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Determining stability of proteins from guanidinium chloride transition curves

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The guanidinium chloride (GdmCl) denaturation of RNAase A, lysozyme and metmyoglobin was investigated at several pH values by using absorbance measurements at 287, 300 and 409 nm respectively. From these measurements the free-energy change on denaturation, $\Delta G_{app.}$, was calculated, assuming a two-state mechanism, and values of $\Delta G_{app.}$ at zero concentration of the denaturant were measured. For each protein all $\Delta G_{app.}$ values were adjusted to pH 7.00 by using the appropriate relationship between $\Delta G_{app.}$ and pH. Dependence of the adjusted $\Delta G_{app.}$ value on GdmCl concentration increases for metmyoglobin and decreases for the other two proteins as the denaturant concentration decreases. It has been shown that these are expected results if the presence of the acid-denatured state during the GdmCl denaturation of proteins is considered.

INTRODUCTION

If the conformational stability ($\Delta G_D^{H_2O}$) of a protein is defined as the Gibbs energy required to convert the native protein in water (or dilute buffer) from its folded conformation into a structureless polypeptide chain (random coil), its evaluation is then connected to the study of reversible unfolding by denaturants that give rise to a random coil. The reason for this is that the equilibrium between the native (N) state and denatured (D) structureless conformation can be established and studied only in the presence of denaturant, but not in its absence. Yet it is the value of $\Delta G_D^{H_2O}$ which is a quantity of fundamental interest in nearly all aspects of protein structure and dynamics.

In those cases in which a chemical denaturant is used to study protein denaturation, three procedures are currently used to evaluate $\Delta G_D^{H_2O}$ [1]. The three procedures are known as the binding model [2], the transfer–free-energy model [3–5] and the linear–free-energy model [6,7]. Application of the three procedures to the same set of data, however, results in $\Delta G_D^{H_2O}$ values that are significantly different [8]. The discrepancies between these estimates of $\Delta G_D^{H_2O}$ for a protein tend to erode confidence in this term as an accurate expression of protein stability. Confidence in the method of evaluation of $\Delta G_D^{H_2O}$ can, however, be increased if the extrapolation region is reduced to zero denaturant concentration.

Pace & Vanderburg [9] have reduced the extrapolation region to zero molar denaturant concentration by studying the guanidinium chloride (GdmCl) denaturation of metmyoglobin (Mb) at several pH values. Adjustment of ΔG_D (free-energy change on denaturation) data to a common pH suggested that ΔG_D is not a linear function in the denaturant concentration. However, all the theoretical [10,11] and experimental considerations [7,12–14] support the linear dependence of free-energy changes on the denaturant concentration. In order to see whether this behaviour of Mb is common to other proteins, we have studied the GdmCl denaturation of RNAase A, lysozyme and Mb at several pH values. In the present paper we address the question of whether, for a protein, ΔG_D values adjusted to a common pH have the property of predictability.

EXPERIMENTAL

Materials

Bovine pancreatic RNAase A (type XII-A), a three-times-crystallized, dialysed and freeze-dried preparation of hen's-egg lysozyme (grade I), and chromatographically purified horse heart myoglobin (type III) were purchased from Sigma Chemical Co. GdmCl was an ultrapure sample from Schwartz–Mann. All other chemicals were analytical-grade reagents.

Methods

Myoglobin was first oxidized by adding 0.1% potassium ferricyanide as described previously [15]. Concentration solutions of proteins were dialysed extensively against 0.1 M-KCl, pH 7.00. Protein concentration of stock solutions was determined experimentally by using the following absorption coefficients ($M^{-1} \cdot cm^{-1}$): RNAase A, $\epsilon_{277.5} = 9800$ [16]; lysozyme, $\epsilon_{280} = 39000$ [17]; and Mb, $\epsilon_{409} = 171000$ [18]. Concentration of the buffered stock solution of GdmCl was determined by refractive-index measurements [19].

All solutions for experimental measurements were prepared in the desired buffer containing 0.1 M-KCl. The pH of solutions was measured with a Consort P-907 pH-meter. Since the pH of individual protein solutions in the presence of GdmCl may change over the course of the transition [9], the pH of each solution was therefore measured.

Spectral measurements were made in a Shimadzu-2100 u.v.–visible spectrophotometer having water-jacketted cell holders. The temperature in the cell was maintained at 25 ± 0.05 °C by circulating water from a thermostatically controlled water bath. Spectra of Mb were recorded in the region 550–350 nm at different GdmCl concentrations. Denaturation transition curves were constructed by plotting ϵ_{409} versus the molar concentration of denaturant, [GdmCl]. GdmCl denaturations of RNAase A and lysozyme were studied by measuring the u.v. difference spectra of the proteins against the native protein at pH 3.00 (RNAase A) and pH 6.00 (lysozyme). The denaturation curves of RNAase A and lysozyme were constructed by plotting the values of $\Delta\epsilon_{287}$ and $\Delta\epsilon_{300}$ respectively against [GdmCl]. For all proteins, each measurement was taken in triplicate at least.

f_d, the fraction of the protein that was denatured, was calculated assuming a two-state transition and using the relationship:

$$f_d = \frac{(y - y_N)}{(y_D - y_N)} \quad (1)$$

Abbreviations used: GdmCl, guanidinium chloride; Mb, metmyoglobin.

where y is the measured optical property at a particular pH and GdmCl concentration, and y_N and y_D are the properties of the protein in the native and fully denatured states respectively under the same conditions. Whenever the spectral properties of the native and denatured states showed dependence on the denaturant concentration, allowances were made for these dependencies in calculating f_d.

The state of equilibrium was expressed in terms of what may be called an apparent equilibrium constant ($K_{app.}$):

$$K_{app.} = \frac{f_d}{(1-f_d)} = \frac{(y-y_N)}{(y_D-y)} \quad (2)$$

The apparent free-energy change on denaturation was calculated by using the relationship:

$$\Delta G_{app.} = -RT\ln\left[\frac{f_d}{(1-f_d)}\right] \quad (3)$$

$\Delta G_{app.}$ values for each protein were plotted against [GdmCl]. For all proteins these plots were linear, and least-squares calculations were used to determine $\Delta G^{H_2O}_{app.}$ and m in the relationship [20]:

$$\Delta G_{app.} = \Delta G^{H_2O}_{app.} - m\,[\text{GdmCl}] \quad (4)$$

where $\Delta G^{H_2O}_{app.}$ is the value of $\Delta G_{app.}$ at [GdmCl] = 0 M, and m is the slope of the straight line.

For each protein all the equilibrium data obtained from the GdmCl denaturation studies at several pH values were adjusted to pH 7.00 using a function that gives the pH-dependence of $K_{app.}$ [3]:

$$F\,(pH) = \frac{\prod_{i=1}^{n}(10^{-pH}+10^{-pK_{i,D}})}{\prod_{i=1}^{n}(10^{-pH}+10^{-pK_{i,N}})} \quad (5)$$

where n is the difference in the number of bound protons between D and N states, and $pK_{i,N}$ and $pK_{i,D}$ represent pK values of the ith group in the native and denatured states respectively.

RESULTS AND DISCUSSION

In terms of optical property (y) which has been used to follow denaturation in this study, the state of equilibrium is expressed by eqn. (2), which assumes that the transition between the native protein and the denatured product obtained by the action of GdmCl, is a two-state process. At lower pH values, if the acid-denatured (X) state of the protein exists, it is expected to contribute to the measured $K_{app.}$, which, for a three-state process ($N \rightleftharpoons X \rightleftharpoons D$) is given by the relationship [21]:

$$K_{app.} = \frac{K_D + \alpha K_X}{1+(1-\alpha)K_X} \quad (6)$$

where K_X and K_D are the equilibrium constants for the reactions $N \rightleftharpoons X$, and $N \rightleftharpoons D$ respectively, and α is the fraction of the total change in y that occurs in going from N to X, i.e.:

$$\alpha = \frac{y_X - y_N}{y_D - y_N} \quad (7)$$

For values of α in the range 0.1–0.9, K_D and $K_{app.}$ will be equal somewhere near the midpoint of the transition, but K_D will be less than $K_{app.}$ below the midpoint and greater than $K_{app.}$ above the midpoint [20]. If optical properties of the X and D states are identical, i.e., $\alpha = 1$, K_D will always be less than $K_{app.}$. Thus it is clear that mechanism of denaturation is very important in analysing the denaturation curves.

It has been suggested that analysis in terms of a two-state mechanism can be used even in those cases where denaturation is known to deviate substantially from a two-state mechanism [8,20,21]. We have therefore analysed GdmCl transitions, obtained at all pH values, in terms of a two-state mechanism, and consequently have used the terms $K_{app.}$ and $\Delta G_{app.}$ rather than K_D and ΔG_D.

RNAase A

It has been observed that RNAase A does not undergo any conformational change in the denaturant concentration range 0–1.25 M at pH 3.00. However, even in the absence of GdmCl, protein is slightly acid-denatured (6%) at pH 2.10 and substantially denatured (38%) at pH 1.30; a value of $-1700\ M^{-1}\cdot cm^{-1}$ for y of the acid-denatured state [21] was used in the determination of the percentage acid denaturation. It is therefore expected that the acid-denatured state will contribute to the measured optical property used to monitor denaturation at two lowest pH values. We have, however, assumed a two-state mechanism at all pH values in the determination of f_d using eqn. (1). In the determination of f_d it is necessary to know y_N and y_D under the same condition in which y has been measured. It has been observed that both y_N and y_D depend only on GdmCl, but not on pH. Values of y_N and y_D were determined from the relations $y_N = 128.65$ [GdmCl] and $y_D = 72.97$ [GdmCl] $- 2510$ respectively. Fig. 1(*a*) shows the plot of f_d versus [GdmCl].

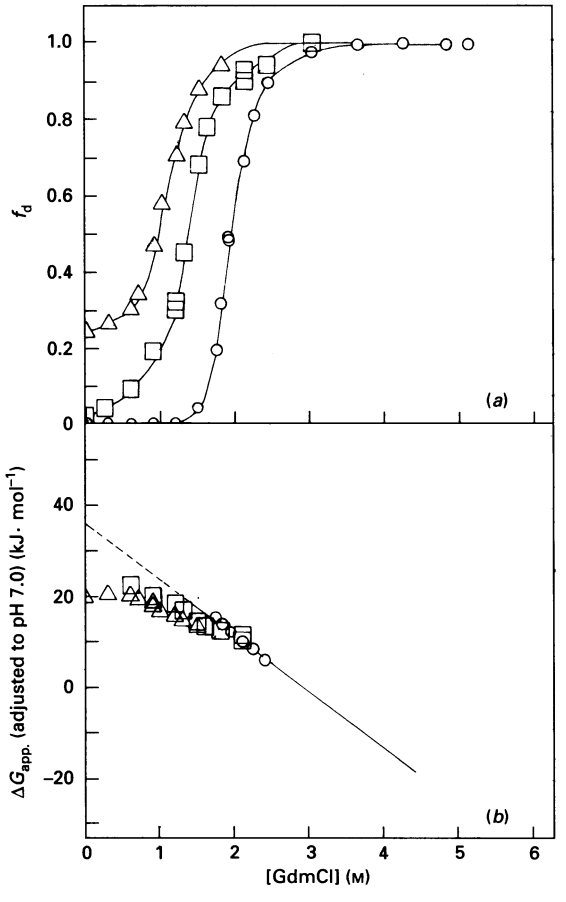

Fig. 1. Denaturation of RNAase

(*a*) Normalized denaturation curves of RNAase A. Curves were calculated from the results of change in $\Delta\epsilon_{287}$ as a function of [GdmCl] at different pH values: ○, pH 3.00; □, pH 2.10, △, pH 1.30. (*b*) As described in text, all $\Delta G_{app.}$ values, calculated from the results in (*a*), were adjusted to pH 7.00. Symbols have the same meaning as in (*a*). The continuous line was drawn using eqn. (3) with $m = 12.27\ kJ\cdot mol^{-1}\cdot M^{-1}$ and $\Delta G^{H_2O}_{app.} = 36.55\ kJ\cdot mol^{-1}$.

Table 1. Stability parameters of RNAase A, lysozyme and Mb at 25 °C

Protein	pH*	$\Delta G^{H_2O}_{app.}$ (kJ·mol^{-1})	m (kJ·mol^{-1}·M^{-1})	C_m (M)
RNAase A	7.00	36.55±0.67	12.27±0.22	2.97
	3.00	23.70±0.41	12.18±0.20	1.94
	2.10	11.01±0.50	8.37±0.34	1.31
	1.30†	7.96±0.26	8.50±0.22	0.93
Lysozyme	7.00	37.18	8.83	4.21
	6.00	35.91±3.11	8.83±0.77	4.05
	2.03	17.05±0.79	7.98±0.35	2.13
	0.64	11.40±0.50	7.17±0.32	1.59
	0.34	10.90±0.37	7.16±0.25	1.52
Mb	5.88	22.41±0.43	18.59±0.04	1.20
	5.52	16.39±1.13	18.34±1.39	0.89
	5.46	12.44±0.02	18.76±0.04	0.66
	5.26	10.47±0.02	21.02±0.57	0.50
	4.92	5.10±0.12	21.27±0.41	0.24
	4.60	−2.06±0.18	21.58±1.78	0.10

* These pH values were used in all calculations; each pH is the average of all pH values in the transition region; the variation in pH was found in the range 0–0.03 for all experiments. †From $\Delta G_{app.}$ values above 0.8 M.

The difference in free energy between the native and denatured conformations was calculated using eqn. (3). For all the results at pH 3.00 and at pH 2.10, $\Delta G_{app.}$ was found to vary linearly with [GdmCl]. At the lowest pH, $\Delta G_{app.}$ was found to be linear in denaturant concentrations above 0.75 M, whereas it is almost independent of [GdmCl] over the range 0–0.75 M. A least-squares analysis was used to fit the data to eqn. (4). Treatment of data in this manner is justified on both experimental [12–14,22] and theoretical [10,11] grounds. Parameters characterizing the GdnHCl denaturation at 25 °C obtained from such treatments are given in Table 1. For comparative purposes we have also included results obtained at pH 7.00 [23].

Results presented in Table 1 demonstrate that the value of m obtained at pH 3.00 is identical with that obtained at pH 7.00. It is also noteworthy that m at pH 3.00 obtained in this study is identical with the one determined by using a different technique [23]. These observations, and the kinetic studies of denaturation of RNAase A by GdmCl [24], suggest that only native and denatured states contribute significantly to the equilibrium under these conditions. This means that values of protein stability over the pH range 7–3 represent $\Delta G^{H_2O}_D$ for the process $N \rightleftharpoons D$. At two lowest pH values, protein is already acid-denatured in the absence of denaturant. As mentioned above, the acid-denatured molecule is expected to contribute to the equilibrium and lead to a value of m which would be lower than that obtained for the transition $N \rightleftharpoons D$, for $\alpha = 0.63$ in our case. As shown in Table 1, results obtained at pH values 2.10 and 1.30 support this prediction.

Pace *et al.* [23] have studied the effect of pH on the values of m obtained from the urea denaturation of RNAase A. They observed that m does not change significantly over the pH range 9.5–6.0, but increases with a decrease in pH from 5.00. They explained that this increase in m with decreasing pH is due to repulsive electrostatic interaction among the positive charges on the unfolded protein. However, this is not the case with the GdmCl results for RNAase A, given in Table 1, for a much higher ionic strength of GdmCl solution would be expected to suppress effects due to electrostatic interactions among charged groups on the protein. At present a good explanation for our results is that the variation of m values reflects deviation from a two-state mechanism.

In order to compare all denaturation results with those at pH 7.00, where GdmCl induces a two-state unfolding, all the $\Delta G_{app.}$ values were adjusted to pH 7.00, using eqn. (5), with $pK_{1,N} = pK_{2,N} = 2.54$, $pK_{1,D} = 3.9$ and $pK_{2,D} = 4.4$ [23–25]. The normalized results are shown in Fig. 1(*b*), where the continuous line was drawn using parameters, at pH 7.00, given in Table 1. The entire data can be put into two sets: those obtained at pH values at which protein is in the native conformation in the absence of the denaturant, falling exactly on the straight line, and those results obtained in the pH region where the starting material is a mixture of the native and acid-denatured conformations, falling below the straight line. It is tempting to conclude that the deviation of results at pH 2.10 and 1.30 from the continuous-line curve mostly represents an incorrectness in the assumption that GdmCl denaturation of RNAase A is a two-state process.

Lysozyme

GdmCl denaturation of lysozyme at various pH values was monitored by measuring the change in the difference spectral intensity at 300 nm. It has been observed: (1) that denaturation is reversible; (2) that lysozyme is an unusually stable protein which undergoes no significant conformational transition in the absence of GdmCl within the pH range covered by the present study; (3) that y_N is independent of pH, and its dependence on [GdmCl] is given by the relationship $y_N = 52.63$ [GdmCl]; and (4) that y_D depends both on pH and [GdmCl]. Dependencies of y_D on [GdmCl] are described by $y_D = 779 + 91.96$ [GdmCl] at pH 6.00, and $y_D = 1564 + 20.22$ [GdmCl] at pH values 2.03, 0.64 and 0.34. These observations were used in the determination of f_d using eqn. (2). A plot of f_d versus [GdmCl] at various pH values are shown in Fig. 2(*a*). It can be seen in this Figure that, in the absence of GdmCl, the protein exists entirely in the N state at pH 2.03 or above, and is, however, slightly acid-denatured at the two lowest pH values.

Assuming a two-state mechanism, values of $\Delta G_{app.}$ were determined by using eqn. (3). It has been observed that $\Delta G_{app.}$ is linear in [GdmCl] at each pH. A least-squares analysis of $\Delta G_{app.}$ results according to eqn. (4) yielded parameters which are listed in Table 1. Values of m measured at pH 2.03 and 6.00 are essentially the same and are in excellent agreement with that reported earlier in the pH range 7–2 (see Fig. 8 in [3]). We have also measured values of m at pH values 0.64 and 0.34 from the study of denaturation of lysozyme. Values of m at the two lowest pH values are slightly lower than that obtained over the pH range 7–2, where a two-state mechanism has been established [2].

In order to compare all denaturation results with those at pH where GdmCl denaturation of lysozyme has been shown to follow a two-state mechanism, all $\Delta G_{app.}$ values were adjusted to pH 7.00, using eqn. (5) with $pK_{1,N} = pK_{2,N} = 1.9$, $pK_{3,N} = 5.1$, $pK_{1,D} = 3.9$, and $pK_{2,D} = pK_{3,D} = 4.4$ [3]. The adjusted $\Delta G_{app.}$ values are shown in Fig. 2(*b*), where the continuous line was drawn using parameters at pH 7.00 given in Table 1. Aune & Tanford [2] have established, on the basis of kinetic studies of denaturation of lysozyme in GdmCl solution at pH values in the range 1.3–5.5 at 25 °C, that the protein denaturation obeys a two-state mechanism. Their results in the denaturant concentration range 1.9–4 M suggest that $\Delta G_{app.}$ can be described by eqn. (4) (see Fig. 8 in [3]). Our results over the pH range 2–6 confirm the earlier finding (see Fig. 2*b*). As Fig. 2(*b*) shows values of $\Delta G_{app.}$ calculated from results at two lowest pH values fall below the straight line. This behaviour of lysozyme represents the incorrectness in the assumption that GdmCl denaturation is a two-state process. The reasons for saying this are that lysozyme is partially acid-denatured in the absence of GdmCl (see Fig. 2*a*) and that $\alpha = 0.7$, determined from the comparison of extra-

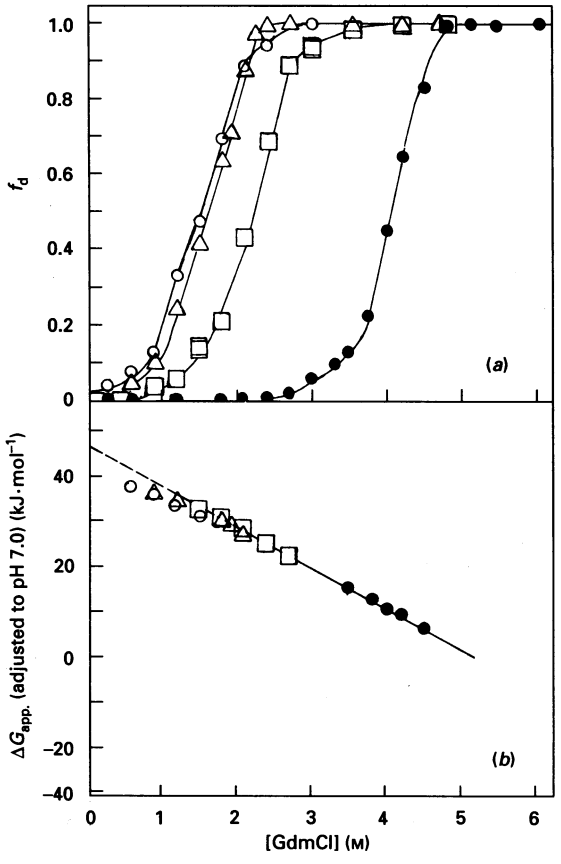

Fig. 2. Denaturation of lysozyme

(*a*) Normalized denaturation curves of lysozyme. Curves were calculated from the results of change in $\Delta\epsilon_{300}$ as a function of [GdmCl] at different pH values: ●, pH 6.00; □, pH 2.03; △, pH 0.64; ○, pH 0.34. (*b*) Relationship between $\Delta G_{app.}$ adjusted to pH 7.00 for the GdmCl concentration. Symbols have same meaning as in (*a*). The continuous line was drawn according to eqn. (3) with values of 8.83 kJ·mol^{-1}·M^{-1} and 37.18 kJ·mol^{-1} for m and $\Delta G^{H_2O}_{app.}$ respectively.

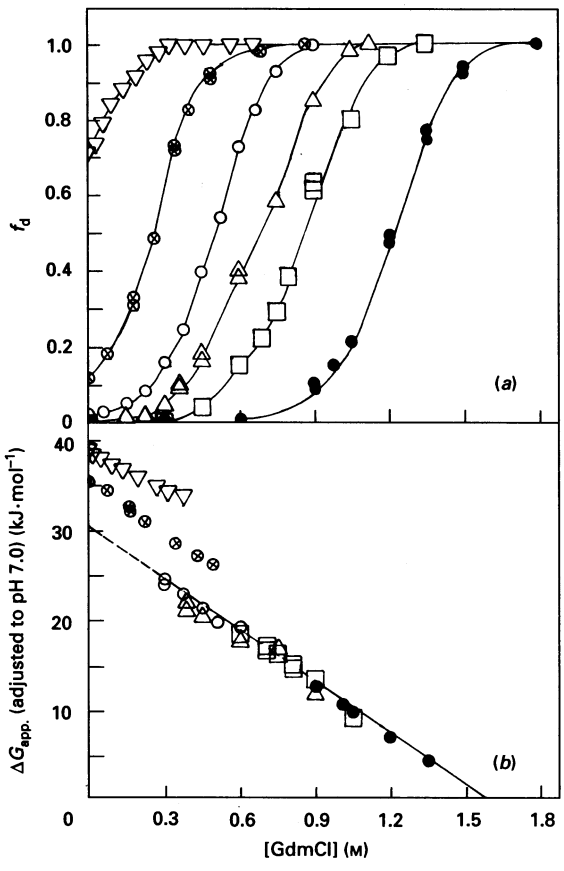

Fig. 3. Denaturation of Mb

(*a*) Normalized denaturation curves of Mb. Curves were calculated from the results of ϵ_{490} as a function of [GdmCl] at pH values 5.88 (●), 5.52 (□), 5.46 (△), 5.26 (○), 4.92 (⊗) and 4.60 (▽). (*b*) All $\Delta G_{app.}$ values calculated from the results in (*a*) were adjusted to pH 7.00. Symbols have the same meaning as in (*a*). The continuous line was drawn using eqn. (3), with m = 18.59 kJ·mol^{-1}·M^{-1} and $\Delta G^{H_2O}_{app.}$ = 31.40 kJ·mol^{-1}.

polated value of $\Delta\epsilon_{300}$ at 25 °C in the absence of denaturant [26] with the value of 1564 M^{-1}·cm^{-1} for $\Delta\epsilon_{300}$ obtained here.

Mb

GdmCl denaturation of Mb was monitored by observing changes in ϵ_{409}. It has been observed: (1) that denaturation is reversible under conditions described earlier [15]; (2) y_N (171000 M^{-1}·cm^{-1}) depends on neither pH nor GdmCl concentration; and (3) y_D measured at different pH values is independent of [GdmCl] but shows a slight dependence on pH; values of y_D are 16000 (pH 5.88), 17600 (pH values 5.52 and 5.46), 17300 (pH 5.26) and 24000 (pH values 4.92 and 4.60) M^{-1}·cm^{-1}. These observations were used in the determination of f_d using eqn. (2) at various pH values. Fig. 3(*a*) shows a plot of f_d against [GdmCl].

Assuming a two-state mechanism, values of $\Delta G_{app.}$ were determined using eqn. (3) at each pH. It has been observed that $\Delta G_{app.}$ is linear in the denaturant concentration. A least-squares analysis according to eqn. (4) gave parameters that are given in Table 1. As this Table shows: (1) $\Delta G^{H_2O}_{app.}$ shows a strong dependence on pH; (2) m is the same, within experimental error, at pH values 5.88, 5.52 and 5.46; and (3) m increases on decreasing the pH below 5.46. It is noteworthy that m measured over the pH range 5.88–5.46 is identical with what had been reported previously [9,13,15,27]. The increase in m with a decrease in pH below 5.46 is, however, less steep than that reported by Pace & Vanderburg [9], who observed an increase in m from 18.84 to a value greater than 36.85 kJ·mol^{-1}·M^{-1} when pH was decreased from 5.85 to 4.60. This discrepancy between results at lower pH values observed here and those reported previously [9] may represent differences in the behaviour of the protein under the two different experimental conditions employed in these studies. For example, Mb at pH 4.60 is 70% acid-denatured in 0.05 M-citrate buffer (see Fig. 3*a*), whereas it is only about 10% acid-denatured in 0.1 M-acetate buffer (see Fig. 1 in [9]). Furthermore, it is also known from the GdmCl denaturation of Mb that the value of m may change on changing the ions in the buffer [27].

Results obtained at different pH values were adjusted to pH 7.00 using eqn. (5), with the constants obtained by Puett [18]: $pK_{1,N}$ = 3.8, $pK_{2,N}$ = 3.7, $pK_{3,N}$ = 6.7, $pK_{1,D}$ = 4.0, $pK_{2,D}$ = 5.97, $pK_{3,D}$ = 6.5 and n = 6. Values of the adjusted $\Delta G_{app.}$ as a function of [GdmCl] are shown in Fig. 3(*b*). The line is a theoretical curve based on parameters determined by the least-squares analysis of the results at pH 5.88 adjusted to

pH 7.00. Pace & Vanderburg [9] reported a value of 31.40 kJ·mol^{-1} for $\Delta G^{H_2O}_{app.}$ in 0.1 M-phosphate buffer at pH 7.00 and 25 °C. It has been observed that 0.1 M-phosphate adds 2.09 kJ·mol^{-1} to $\Delta G^{H_2O}_{app.}$ term of proteins which do not have any specific binding sites on them [27]. This finding and results obtained here suggest that $\Delta G^{H_2O}_{app.}$ of Mb in 0.1 M-phosphate buffer, pH 7.0, should be 29.48 + 2.09 = 31.57 kJ·mol^{-1}. This is in excellent agreement with $\Delta G^{H_2O}_{app.}$ value obtained by Pace & Vanderburg [9].

As Fig. 3(*b*) shows, the corrected $\Delta G_{app.}$ values measured over the pH range 5.88–5.46 cluster around a single curve determined by the parameters $\Delta G^{H_2O}_{app.} = 29.48$ kJ·mol^{-1} and $m = 18.59$ kJ·mol^{-1}·M^{-1}. This suggests that m, within experimental error, is independent of GdmCl concentration over the range 1.4–0.4 M. Fig. 3(*b*) also shows that all corrected $\Delta G_{app.}$ values measured over the pH range 5.26–4.60 lie above the theoretical curve. This is an expected behaviour, because Mb is partially acid-denatured over this pH range (see Fig. 3*a*). The acid-denatured state is expected to make $\Delta G_{app.}$ greater than ΔG_D, for $\alpha = 1$ under the experimental conditions, for the properties of both acid- and GdmCl-denatured states are the same (16000 M^{-1}·cm^{-1}) [18]. The same argument can also be used to explain the non-linearity observed at lower concentrations in the plot of $\Delta G_{app.}$ of Mb versus [GdmCl], reported by Pace & Vanderburg [9], who interpreted their results in terms of binding of denaturants to the proteins, for which no experimental evidence has been presented so far [6].

In summary, we are sure of one thing: When free-energy changes on GdmCl denaturation of proteins are estimated by using eqn. (3) at various pH values, the existence of the acid-denatured state will complicate the analysis of the plot of $\Delta G_{app.}$ values, adjusted to a common pH, versus denaturant concentration.

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