolation to 0 M denaturant concentration, using different denaturation models, namely, the linear free energy model (*9*, *10*), the binding site model (*11*, *12*), and the transfer free energy model (*4*, *11*). It has been observed that, for a protein, different extrapolation procedures give different values of protein stability (ΔG_D^0 [1]), the value of ΔG_D at zero concentration (or activity) of the denaturant. The fact that a given equilibrium, N (native) conformation ↔ D (denatured) conformation, can have only one value of ΔG_D^0 brings into question the validity of extrapolation procedures.

Two approaches have widely been used to test the validity of various extrapolation methods. One approach is to study the isothermal denaturation of several proteins by two (*9*) and more (*3*) chemical denaturants inducing the same transition between N and D states at constant temperature and pH, and demonstrate that all denaturants give the same value of ΔG_D^0, as they should. Another method developed by Bolen and Santoro (*13*) is to (i) study urea and Guanidine hydrochloride (GdnHCl[1]) denaturation of a protein at two different pH values, (ii) incorporate ΔG_D^0 values at these pH values into the thermodynamic cycle involving Gibbs energy change for titration of native and denatured protein molecules from one pH to another, and (iii) demonstrate that ΔG_D^0 has the property of predictability and is independent of path as required for all thermodynamic functions. A linear dependence of ΔG_D on [denaturant], the molar concentration of the denaturant, has been supported by both approaches. It should, however, be noted that the agreement between estimates of ΔG_D^0 from GdnHCl, the ionic denaturant, and urea, the nonionic denaturant, could be coincidental, for the isothermal denaturation of proteins has been carried out at a low ionic strength. Indeed, it has been observed in several cases that, for a protein, ΔG_D^0 from urea denaturation not only shows a strong dependence on the ionic strength of the protein solution but also is different from that associated with the GdnHCl-induced transition (*3*, *8*, *9*, *14*−*17*). If ΔG_D^0 shows the denaturant dependence, then this means that ΔG_D^0 associated with the equilibrium, N ↔ D, cannot be regarded as the property of the protein alone.

An unequivocal value of ΔG_D^0 of a protein can be obtained if the extrapolation region is reduced to 0 M denaturant

† Supported by the Department of Science and Technology Grant (SP/SO/D-26/96).

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[1] Abbreviations: ΔG_D, Gibbs energy of denaturation; ΔG_D^0, Gibbs energy of denaturation in absence of the denaturant; GdnHCl, guanidine hydrochloride; RNase-A, ribonuclease-A; cyt-c, ferric cytochrome-c; m_g, dependence of ΔG_D on GdnHCl concentration; m_u, dependence of ΔG_D on urea concentration; C_m, the denaturant concentration at which ΔG_D is zero.

10.1021/bi982078m CCC: $18.00 © 1999 American Chemical Society
Published on Web 01/30/1999

concentration. Several ways have been suggested to reduce the extrapolation region to zero concentration. The earlier approach (*18*) involves the measurements of isothermal GdnHCl-induced denaturation of a protein at several pH values and the normalization of ΔG_D values measured at different pH values to a common pH using a standard method. It has been shown that this approach is problematic (*6*). The reason for this is that, at extreme pH values, proteins are acid-denatured in the absence of GdnHCl; hence, the acid-denatured state contributes to the equilibrium between N and D states leading to curvature in the plot of ΔG_D determined using a two-state assumption, versus [denaturant] at these pH values. Another method to reduce the extrapolation region to 0 M denaturant combines the ΔG_D data at 25 °C from GdnHCl-induced denaturation followed by a conformational technique and differential scanning calorimetry measurements at low denaturant concentrations (*19*). It has been argued that this method will not work for all proteins (*7*). Another approach that seems to make the ΔG_D determination in the pretransition region of a GdnHCl-induced denaturation curve of the protein possible, exploits the measurements of the rate of exchange of the slowest peptide group hydrogens (*20*−*22*). However, it has been argued that several factors can make ΔG_D from exchange experiments differ from the value of ΔG_D measured by classical techniques such as fluorescence spectroscopy (*21*, *22*). We have recently suggested another procedure to reduce the extrapolation region to 0 M GdnHCl (*7*). It involves measurements of GdnHCl-induced denaturations of the protein in the presence of different concentrations of urea at constant temperature and pH. ΔG_D values from these measurements in the presence of GdnHCl−urea mixture were corrected for the effect of urea on the GdnHCl-induced denaturation using an appropriate relation. Although this method has at least two advantages, namely, both urea and GdnHCl give the same unfolded states of the protein and all measurements are made at the same temperature and pH, one assumption that the functional dependence of ΔG_D is linear over the full [urea] range was introduced.

Although a linear dependence of ΔG_D on [urea] has support from the theoretical models (*10*, *23*, *24*), there are some reports of curvature in such plots (*25*, *26*). To the best of our knowledge there are only two denaturation studies in which measurement of ΔG_D has been extended to 0 M urea (*8*, *26*). These studies, however, led to different conclusions regarding the dependence of ΔG_D on [urea]; ΔG_D of Barnase (*26*) and that of metmyoglobin (*8*) showed, respectively, nonlinear and linear dependence on [urea]. (The source(s) of the discrepancy between these two proteins will be considered in the Discussion.) We have investigated the denaturation of three proteins in the full [urea] range by measuring optical transitions induced by GdnHCl, urea, and urea-GdnHCl mixture, and have arrived at two important conclusions: (i) at least for the proteins studied here ΔG_D versus [urea] plot is linear in the full [urea] range, and (ii) identical ΔG_D^0 values from GdnHCl and urea denaturations of a protein are obtained when the effect of salt on urea-induced denaturation is taken into account.

MATERIALS AND METHODS

Thrice crystallized, dialyzed, and lyophilized preparation of lysozyme (lot 111H-7010) from chicken egg white, ribonuclease-A (Type III A, lot 77H-7009) from bovine pancreas, and cytochrome-c (lot 61H-7001) from horse heart were purchased from Sigma Chemical Co. GdnHCl (lot 54408) and urea (lot 60963) were ultrapure grade samples obtained from ICN Biomedical, Inc. Analytical grade sodium salt of cacodylic acid was from Aldrich Chemical Co. KCl and glycine were from Merck (India) Ltd. All other chemicals were reagent grade and were used without further purification.

Cytochrome-c was first oxidized by adding 0.01% potassium ferricyanide. The oxidized preparations of cytochrome-c (∼2 mg/mL), lysozyme (∼4 mg/mL), and ribonuclease-A (∼5 mg/mL) were dialyzed in cold against several changes of 0.1 M KCl (pH 7.0). Protein concentration of the stock solution was determined from the absorbance of an appropriately diluted aliquot, using molar absorption coefficient (ϵ) values of 39 000 M^{-1} cm^{-1} at 280 nm for lysozyme (*27*); 9800 M^{-1} cm^{-1} at 278 nm for ribonuclease-A (RNase-A[1]) (*28*); and 106 000 M^{-1} cm^{-1} at 410 nm for cytochrome-c (cyt-c[1]) (*29*). The concentrations of urea and GdnHCl in buffered solutions were determined refractometrically using tabulated values of the solution refractive index (*30*).

The buffers used throughout the denaturation study were KCl−HCl (pH 2.0) for lysozyme, 0.05 M Gly-HCl (pH 3.0) for RNase-A, and 0.03 M cacodylic acid buffer (pH 6.0) for cyt-c. All buffers contained 0.1 M KCl unless otherwise stated. Details of the preparation of protein solutions for denaturation and renaturation experiments were reported earlier (*3*). Absorption spectra in the near-UV region (350−240 nm) in case of lysozyme and RNase-A and visible region (500−360 nm) in case of cyt-c were obtained in a Shimadzu-2100 UV−vis spectrophotometer. The temperatures of solutions in 1 cm quartz cuvettes were controlled at 25 ± 0.05 °C by circulating water from thermostated water bath (Shimadzu-TB-85) through water-jacketed cell holders. Denaturation transition curves were constructed by plotting ϵ versus [denaturant].

RESULTS

The results described below are to determine the functional dependence of ΔG_D on [urea] in the full concentration range of the denaturant. For this purpose denaturations of three proteins, namely, lysozyme, RNase-A, and cyt-c by urea, GdnHCl, and urea-GdnHCl mixture, have been measured at least in triplicate by following changes in the molar absorption coefficient as a function of [denaturant] at 25 °C. It has been observed that denaturations of all proteins are reversible. Assuming a two-state model of denaturation of these proteins, the optical data were converted into ΔG_D using the relation,

$$\Delta G_D = -RT \ln\{(y - y_N)/(y_D - y)\} \quad (1)$$

where y is the observed optical property and y_N and y_D are, respectively, the properties of the native and denatured protein molecules under the same experimental condition in which y has been determined. It has been observed that, in each case, the plot of $-1.3 \leq \Delta G_D$ (kcal mol^{-1}) ≤ 1.3 versus [denaturant] is linear in the transition region. A least-squares analysis has been used to fit the data (ΔG_D, [denaturant]) according to the relation (*9*),

$$\Delta G_D = \Delta G_D^{\,0} - m_d[\text{denaturant}] \quad (2)$$

where ΔG_D^0 is the value of ΔG_D at 0 M denaturant, and m_d[1] gives the dependence of ΔG_D on the [denaturant], i.e., $m_d = (\delta \Delta G_D/\delta[\text{denaturant}])_{p,pH,T}$. In the case of the mixed denaturant system, each value of ΔG_D obtained from the urea-induced denaturation of the protein in the presence of a fixed [GdnHCl] has been corrected for the effect of GdnHCl on ΔG_D associated with the urea-induced transition between N and D states, using the relation (*7*),

$$\Delta G_D^{cor} = \Delta G_D + m_g[\text{GdnHCl}] = \Delta G_D^{\ 0} - m_u[\text{urea}] \quad (3)$$

where subscripts u and g give the dependence of ΔG_D on [urea] and [GdnHCl], respectively.

Figure 1A shows the GdnHCl-induced and urea-induced denaturations of lysozyme followed by observing changes in $\Delta\epsilon_{300}$, the difference molar absorption coefficient at 300 nm, as a function of [denaturant]. All measurements of $\Delta\epsilon_{300}$, which monitors the change in the environment of tryptophan in the protein (*31*), were made in KCl−HCl buffer (pH 2.0) at 25 °C. It is seen in Figure 1A (i) that the dependence of y_N and y_D on [GdnHCl] and [urea] are independent of [KCl] in the range 0.1−1.0 M, (ii) that the neutral salt has no effect on the GdnHCl-induced denaturation, and (iii) that the urea-induced denaturation shows strong dependence on [KCl]. It is interesting to note that our results are in excellent agreement with those reported earlier (see Figure 20 in ref *32*). Values of ΔG_D associated with each transition curve shown in Figure 1A have been determined using eq 1.

Figure 1B shows the plot of ΔG_D as function of [denaturant]. Analysis of each plot according to eq 2 gave thermodynamic parameters which are entered in Table 1. In this table C_m[1] represents the concentration of the denaturant at which ΔG_D is 0. It is seen in Table 1 that for lysozyme (i) both modes of denaturation give, within experimental error, identical ΔG_D^0 values, values are 4.18 ± 0.21 kcal mol^{-1} from GdnHCl denaturation and 4.09 ± 0.26 kcal mol^{-1} from urea denaturation; (ii) KCl does not have any effect on the ΔG_D^0 associated with GdnHCl-induced denaturation; (iii) in the case of urea-induced denaturation, KCl addition increases the ΔG_D^0; the values are 4.09 kcal mol^{-1} in the presence of 0.1 M KCl, 4.38 kcal mol^{-1} in the [KCl] range 0.3−0.5 M, and 5.29 kcal mol^{-1} in the [KCl] range 0.75−1.00 M; and (iv) KCl has no significant effect on m_u, and (v) KCl increases C_m.

Figure 1C shows the urea-induced denaturation of lysozyme in the presence of 0.90, 1.23, 1.84, and 1.98 M GdnHCl. It is seen in the inset of this figure that the slope of the pretransition baseline does not depend on the presence of GdnHCl in the concentration range 0−0.9 M. These results gave the following dependence of y_N on the composition variables (urea and GdnHCl),

$$y_N, \text{M}^{-1}\,\text{cm}^{-1} = 87.40(\pm 20.60)[\text{GdnHCl}] + 100.214(\pm 17.470)[\text{urea}] \quad (4)$$

Figure 1C also shows (i) that [urea] dependence of y_D in 0.9 M GdnHCl is the same as that in the absence of GdnHCl, and (ii) that the post-transition baselines observed for the urea-induced denaturations at 1.23, 1.84, and 1.98 M GdnHCl are independent of [urea]; values of y_D (M^{-1} cm^{-1}) are 1676 at 1.23 M GdnHCl and 1750 at 1.84 and 1.98 M GdnHCl.

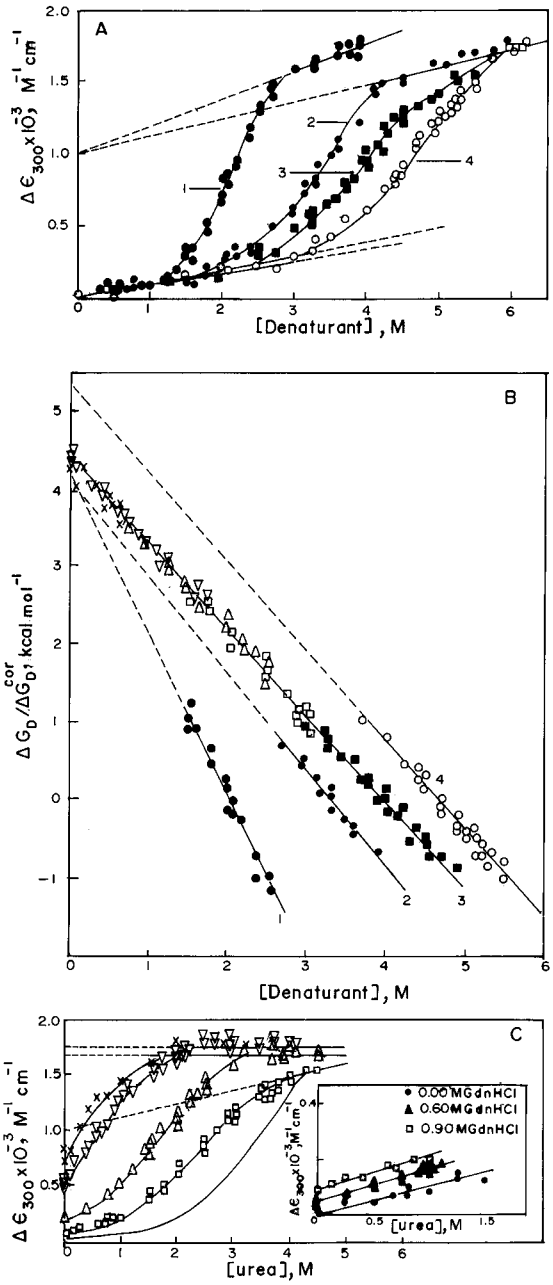

FIGURE 1: Denaturation of lysozyme by GdnHCl and urea at pH 2.0 and 25 °C. (A) Curve 1: GdnHCl denaturation in the presence of 0.10−1.0 M KCl; the least-squares fit of data in the pre- and post-transition regions gave the baselines, y_N, M^{-1} cm^{-1} = 87.40 (±20.60)[GdnHCl] and y_D, M^{-1} cm^{-1} = 990(±109) + 196.36 (±30.82)[GdnHCl], respectively. Urea denaturation in the presence of 0.10 M (curve 2), 0.30−0.50 M (curve 3), and 0.75−1.00 M (curve 4) KCl; the least-squares fit of data in the pre- and post-transition regions gave the baselines y_N, M^{-1} cm^{-1} = 100.214 (±17.470)[urea] and y_D, M^{-1} cm^{-1} = 1000(±67) + 121.33(±23.83) [urea], respectively. Values in parentheses are standard deviations of the fit. To maintain clarity all data points of curves 3 and 4 are not shown. (B) Plot of ΔG_D as a function of [denaturant]. The curve numbers have the same meaning as in (A), and solid lines are drawn using eq 2 with the parameters given in Table 1. ΔG_D^{cor} values are obtained from the measurements of the urea-induced denaturation of protein at various concentrations of GdnHCl: □, 0.90; △, 1.23; ▽, 1.84; and ×, 1.98 M (see the text) and are mapped onto curve 3. (C) Urea-induced denaturation in the presence of different concentrations of GdnHCl. All solutions contained 0.1 M KCl. Symbols have the same meaning as in (B). The transition curve in the presence of 0.1 M KCl (curve 2 in (A)). The inset shows the pretransition baseline dependence on [urea] and [GdnHCl].

Table 1: Thermodynamic Parameters for Unfolding of Proteins at 25 °C[a]

proteins	conditions	ΔG_D° (kcal mol^{-1})	m (kcal mol^{-1} M^{-1})	C_m (M)
lysozyme (pH 2.0)				
		GdnHCl Denaturation		
	0.1−1.0 M KCl	4.18 (±0.21)	2.03 (±0.10)	2.06
		Urea Denaturation		
	0.1 M KCl	4.09 (±0.26)	1.23 (±0.08)	3.32
	0.30−0.50 M KCl	4.38 (±0.26)	1.10 (±0.06)	3.98
	0.75−1.00 M KCl	5.29 (±0.44)	1.13 (±0.09)	4.68
	0.90 M GdnHCl	2.56 (±0.14)	1.10 (±0.05)	2.33
	1.23 M GdnHCl	1.72 (±0.06)	1.00 (±0.03)	1.72
	1.84 M GdnHCl	0.70 (±0.03)	1.06 (±0.03)	0.66
	1.98 M GdnHCl	0.30 (±0.05)	1.12 (±0.10)	0.27
RNase-A (pH 3.0)				
		GdnHCl Denaturation		
	0.1−1.0 M KCl	5.03 (±0.21)	2.83 (±0.12)	1.78
		Urea Denaturation		
	0.10 M KCl	4.07 (±0.16)	1.98 (±0.08)	2.05
	0.50 M KCl	4.10 (±0.25)	1.71 (±0.10)	2.40
	1.00 M KCl	4.49 (±0.19)	1.59 (±0.07)	2.82
	1.25 M KCl	4.99 (±0.13)	1.65 (±0.05)	3.02
	1.50 M KCl	5.26 (±0.21)	1.54 (±0.06)	3.41
	0.63 M GdnHCl	3.01 (±0.11)	1.58 (±0.06)	1.90
	0.90 M GdnHCl	2.45 (±0.11)	1.76 (±0.08)	1.39
	1.30 M GdnHCl	1.24 (±0.05)	1.57 (±0.06)	0.79
	1.50 M GdnHCl	0.82 (±0.04)	1.73 (±0.05)	0.47
	1.70 M GdnHCl	0.16 (±0.03)	1.78 (±0.07)	0.09
Cyt-c (pH 6.0)				
		GdnHCl Denaturation		
	0.1−1.0 M KCl	8.18(±0.42)	3.31(±0.17)	2.47
		Urea Denaturation		
	0.1−1.0 M KCl	8.02(±0.43)	1.28(±0.07)	6.26
	0.60 M GdnHCl	6.09(±0.29)	1.32(±0.06)	4.61
	1.21 M GdnHCl	3.87(±0.13)	1.25(±0.04)	3.10
	2.20 M GdnHCl	0.79(±0.06)	1.37(±0.07)	0.58
	2.35 M GdnHCl	0.32(±0.04)	1.37(±0.05)	0.23

[a] The value of each parameter in parentheses is the standard deviation of the least-squares fit.

These observations on y_N and y_D for urea-GdnHCl mixture were used in the estimation of ΔG_D using eq 1. A linear least-squares analysis of ΔG_D versus [urea] plots at different GdnHCl concentrations (not shown here) gave the values of thermodynamic parameters that are listed in Table 1. It is interesting to note that the addition of GdnHCl to the urea-induced denaturation of lysozyme has no significant effect on m_u, but values of ΔG_D^0 and C_m decrease with an increase in [GdnHCl] (see Table 1).

The values of ΔG_D obtained in the presence of the urea-GdnHCl mixture were corrected for the effect of GdnHCl on the Gibbs energy change associated with the urea-induced transition using eq 3; a value of 2.03 kcal mol^{-1} M^{-1} for m_g was used for this purpose, for it has been shown earlier that m_g depends neither on [urea] (*7*) nor on pH (*6*). Figure 1B (curve 3) shows the plot of ΔG_D^{cor} versus [urea].

Figure 2A shows the GdnHCl-induced (curve 1) and urea-induced (curve 2) denaturations of RNase-A in Gly-HCl buffer (pH 3.0), followed by observing changes in $\Delta\epsilon_{287}$ as a function of [denaturant] at 25 °C. The effect of KCl on the GdnHCl-induced and urea-induced denaturations was also studied at the same temperature and pH. It is seen in this figure (curve 1) that [KCl] in the range 0.1−1.0 M has no effect on the denaturation of the protein by GdnHCl. On the other hand, urea-induced denaturation showed strong dependence on [KCl] (see curves 2−6 in Figure 2A). It is seen in Figure 2A that the pretransition baseline of the urea-induced denaturation does not depend on [urea], whereas the post-transition baseline shows dependence on [urea]. By using the observed [denaturant] dependencies of y_N and y_D of RNase-A (see Figure 2A) in eq 1, we determined values of ΔG_D.

Plots of ΔG_D versus [GdnHCl] (curve1) and ΔG_D versus [urea] at different KCl concentrations (curves 2−6) are shown in Figure 2B. A linear least-squares analysis of ΔG_D versus [denaturant] plots according to eq 2 gave values of thermodynamic parameters that are entered in Table 1. It is seen in this table (i) that the neutral salt has no effect on the thermodynamic parameters associated with GdnHCl-induced denaturation, (ii) that addition of KCl to urea-induced denaturation of RNase-A increases ΔG_D^0 from 4.07 to 5.26 kcal mol^{-1} and C_m from 2.05 to 3.41 M when [salt] is raised from 0.1 to 1.5 M, and (iii) that there is a very small change in m_u value when [KCl] is increased from 0.1 to 1.5 M.

Figure 2C shows the effect of GdnHCl on urea-induced denaturation of RNase-A in the presence of 0.1 M KCl at pH 3.0 and 25 °C. The inset in this figure shows the [urea] dependence of y_N in the presence of different concentrations of GdnHCl. These measurements show that the [urea] dependence of y_N remains unchanged in the [GdnHCl] range 0−0.63 M. It has therefore been assumed that this is true at all concentrations of urea, that is, the relation describing the

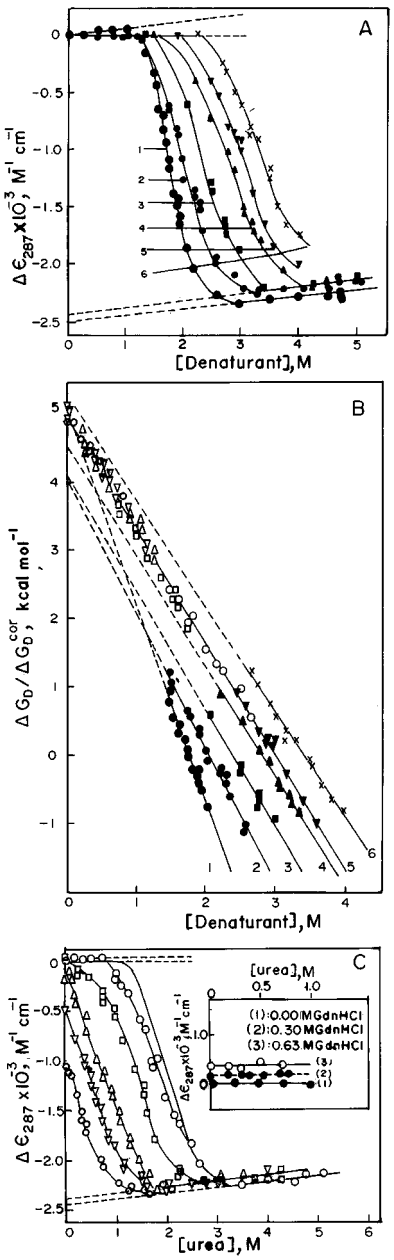

FIGURE 2: (A) GdnHCl-induced denaturation of RNase-A in the presence of 0.1−1.0 M KCl at pH 3.0 and 25 °C (curve 1). Results in the pre- and post-transition regions are described by the relations, y_N, M^{-1} cm^{-1} = 64.80(±21.60)[GdnHCl] and y_D, M^{-1} cm^{-1} = −2523(±74) + 56.88(±17.60)[GdnHCl], respectively. Urea-induced unfolding of RNase-A in the presence of 0.10 M (curve 2), 0.50 M (curve 3), 1.00 M (curve 4), 1.25 M (curve 5), and 1.50 M (curve 6) KCl at pH 3.0 and 25 °C. Results in the post-transition region gave y_D, M^{-1} cm^{-1} = −2454(±77) + 62.54(±18.49)[urea]. (B) ΔG_D versus [denaturant] plots. The curve numbers have the same meaning as in (A), and the solid lines are drawn according to eq 2 with the parameters given in Table 1. Values of ΔG_D^{cor} obtained from the urea-induced denaturation of protein in the presence of various concentrations of GdnHCl: ○, 0.63; □, 0.90; △, 1.30; ▽, 1.50; o, 1.70 M are mapped onto ΔG_D versus [urea] plots at different KCl concentrations. For clarity, all ΔG_D^{cor} data points and results on curves 3 and 4 are not shown. (C) Effect of GdnHCl on urea-induced denaturation of RNase-A in Gly-HCl buffer (pH 3.0) at 25 °C. Symbols have the same meaning as in (B), and the transition curve without data points is curve 2 in (A). The [urea] dependence of y_D in the presence of [GdnHCl] range 0.90−1.70 M is given by the relation, y_D, M^{-1} cm^{-1} = −2391 (±71) + 61.54(±23.25)[urea]. The inset shows the [urea] dependence of y_N on GdnHCl concentrations.

[GdnHCl] dependence of y_N given in Figure 2A was used to estimate y_N in the urea-GdnHCl mixture. It is seen in Figure 2C that (i) the dependence of y_D on [urea] in the presence of 0.63 M GdnHCl is the same as that observed in its absence (see Figure 2A), and (ii) the dependence of y_D on [urea] in the presence of 0.90, 1.30, 1.50, and 1.70 M GdnHCl is described by the relation given in the figure. The optical data shown in this figure were converted into ΔG_D using the observed dependencies of y_N and y_D on [urea] in eq 1. A ΔG_D versus [urea] plot was constructed at each [GdnHCl] (results not shown). A least-squares analysis of (ΔG_D, [urea]) data at each [GdnHCl] according to eq 2 gave values of thermodynamic parameters that are given in Table 1. It is seen in this table that both ΔG_D^0 and C_m of urea-induced denaturation decrease in the presence of GdnHCl, whereas m_u is unchanged in GdnHCl. ΔG_D values were corrected for the effect of GdnHCl on the urea-induced denaturation of RNase-A using eq 3 with a value of m_g = 2.83 kcal mol^{-1} M^{-1} (see Table 1). ΔG_D^{cor} values thus obtained are plotted as a function of [urea] in Figure 2B. It is seen in this figure that all values of ΔG_D^{cor} fall on the curve obtained from the studies of urea-induced denaturation in the presence of 1.25 M KCl.

Figure 3A shows the GdnHCl-induced (curve1) and urea-induced (curve 2) denaturations followed by measuring changes in $\Delta\epsilon_{405}$ of cyt-c in 0.03 M cacodylic acid buffer containing 0.1M KCl at pH 6.0. The effect of [KCl] in the range 0.1−1.0 M on both denaturations suggested that the neutral salt has no effect on the denaturation of cyt-c at this pH. It is seen in Figure 3A that (i) y_N shows dependence on [GdnHCl] and [urea], and (ii) y_D does not depend on [denaturant]. These observations are in excellent agreement with those reported earlier (*33*, *34*). To estimate ΔG_D values, we used these observations on y_N and y_D in the analysis of the transition curves shown in Figure 3A, using eq 1.

Figure 3B shows the ΔG_D versus [denaturant] plots of cyt-c. A least-squares analysis of these plots gave the thermodynamic parameters that are entered in Table 1. These values of the thermodynamic parameters are in excellent agreement with those reported earlier (*7*). Figure 3C presents urea-induced unfolding profiles of cyt-c at fixed GdnHCl concentrations in the range 0.60−2.35 M. It is seen in the inset of this figure that the slope of pretransition baselines of the urea-induced denaturation does not depend on [GdnHCl]. It has been observed that the dependence of y_N in the urea-GdnHCl mixture is described by the relation,

$$y_N,\ M^{-1}\ cm^{-1} = 1312.22(\pm 194.86)[\text{GdnHCl}] + 406.08(\pm 67.91)[\text{urea}] \quad (5)$$

In the case of the urea-GdnHCl mixture, y_D does not depend on [urea]. However, its value varies with [GdnHCl]; y_D values are 23 500, 24 500, 24 060, and 24 500 M^{-1} cm^{-1} at 0.60, 1.21, 2.20, and 2.35 M GdnHCl, respectively (see Figure 3C). Making use of the above observations on y_N and y_D, we estimated values of ΔG_D using eq 1, and they were plotted against [urea] (results not shown). A least-squares analysis according to eq 2 gave the parameters which are entered in Table 1. It has been observed that m_u of cyt-c remains unchanged in the presence of GdnHCl (see Table 1). Values of ΔG_D^{cor} were determined using eq 3 with m_g = 3.31 kcal mol^{-1} M^{-1}. These ΔG_D^{cor} values are shown in

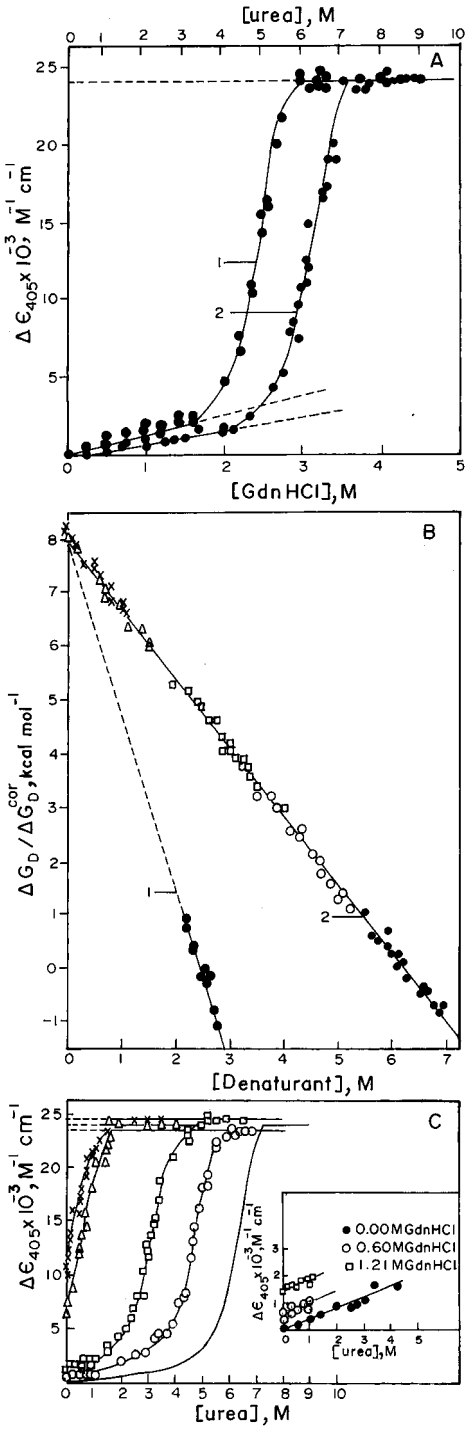

FIGURE 3: (A) GdnHCl-induced (curve 1) and urea-induced (curve 2) denaturations of cyt-c in the presence of 0.1−1.0 M KCl at pH 6.0 and 25 °C. A least-squares fit of data in the pretransition region gave y_N, M^{-1} cm^{-1} = 1312.22(±194.86)[GdnHCl] and y_N, M^{-1} cm^{-1} = 406.08(±67.91)[urea] for GdnHCl and urea unfoldings, respectively. (B) The curve numbers have the same meaning as in (A). Lines were drawn with the parameters given in Table 1. The ΔG_D^{cor} values obtained from the urea-induced denaturation of protein in the presence of ○, 0.60; □, 1.21; △, 2.20; ×, 2.35 M GdnHCl are mapped onto curve 2. (C) Urea-induced denaturation of cyt-c in the presence of 0.1 M KCl at different fixed GdnHCl concentrations at pH 6.0 and 25 °C. Symbols have the same meaning as in (B). The transition curve without data points represents the urea-induced denaturation of protein in the presence of 0.1 M KCl shown in (A). The inset in this figure shows the pretransition baseline dependence on [urea] in the presence of different GdnHCl concentrations.

Figure 3B where it is seen that they fall on the ΔG_D versus [urea] plot in the absence of GdnHCl (see curve 2).

DISCUSSION

Estimation of ΔG_D^0 from the conformational transition curve is possible only if the protein operates under certain constraints. A few comments are therefore necessary. First, since accurate data in the pre- and post-transition regions are as important as those in the transition region in the determination of ΔG_D (see eq 1), an attempt has been made to accurately determine the dependencies of y_N and y_D on [denaturant] under all experimental conditions. It should be noted that these dependencies are accurately known from the experimental data in all conditions except the [urea] dependence of y_N in the presence of high GdnHCl concentrations which by themselves denature the protein in the absence of urea. Dependence of y_N on [urea] could, however, be accurately determined at low GdnHCl concentrations which by themselves do not perturb the native structure of the protein (see insets in Figures 1−3). An assumption that the observed dependence of y_N on [urea] in the presence of these low GdnHCl concentrations is also applicable at all compositions of the mixed denaturant system was introduced in the determination of ΔG_D of the protein using eq 1.

Second, to estimate the ΔG_D value from denaturation curves, we assumed a two-state mechanism in the derivation of eq 1. All available data (agreement between denatured fraction versus [denaturant] profiles, and kinetic studies of denaturation) strongly support that this assumption is valid in the case of urea-induced and GdnHCl-induced denaturations of lysozyme (reviewed in ref *35*), RNase-A (reviewed in ref *14*), and cyt-c (reviewed in refs *6*, *33*). However, no such data are available for denaturation of proteins by the mixed denaturant system (urea-GdnHCl mixture). Since the effect of urea and GdnHCl on denaturation is additive, we have assumed that a two-state mechanism also holds for the protein denaturation by the mixed denaturant system.

Finally, ΔG_D can be measured only in a narrow region of the denaturation curve induced by a chemical denaturant at constant temperature and pH. It is well-known fact that, although the linear free energy, binding site, and transfer free energy models are equally appropriate to describe the functional dependence of ΔG_D on [denaturant] in this narrow transition region, these extrapolation methods yield different values of ΔG_D at denaturant concentrations in the pretransition region (*9*, *30*). This is a practical issue involving the evaluation of ΔG_D^0. A solution to this problem is to extend the ΔG_D measurements to 0 M of the denaturant. There are only a few ways of doing this (see Introduction). To extend the ΔG_D measurements to 0 M urea, we have studied urea-induced denaturation of each protein in the presence of different fixed GdnHCl concentrations at which the dependence of ΔG_D on [GdnHCl] has already been measured (e.g., [GdnHCl] range 1.5−2.6 M in the case of lysozyme). In such mixed denaturant systems eq 3 can be used safely for correcting the effect of GdnHCl on urea-induced denaturation of all proteins. There are two reasons for saying this. First, all parameters of eq 3 are determined from the experimental solvent denaturation data. Second, measurements of denaturation of a protein by a mixed denaturant system suggest that m_u is independent of [GdnHCl] (see Table 1) and m_g is independent of [urea] (*7*).

In principle, different effects of neutral salts such as KCl on the GdnHCl-induced and urea-induced denaturations of a protein should be observed, if the native protein contains stabilizing (or destabilizing) electrostatic interaction(s) and/or specific binding site(s) for the neutral salt ion(s). This disagreement is mainly due to the ionic property of GdnHCl and its absence in urea (*16*, *17*). Previous studies of the effect of neutral salts on the GdnHCl-induced denaturations of several proteins have shown that the neutral salts have no effect on the transition between N and D states (*8*, *16*, *17*, *36*). As an explanation of this observation, it has been argued that the ionic strength due to low GdnHCl concentrations in the pretransition region is so high that it completely eliminates all kinds of electrostatic interaction (*14*). This argument may be used to understand our results of the effect of KCl on the GdnHCl-induced denaturation of lysozyme, RNase-A, and cyt-c, for KCl does not affect the stability of these proteins (see Table 1).

Unlike GdnHCl, urea cannot eliminate any kind of electrostatic interaction, for it is an uncharged molecule. It is obvious that the effect of the neutral salt on urea-induced denaturation may be understood if detailed information about the molecular architecture such as the geometric relationship of charged groups and information about the existence of specific salt ions binding site(s) are available. Examination of the known three-dimensional structure has revealed (i) that charged groups of cyt-c and RNase-A are distributed in such a way that electrostatic interaction makes no significant contribution to the ΔG_D^0 value, and (ii) that there exists destabilizing electrostatic interaction in lysozyme in the pH range 1−7 (*37*−*39*). Our observation that the addition of KCl has no effect on the urea-induced denaturation of cyt-c may be understood in light of this structural information and the observation that the salt does not bind to the protein near neutral pH (*40*). Although electrostatic interaction has no significant effect on RNase-A stability (*39*), our results suggest that KCl stabilizes RNase-A against urea-induced denaturation (see Figure 2A and Table 1). This observation may be understood in light of the earlier findings that Cl^- ion stabilizes RNase-A which contains anion binding sites (*41*, *42*). It is seen in Figure 1A that KCl increases the stability of lysozyme against urea denaturation. A possible explanation of the effect of this salt on protein stability is that KCl shields the electrostatic repulsion between positively charged Arg 45 and Arg 68 which are less than 3 Å apart in the native protein, by general ionic strength effects, and hence the protein is stabilized.

Unlike ΔG_D^0 values of lysozyme and cyt-c, ΔG_D^0 values from urea-induced and GdnHCl-induced denaturation of RNase-A in the presence of 0.1 M KCl do not agree with one another. As mentioned above, the most likely candidate for this discrepancy is the Cl^- binding to native RNase-A (*41*, *42*). To see that this is indeed true, we have studied urea-induced denaturation of the protein in the presence of different concentrations of KCl. It has been observed that the value of ΔG_D^0 from urea denaturation increases from 4.07 kcal mol^{-1} in 0.1 M KCl to 5.26 kcal mol^{-1} in 1.50 M KCl (see Table 1). Furthermore, a comparison of our results with those of Yao and Bolen (*14*), who studied the GdnHCl-induced and urea-induced denaturation of RNase-A in the presence of different NaCl concentrations at pH 3.0 and 25 °C, suggests that agreement is excellent. There is, however, one quantitative difference in the effects of KCl and NaCl on urea-induced denaturation of the protein. The agreement between ΔG_D^0 values from GdnHCl and urea denaturation is reached only when urea denaturation is carried out at 1.25 M KCl (this study) or 0.5 M NaCl (*14*). We have no explanation for the difference in effectiveness of these salts in the stabilization of RNase-A.

Measurements of ΔG_D from the conformational transition curve induced by a chemical denaturant can be carried out only in a narrow range of [denaturant] at constant pH and temperature. To extend ΔG_D measurements to 0 M urea, we have studied urea-induced denaturations of lysozyme in the presence of 1.84 and 1.98 M GdnHCl, RNase-A in the presence of 1.50 and 1.70 M GdnHCl, and cyt-c in the presence of 2.20 and 2.35 M GdnHCl (see Figures 1−3). These concentrations of GdnHCl were chosen because eq 3 can be used safely to correct the effect of GdnHCl on the urea-induced denaturations. There are two reasons for saying this. One is that the [GdnHCl] dependence of ΔG_D is independent of the denaturation models used to analyze (ΔG_D, [GdnHCl]) data in the transition region (*30*). Another one is that m_g does not depend on [urea] (*7*). For each protein, when ΔG_D values measured in the presence of the mixed-denaturant system were corrected for the effect of these high GdnHCl concentrations on the urea-induced denaturation using eq 3 and when ΔG_D^{cor} values were mapped onto the ΔG_D versus [urea] plot, ΔG_D^{cor} values were found to fall on the linear curve obtained from the urea-induced denaturation in absence of GdnHCl (see curves 2, 3, and 5 in Figures 1B, 2B, and 3B, respectively). We have also studied urea-induced denaturation of proteins in the presence of low GdnHCl concentrations which by themselves do not perturb the native structure. It has been observed that ΔG_D^{cor} values of a protein derived from these measurements fall on the linear plot of ΔG_D versus [urea] only when it is assumed that m_g is not a function of [urea] (*7*). Thus our results provide convincing evidence for the linear dependence of ΔG_D of lysozyme, RNase-A, and cyt-c on [urea] over the full concentration range. It should, however, be noted that we have no explanation for the mixed denaturant data falling on the ΔG_D versus [urea] plot only when urea-induced denaturation of lysozyme and RNase-A are studied in the presence of 0.40 ± 0.10 and 1.25 M KCl, respectively.

There are only two other proteins, namely, myoglobin and barnase, whose urea-induced denaturations have been studied in the full denaturant concentration range. In the case of metmyoglobin, the extrapolation region of the ΔG_D versus [urea] plot was reduced to 0 M of the denaturant by studying it in the presence of different concentrations of GdnHCl at constant pH and temperature, and it has been observed that the ΔG_D of the protein varies linearly with [urea] (*8*). In case of barnase, combination of the ΔG_D results from the isothermal urea-induced optical transition curve with those from the differential scanning calorimetric measurements in the presence of low concentrations of urea suggested that the ΔG_D versus [urea] plot is nonlinear (*26*). Our earlier studies of the combined effect of two denaturants (a strong denaturant, urea, and one of the weak denaturants, LiCl, $LiClO_4$, and $CaCl_2$) on RNase-A led to several important conclusions (*43*). One of them is that, if low concentrations of urea which by themselves do not disrupt the native structure of the protein (i.e., pretransition range of the urea-

induced denaturation) are added to the salt-induced transition, N ↔ A (the salt-denatured state), one finds that urea destabilizes the native protein (i.e., it causes the transition to occur at lower concentrations of the salt), but it does not fundamentally change the transition (i.e., the transition is still between N and A states). Higher concentrations of urea, which by themselves disrupt the N state of RNase-A, continue destabilization, but the denaturation process is altered so that now one observes the transition between N and D states. One question of interest is then whether the thermal denaturation of barnase in the presence of low concentrations of urea (which by themselves do not disrupt its native conformation) is between N and heat-denatured states or between N and D (the urea-denatured) states. If heat- and urea-denatured states are structurally different, mapping of calorimetric ΔG_D in the presence of these low concentrations of urea onto the ΔG_D versus [urea] plot obtained from optical transition curve may then not be valid. It is interesting to note that calorimetric and equilibrium results are in agreement at high urea concentrations which belong to the transition region of the isothermal urea-induced denaturation of barnase (see Figure 7 in ref *26*). The most probable explanation for this observation is that the thermal denaturation of the protein in the presence of these high concentrations of urea is between N and D states. Whether these are the correct explanations for the nonlinearity in the ΔG_D versus [urea] plot of barnase or the protein's behavior is an exception is not clear without additional data.

Our results support the use of linear free energy model to analyze the urea denaturation of proteins, which has been justified on theoretical and experimental grounds (*10*, *23*, *24*). In several investigations urea denaturation curves have been analyzed using two other models, namely Tanford's model (*11*) and the binding site model (*11*). We shall now discuss why these models in their present forms should not be used for analyzing urea denaturation curves.

Tanford's (*11*) model interprets chemical denaturation in terms of transfer free energies of protein groups that make up the protein from water to denaturant solutions. The basic relationship is

$$\Delta G_D = \Delta G_D^{\,0} + \Delta\alpha(\Sigma n_i \Delta g_{tr,i}) \qquad (6)$$

where n_i is the total number of the ith kind of group present in the protein, $\Delta\alpha$ the fractional increase in the exposure of each type of group when protein unfolds, and $\Delta g_{tr,i}$ is the transfer free energy of the ith kind of group from water to solutions of the denaturant such as urea. Tanford has interpreted $\Delta g_{tr,i}$ values in terms of transfer free energies of similar groups attached to free amino acids and oligopeptides. As discussed earlier (*4*, *5*), the problem with this approach is that it ignores the effect of steric bulk of the peptide backbone on $\Delta g_{tr,i}$ (*44*), and it regards the unfolded state of a protein as dilute solutions of constituent groups (*32*). It is interesting to note that, when Pace (*30*) analyzed urea and GdnHCl denaturations of several proteins, he observed that, for a protein value of ΔG_D^0 derived from GdnHCl, denaturation is 20−50% higher than that from urea denaturation, suggesting that eq 6 is not appropriate to analyze chemical denaturation results. Furthermore, eq 6 predicts a curvilinear relationship between ΔG_D of all proteins and [urea], for individual $\Delta g_{tr,i}$ varies nonlinearly with [urea] (*4*). This prediction is consistent neither with the finding that observed ΔG_D values of lysozyme, RNase-A, cyt-c, and myoglobin (*8*) vary linearly with [urea] in the full denaturant concentration range, nor with the recent observations that the calorimetric $\Delta g_{tr,i}$ of dissolution of homologous series of cyclic dipeptides into solutions of varying concentrations of urea is a linear function of [urea] (*24*). In summary, the use of Tanford's model in the present form (eq 6) to analyze urea denaturation of proteins cannot be justified.

Schellman and Hawkes (*45*) pointed out that the use of the binding site model to account for denaturation by a chemical denaturant is "permissible in principle". Assuming that the denatured state has more sites at which denaturant can bind and that all sites on the protein are identical and independent, Tanford (*11*) derived the relation,

$$\Delta G_D = \Delta G_D^{\,0} + \Delta nRT \ln(1 + ka) \qquad (7)$$

where k is the binding constant for denaturant to site on the protein, a is the denaturant activity, and Δn is the additional number of binding sites in the denatured protein relative to the native state. However, there are several serious problems with using this approach in analyzing protein denaturation curves. First is the assumption that denaturants such as urea act through specific binding to the protein, for there is no experimental evidence for the stoichiometric binding of urea and GdnHCl to proteins (*10*, *24*). Second is the assumption that all sites have identical binding constants, for it has been recently argued that interactions of denaturants such as urea with hydrophobic groups and with the peptide group result from different mechanisms (*24*). Third is that, whenever denaturation results are analyzed using eq 7, the resulting parameters such as Δn are far less than those calculated from X-ray data (*45*). Fourth is that, for a protein value of ΔG_D^0 from GdnHCl, denaturation is 17−50% higher than that from urea denaturation (*30*). In summary, the use of the binding site model for analyzing urea-induced denaturation of proteins is consistent neither with experimental results such those presented here and elsewhere (*8*) nor with theoretical (*10*) and empirical considerations (*23*, *24*). In light of the above problems it is recommended that the use of eq 7 to analyze urea transition curves should be abandoned in the present form (*8*, *9*). Instead of eq 7, a more realistic treatment of the denaturational data must involve both solvation and binding contributions of the denaturant (see eq 45 in ref *46*).

The experimental evidence presented in this paper and in an earlier report (*8*) clearly shows that the unfolding free energy changes of proteins depend linearly on [urea] over an extended concentration range. Finally the experimental method to reduce the extrapolation region to 0 M urea (or GdnHCl) not only is simpler but also has at least two advantages: (i) both urea and GdnHCl induce the same conformational transition between N and D states, and (ii) measurements of urea-induced and GdnHCl-induced denaturation can be made at the same temperature and pH.

ACKNOWLEDGMENT

We gratefully acknowledge the comments on the manuscript by Drs. A. R. Fersht & C. Johnson, Cambridge University Chemical Laboratory, Cambridge, U.K.

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BI982078M