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Thermodynamics of alligator metmyoglobin unfolding

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The conformational free energy of alligator metmyoglobin was examined over a pH range of 4.4–8.0, a temperature range of 18–48 °C, and a guanidinium chloride concentration of 0–2.3 M. For isothermal unfolding at 25 °C essentially the same value was obtained for the conformational free energy from all the data: 7.0 ± 0.5 kcal/mol. The cooperativity of the unfolding with respect to denaturant is considerably less than for mammalian myoglobins. On unfolding three to four side chains with a pK_a of 6.3 in the unfolded protein are protonated instead of the six expected. The temperature at which ΔH(unfolding) is zero is much lower than for previously characterized myoglobins. Alligator metmyoglobin, considerably less stable than other previously characterized myoglobins, may not be as compactly folded.

1. Introduction

The folding of a polypeptide chain to yield a stable, well-defined compact structure, which has been appropriately called the second translation step [1], involves a wide variety of intramolecular forces whose relative contributions are only poorly understood. Recently, the first attempts at producing 'designer' proteins have begun with the construction of a synthetic protein composed of four amphipathic α-helical segments [2]. Studies of homologous proteins have revealed just how sensitive the overall conformational stability is to small changes in the sequence [3–8]. Myoglobin has been the subject of much of this work because the tertiary structure of sperm whale myoglobin is known precisely [9], and the unfolding of sperm whale myoglobin is known to follow closely the two-state model [10,11], although some very small amounts of intermediates may exist [12]. Recent work on sperm whale myoglobin has focused on the importance of electrostatic interactions both within helical segments and between different helical segments [13–15]. Chicken myoglobin studies have suggested that in this species the D helix may not even form [16]. The recent elucidation of the sequences of myoglobins from a number of nonmammalian species as well as the growing library of mammalian sequences allows the investigation of sequence-stability relationships over considerable distances in the phylogenetic tree.

One intensively studied facet of myoglobin structure involves a number of histidyl residues that are not exposed to the solvent in the native structure. Carp metmyoglobin has been found to expose five histidyl residues on acid unfolding, suggesting that its tertiary structure in solution is analogous to that of the mammalian myoglobins [17]. Acid unfolding studies indicate that in both horse and sperm whale myoglobin, six histidyl residues are exposed on unfolding [4]. A detailed NMR study on sperm whale myoglobin shows

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that His 24, 64, 82, 93 and 97 do not titrate in native sperm whale myoglobin [18]. His 36 shows anomalous behavior in that the H-2 and H-4 resonances have different pH dependences. The sequence [19] of alligator (*Alligator mississipiensis*) myoglobin reveals nine histidyls at positions 9, 24, 26, 36, 48, 64, 82, 93 and 97. If the supersecondary structure of myoglobin has been conserved since the divergence of sperm whale and alligator, the pK_a of six of these residues should change on unfolding.

This study examines the free energy change on denaturation of alligator metmyoglobin as a function of guanidinium chloride concentration, temperature, and H^+ activity. The free energy of unfolding of the protein at pH 8, and the dependence of the apparent free energy of unfolding upon both guanidinium ion and H^+ activities were determined to ascertain how many ionizable groups with a pK_a of about 6 in the unfolded protein are buried in the native structure. The temperature dependence of the unfolding was examined in order to compare the temperature at which ΔH is zero and the change in heat capacity on unfolding of alligator to homeotherm myoglobins.

2. Materials and methods

2.1. Reagents

Guanidinium chloride was ultrapure grade purchased from Heico, Inc. Microgranular preswollen DEAE-cellulose was purchased from Sigma. Sephadex G-100 was a product of Pharmacia, Inc. Sperm whale myoglobin was from Sigma. Other salts and common reagents were analytical grade. Purified water with a conductivity of less than 60 $\mu\Omega^{-1}$ was used.

2.2. Isolation of alligator cardiac metmyoglobin

Freshly frozen alligator hearts were obtained from Sauros, Inc. (Bell City, LA) and were kept at $-38°C$ until use. Portions of about 600 g were thawed for 1 day at 4°C, ground with two passes through a meat grinder and homogenized in 70% of saturation ammonium sulfate as described previously [16]. The homogenate supernatant was filtered through glass wool and the heme oxidized by the addition of a 2-fold molar excess of $K_3Fe(CN)_6$ over 1 h in the cold [5]. The supernatant was dialyzed exhaustively against deionized water to remove any traces of CN^-, concentrated against 50% poly(ethylene glycol), and aliquots were chromatographed on a 2.5×105 cm column of Sephadex G-100 in 20 mM Tris-Cl, 1 mM EDTA (pH 8) in the cold. The indicated fractions were pooled, concentrated by dialysis against concentrated poly(ethylene glycol), and dialyzed against 20 mM Tris-Cl (pH 8). Subsequent purification was on a 1.5×45 cm microgranular DEAE-cellulose column equilibrated with 20 mM Tris-Cl (pH 8.0) at room temperature. The myoglobin was eluted at room temperature with an 800 ml linear NaCl gradient (0–200 mM) in 20 mM Tris-Cl (pH 8.0). Indicated fractions were pooled, dialyzed against 30 mM sodium phosphate (pH 8.0) and aliquots frozen at $-38°C$. The final yield was about 17 mg per 100 g tissue.

SDS-10% polyacrylamide gels were stained with Coomassie blue [20]. Discontinuous 7.5% polyacrylamide gel electrophoresis was performed using a stacking gel at pH 8.3 and a separating gel at pH 9.5 [21]. Gels were stained with Coomassie blue R-250.

2.3. Solution preparation

Guanidinium chloride stock solutions were prepared by mass from solute dried over P_2O_5 and checked by density [22]. Aliquots of the stock solutions were singly thawed on the day of use and centrifuged at $15000 \times g$ for 10 min to remove damaged protein. Final metmyoglobin solutions scanned contained 80–100 μg myoglobin per ml. Solutions at pH 8 contained 10 mM sodium phosphate. 0 M denaturant solutions at pH 8 contained 0.25 M KCl. Solutions at acidic pH were prepared from isoionic stock myoglobin solutions and contained either sodium acetate buffer with 0, 0.3, or 0.6 M guanidinium chloride or sodium cacodylate buffer with 0.8 M guanidinium chloride. NaCl was added to give a buffer ionic strength increment (in addition to the denaturant) of 0.05 M.

The visible absorption spectrum of alligator metmyoglobin at pH 8 in the absence of denaturant was identical with that of sperm whale metmyoglobin within experimental error. The visible absorption spectrum did not change with exposure to air, and was independent of pH over the range pH 8–6.

2.4. Data acquisition

Preliminary studies showed that apparent equilibrium was reached in less than 10 min. At longer times a slow decrease in absorbance was observed which probably resulted from irreversible aggregation of denatured globin and heme. Spectra were recorded 15 min after solution preparation using 1-cm cuvettes in a Hitachi 110A spectrophotometer with a slit width of 0.5 nm and time constant of 0.5 s from 700 to 300 nm. A jacketed cell holder and thermostatted circulating water bath were used. Data at variable temperature at pH 8 were acquired as described previously [16].

2.5. Analysis of pH 8, 25 °C data

The height of the Soret peak was used to calculate F_N, the fraction of native protein, K_D, the equilibrium denaturation constant, and ΔG_D, the apparent change in free energy according to the two-state model. Estimates for the change in free energy of unfolding at pH 8 and 25 °C were calculated as described previously [16,17].

2.6. Analysis of 25 °C data at variable pH

Data obtained at 25 °C at various pH values and denaturant concentrations were analyzed by fitting ΔG_D values (calculated using the two-state model) vs. H^+ and guanidinium ion activities using the Simplex algorithm [23] implemented in Turbo Pascal [24] to the previously derived equation [17]:

$$\Delta G_D = \Delta G_D^0 - F_G - F_H \quad (1)$$

where ΔG_D^0 is the unfolding free energy at pH 8 in the absence of denaturant, and where:

$$F_H = \Delta n_H RT \ln[(a_H/K_U + 1)/(a_H/K_N + 1)] \quad (1a)$$

where Δn_H denotes the increase in the number of H^+ bound on unfolding, a_H is H^+ activity (determined immediately following solution scanning), and K_U and K_N are the acid dissociation constants for the groups binding additional H^+ for the unfolded and native forms of the protein, respectively, and

$$F_G = \Delta n_G RT \ln(1 + ka_+) \quad (1b)$$

where Δn_G is the increase in the number of guanidinium ions bound on unfolding, k the binding constant for the guanidinium ion (taken to be 0.6 as suggested by Pace and Vanderburg [26]), and a_+ the guanidinium ion activity (calculated from isopiestic data [27]).

In the analysis of the acid denaturation data, the examination of three-dimensional cross-sections of the six-dimensional sum of squares surface showed that some parameter estimates were highly correlated (e.g., Δn_H with K_U). Thus, a set of joint confidence intervals was estimated from the contours of all ten three-dimensional cross-sections of the six-dimensional sum of squared residuals surface.

2.7. Analysis of variable temperature data at pH 8

The absorbances of the metmyoglobin in 0.25 M KCl, 10 mM sodium phosphate and in 3.12 M guanidinium chloride, 10 mM sodium phosphate were determined as a function of temperature. These data were used to estimate A_N and A_D as a function of temperature. The normalized absorbances at variable temperature and denaturant concentrations were used along with estimates for A_N and A_D to calculate the denaturation equilibrium constant, K_D. The data obtained at pH 8 at various temperatures and denaturant concentrations were analyzed by assuming that ΔC_P is independent of temperature and by realizing that if ΔC_P is sufficiently large that at some temperature ΔH(unfolding) will be zero. Call the temperature at which ΔH(unfolding) is zero T'. Then:

$$\Delta H = \Delta C_P(T - T') \quad (2a)$$

and

$$d(\ln(K_D^0)) = (1/R)\Delta C_P(T' - T)\, d(1/T) \quad (2b)$$

by integration from $T = 298.15$ K to $T = T$ then:

$$\ln(K_D) = \ln(K_D^0) + (1/R)\Delta C_P[T'(1/T - 1/298.15) + \ln(T/298.15)] \quad (2c)$$

where K_D^0 is K_D at 298 K, ΔC_P the change in heat capacity, and T the absolute temperature. The fit of the experimental data $\ln(K_D)$ vs. T at each denaturant concentration to eq. 2c was carried out using the Simplex algorithm.

A set of approximate 95% confidence intervals for $\ln(K_D^0)$, T' and ΔC_P was obtained by examining the residual sum of squares surface along each parameter axis, holding all other parameters at their best values. The ends of the confidence intervals were taken as the parameter values giving a sum of squared residuals for which the lack of fit $F(p, n-p)$ test just exceeded the 0.95 value [25].

3. Results and discussion

Fig. 1 shows the elution profile of the concentrated alligator supernatant on the Sephadex G-100 column. Fig. 2 shows the chromatogram of the Sephadex G-100 peak after passage through the DEAE-cellulose column together with native

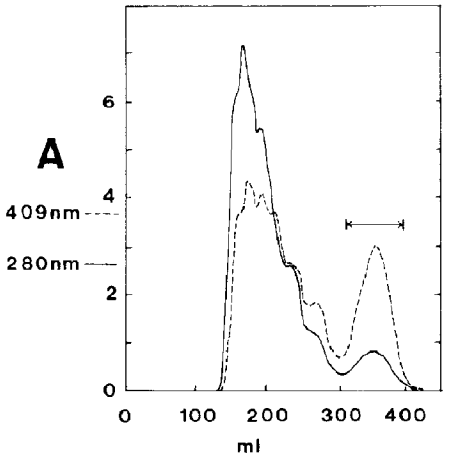

Fig. 1. Elution pattern of homogenate ammonium sulfate supernatant on a 2.5×105 cm Sephadex G-100 column equilibrated and eluted with 20 mM Tris-Cl (pH 8) at 4°C.

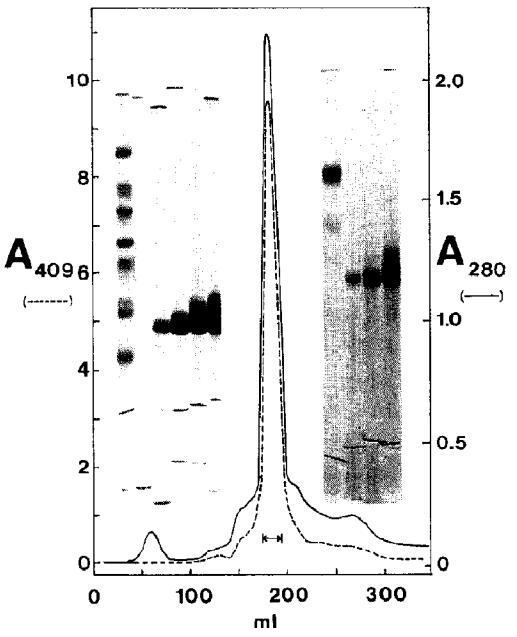

Fig. 2. Elution pattern of concentrate (pooled from 3–4 Sephadex G-100 columns) over a 1.5×49 cm DEAE-cellulose bed with a linear salt gradient of 4.7 column volumes of 20 mM Tris-Cl and 4.7 column volumes of 20 mM Tris-Cl, 200 mM NaCl, both at pH 8.0. (Left inset) SDS-polyacrylamide gel electrophoresis of purified alligator myoglobin. 10% gels were stained with Coomassie blue. Samples were (from left to right): 7 μg standard proteins, blank gel, 7, 28, 70 and 140 μg alligator myoglobin. (Right inset) Native discontinuous buffer, 7.5% gel electrophoresis of purified alligator myoglobin. Samples were (from left to right) 10 μg sperm whale myoglobin, 2.5, 5 and 15 μg alligator myoglobin.

and SDS-polyacrylamide gels stained with Coomassie blue. Polyacrylamide gel electrophoresis demonstrates that the alligator myoglobin is better than 99% homogeneous by mass, with a small fraction of the protein having a slightly lower negative charge at alkaline pH.

Fig. 3 shows the isothermal guanidinium chloride unfolding data at pH 8. Reversibility of denaturation (□) was excellent provided dilution of concentrated denaturant-protein solution was done shortly after apparent equilibrium was reached. Table 1 gives the least-squares estimates resulting from the three models used to extrapolate pH 8, 25°C data to 0 M denaturant.

Fig. 4 shows the experimental values of A/A_0

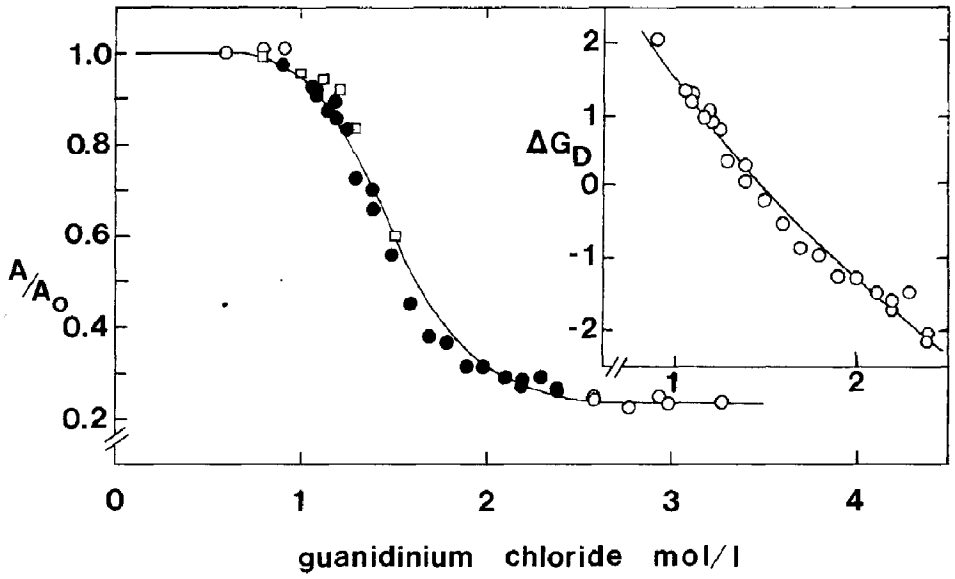

Fig. 3. Isothermal unfolding profile of alligator metmyoglobin at 25.0 ° C in 10 mM sodium phosphate at pH 8.0. The absorbance at 409 nm divided by the absorbance at 0 M denaturant is plotted vs. the molar concentration of denaturant. Squares represent reversal points and filled circles are points used to calculate free energy changes. (Inset) The experimental free energy change (in kcal/mol) calculated from the filled circles is plotted vs. the molar concentration of guanidinium chloride. In both plots the curves shown are calculated using the Aune and Tanford binding model linear least-squares estimates for ΔG_D and Δn_G.

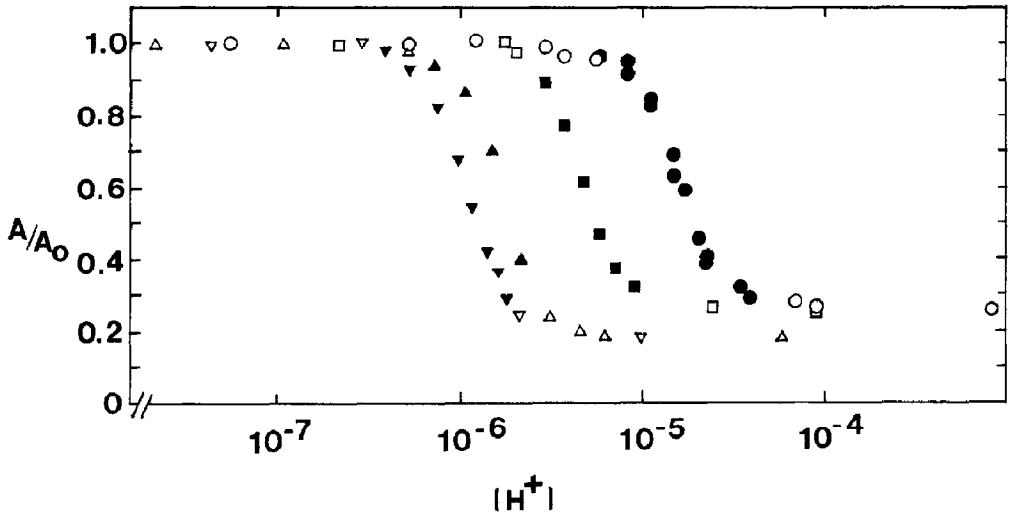

Fig. 4. The absorbance at 409 nm divided by the absorbance of the native protein (from data in the pH regions above the transition zone) is plotted vs. H^+ concentration at 25 ° C. Circles, 0 M guanidinium chloride, 0.050 M sodium acetate buffer; squares, 0.300 M guanidinium chloride, 0.050 M sodium acetate buffer; triangles, 0.600 M guanidinium chloride, 0.050 M sodium cacodylate buffer; inverted triangles, 0.800 M guanidinium chloride, 0.050 M sodium cacodylate buffer.

Table 1

Analysis of isothermal unfolding data at pH 8 and 25 °C

	Transfer model	Binding models	
		$RT \ln(1+0.6a_+)$	$RT \ln(1+0.8$ [Gdm. Cl])
ΔG_D^0 (kcal/mol)	6.6 ± 0.2	6.6 ± 0.2	5.9 ± 0.2
Fitting parameter	$\Delta\alpha = 0.16 \pm 0.01$	$\Delta n_G = 30 \pm 1$	$\Delta n = 12.6 \pm 0.4$

below pH 8 at 25 °C as a function of H^+ concentration. The filled points in figs. 3 and 4 were used to calculate ΔG_D values. Table 2 gives parameter estimates and approximate confidence intervals obtained by least-squares fitting of these 55 data points ranging from pH 4.4 to 8 with guanidinium chloride concentrations ranging from 0 to 2.3 M to eq. 1.

Fig. 5 shows the experimental and theoretical free energy changes. Since there are two independent variables in the data, two plots are shown with both panels giving a theoretical line slope of −1. Each plot gives ΔG_D plus one half of the fitting function vs. the other half of the fitting function. The mean root squared residual error for the curve fitting is 0.27 kcal/mol, close to that found earlier for carp myoglobin data [17]. Addition of more classes of ionizable groups (using as initial estimates the values in column d of table II in ref. 4) to eq. 1a as was previously done for two mammalian metmyoglobins failed to produce a statistically significant reduction in the residual sum of squares.

Table 2

Analysis of 25 °C data at variable pH and denaturant concentrations

Parameter	Estimate	Approximate 95% joint confidence intervals
ΔG_D^0	7.2 kcal/mol	7.0– 7.4
Δn_G	32.4 sites	30.6–33.3
Δn_H	3.4 sites	3.1– 3.8
pK_U	6.35	6.2– 6.5
pK_N	3.4	< 4.0

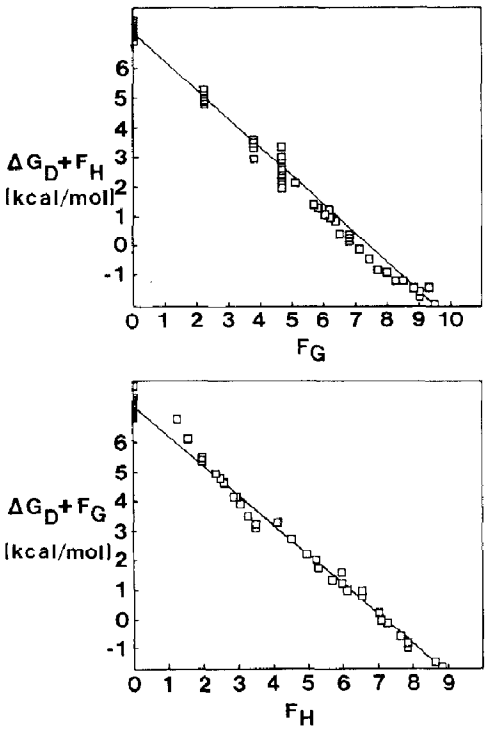

Fig. 5. Experimental data points obtained at 25 °C are shown as squares. The lines shown are the theoretical lines calculated according to eqs. 1, 1a and 1b with the parameter estimates in table 2. F_G is dependent upon the activity of guanidinium ion given by eq. 1b; F_H is dependent upon the activity of H^+ shown in eq. 1a; and ΔG_D is the apparent free energy of unfolding at various pH values and denaturant concentrations. Both F_G and F_H are dependent on the parameter estimates in table 2.

Fig. 6 shows experimental data obtained at pH 8 at varying temperatures. Table 3 gives the least-squares parameter estimates obtained by fitting $\ln(K_D)$ vs. T data to eq. 2c. Fig. 7 shows the calculated $\ln(K_D)$ points and theoretical curves generated from the parameter estimates in table 3 and eq. 2c.

Both isothermal data at pH 8 and acid unfolding data suggest that the conformational free energy of alligator metmyoglobin is in the range of 6–7 kcal/mol. Aligator metmyoglobin is thus the least stable of all the characterized myoglobins [4–7,16,17,28]. It is, of course, possible that alligator metmyoglobin unfolds via a multistate

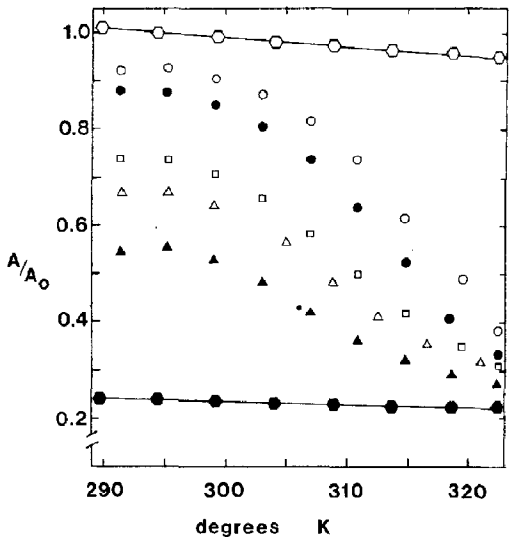

Fig. 6. The absorbance at pH 8 and 409 nm divided by the absorbance at 25 °C and 0 M denaturant is plotted vs. sample temperature in Kelvin. (○) 1.095 M guanidinium chloride, (●) 1.194 M guanidinium chloride, (□) 1.294 M guanidinium chloride, (△) 1.393 M guanidinium chloride, (▲) 1.493 M guanidinium chloride, (○) 0.25 M KCl (no denaturant), (●) 3.12 M guanidinium chloride. The lines shown were used to estimate the absorbances of the fully native and fully denatured protein as a function of temperature.

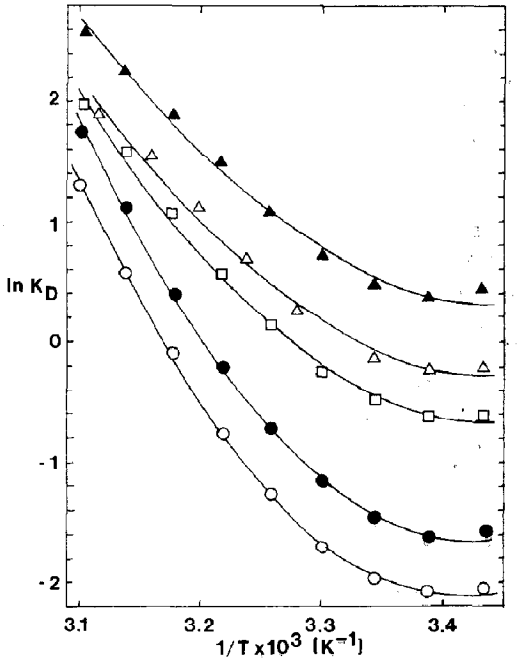

Fig. 7. Van't Hoff plots for alligator metmyoglobin. The experimental points calculated from the data in fig. 6 are shown along with the theoretical curves calculated using eq. 2c with the parameter estimates given in table 3. The symbols are identical to those of fig. 6.

mechanism which would give a lowered value for ΔG_D^0. The fact that essentially the same estimate for ΔG_D^0 is obtained from data over such a wide range of conditions seems to argue against a multistate unfolding mechanism, since the relative amounts of intermediates would have to remain essentially constant under widely varying solution conditions.

Table 3

Analysis of thermal unfolding data at variable denaturant concentrations at pH 8

Reversibility ranged from 87 to 92% on cooling back to 25 °C.

[Gdm.Cl] (mol/l)	$\ln(K_D)$ (at 298 K)	T' (at $\Delta H = 0$)	ΔC_p (kcal/mol per K)
1.095	-1.99 ± 0.09	292.5 ± 0.9	1.46 ± 0.08
1.194	-1.48 ± 0.09	292.4 ± 0.8	1.39 ± 0.07
1.294	-0.48 ± 0.10	288.6 ± 1.7	0.91 ± 0.08
1.393	-0.10 ± 0.10	288.8 ± 2.0	0.84 ± 0.12
1.493	0.52 ± 0.13	287.3 ± 2.7	0.76 ± 0.10

The estimates obtained in this study for the acid dissociation constants of the groups which bind protons on unfolding are in excellent agreement with those for horse [4], sperm whale [4], and carp myoglobins [17]. If alligator myoglobin were folded analogously to mammalian myoglobins, six histidyls should be exposed on unfolding. The finding that only three or four side chains with a pK_A of 6.3 in the unfolded protein become exposed on unfolding suggests that alligator myoglobin may not be as compactly folded as other myoglobins.

There are several other lines of evidence which also suggest that alligator myoglobin is not folded as compactly as other myoglobins. For a number of globular proteins which denature according to the two-state model the extrapolated value for ΔH at about 110 °C converges to a common value of

about 13 cal/g [29]. In addition, for these compact proteins the change in heat capacity is temperature independent and is strongly correlated with the saturation of nonpolar contacts [29]. It then follows that the temperature at which ΔH becomes zero also linearly correlates with the relative saturation of nonpolar contacts. Note that the temperature at which ΔH becomes zero changes little with changes in denaturant concentration in this and all prior studies [16,30–32]. The observation that the temperature at which ΔH is zero for alligator myoglobin is some ten degrees less than that for sperm whale [11] and chicken myoglobin [16] strongly suggests that the degree of saturation of nonpolar contacts is less for the alligator protein. The change in heat capacity on alligator myoglobin unfolding is two-thirds that for chicken myoglobin at equivalent denaturant concentrations. This observation again suggests that the saturation of nonpolar contacts is less in the alligator protein.

Finally, the marked decrease in the cooperativity of guanidinium chloride-induced unfolding suggests a less compact native structure for the native protein. The estimates in table 1 for Δn (the increase in bound guanidinium ions on unfolding, Aune and Tanford binding model) and for $\Delta\alpha$ (the average increase in residue exposure, transfer model) for alligator myoglobin are 63% of the values observed for sperm whale myoglobin [16].

One possibility for the lessened stability and compactness of alligator myoglobin can be postulated when conservation of side chain charge patterns is compared on a helix by helix basis for sperm whale and alligator myoglobin. It has been recently suggested that alignment of side-chain dipoles with the α-helix dipole is important to the stability of helical segments [33–35]. Only the B helix does not conserve a side-chain charge pattern aligned with the helix moment. If the conformation of the B helical segment is markedly altered in alligator myoglobin, the exposure of His 24 and 36 to the solvent might result. It is clear from recent work on the 32–139 fragment of horse cardiac myoglobin that the entire sequence need not be present and folded for oxygen binding [36]. This 'mini-myoglobin', lacking the A, B, and most of the H helical segments, shows binding kinetics remarkably similar to the intact protein.

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