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Determining Globular Protein Stability: Guanidine Hydrochloride Denaturation of Myoglobin†

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ABSTRACT: The guanidine hydrochloride (Gdn·HCl) denaturation of horse myoglobin has been investigated at several pH values using absorbancy measurements at 409 nm. From these data the free energy of denaturation, ΔG_D, can be calculated and ΔG_D values have been measured to zero concentrations of denaturant. The dependence of ΔG_D on Gdn·HCl concentration, $d(\Delta G_D)/d(\text{Gdn·HCl})$, increases markedly as the denaturant concentration decreases. This indicates that an increase in the number of Gdn·HCl binding sites on unfolding is the major driving force for denaturation by Gdn·HCl. An equation based on denaturant binding which fits the experimental data for myoglobin at pH 7 is $\Delta G_D = \Delta G_D^{H_2O} - \Delta nRT \ln (1 + ka_\pm)$, where $\Delta G_D^{H_2O}$ (= 10.1 kcal/mol) is the free energy of denaturation in the absence of denaturant, Δn (= 42.8) is the difference in the number of Gdn·HCl binding sites on the native and denatured states of the protein, k (= 0.6) is the average denaturant binding constant, and $a_\pm$ is the mean ion activity of Gdn·HCl. Several lines of evidence are presented which show convincingly that $k = 0.6$ should be used in the analysis of Gdn·HCl denaturation curves rather than $k = 1.2$ which is currently in widespread use. In addition, for myoglobin and four other proteins, an equivalent and more convenient method of analyzing Gdn·HCl denaturation curves is to use Gdn·HCl molarities rather than mean ion activities and a denaturant binding constant of 0.8.

Guanidine hydrochloride (Gdn·HCl),[1] urea, and sodium dodecyl sulfate are the three most popular protein denaturants. Gdn·HCl and urea produce a randomly coiled denatured state (Tanford, 1968) which, for many purposes, is a more useful denatured state than the helical, rod-like denatured state (Reynolds & Tanford, 1970) produced by sodium dodecyl sulfate. The use of Gdn·HCl as a denaturant has increased substantially over the past 10 years. Gdn·HCl is a more potent denaturant than urea, unfolding proteins at two to three times lower concentrations (Greene & Pace, 1974), and Gdn·HCl is chemically stable, while urea slowly decomposes to form cyanate and ammonia.

One of the interesting results from a study of the Gdn·HCl denaturation of a globular protein is an estimate of the stability of the protein; i.e., it is possible to estimate the free energy change, ΔG_D, for the reaction, globular conformation $\rightleftharpoons$ randomly coiled conformation, in the absence of denaturant, $\Delta G_D^{H_2O}$. This is done by measuring ΔG_D as a function of Gdn·HCl concentration and extrapolating to zero concentration. Several different approaches have been used for making this extrapolation, but, unfortunately, they lead to estimates of $\Delta G_D^{H_2O}$ which differ by as much as 20–50%. Thus it has been possible to show that the stability of globular proteins is remarkably low, but it has not been possible to determine with certainty small differences in stability between, for example, homologous proteins or chemically modified proteins (Pace, 1975).

Aune & Tanford (1969) made the first serious attempt to estimate $\Delta G_D^{H_2O}$ using data from a Gdn·HCl denaturation study. They state that the fact that Gdn·HCl promotes unfolding "... requires that more guanidinium and/or chloride ions are bound to the denatured form than to the native form; or that more water is bound to the native form than to the denatured forms; or a combination of these effects." They showed that several different models for analyzing denaturization in terms of binding were consistent with their data and could not be distinguished. The model used subsequently by Tanford's laboratory (Salahuddin & Tanford, 1970) and many other groups (Pace, 1975; Ahmad & Salahuddin, 1976; McLendon & Sandburg, 1978; Ahmad & McPhie, 1978) ignored the contribution of changes in water binding (Tanford, 1970) and assumed that denaturation results from an increase in the number of binding sites for guanidinium ions on unfolding. This model led to an equation of the form:

$$\Delta G_D = \Delta G_D^{H_2O} - \Delta nRT \ln (1 + ka_\pm) \quad (1)$$

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[1] Abbreviation used: Gdn·HCl, guanidine hydrochloride.

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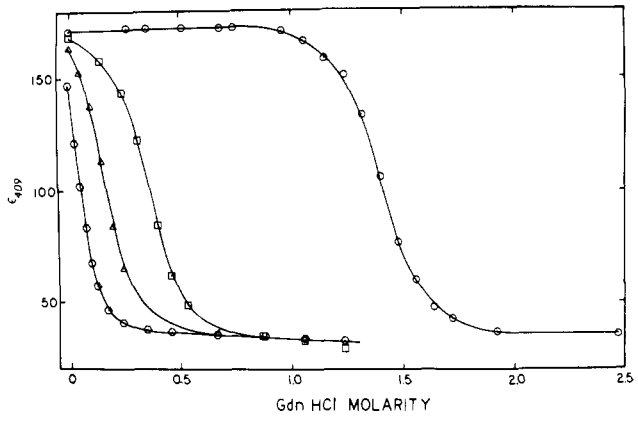

FIGURE 1: Millimolar absorption coefficient at 409 nm as a function of Gdn·HCl molarity for horse myoglobin at 25 °C, 0.1 M KCl, 0.1 M phosphate (pH 6) or acetate buffer. pH 6 (○), 4.9 (□), 4.7 (△), and 4.6 (○).

where Δn is the difference in the number of binding sites between the denatured and native states, k is the average binding constant of these sites, and $a_{\pm}$ is the mean ion activity of Gdn·HCl. The fit of the experimental data is not very sensitive to the value of k and a value of $k = 1.2$ which was found to give the best fit to data for the denaturation of lysozyme by Aune & Tanford (1969) was used in most subsequent papers (Pace, 1975).

Since the publication of Aune & Tanford's paper, several interesting experimental studies have led to results bearing on the applicability of eq 1. In this paper we use these results and our studies of the Gdn·HCl denaturation of horse myoglobin to show convincingly that eq 1 is well-suited for analyzing Gdn·HCl denaturation curves but that the binding constant for Gdn·HCl should be reduced from 1.2 to 0.6.

Materials and Methods

Type I horse myoglobin (lot 25C-7640) was purchased from Sigma Chemical Co. No purification of the myoglobin was attempted since the midpoint of the neutral pH Gdn·HCl denaturation curve is in good agreement with previous studies (Puett, 1973), and the observed dependence of ΔG_D on Gdn·HCl concentration (4500 cal mol^{-1} M^{-1}) is the largest observed for any protein and in agreement with the results of McLendon (1977). Myoglobin was oxidized by adding 0.1% potassium ferricyanide and then dialyzed extensively against 0.1 M KCl. Concentrations of myoglobin stock solutions were determined using a molar absorption coefficient at 409 nm of 1.71×10^5 M^{-1} cm^{-1} (Puett, 1973). This solution was then buffered at the required pH with 0.1 M phosphate or 0.1 M acetate.

Guanidine hydrochloride (lot 217018) was obtained from Heico Inc. All Gdn·HCl stock solutions contained 0.1 M KCl and 0.1 M acetate or phosphate buffer. Concentrations were determined from refractive index measurements using an equation given by Nozaki (1972).

Absorbances were measured in thermostated 1-cm cuvettes at 25.0 °C with a Cary Model 15 spectrophotometer after equilibrium was attained (30–90 min). The pH of each solution was then measured on a Radiometer Model 26 pH meter.

Results

The denaturation of myoglobin by Gdn·HCl at four pH values is shown in Figure 1. It has been shown previously that denaturation under these conditions is reversible and that the mechanism is close to a two-state mechanism (Puett, 1973, and references therein). Consequently, the free energy of denaturation, ΔG_D, can be determined from the experimental data using

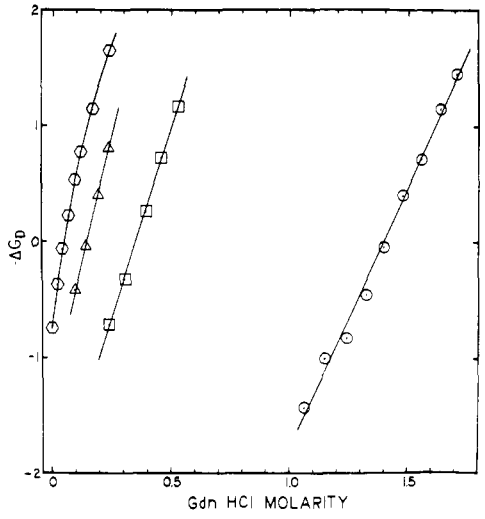

FIGURE 2: ΔG_D as a function of Gdn·HCl molarity for the denaturation of horse myoglobin at 25 °C. ΔG_D was calculated with eq 1 using the data in Figure 1 and adjusted to a constant pH as described in the text. pH 6.0 (○), 4.9 (□), 4.7 (△), and 4.6 (○).

$$\Delta G_D = -RT \ln \frac{\epsilon_N - \epsilon}{\epsilon - \epsilon_D} \qquad (2)$$

where ϵ is the observed molar absorption coefficient, and ϵ_N and ϵ_D are the molar absorption coefficients of the native and denatured states under the same conditions (Pace, 1975). When eq 2 is used to analyze the data in Figure 1, the results shown in Figure 2 are obtained. The ΔG_D values are strongly pH dependent and Gdn·HCl concentration influences the pH of the solutions even in the presence of 0.1 M buffer (Nozaki, 1972). Consequently, the pH values of the individual solutions used for determining the points shown in Figure 1 decrease over the course of the transition; e.g., for the pH 6 experiment, the pH decreases from 6.07 to 5.85 between 1.06 and 1.71 M Gdn·HCl. Since we are interested only in the dependence of ΔG_D on Gdn·HCl concentration, it is essential to correct the observed values of ΔG_D to a common pH. This was done using an equation and parameters[2] determined by Puett (1973). It will be seen below that our data, though more limited, are in reasonable agreement with values calculated using Puett's equation.[2] It can be seen in Figure 2 that the dependence of ΔG_D on Gdn·HCl concentration increases as the denaturant concentration decreases. The slope of the data at pH 6 yields a dependence of 4500 cal mol^{-1} M^{-1} near 1.4 M Gdn·HCl, but this increases to 6600 cal mol^{-1} M^{-1} near 0.4 M Gdn·HCl (pH 4.9 data) and to 8800 cal mol^{-1} M^{-1} near 0.2 M Gdn·HCl (pH 4.7 data). The results at pH 4.6 show that dependence increases even further as the Gdn·HCl concentration is decreased below 0.1 M.

The data in Figure 2 have been adjusted to pH 7 by adding a constant to all of the data points at a given pH and the results are shown in Figure 3 (see Tanford, 1970, for a discussion of the additivity of contributions to ΔG_D). The constants added at low pH, 9.56 (pH 4.6), 8.69 (pH 4.7), and 7.05 kcal/mol (pH 4.9), were selected so that the data coincided near 0.24 M Gdn·HCl where ΔG_D values had been determined for all

[2] The equation determined by Puett (1973) is $\Delta G_D = 8.22 - 6RT \ln (H^+ + 10^{-3.7})(H^+ + 10^{-3.8})(H^+ + 10^{-6.7})/(H^+ + 10^{-4.0})(H^+ + 10^{-5.97})(H^+ + 10^{-6.5})$. This equation was used only for determining the differences in ΔG_D values between various pH values.

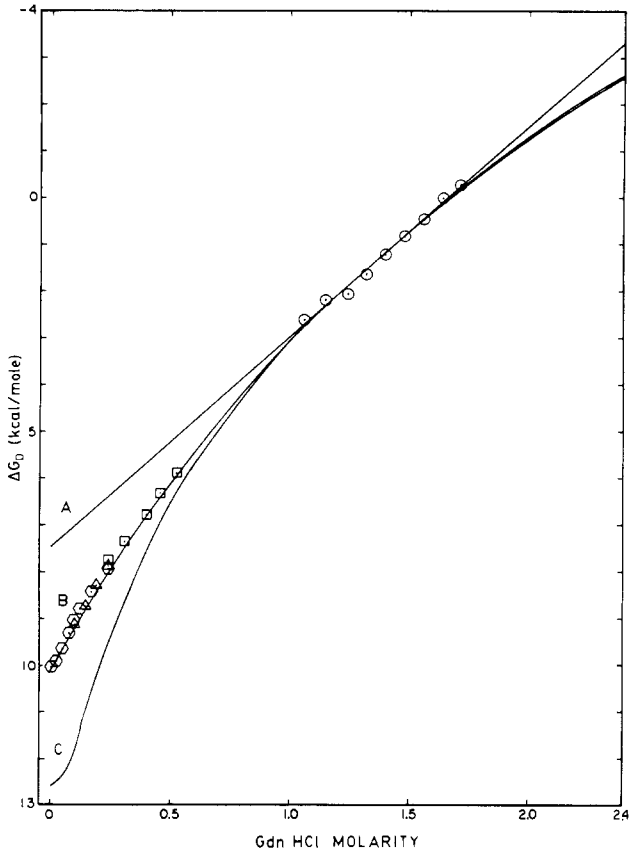

FIGURE 3: ΔG_D for the denaturation of horse myoglobin as a function of Gdn·HCl molarity at 25 °C, pH 7. The data from Figure 2 have been adjusted to pH 7 as described in the text. The solid lines are theoretical curves. (Curve A) Equation 4 with m = 4.50 kcal mol^{-1} M^{-1} and $\Delta G_D^{H_2O}$ = 7.5 kcal/mol. (Curve B) Equation 1, with k = 0.6, Δn = 42.8, and $\Delta G_D^{H_2O}$ = 10.1 kcal/mol. (Curve C) Equation 1 with k = 1.2, Δn = 31.56, and $\Delta G_D^{H_2O}$ = 12.56.

three pH values. These constants agreed to better than 0.5 kcal/mol with constants calculated using Puett's equation.[2] The constant term added to the pH 6 data was 1.18 kcal/mol calculated using Puett's equation.[2] The marked departure from linearity at lower Gdn·HCl concentrations is clearly evident in Figure 3.

Discussion

The results of most Gdn·HCl denaturation studies are being analyzed using eq 1 with an average denaturant binding constant of 1.2. Several different lines of evidence suggest that a binding constant of 1.2 is too high and that a value of 0.6, or perhaps lower, is more reasonable. This evidence is summarized below.

The extent of binding of Gdn·HCl to proteins has now been measured in several laboratories (Hade & Tanford, 1967; Warren & Gordon, 1970; Lee & Timasheff, 1974; Span et al., 1974; Bull & Breese, 1976). In the most comprehensive of these studies, Lee & Timasheff show that the number of Gdn·HCl molecules bound by proteins in 6 M Gdn·HCl correlates well with the total of the number of peptide bonds/2 plus the number of aromatic amino acids. This indicates that peptide bonds and the aromatic amino acids are the most important Gdn·HCl binding sites, a conclusion in accord with results from solubility studies (see below). Using only one-half the number of peptide bonds suggests that two peptide bonds form a binding site for Gdn·HCl, a proposal originally made by Robinson & Jencks (1965). Thus, the use of a model based on denaturant binding for analyzing Gdn·HCl denaturation curves is now based on solid experimental evidence.

Table I: Estimation of Denaturant Binding Constant from Analysis of Gdn·HCl Binding to Lysozyme

Gdn·HCl bound to lysozyme (mol of Gdn·HCl/mol of lysozyme)			
technique	3.2 M Gdn·HCl	5 M Gdn·HCl	difference (Δn)
density measurements[a]	33	61	28
equilibrium dialysis[b]	24	52	28

Gdn·HCl denaturation curve[c] anal. with eq 1		
k	$\Delta G_D^{H_2O}$	Δn
0.6	9.3	28.0
1.2	10.7	19.8

[a] Lee & Timasheff (1974). [b] Span et al. (1974). [c] Greene & Pace (1974).

Table II: Calculation of Denaturant Binding Constant from Free Energy of Transfer of Unfolded RNase

RNase (native, H_2O) ⇌ RNase (denatured, 6 M Gdn·HCl)	$\Delta G = -18.3$ kcal[a]/mol
RNase (native, H_2O) ⇌ RNase (denatured, H_2O)	$\Delta G = 16.1$ kcal[b]/mol
RNase (denatured, H_2O) ⇌ RNase (denatured, 6 M Gdn·HCl)	$\Delta G = -34.4$ kcal/mol

in 6 M Gdn·HCl RNase binds 72 mol of Gdn·HCl/mol[c]

$$-\Delta g_{tr} = \Delta nRT \ln (1 + ka)$$
$$34\,400 = (72)RT \ln (1 + ka)$$
$$\therefore k = 0.51 \text{ M}^{-1}$$

[a] Schrier & Schrier (1976). [b] Greene & Pace (1974). [c] Lee & Timasheft (1974). The total number of binding sites for Gdn·HCl may well be greater than this estimate of the amount of Gdn·HCl actually bound in 6 M Gdn·HCl. If so, the value of k would be even lower than the value of 0.51.

Table III: Analysis of Free Energy of Transfer Data in Terms of Denaturant Binding

Gdn·HCl molarity	k for binding[a] of Gdn·HCl to Trp[b]	Tyr	Phe	two peptide groups	Met	Leu	His	Asn
1	0.76	0.92	0.83	0.61	0.54	0.54	0.67	0.76
2	0.75	0.97	0.87	0.61	0.54	0.45	0.66	0.76
4	0.80	1.11	1.04	0.63	0.60	0.51	0.57	0.80
6	0.78	1.10	1.11	0.53	0.61	0.52	0.43	0.81
av[f]	0.78	1.03	0.96	0.60	0.57	0.51	0.58	0.78

[a] Calculated from Δg_{tr} values[c-e] using eq 3. [b] Calculated assuming two binding sites per Trp side chain. [c] Nozaki & Tanford (1970). [d] Robinson & Jencks (1965). [e] Pace (1975). [f] Some of these values were listed previously in Tanford (1970).

For one protein, lysozyme, Lee & Timasheff (1974) measured the extent of Gdn·HCl binding as a function of Gdn·HCl concentration. As shown in Table I, their results suggest that the number of Gdn·HCl molecules bound by lysozyme increases by 28 between 3.2 and 5.0 M Gdn·HCl where lysozyme unfolds. When we analyze results presented by Span et al. (1974) in the same way, the magnitude of the binding is different, but the change in the number of Gdn·HCl molecules bound is again 28. Also shown in Table I are the Δn values obtained from an analysis of the Gdn·HCl denaturation of lysozyme using eq 1 and k values of 1.2 and 0.6. It is clear that a value of k = 0.6 leads to better agreement between results from direct studies of Gdn·HCl binding and results from studies of the Gdn·HCl denaturation of lysozyme.

Using isopiestic vapor pressure measurements, Schrier & Schrier (1976) have determined the free energy for transfer

Table IV: Values of $\Delta G_D^{H_2O}$ and Δn Calculated Using Equation 1

protein	$a_\pm(k = 1.2)$		$a_\pm(k = 0.6)$		$M(k = 0.8)$		Tanford's model
	Δn	$\Delta G_D^{H_2O}$	Δn	$\Delta G_D^{H_2O}$	Δn	$\Delta G_D^{H_2O}$	$\Delta G_D^{H_2O}$
myoglobin (pH 6.0)	29.2	10.7	45.1	9.5	19.9	8.8	9.7
lysozyme (pH 2.9)[a]	19.8	10.7	28.0	9.3	12.8	9.1	9.1
α-chymotrypsin (pH 4.3)[a]	32.2	13.7	47.3	11.9	21.3	11.4	10.4
ribonuclease (pH 6.6)[a]	34.2	18.9	47.6	16.1	22.4	16.3	14.8
β-lactoglobulin (pH 3.2)[a]	45.5	26.0	62.3	21.9	29.5	22.3	18.4

[a] Green & Pace (1974). [b] $\Delta G_D^{H_2O}$ values are in kcal/mol.

of ribonuclease from 0 to 6 M Gdn·HCl to be −18.3 kcal/mol. Using the approach developed in this paper and the data of Greene & Pace (1974) leads to an estimate of ΔG_D = 16.1 kcal/mol for the unfolding of ribonuclease in H_2O. As shown in Table II, these data can be used to calculate that the free energy for transfer of *unfolded* ribonuclease from 0 to 6 M Gdn·HCl is −34.4 kcal/mol. Lee & Timasheff (1974) estimate that 72 mol of Gdn·HCl is bound per mole of ribonuclease in 6 M Gdn·HCl. Combining these data as illustrated in Table II leads to an estimate of the average binding constant for Gdn·HCl of 0.51.

Gdn·HCl increases the solubility of the peptide group and almost all of the amino acid side chains in aqueous solutions (Nozaki & Tanford, 1970). The free energies of transfer from water to an aqueous solution of denaturant, Δg_{tr}, for both urea and Gdn·HCl have been determined for most of the constituent parts of a protein and are summarized by Pace (1975). In most cases it is possible to account for these Δg_{tr} values in terms of a denaturant binding mechanism. This is illustrated in Table III where binding constants, k, have been calculated from Δg_{tr} values at four Gdn·HCl concentrations using

$$\Delta g_{tr} = RT \ln (1 + ka_\pm) \quad (3)$$

A molarity based mean ion activity was used for $a_\pm$ (see footnote 3, below). It can be seen that, for a dipeptide group and for a variety of amino acid side chains, including the most nonpolar side chains, binding constants in the range 0.5–1.0 M^{-1} will account for the observed Δg_{tr} values.

The range of k values is not large, and the peptide groups should predominate in determining the average value of k for a given protein. Thus, the use of k = 0.6 might be somewhat low for a protein in which a high proportion of aromatic side chains are exposed on unfolding and it might be somewhat too high for other proteins, but a value of 0.6 is reasonable based on these data and is clearly preferable to a value of 1.2.

Our results with myoglobin provide further support for the ideas presented above. Using eq 1 with k = 1.2 and determining Δn by a least-squares fit of the pH 6 data for myoglobin lead to Δn = 31.6 and $\Delta G_D^{H_2O}$ = 12.6 kcal/mol (pH 7). It can be seen in Figure 3 (curve C) that, while these parameters provide an excellent fit of the data at higher Gdn·HCl concentrations, they lead to a marked deviation from the experimental data at lower concentrations. Part of the difficulty lies in the equation given by Aune & Tanford (1969) for calculating activities for Gdn·HCl solution.[3] When the equation given in footnote 3 is used for calculating Gdn·HCl activities, the resulting curve still deviates substantially from the experimental data, but the upward bend in curve C at low Gdn·HCl activities is no longer observed.

When eq 1 is used with k = 0.6 to best fit the pH 6 data for myoglobin, Δn is found to be 42.84 and $\Delta G_D^{H_2O}$ = 10.1 kcal/mol (pH 7). Curve B in Figure 3 was calculated using these values and it follows the experimentally observed values of ΔG_D quite closely. This is somewhat surprising. Hermans et al. (1969) have shown that acid-denatured sperm whale myoglobin possesses considerable α-helical structure and 2–4 M urea is required to disrupt this secondary structure. Consequently, it seems likely that as low Gdn·HCl concentration is approached the denatured state will form increasing amounts of α helix. This would be expected to decrease the number of peptide groups available to bind Gdn·HCl and decrease the dependence of ΔG_D on Gdn·HCl concentration. In spite of thus, the dependence of ΔG_D on Gdn·HCl is observed to increase markedly at low Gdn·HCl concentrations. Thus, this is good evidence for the importance of denaturant binding in the unfolding of myoglobin.

A lower limit of the stability of a protein can be obtained by assuming that the linear dependence of ΔG_D on Gdn·HCl concentration observed at high denaturant concentration continues to zero concentration of denaturant. This leads to an equation of the form

$$\Delta G_D = \Delta G_D^{H_2O} - m(\text{Gdn·HCl}) \quad (4)$$

This straightforward approach has been used to estimate $\Delta G_D^{H_2O}$ in a number of papers (Knapp & Pace, 1974; Greene & Pace, 1974; McLendon & Sandburg, 1978). However, it is clear from Figure 3 that in the case of myoglobin this leads to an underestimate of the stability of the protein by almost 30%.

In a previous study (Greene & Pace, 1974) we compared the urea and Gdn·HCl denaturation of four proteins. The data from that study have been analyzed using eq 1 and k values of 0.6 and 1.2. The results are presented in Table IV along with the results for myoglobin. The fit of the experimental data to eq 1 does not differ significantly for the two values of k. The estimates of $\Delta G_D^{H_2O}$ are from 12 to 20% lower when the smaller binding constant is used. Estimates of $\Delta G_D^{H_2O}$ from another approach which uses the free energies of transfer referred to above and a theory developed by Tanford (1970) are also included in Table IV. These estimates are in better agreement with the $\Delta G_D^{H_2O}$ values obtained using a binding constant of 0.6 than with the estimates obtained using 1.2.

Because mean ion activities provide at best an approximation of the activity of the guanidinium ion and because of the

[3] The equation given by Aune & Tanford (1969) for calculating Gdn·HCl activities is based on calculations by K. A. Aune using unpublished experimental data determined by E. P. K. Hade, Jr. Dr. Aune has confirmed that this equation is only applicable above 0.5 M Gdn·HCl and has kindly supplied us with the original experimental data—results from vapor pressure osmometry between 0.05 and 0.5 *m* Gdn·HCl and results from the isopiestic method at higher concentrations. The vapor pressure osmometry data appear unreliable at low Gdn·HCl concentrations and we have assumed that the Gdn·HCl solutions approach Debye theory at low concentration, as they must, in determining an equation usable at both high and low Gdn·HCl concentrations. The equation so determined is $a_\pm = 0.6761\ M - 0.1468\ M^2 + 0.02475\ M^3 + 0.001318\ M^4$, where $a_\pm$ is the molarity based mean ion activity and M the molarity of Gdn·HCl solutions. The use of this equation to calculate activities at molarities below 1 M, while clearly better than the equation give by Aune & Tanford, should be regarded with skepticism until more reliable experimental data are available at low Gdn·HCl concentrations. This equation reproduces the experimental data above 0.5 M Gdn·HCl with an average deviation of 0.007 and was used for all of the calculations in this paper.

shortcomings of the activity data noted above, we attempted to use molarities rather than mean ion activities in eq 1 for analyzing the results for myoglobin. Using $k = 0.8$, we obtain $\Delta n = 19.9$ and $\Delta G_D^{H_2O} = 10.0$ kcal/mol (pH 7). These parameters lead to a curve which almost superimposes curve B, Figure 3, and gives an even better fit to the experimental data at low Gdn·HCl concentrations. In addition, for all of the proteins in Table IV it can be seen that this method yields estimates of $\Delta G_D^{H_2O}$ almost identical with those obtained using mean ion activities and $k = 0.6$. This method offers a more convenient procedure for analyzing Gdn·HCl denaturation curves since mean ion activities need not be calculated.

The evidence presented in this paper shows clearly that an increase in the number of binding sites for Gdn·HCl on unfolding is the major driving force for Gdn·HCl denaturation. The evidence is also good that a binding constant of 0.6 rather than 1.2 should be used in eq 1 when analyzing the results of a Gdn·HCl denaturation study.

Added in Proof

Dr. Eugene E. Schrier called to our attention that activity coefficients have been reported for guanidine hydrochloride solutions by Schrier & Schrier (1977) and by Bonner (1976). The activities calculated with the equation given in footnote 3 agree to better than ±0.01 with results reported by Bonner at 0.5 *m* and lower concentrations.

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