Protein Stability: Urea-Induced versus Guanidine-Induced Unfolding of Metmyoglobin†

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ABSTRACT: We have studied the denaturation of metmyoglobin at pH 6.0 and 25 °C by urea and guanidine hydrochloride (GdnHCl) which are known to unfold the protein to the same extent. It has been observed that estimates of protein stability ($\Delta G^0_{N\text{-}U}$) from urea-induced and GdnHCl-induced denaturations do not agree with one another; the linear extrapolation method gave $\Delta G^0_{N\text{-}U}$ values of 7.59 ± 0.33 and 5.35 ± 0.10 kcal mol^{-1} for urea and GdnHCl denaturations, respectively. Measurements of the effect of the addition of KCl in the concentration range 0.1−1.0 M to urea denaturation have suggested that this disagreement is not due to the nonionic and ionic characters of urea and GdnHCl, respectively. The functional dependence of the free energy change of unfolding ($\Delta G_{N\text{-}U}$) on [denaturant], the molar concentration of the denaturant, has been investigated for understanding the cause(s) of the disagreement between the two estimates of $\Delta G^0_{N\text{-}U}$ of metmyoglobin. For this purpose, we have studied the GdnHCl-induced denaturation of the protein in the presence of different urea concentrations at pH 6.0 and 25 °C and vice versa. These measurements yield $\Delta G_{N\text{-}U}$ values in the full concentration range [Ahmad et al. (1994) *J. Biochem. 115,* 322−327], and these results provide strong evidence that the $\Delta G_{N\text{-}U}$ dependence on [urea] is linear (linear free energy model of denaturation) and the relation between $\Delta G_{N\text{-}U}$ and [GdnHCl] is curved (binding model of denaturation). It has been observed that the extrapolated value of $\Delta G_{N\text{-}U}$ in urea using the linear free energy model becomes identical to the extrapolated value of $\Delta G_{N\text{-}U}$ in GdnHCl using the binding model.

Determination of protein stability, defined as the decrease in Gibbs energy of a structureless polypeptide chain when it folds to give a native protein molecule in water (or dilute buffer), from the isothermal transition curves induced by chemical denaturants is problematic. There are at least two reasons for this. First, the magnitude of this quantity shows a dependence on the denaturation models used to extrapolate the same set of values of $\Delta G_{N\text{-}U}$,[1] the change in Gibbs energy of denaturation which is measured in the narrow range of denaturant concentration, to the native condition (Pace, 1986; Ahmad, 1993). That is, for a protein, different extrapolation procedures, namely, the linear free energy model (Pace, 1975; Schellman, 1978), the binding-site model (Tanford, 1970; Schellman, 1975), and the transfer free energy model (Tanford, 1970), give different values of $\Delta G^0_{N\text{-}U}$,[1] the value of $\Delta G_{N\text{-}U}$ at zero molar denaturant concentration (or activity). The fact that a given equilibrium, N (native) conformation ↔ U (unfolded) conformation, can have only one value of $\Delta G^0_{N\text{-}U}$ brings into question the validity of extrapolation procedures. Second, it has been shown in cases of myoglobin (Pace, 1975; Ahmad & Bigelow, 1982) and a few more proteins (Ropson et al., 1990; Monera et al., 1993, 1994; Yao & Bolen, 1995) that the $\Delta G^0_{N\text{-}U}$ value shows denaturant-dependence. That is, for a protein, extrapolation of $\Delta G_{N\text{-}U}$ measured in a narrow concentration range to the native condition gives different values of $\Delta G^0_{N\text{-}U}$ for guanidine hydrochloride (GdnHCl)[1] unfolding in comparison to urea-induced unfolding. This means that $\Delta G^0_{N\text{-}U}$ associated with the N ↔ U transition cannot be regarded as the property of the protein alone. An unequivocal value of $\Delta G^0_{N\text{-}U}$ can, however, be obtained if the extrapolation region is reduced to zero molar concentration of chemical denaturant (Pace & Vanderburg, 1979; Santoro & Bolen, 1992; Ahmad et al., 1994).

In the case of GdnHCl, measurements of $\Delta G_{N\text{-}U}$ of several proteins were extended to 0 M of the denaturant, and it was observed that in each case the functional dependence of $\Delta G_{N\text{-}U}$ was linear with [GdnHCl], the molar concentration of the denaturant (Santoro & Bolen, 1992; Swint & Robertson, 1993; Ahmad et al., 1994). The only clear-cut exception seems to be the dependence of $\Delta G_{N\text{-}U}$ of metmyoglobin on [GdnHCl] reported by Pace and Vanderburg (1979), who studied the GdnHCl-induced denaturation of the protein at different pH values. Assuming a two-state transition at all pH values, they calculated $\Delta G_{N\text{-}U}$ values and obtained separate $\Delta G_{N\text{-}U}$ versus [GdnHCl] plots. They then pieced together the separate data from each curve at different pH values into one normalized curve. They observed that the slope of the plot of $\Delta G_{N\text{-}U}$ versus [GdnHCl] increased with decreasing denaturant concentration. This finding was taken as evidence to support the binding-site model.

We have recently shown that the method of reducing the extrapolation region to zero molar denaturant concentration which involves the isothermal GdnHCl-induced denaturation of proteins at different pH values (Pace & Vanderburg, 1979) is problematic (Ahmad et al., 1992). The reason for this is that at extreme pH values proteins are acid-denatured in the absence of GdnHCl; hence, the acid-denatured state con-

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[1] Abbreviations: $\Delta G_{N\text{-}U}$, Gibbs energy of denaturation; $\Delta G^0_{N\text{-}U}$, Gibbs energy of denaturation in the absence of denaturant; GdnHCl, guanidine hydrochloride; Mb, metmyoglobin.

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tributes to the equilibrium between the N and U states, leading to a curvature in the $\Delta G_{N\text{-}U}$ versus [GdnHCl] plot at these pH values. Whether the curvature is upward or downward depends on the difference in the values of the optical properties of the GdnHCl- and acid-denatured states. It has been shown that in the case of metmyoglobin, this curvature is upward, for the magnitude of the optical property, namely, the molar absorption coefficient at 409 nm (ϵ_{409}), used to follow the denaturation is the same for both U and acid-denatured states (Puett, 1973). In order to understand whether the exceptional behavior of the $\Delta G_{N\text{-}U}$ versus [GdnHCl] plot of myoglobin is real or whether it is due to the presence of the acid-denatured state at extreme pH values, we have reinvestigated the problem of the functional dependence of $\Delta G_{N\text{-}U}$ of metmyoglobin on [GdnHCl] using a different approach for extending the $\Delta G_{N\text{-}U}$ measurements to zero molar denaturant concentration (Ahmad et al., 1994). From the study of the GdnHCl-induced denaturation of myoglobin in the presence of different concentrations of urea, instead of different pH values, it has been observed that the functional dependence of $\Delta G_{N\text{-}U}$ is indeed nonlinear over the full GdnHCl denaturant concentration range.

Although a linear dependence of $\Delta G_{N\text{-}U}$ on [urea] has some support from the theoretical models (Alonso & Dill, 1991; Schellman, 1987), there are reports of curvature in the $\Delta G_{N\text{-}U}$ versus [urea] plots of a few proteins (Shortle et al., 1989; Johnson & Fersht, 1995). In order to test the functional dependence of $\Delta G_{N\text{-}U}$ of metmyoglobin, we have studied the urea-induced denaturation in the presence of different concentrations of GdnHCl. It has been observed (1) that contrary to the GdnHCl results, the functional dependence of $\Delta G_{N\text{-}U}$ on [urea] is linear over the full urea concentration range, and (2) that contrary to the earlier reports (Pace, 1975; Ahmad & Bigelow, 1982), the value of $\Delta G^0_{N\text{-}U}$ for urea unfolding is identical to that for GdnHCl unfolding.

MATERIALS AND METHODS

A thrice-crystallized, lyophilized, and chromatographically purified preparation of horse heart myoglobin (type III, lot 90F-7085) was purchased from Sigma Chemical Co. Ultrapure samples of GdnHCl (lot 43826) and urea (lot 60963) were obtained from Schwarz/Mann Biotech, Division of ICN Biomedicals, Inc. Analytical grade sodium salt of cacodylic acid and KCl were from Aldrich Chemical Co. and Merck (India) Ltd., respectively.

Myoglobin was first oxidized by adding 0.1% potassium ferricyanide and then dialyzed in the cold against several changes of 0.1 M KCl (pH 6.5). This preparation of myoglobin is abbreviated as Mb[1] which showed a single band on polyacrylamide gel electrophoresis. The concentration of the protein stock solution was determined using a value of 171 000 M^{-1} cm^{-1} for ϵ_{409}, the molar absorption coefficient at 409 nm (Puett, 1973). Concentrations of stock solutions of GdnHCl and urea were determined by refractive index measurements (Pace, 1986).

All stock solutions for denaturation studies were prepared in 0.03 M cacodylic buffer containing a fixed concentration of KCl at pH 6.0. A known amount of stock protein solution (1.5−1.6 mg/mL), buffer, and denaturant (single or mixed) were taken in a 1-mL volumetric flask and incubated for sufficient time for completion of the reaction. The visible absorption spectrum (500−360 nm) of each protein solution was measured in a Shimadzu-2100 UV−VIS spectrophotometer using matched 1-cm quartz cuvettes whose temperature was maintained at 25 ± 0.05 °C by circulating water from a thermostated water bath (Shimadzu TB-85). Denaturation transition curves were constructed by plotting ϵ_{409} versus the molar concentration of the denaturant. The reversibility of the transition between native and denatured states was checked using the procedure described earlier (Ahmad, 1985).

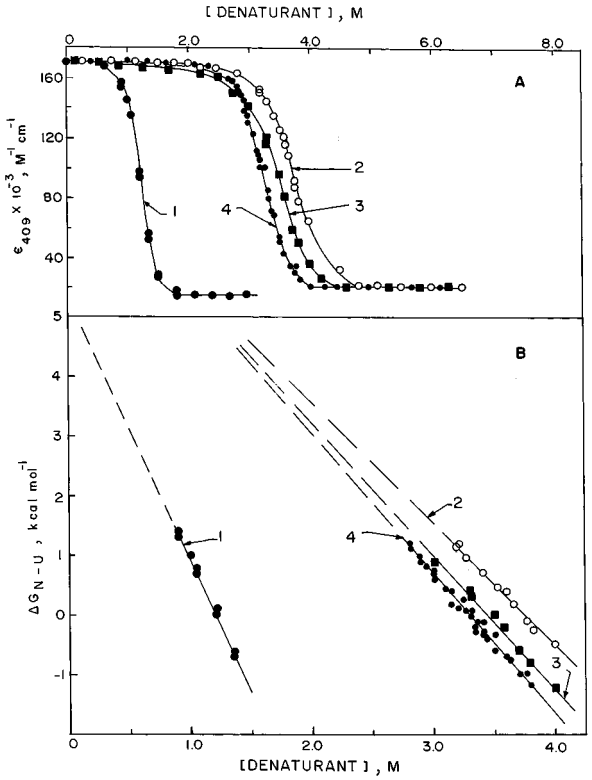

FIGURE 1: (A) Denaturation of Mb by GdnHCl in cacodylic acid buffer (0.03 M cacodylic acid−0.1 M KCl) at pH 6.0 and 25 °C (curve 1). Transition curves (2−4) show the unfolding of Mb by urea in 0.03 M cacodylic acid buffer which contained 0.10 (curve 2), 0.26 (curve 3), and 0.43−1.10 (curve 4) M KCl at pH 6.0 and 25 °C. In order to maintain clarity, all data are not shown on curve 4. (B) $\Delta G_{N\text{-}U}$ versus [denaturant] plots. Lines were drawn with the parameters given in Table 1. The curve numbers have the same meaning as in (A). 58 data points ($\Delta G_{N\text{-}U}$, [urea]) were used to determine curve 4.

RESULTS AND DISCUSSION

Curve 1 in Figure 1A shows the GdnHCl-induced denaturation of metmyoglobin (Mb) in 0.03 M cacodylic acid−0.1 M KCl buffer at pH 6.0 and 25 °C. The urea-induced denaturation of the protein under the same experimental conditions is also shown in this figure (see curve 2). From these measurements, values of $\Delta G_{N\text{-}U}$ for a two-state process were determined using the relation:

$$\Delta G_{N\text{-}U} = -RT \ln \frac{y - y_N}{y_D - y} \qquad (1)$$

where y is the measured optical property in a particular denaturing condition and y_N and y_D are respectively the properties of the protein in the fully native and denatured states under the same condition. It should be noted that

Table 1: Thermodynamic Parameters for Mb Unfolding at pH 6.0 and 25 °C[a,b]

conditions	ΔG^0_{N-U} (kcal mol^{-1})	m (kcal mol^{-1} M^{-1})	C_m (M)
GdnHCl Denaturation			
control	5.35 (±0.10)	4.44 (±0.09)	1.20
0.1−1.0 M KCl	5.35 (±0.10)	4.44 (±0.09)	1.20
0.49 M urea	5.38 (±0.12)	5.67 (±0.12)	0.95
1.00 M urea	5.17 (±0.07)	6.66 (±0.09)	0.78
2.30 M urea	2.39 (±0.05)	7.04 (±0.13)	0.34
3.67 M urea	0.17 (±0.05)	9.10 (±0.45)	0.02
Urea Denaturation			
control	7.59 (±0.33)	2.02 (±0.09)	3.76
0.16 M KCl	7.57 (±0.35)	2.19 (±0.10)	3.45
0.33−1.0 M KCl	7.98 (±0.22)	2.44 (±0.07)	3.27
0.16 M GdnHCl	6.39 (±0.03)	2.32 (±0.01)	2.75
0.33 M GdnHCl	4.25 (±0.05)	1.96 (±0.02)	2.17
0.50 M GdnHCl	2.82 (±0.17)	1.79 (±0.11)	1.58
0.67 M GdnHCl	2.66 (±0.06)	2.45 (±0.06)	1.08
1.00 M GdnHCl	0.69 (±0.02)	2.19 (±0.06)	0.32

[a] Value of each parameter in parentheses is the standard deviation. [b] All solutions contained 0.03 M cacodylic acid−0.1 M KCl adjusted to pH 6.0.

whenever the spectral properties of the native state (y_N) and the denatured state (y_D) showed a dependence on denaturant concentration, allowances were made for these dependencies in estimating ΔG_{N-U}.

The values of the Gibbs energy change for $-1.3 < \Delta G_{N-U}$ (kcal mol^{-1}) < 1.3, determined from the measurements shown in Figure 1A, are plotted in Figure 1B. All the plots of ΔG_{N-U} versus denaturant concentration were analyzed using LEM, the linear free energy model (eq 2) where m is

$$\Delta G_{N-U} = \Delta G^0_{N-U} - m[\text{denaturant}] \qquad (2)$$

the slope of the ΔG_{N-U} versus [denaturant] plot and ΔG^0_{N-U} is the value of ΔG_{N-U} at [denaturant] = 0 M. A least-squares analysis of the GdnHCl-induced denaturation results gave values of 5.35 ± 0.10 kcal mol^{-1} and 4.44 ± 0.09 kcal mol^{-1} M^{-1} for ΔG^0_{N-U} and m, respectively. These values of ΔG^0_{N-U} and m are in excellent agreement with what had been reported previously [see Ahmad et al. (1992) and references cited therein]. In the case of urea-induced denaturation, analysis of the ΔG_{N-U} versus [urea] plot (see curve 2 in Figure 1B) using eq 2 gave values of 7.59 ± 0.33 kcal mol^{-1} and 2.02 ± 0.09 kcal mol^{-1} M^{-1} for ΔG^0_{N-U} and m, respectively. These values are in good agreement with those reported earlier (Ahmad & Bigelow, 1982; Pace, 1975).

All available data suggest that the concentrated solutions of GdnHCl and urea unfold Mb to the same extent (Lapanje, 1978). It is then expected that the same amount of ΔG^0_{N-U} will be associated with the transition between N and U states induced by these denaturants. Results shown in Figure 1B (also see Table 1), however, suggest that the estimate of ΔG^0_{N-U} from urea-induced denaturation is significantly larger than that from the GdnHCl-induced denaturation. It is not surprising to find proteins which give different values of ΔG^0_{N-U} associated with urea- and GdnHCl-induced transitions (Pace, 1975; Ahmad & Bigelow, 1982; Ropson et al., 1990; Monera et al., 1993, 1994; Yao & Bolen, 1995). What seems to be surprising is that the estimate of ΔG^0_{N-U} of Mb from urea denaturation is larger than that obtained from GdnHCl denaturation, for the net charge on Mb having an isoelectric point of 7 would be positive at pH 6.0, and urea being a neutral molecule cannot mask the electrostatic repulsion that should destabilize the N state.

It is interesting to note the study of acid-induced denaturation of Mb at different NaCl concentrations reported by Friend and Gurd (1979). They observed that the midpoint of acid denaturation of the protein shifted to lower pH on addition of the salt; i.e., the native form is relatively destabilized by increased ionic strength. Since the titration behavior of the denatured form was found to be less sensitive to ionic variation than that of the native form, they argued that the native form experiences net stabilization from intramolecular electrostatic interactions. If one accepts this argument, it seems that the observed discrepancy between the estimate of ΔG^0_{N-U} of Mb from GdnHCl and urea stems from the ionic and nonionic behaviors of GdnHCl and urea, respectively. In order to investigate this possibility, we have studied the urea-induced denaturation of the protein in the presence of various concentrations of KCl. Curves 2−4 in Figure 1A show these measurements. As it has been observed in the case of acid-induced denaturation of Mb in the presence of different salt concentration (Friend & Gurd, 1979), the addition of KCl in the range 0.0−0.3 M reduces the midpoint of the urea-induced denaturation of the protein. It is also seen in Figure 1A that addition of KCl above 0.3 M KCl has no effect on the protein stability. The optical data (curves 2−4 in Figure 1A) were converted into ΔG_{N-U} values using eq 1, and these values of the free energy change of denaturation were plotted as a function of urea concentration. These plots are shown in Figure 1B. At least-squares analysis of ΔG_{N-U} versus [urea] at each concentration of KCl gave values of ΔG^0_{N-U}, m, and C_m which are given in Table 1. It is seen in this table that the value of ΔG^0_{N-U} is, within experimental error, independent of the addition of KCl. Thus, this observation rules out the possibility that the cause of discrepancy between the estimates of protein stability from urea and GdnHCl denaturations is due to electrostatic interaction.

Theoretical arguments were presented for the nonlinear dependence of ΔG_{N-U} on [GdnHCl] and the linear dependence of ΔG_{N-U} on [urea] (Alonso & Dill, 1991). Thus, another possibility for the difference in the estimates of protein stability from urea and GdnHCl denaturations could be due to this difference in the functional dependence of ΔG_{N-U} on the concentrations of these chemical denaturants. In order to investigate this possibility, studying the Mb denaturation in the full concentration range of the denaturant is required so that the extrapolation region can be reduced to 0 M denaturant. There are several ways to do it. The earliest approach involves measurements of GdnHCl-induced denaturation of the protein at different pH values (Pace & Vanderburg, 1979). In this approach using a standard procedure, all the ΔG_{N-U} data are normalized to a common pH. Another method to extend the GdnHCl measurements of protein denaturation to 0 M of the denaturant combines the differential scanning calorimetric measurements in the presence of the chemical denaturant with the isothermal denaturation of the protein followed by observing changes in a suitable conformational property (Santoro & Bolen, 1992). Using the Gibbs−Helmoltz equation, Santoro and Bolen used the calorimetric measurements to calculate the apparent ΔG_{N-U} in the presence of GdnHCl at a temperature at which equilibrium measurements were made. We have recently suggested another procedure that involves measurements of

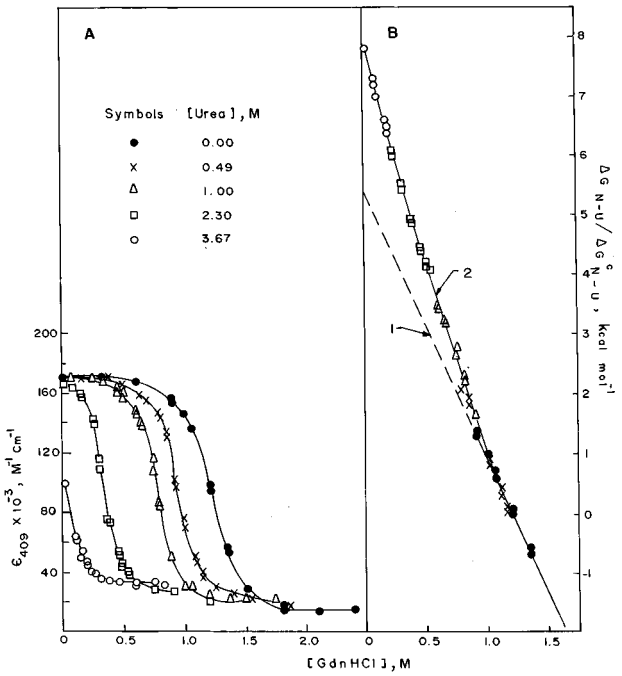

FIGURE 2: (A) GdnHCl-induced denaturation of Mb at pH 6.0 and 25 °C in the presence of different concentrations of urea. Each solution contained 0.03 M cacodylic acid−0.1 M KCl. (B) Symbols have the same meaning as in (A), and values of $\Delta G^{c}_{N\text{-}U}$ were determined as described in the text. Curve 1 is drawn with $\Delta G^{0}_{N\text{-}U}$ = 5.35 kcal mol^{-1} and m = 4.44 kcal mol^{-1} M^{-1} (control experiment) using LEM. Curve 2 is drawn with $\Delta G^{0}_{N\text{-}U}$ = 8.03 kcal mol^{-1}, $\Delta n = 20$, and $k = 0.8$ according to the binding model (eq 5).

the GdnHCl-induced denaturation of the protein in the presence of different concentrations of urea and vice versa at constant temperature and pH (Ahmad et al., 1994). There are at least two advantages of this procedure: (1) Both urea and GdnHCl give the same unfolded state of the protein. (2) All measurements are made at the same temperature and pH.

In order to investigate the problem of the functional dependence of $\Delta G_{N\text{-}U}$ on [GdnHCl] in the full concentration range, we have studied GdnHCl-induced denaturation of Mb in the presence of different concentrations of urea at pH 6.0 and 25 °C (see Figure 2A). It should be noted that we did not maintain the ionic strength of the protein solution during denaturation by the mixed denaturant system (GdnHCl−urea). The reason for this is that we have observed that GdnHCl-induced denaturation of Mb is independent of the addition of KCl in the concentration range 0−1.0 M (see Table 1). All the $\Delta G_{N\text{-}U}$ values calculated from the results shown in Figure 2A are plotted as a function of [GdnHCl]. It has been observed that at each urea concentration the $\Delta G_{N\text{-}U}$ versus [GdnHCl] plot is linear (results not shown here). A least-squares analysis of these plots according to eq 2 gave values of $\Delta G^{0}_{N\text{-}U}$, m, and C_m which are given in Table 1. As expected, values of C_m and $\Delta G^{0}_{N\text{-}U}$ decreased with increasing concentration of urea. On the other hand, the value of m_g, the slope of the $\Delta G_{N\text{-}U}$ versus [GdnHCl] plot, showed a strong dependence on [urea], and its values increased from 4.44 to 9.10 kcal mol^{-1} M^{-1}. Interestingly, these findings are in agreement with those obtained from the equilibrium measurements of GdnHCl-induced denaturation of Mb at different pH values (Pace & vanderburg, 1979).

We may write the free energy change of denaturation in the presence of a GdnHCl−urea mixture at constant temperature and pH as the sum of contributions of GdnHCl and urea (Ahmad et al., 1994), i.e.

$$\Delta G_{N\text{-}U} = \Delta G^{0}_{N\text{-}U} - m_g[\text{GdnHCl}] - m_u[\text{urea}] \quad (3)$$

where $\Delta G^{0}_{N\text{-}U}$ is the value of $\Delta G_{N\text{-}U}$ at 0 M of both chemical denaturants and m_g[GdnHCl] and m_u[urea] are the free energy contributions of GdnHCl and urea, respectively. The subscripts g and u denote the fact that the values of m are determined from the measurements of GdnHCl- and urea-induced denaturations of the protein, respectively. It should be noted that the inherent assumption in eq 3 is that the dependence of $\Delta G_{N\text{-}U}$ on each [denaturant] is linear. It is obvious from this equation that all the $\Delta G_{N\text{-}U}$ values measured from the GdnHCl-induced denaturation at a fixed urea concentration can be corrected for the denaturing effect of urea if the value of m_u is known and vice versa. The corrected free energy change ($\Delta G^{c}_{N\text{-}U}$), given by eq 4,

$$\Delta G^{c}_{N\text{-}U} = \Delta G_{N\text{-}U} + m_u[\text{urea}] = \Delta G^{0}_{N\text{-}U} - m_g[\text{GdnHCl}] \quad (4)$$

depends only on [GdnHCl]. Contrary to m_g, the magnitude of m_u showed a dependence on KCl concentration (see Table 1). It is seen in Figure 2A that values of $\Delta G_{N\text{-}U}$ [$-1.3 < \Delta G_{N\text{-}U}$ (kcal mol^{-1}) < 1.3] can be measured in the ionic denaturant concentration ranges of 0.8−1.2, 0.6−0.9, 0.2−0.5, and 0−0.15 at 0.49, 1.00, 2.30, and 3.67 M urea, respectively. These values of $\Delta G_{N\text{-}U}$ were normalized using eq 4 with appropriate values of m_u. A value of 2.44 kcal mol^{-1} M^{-1} for m_u was used to determine $\Delta G^{c}_{N\text{-}U}$ at two fixed concentrations of 0.49 and 1.00 M urea, for m_u does not depend on KCl concentration in the range 0.3−1.0 M (see Table 1). Since values of $\Delta G_{N\text{-}U}$ associated with the GdnHCl-induced denaturation in the presence of 2.30 M urea could be measured in the ionic denaturant concentration range of 0.2−0.5 M, $\Delta G^{c}_{N\text{-}U}$ values were estimated using a value of 2.32 kcal mol^{-1} M^{-1} for m_u, which is an average of the m_u values obtained from urea-induced denaturation at KCl concentrations of 0.16 M and greater than 0.3 M (see Table 1). In order to correct $\Delta G_{N\text{-}U}$ for GdnHCl-induced denaturation in the presence of 3.67 M urea, we used a value of 2.12 kcal mol^{-1} M^{-1} for m_u, which is the mean of the values of m_u obtained from the urea-induced denaturation at 0 M (control experiment) and 0.16 M KCl additions.

All the $\Delta G^{c}_{N\text{-}U}$ values estimated from the results shown in Figure 2A, using the procedure described in the preceding paragraph, are plotted as a function of [GdnHCl] in Figure 2B. It is seen in Figure 2B that all the data do not fall on curve 1 which describes the linear dependence of $\Delta G_{N\text{-}U}$, obtained from measurements of GdnHCl-induced denaturation of Mb in the absence of urea, on [GdnHCl] (curve 1 in Figure 1B). These results suggest that the functional dependence of $\Delta G_{N\text{-}U}$ on [GdnHCl] is nonlinear. Since the relationship between $\Delta G_{N\text{-}U}$ and [GdnHCl] is nonlinear, and the observed upward curvature is expected for the denaturation described by the binding model (Tanford, 1970; Pace & Vanderburg, 1979; Alonso & Dill, 1991), we fitted the entire ($\Delta G^{c}_{N\text{-}U}$, [GdnHCl]) data to eq 5 (Pace & Vanderburg, 1979), where Δn is the number of specific binding sites on the protein for the denaturant which binds noncooperatively

$$\Delta G^{c}_{N\text{-}U} = \Delta G^{0}_{N\text{-}U} - (\Delta n)RT \ln (1 + k[\text{GdnHCl}]) \quad (5)$$

with an average association constant k. Results of the least-squares analysis of the $\Delta G^{c}_{N\text{-}U}$ versus [GdnHCl] plot according to eq 5 are shown in Figure 2B. It is seen in this figure (see curve 2) that the value of $\Delta G^{0}_{N\text{-}U}$ is 8.03 ± 0.04 kcal mol^{-1} (see curve 2), which is, within experimental error, identical to that observed for urea denaturation (see Table 1). Furthermore, we obtained values of 20 for Δn and 0.8 for k, which are in excellent agreement with those reported by Pace and Vanderburg (1979).

Results of the analysis of data of Mb presented in Figure 2B are consistent with the theoretical (Alonso & Dill, 1991) and experimental (Pace & Vanderburg, 1979) considerations. A few comments are, however, necessary. First, the theoretical support of the nonlinear dependence of $\Delta G_{N\text{-}U}$ on [GdnHCl] is based on the transfer free energy data of amino acids and oligopeptides (Pace, 1975). The problem with the theoretical treatment is that the steric bulk of the peptide backbone which reduces the number of solvent molecuels in contact with the exposed side chains has not been considered. In fact, on comparing the value of hydrocarbon for the amino acid side chain, Nemethy (1967) remarked: "one must bear in mind that the parameters for the transfer of a side chain from one medium to another must always be smaller in magnitude than for the dissolution of free hydrocarbon with the same number of carbon atoms". It is then possible that the nonlinearity between $\Delta G_{N\text{-}U}$ and [GdnHCl] predicted from the theoretical treatment may stem from the observation that for each protein group the relationship between transfer free energy and [GdnHCl] is nonlinear (Ahmad & Bigelow, 1986, 1990). Second, a value of 20 for Δn is several times smaller than the number of putative binding sites exposed on denaturation. Third, instead of eq 5, a more realistic treatment of the data shown in Figure 2B must involve both solvation and binding contributions of the denaturant [see eq 45 in Schellman (1978)]. We have observed that treatment of data using this model fits the entire results very well. Finally, in correcting the observed $\Delta G_{N\text{-}U}$ for the presence of [urea] during GdnHCl-induced denaturation using eq 4, it has been assumed that the functional dependence of $\Delta G_{N\text{-}U}$ on [urea] is linear (Alonso & Dill, 1991). However, there are a few reports of $\Delta G_{N\text{-}U}$ to be a nonlinear function of [urea] (Shortle et al., 1989; Johnson & Fersht, 1995).

In order to determine the exact functional relationship between $\Delta G_{N\text{-}U}$ and [urea] in the full denaturant concentration range, we measured the urea-induced denaturation of Mb in the presence of different concentrations of GdnHCl at pH 6.0 and 25 °C. Results of such measurements are shown in Figure 3A. From these measurements, values of $\Delta G_{N\text{-}U}$ were estimated using eq 1, and plotted against [urea]. It has been observed that each $\Delta G_{N\text{-}U}$ versus [urea] plot is linear (result not shown). A least-squares analysis according to eq 2 of these plots yielded values of thermodynamic parameters which are entered in Table 1. At each fixed concentration of the ionic denaturant, $\Delta G_{N\text{-}U}$ values obtained from the measurements shown in Figure 3A were corrected for the denaturational effect of GdnHCl using eq 6 where $\Delta G_{N\text{-}U}$ is

$$\Delta G^{c}_{N\text{-}U} = \Delta G_{N\text{-}U} + 20\,RT \ln (1 + 0.8[\text{GdnHCl}]) = \Delta G^{0}_{N\text{-}U} - m_u[\text{urea}] \quad (6)$$

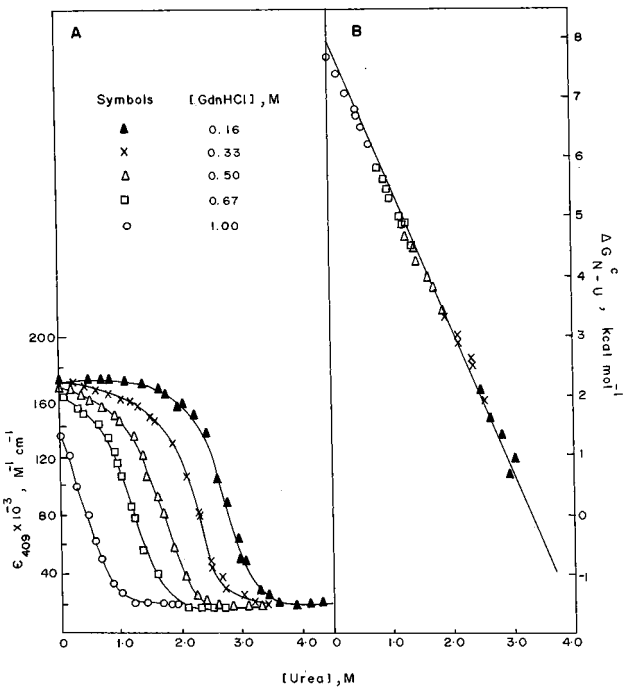

FIGURE 3: (A) Urea-induced denaturation of Mb at pH 6.0 and 25 °C in the presence of different values of [GdnHCl] shown in the figure. Each solution contained 0.03 M cacodylic acid−0.1 M KCl. (B) The solid line is drawn with $\Delta G^{0}_{N\text{-}U}$ = 7.98 kcal mol^{-1} and m_u = 2.44 kcal mol^{-1} M^{-1}. Symbols have the same meaning as in (A), and represent $\Delta G^{c}_{N\text{-}U}$ values obtained from measurements of the urea-induced denaturation of protein at different GdnHCl concentrations (see the text).

the value of the free energy change in the presence of the urea−GdnHCl mixture. Figure 3B shows the mapping of $\Delta G^{c}_{N\text{-}U}$ on the $\Delta G_{N\text{-}U}$ versus [urea] plot obtained from measurements of urea-induced denaturation in the presence of [KCl] > 0.3 M (see the solid line). It is seen in this figure that the value of $\Delta G^{c}_{N\text{-}U}$ in the lower [urea] range falls slightly below the solid line. However, this variation is not significant. The reason for saying this is that a least-squares analysis of all the data ($\Delta G^{c}_{N\text{-}U}$, [urea]) given in Figure 3B, according to eq 2, gave values of 7.68 ± 0.04 kcal mol^{-1} and 2.29 ± 0.08 kcal mol^{-1} M^{-1} for $\Delta G^{0}_{N\text{-}U}$ and m_u, respectively, which are, within the error of experiments, identical to those observed for the urea-induced denaturation in the presence of [KCl] in the range 0.33−1.00 M (see Table 1).

It is interesting to note the findings of the study of denaturation *of Escherichia coli* derived rat intestinal fatty acid binding protein by GdnHCl and urea (Ropson et al., 1990). These investigators used LEM to analyze both urea and GdnHCl denaturation results. It has been observed (1) that the $\Delta G^{0}_{N\text{-}U}$ value of urea is significantly larger than that for GdnHCl denaturation and (2) that addition of 1 M NaCl to urea-induced denaturation reduces the value of $\Delta G^{0}_{N\text{-}U}$. We have analyzed their GdnHCl results according to eq 5, and observed that the discrepancy between the estimates of $\Delta G^{0}_{N\text{-}U}$ from urea and GdnHCl is reduced, and they are very close to one another.

In conclusion, we are sure of three things: (1) The electrostatic interaction stabilizes the native structure of Mb. (2) The Gibbs energy change varies nonlinearly with the concentration of GdnHCl. (3) $\Delta G_{N\text{-}U}$ is a linear function of urea concentration.

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