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DENATURATION THERMODYNAMICS OF CHICKEN CARDIAC METMYOGLOBIN

Leslie A. HOLLADAY

Department of Chemistry, Louisiana Tech University, Ruston, LA 71272, U.S.A.

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The unfolding at pH 8 of chicken cardiac aquometmyoglobin was examined as a function of temperature and concentration of guanidinium chloride using the two-state model. The isothermal unfolding data at 25°C were fitted to Tanford's transfer model and the binding model of Aune and Tanford. The estimates obtained for ΔG_D were virtually identical, viz., 8.3 ± 0.3 kcal mol^{-1}. The chicken metmyoglobin is thus some 5.3 kcal mol^{-1} less stable than that of sperm whale metmyoglobin. The unfolding parameters α and Δn were decreased 20% from those of mammalian myoglobins thus far examined, suggesting nonidentity of native conformations. The apparent enthalpy change on unfolding was dependent on both temperature and denaturant concentration. The decreases in the isothermal unfolding parameters from those of sperm whale are principally assigned to three of the 46 sequence changes.

1. Introduction

It is not currently possible to ascertain whether an arbitrary polypeptide sequence will fold to give a stable structure. At the moment, the study of the folding properties of a series of homologous proteins seems to provide one of the better avenues for attempts at arriving at a detailed methodology for predicting folding patterns. Myoglobin has become a favorite for such studies because the tertiary structure of that of sperm whale is known precisely [1], the sequences of more than 60 vertebrate myoglobins have been elucidated, and the unfolding of sperm whale metmyoglobin is known to be essentially a two-state process [2,3], although some very small amounts of intermediates may exist [4]. Since the initial observation by Schecter and Epstein [5] that the folded structure of horse metmyoglobin was less stable than that of the sperm whale, a series of studies have compared the stabilities of metmyoglobins from different species [6–9]. A detailed comparison of horse and sperm whale myoglobins by Puett [6] suggested that much of the difference in stabilities arose from a lack of two salt bonds in horse metmyoglobin. Other studies on several mammalian myoglobins [7] and a detailed study on a number of cetacean myoglobins [9] have confirmed the relative importance of electrostatic interactions in determining the stability of the folded protein. Indeed, a number of residues invariant in vertebrate myoglobins are involved in salt bonds [1,10]. So far no detailed reports have appeared on the reversible unfolding equilibria of nonmammalian myoglobins. The recent elucidation of the sequences of a number of nonmammalian myoglobins now make such studies attractive, since the large number of sequence differences between these myoglobins and that of the sperm whale may give new insights into myoglobin stability. In this paper are presented studies on the reversible unfolding of chicken cardiac metmyoglobin and inferences drawn from the unfolding parameters.

2. Materials and methods

2.1. Reagents

Guanidinium chloride was ultrapure grade purchased from Heico, Inc. Other salts and common reagents were analytical grade. Ion-exchange and gel filtration gels were from Pharmacia. Sperm whale metmyoglobin was obtained from Sigma, and purified by ion-exchange chromatography before use [6]. Purified water with a conductivity of less than 60 $\mu\Omega^{-1}$ was used.

2.2. Isolation of chicken cardiac metmyoglobin

Chicken hearts were obtained from a local processing plant and were frozen until use. The aquometmyoglobin was isolated essentially by the method of Deconinck et al. [11] except that gel filtration chromatography was done with Sephadex G-100 using 0.05 M sodium phosphate, pH 6.0, and myoglobin solutions were concentrated by dialysis against concentrated polyethylene glycol. The ion-exchange chromatograph obtained during isolation was identical to that previously shown [11]. The purified metmyoglobin was stored as frozen aliquots in 50 mM sodium phosphate at pH 8.0.

2.3. Solution preparation

Guanidinium chloride stock solutions were prepared by mass and checked by density [12]. Sperm whale metmyoglobin solutions contained 10 mM sodium phosphate, pH 8. Zero molar denaturant solutions contained 0.1 M KCl [6]. Chicken cardiac metmyoglobin solutions contained 17 mM sodium phosphate, pH 8. Zero molar denaturant solutions contained 0.25 M KCl. It was found that native chicken metmyoglobin solutions were not stable at ionic strengths of less than about 0.2 at room temperature. The increased ionic strength permitted solutions of the native protein to remain stable for at least 3 days at room temperature. All solutions contained 80–100 μg myoglobin per ml. Solutions were prepared, stored overnight in the cold, warmed for 2 h, and scanned. Chicken metmyoglobin solutions throughout the transition region exhibited greater than 97% stability at room temperature over a 24 h period.

2.4. Data aquisition and analysis

Spectra were recorded using 1 cm cuvettes on a Hitachi 110A spectrophotometer with 0.5 nm slit width and a time constant of 0.5 s from 700 to 380 nm. A thermostatted cell holder and circulating water bath were used. Observed water bath temperatures were corrected to actual cell temperatures. The height of the Soret peak was used to calculate f_N, K_D, and the apparent change of free energy of denaturation according to a two-state model. Two models were used for extrapolation of the apparent free energy change to zero molar denaturant. The binding model of Aune and Tanford [13] was used with a binding constant of 0.6 as suggested by Pace and Vanderburg [14] and guanidinium ion activities calculated from the equation of Aune and Tanford [13]. The second model, based on side chain solubilities in water and denaturant, used Puett's protocol [15] for estimating transfer free energies for side chains for which no data are available. For both models, least-square fits of the apparent free energy change to the model's fitting function were done.

2.5. Temperature studies

For the temperature studies, the cell temperature was varied from around 18 to 48°C. After temperature equilibrium had been reached at each new temperature, the absorbance at 409 nm was monitored until no further change occurred (15–25 min for a 3°C change). After 48°C had been reached, the solution was cooled. Reversibility of thermal denaturation averaged 94%. The ab-

Table 1
Analysis of denaturation data by two methods

	Binding model		Transfer model	
	ΔG_D (kcal/mol)	Δn	ΔG_D (kcal/mol)	α
Sperm whale	13.8±0.6	48±2	13.3±0.6	0.25±0.01
Chicken	8.2±0.3	36±1	8.3±0.3	0.20±0.02

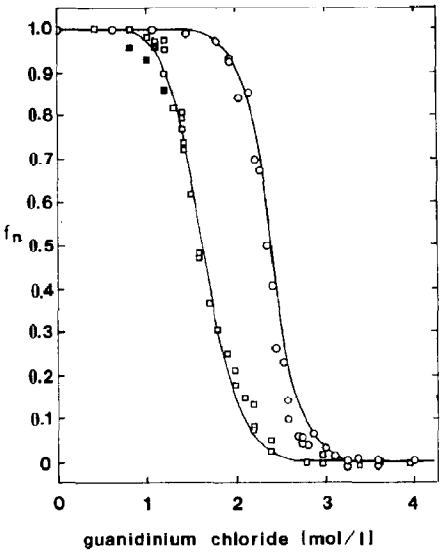

Fig. 1. The fraction of native sperm whale (○) and chicken (□) metmyoglobin calculated from $A_{409\ nm}$ vs. guanidinium chloride molar concentration at 25°C. The theoretical curves were drawn from the binding model parameters given in table 1. The filled squares are reversibility tests.

sorbances of both the native metmyoglobin in 0.25 M KCl in 0, 0.6 and 0.8 M denaturant and that of the denatured metmyoglobin in 3.4 M denaturant were obtained as a function of temperature and least-squares lines determined. Values for $\ln(K_D)$ were computed from the $A_{409\ nm}$ data and the

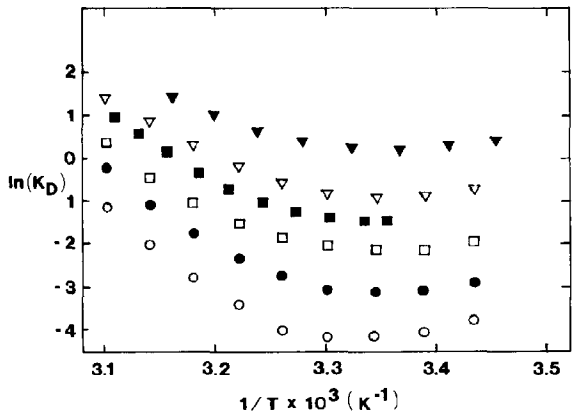

Fig. 2. Van't Hoff plots for chicken metmyoglobin at pH 8 in 1.00 M (○), 1.10 M (●), 1.20 M (□), 1.30 M (■), 1.40 M (▽) and 1.60 M (▼) guanidinium chloride.

least-squares lines giving the absorbances of native and denatured protein as a function of temperature. Apparent values of ΔH were computed as the slope at the center point of three data points fitted to a parabola. Within experimental error, identical estimates were obtained from both cooling and heating curves. The apparent values of ΔH were plotted against temperature and the values of ΔC_p and ΔH_T estimated by least squares.

3. Results and discussion

Fig. 1 gives guanidinium chloride unfolding curves at 25°C for sperm whale and chicken metmyoglobin. The sperm whale data are in excellent agreement with previous work [6]. Table 1 gives estimates obtained for the free energy of unfolding in buffer alone. Fig. 2 shows the van't Hoff plots for chicken metmyoglobin at several denaturant concentrations. Table 2 lists the estimates obtained at 40°C for apparent values of the thermodynamic quantities obtained at denaturant concentrations from 1.0 to 1.6 M. The correlation coefficients for plots of apparent enthalpy change vs. temperature exceeded 0.96.

Sperm whale myoglobin unfolding equilibria have been studied by a variety of workers and methods. The estimate obtained herein for the free energy change on unfolding of 13.5 ± 0.6 kcal/mol is close to that obtained from acid unfolding [2] of 13.6 kcal/mol, from microcalorimetry [3] of 12.0 ± 0.8 kcal/mol, and from guanidinium chloride unfolding [6] of 13.6 ± 0.7 kcal/mol. For both proteins, identical estimates, within experimental

Table 2

Thermodynamics of chicken metmyoglobin denaturation

Guanidinium chloride (mol l^{-1})	ΔH_{313} (kcal mol^{-1})	ΔC_p (kcal mol^{-1} K^{-1})	ΔS_{313} (cal mol^{-1} K^{-1})
1.0	31.9 ± 1.7	2.37 ± 0.17	96 ± 5
1.1	32.2 ± 1.7	2.14 ± 0.17	99 ± 5
1.2	24.6 ± 0.7	1.65 ± 0.07	76 ± 2
1.3	26.2 ± 0.4	1.83 ± 0.06	83 ± 1
1.4	25.0 ± 1.5	1.55 ± 0.15	80 ± 5
1.6	20.1 ± 0.6	1.29 ± 0.49	66 ± 2

error, for the free energy change on unfolding are given by both models. All these results suggest that fairly exact measurements of the free energy change of unfolding for the reversible, two-state case are feasible.

Measurement of other thermodynamic quantities from thermal studies of proteins in guanidinium chloride and their extrapolation to zero molar denaturant are considerably more difficult. The pioneering work of Tanford and his students began with the assumption that the enthalpy change on denaturation did not depend appreciably on the denaturant concentration. Upon examining the van't Hoff plots obtained in this and prior studies, such a conclusion appears reasonable. Yet, detailed microcalorimetric studies by Privalov and co-workers [3,16,17] have shown that such is not the case. A close examination of the data of Pfiel and Privalov for lysozyme strongly suggests that, provided the pH is kept constant, the preferential binding enthalpy change is a nearly linear function of guanidinium chloride concentration. If so, then a valid estimate for ΔH_{313} in buffer alone could be obtained by linear extrapolation of ΔH_{app} vs. denaturant concentration. The data obtained in this study over a more limited range of denaturant concentration suggest that ΔH_{app} may be to a first approximation linearly dependent on denaturant concentration.

It is known from recent work that the replacement of lysine at position 118 by arginine increases the free energy of unfolding by 1.1 kcal/mol [9]. This appears to be due to the additional salt bond linking the B helix to the GH turn. This result, taken with earlier data on the unfolding of horse metmyoglobin [6], suggests that the replacement of Lys 45 by arginine also adds up to 1.7 kcal/mol to the stabilization of the sperm whale conformation. Chicken myoglobin has lysine at both positions 45 and 118. In addition to these two changes, a significant change occurs within the D helix (positions 51–57). In sperm whale myoglobin a salt bond between the side chains at position 52, glutamate, and position 56, lysine, stabilizes the D helix [1]. At position 52 in chicken myoglobin, proline occurs. Not only is the salt bond lost, but the D helix must be shorter due to Pro 52. It is entirely possible that in chicken myoglobin the D helix does not form, given a Chou-Fasman $\langle P_\alpha \rangle$ of 0.94 for the 52–57 segment. In soybean leghemoglobin, a protein whose overall supersecondary structure strongly resembles myoglobin, the area of the D helix is not helical [19]. Of considerable interest in this regard is the change in the unfolding parameters on going from whale to chicken myoglobin. Both the values of Δn, the change in the number of guanidinium ion binding sites, and α, the parameter giving the overall average change in exposure of peptide and side chains on denaturation, are decreased by 20%. Earlier studies on several mammalian myoglobins failed to find any variation in these two parameters [6,7]. The conversion of the D helix to aperiodic structure might well account for the observed decreases in these unfolding parameters in addition to a portion of the decrease in the free energy change on unfolding.

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