The Stability of Holomyoglobin Is Determined by Heme Affinity†

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ABSTRACT: The properties of wild-type, V68T, and H97D sperm whale myoglobins were compared to determine the relative importance of heme affinity and globin stability on the resistance of the holoprotein to denaturation. The V68T mutation decreases apoglobin stability by placing a polar side chain in the interior heme pocket. However, this substitution increases hemin affinity by formation of a strong hydrogen bond between coordinated water and the Thr[68](E11) side chain. The H97D substitution disrupts favorable contacts with Ser[92](F7) and the heme-7-propionate and causes a large increase in the rate of hemin dissociation. The Asp replacement has little affect on apoglobin stability because His[97](FG3) is a surface residue. The aquomet, cyanomet, deoxyferrous, and apoglobin forms of each mutant and wild-type myoglobin were unfolded by titration with guanidinium chloride. Even though holomyoglobin denaturation involves the dissociation of heme and should be dependent on protein concentration, nonspecific heme binding to unfolded states makes the overall process appear to be a simple, unimolecular unfolding transition. The equilibrium constants for the denaturation of the holomyoglobin mutants correlate almost exclusively with heme affinity and not with the stability of the globin portion of the molecule. The strong correlation with heme affinity explains quantitatively why the stability of myoglobin is enhanced ∼60-fold by reduction of iron to the ferrous deoxy state and by another ∼100-fold with CO coordination. Parameters measured for GdmCl-, urea- , acid-, and heat-induced denaturation of holomyoglobins and hemoglobins reflect heme affinity and not the folding properties of the corresponding apoproteins. This conclusion suggests that (1) many previous studies of the denaturation of intact heme proteins need to be reevaluated in terms of heme affinity and (2) measurements with apoproteins are required for unambiguous determinations of the stability of globin structures.

Myoglobin and hemoglobin are perhaps the most thoroughly studied proteins with respect to structure and function and yet the complex interaction between heme and globin prevents a simple understanding of holoprotein stability. The development of extracellular heme proteins for use as O_2-delivering pharmaceuticals has generated even more interest in understanding heme binding and globin folding. *In vivo* and *in vitro* stabilities of potential blood substitute products are determined by the resistance of the holoprotein to heme loss and subsequent denaturation. On the other hand, expression yields appear to be determined primarily by the rate and extent of apoglobin folding, which need to be optimized for commercial production of recombinant hemoglobins (Hargrove *et al*., 1994b).

Heme clearly stabilizes intact myoglobins and hemoglobins with respect to their corresponding apoglobins (Crumpton & Polson, 1965; Kawahara *et al*., 1965). Removal of heme from myoglobin results in a decrease in helicity, swelling of the resulting apoprotein, and a marked decrease in resistance to denaturation (Schechter & Epstein, 1968; Hughson & Baldwin, 1989). Baldwin, Wright, and coworkers have characterized the acid- and urea-induced unfolding pathway of apomyoglobin in terms of a three-state model (Hughson *et al*., 1991; Barrick & Baldwin, 1993; Jennings & Wright, 1993). The native (N) apoglobin state unfolds first into a molten globule intermediate (I) in which the A, G, and H helices are still intact and appear to form a hydrophobic core. This intermediate then unfolds completely to form the U state at low pH or high concentrations of denaturant. This model has been successful for explaining the effects of mutagenesis on apomyoglobin stability (Hughson & Baldwin, 1989; Hargrove *et al*., 1994b; Kiefhaber & Baldwin, 1996).

In contrast to the work with apoglobins, recent and past studies have not been successful at correlating effects such as helix propensity, helix pairing, and electrostatic interactions with holomyoglobin stability (Flanagan *et al*., 1983; Garcia-Moreno *et al*., 1985; Hughson & Baldwin, 1989; Pinker *et al*., 1993; Lin *et al*., 1993, 1994). Most evidence suggests that the unfolding of myoglobin results in heme dissociation. For example, Schechter and Epstein (1968) showed that quenching of tryptophan fluorescence by heme is lost when myoglobin is unfolded. However, despite the large number of previous studies, the contribution of heme to the stability of myoglobin has not been determined quantitatively (Goto & Fink, 1994).

To address this problem, we compared the denaturation properties of three recombinant sperm whale myoglobins with very different heme affinities and apoglobin stabilities. The relative contributions of these two properties to the overall stability of the holoprotein were determined quantitatively. Wild-type myoglobin served as a control. The internal Val[68](E11) residue was replaced with a polar Thr

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residue which is known to decrease apoglobin stability but, at the same time, increase resistance to hemin loss by forming a strong hydrogen bond to the water molecule coordinated to the iron atom (Smerdon *et al*., 1991; Hargrove *et al*., 1994b). The external His^{97}(FG3) residue was replaced with Asp to disrupt favorable interactions at the solvent−heme interface. This surface replacement causes a large decrease in heme affinity without significantly affecting apoglobin stability. The aquomet, cyanomet, deoxy ferrous, and apoglobin forms of each mutant were unfolded by titration with guanidinium chloride. In combination, these experiments show unambiguously that denaturation parameters obtained from analyses of holoprotein unfolding reflect primarily heme affinity and not the stability of the globin portion of the molecule.

MATERIALS AND METHODS

Preparation of Proteins. Recombinant wild-type and mutant sperm whale myoglobins were expressed and purified as described by Springer and Sligar (1987) and Carver *et al*. (1992). The V68T mutation in sperm whale myoglobin was constructed using cassette mutagenesis of the wild-type sperm whale myoglobin gene. H97D was constructed using the Kunkel method of oligonucleotide-directed mutagenesis. Apomyoglobins were prepared using the methyl ethyl ketone method described by Ascoli *et al*. (1981) and Hargrove *et al*. (1994a). Protein concentrations were determined using the following extinction coefficients: apomyoglobin, $\epsilon_{280} = 15.2\ mM^{-1}\ cm^{-1}$ (Light, 1987); metmyoglobins, $\epsilon_{409} = 157\ mM^{-1}\ cm^{-1}$; cyanomyoglobins, $\epsilon_{423} = 187\ mM^{-1}\ cm^{-1}$; deoxymyoglobins, $\epsilon_{434} = 115\ mM^{-1}\ cm^{-1}$ (Antonini & Brunori, 1971).

Denaturation Conditions and Equilibration Problems. All experiments were carried out in 50 mM Tris and 50 mM NaCl, pH 8.0, in order to compare the results with heme binding studies under the same conditions (Hargrove *et al*., 1996a). A stock 6.5 M GdmCl solution was prepared in this buffer as described by Pace *et al*. (1990). In each unfolding experiment the appropriate volume of buffer and stock GdmCl were mixed to achieve the desired GdmCl concentration (0−6.5 M) in a final volume of 1 mL. A 10-μL aliquot of concentrated myoglobin (∼1 mM) was then added to 1 mL of each GdmCl concentration. The resultant solutions were equilibrated for 1 h prior to measurement. All absorbance spectra were measured with a SLM/Aminco 3000 diode-array spectrophotometer. Steady-state fluorescence emission was measured with a SLM 8100 spectrofluorometer. Circular dichroism was measured with an Aviv 62DS spectrapolarimeter.

Native and H97D metmyoglobin reached equilibrium in a few minutes after dilution into the denaturant, and no further absorbance or fluorescence changes occurred on longer time scales. In contrast, all the cyanomet- and deoxymyoglobins and V68T metmyoglobin required at least 30−45 min to reach equilibrium, and reversibility of these reactions was compromised by aggregation of free heme and nonspecific heme binding to the apoprotein (Shen & Hermans, 1972a−c; Hargrove *et al*., 1996a). Unfolding of CO-myoglobins was attempted, but extremely long incubation times were required to reach equilibrium, and often oxidation of dissociated CO heme occurred. Problems with reversibility are very severe for acid- and heat-induced denaturation of holomyoglobin since these conditions almost always cause precipitation of both globin and hemin. GdmCl has the advantage of inducing denaturation and, at the same time, solubilizing both the unfolded protein and the amphipathic prosthetic group. However, in general, caution must be exercised when using results for holoprotein denaturation to obtain thermodynamic parameters (Acampura & Hermans, 1967).

Hemin Loss. Rate constants for hemin loss from wild-type and mutant myoglobins were measured as described in Hargrove *et al*. (1994a). The reaction conditions at pH 5.0 were 0.15 M potassium phosphate and 0.45 M sucrose, at 37 °C. Hemin loss experiments at pH 8.0 were carried out in 50 mM Tris and 50 mM NaCl at 37 °C without sucrose. The rate constant for hemin loss, k_{-H}, was obtained from fits of each time course to a single-exponential expression with an offset.

Unfolding Reactions: Apomyoglobins. Total tryptophan fluorescence emission was measured with a 320-nm cutoff filter, and excitation was at 285 nm. Formation of the I state is seen clearly as a peak in plots of fluorescence intensity vs [GdmCl] (Hargrove *et al*., 1994b). CD at 222 nm was also monitored to follow changes in helicity during unfolding (Barrick & Baldwin, 1993). These changes are completely reversible (Hargrove *et al*., 1994b).

Metmyoglobins. Soret absorbance spectra were measured at each GdmCl concentration. The spectra of free hemin in 6.5 M GdmCl was recorded as a control (Figure 1A). Absorbance changes at 409 nm were plotted for analysis. The fluorescence of holomyoglobin is highly quenched when heme is bound in the heme pocket, and measurement of holomyoglobin fluorescence during unfolding monitors primarily heme dissociation (Schechter & Epstein, 1968). Excitation and emission were measured as described for apomyoglobin, and CD was monitored at 222 nm.

Cyanomyoglobins. Unfolding of cyanomyoglobins was carried out as described above except that the buffer and GdmCl stock solutions contained 0.5 mM KCN and absorbance was measured at 423 nm (Pinker *et al*., 1993). In addition, the myoglobin sample was mixed with KCN prior to dilution into GdmCl to ensure that cyanide was bound before the protein was unfolded. An absorbance spectrum was measured at each GdmCl concentration, and a spectrum of free cyanohemin in 6.5 M GdmCl was recorded as a control (Figure 1B).

Deoxymyoglobins. Unfolding was carried out anaerobically in the presence of a small amount of sodium dithionite to prevent oxygenation and oxidation. The buffer and GdmCl/buffer solutions were equilibrated with 1 atm of nitrogen prior to mixing with myoglobin, and the mixtures were sealed in airtight cuvettes that had been flushed with nitrogen. CD and fluorescence measurements of deoxymyoglobin were not possible due to the large absorbance of sodium dithionite at wavelengths less than ∼350 nm. An absorbance spectrum of each GdmCl/deoxymyoglobin mixture was recorded, and a spectrum of free deoxyheme in 6.5 M GdmCl was measured as a control (Figure 1C).

Thermodynamic Analysis. Apomyoglobin unfolding curves were fitted to the three-state model of Barrick and Baldwin (1993). The same model was modified for analysis of fluorescence data as described by Hargrove *et al*. (1994b). Each holomyoglobin unfolding curve was initially fitted to

a two-state unfolding transition as described by Pace *et al*. (1990). A rationale for this method is given at the beginning of the results section. In these analyses, each unfolding constant, $K_{(X)}$, depends exponentially on denaturant concentration:

$$K_{(X)} = K \exp(mX) \quad (1)$$

where X = [GdmCl], $K_{(X)}$ = [denatured myoglobin]/[holomyoglobin] at various [GdmCl], K is the unfolding equilibrium constant at [GdmCl] = 0, and m is a fitting constant representing differential binding between the native and unfolded protein states. Initially, pre- and posttransitional slopes were fixed at 0, and K and m were allowed to vary. In each case, m was near 4.1 M^{-1}, the value for the GdmCl-induced N to I transition of apomyoglobin determined by Hargrove *et al*. (1994b). In subsequent analyses, m was fixed at 4.1 M^{-1} and K was varied. In the cases of V68T deoxy, cyano, wild-type, and H97D myoglobins, the pretransitional slope was allowed to vary. Wild-type and H97D metmyoglobin unfolding curves measured by CD were fitted to the three-state model described by Barrick and Baldwin (1993), allowing the CD signal of the intermediate to vary. The m value for the second transition was fixed at 2.8 M^{-1} on the basis of the results for the I to U transition described by Hargrove *et al*. (1994b).

RESULTS

Denaturation of Holomyoglobin Results in Heme Dissociation. The Soret spectra of free hemin, cyanohemin, and deoxyheme in 6.5 M GdmCl are similar, with the exception of the protein peak at 280 nm, to the final spectra observed when the corresponding complex of myoglobin is titrated with this denaturant (Figure 1). As [GdmCl] is increased, the fluorescence emission intensity of each complex increases (data not shown) concomitant with the decreases in Soret absorbance. The fluorescence increase is due to loss of tryptophan quenching by the heme group as the latter dissociates from the protein. The correspondence between the protein fluorescence changes, the heme absorbance changes, and the initial portion of the CD change indicates that heme dissociation occurs at the same time as or prior to denaturation of the globin.

Guanidinium chloride does not promote hemin dissociation by direct coordination to the iron atom. The spectra of free hemin and deoxyheme in 6.5 M GdmCl are similar to the spectra of these compounds in buffer (Figure 1). The Soret absorption bands are very broad, showing no indication of coordination with nitrogenous bases (*i.e*., narrow peaks in the 410−440-nm wavelength region). It is also unlikely that the positively charged guanidinium cation can coordinate with ferric iron which has a formal +1 charge in hemin.

Thermodynamic Analysis of GdmCl-Induced Heme Dissociation. Thermodynamic analyses of holomyoglobin denaturation are complex because of heme aggregation and nonspecific interactions between heme and the different folded and unfolded states of the apoprotein (Shen & Hermans, 1972a−c). Because heme dissociation accompanies myoglobin denaturation, a model for the denaturation of holomyoglobin must take into account the effects of heme affinity on stability. The simplest expression for this

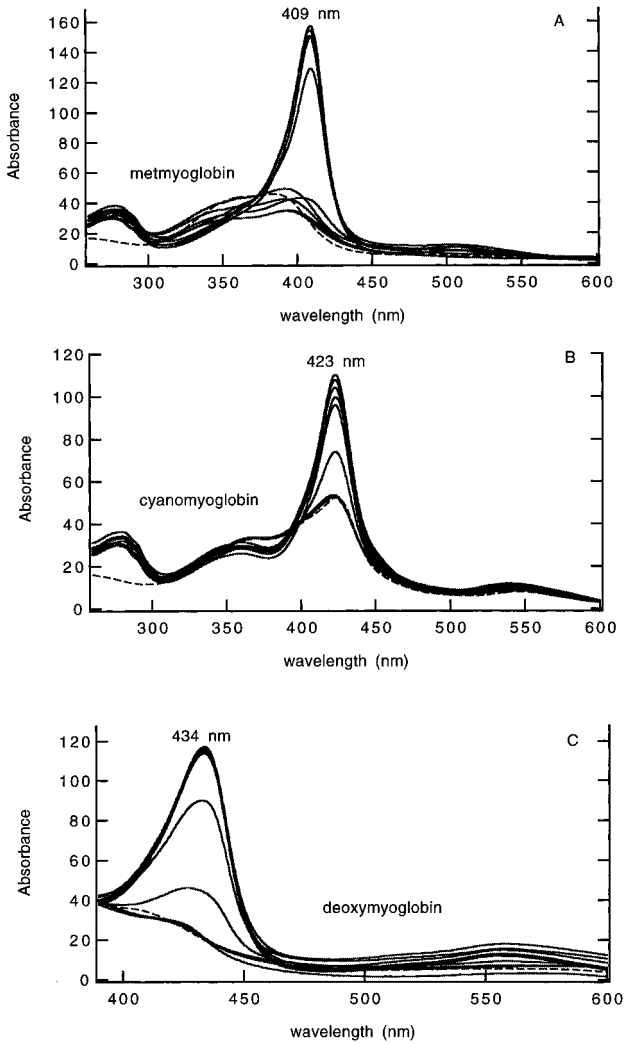

FIGURE 1: Absorbance spectra for the titration of aquomet, cyano, and deoxy forms of sperm whale myoglobin with GdmCl. (A) Metmyoglobin; (B) cyanometmyoglobin; (C) deoxymyoglobin. In each case, the range of [GdmCl] was 0−6.5 M. The dotted line in each figure is a spectrum of free hemin, cyanohemin, or deoxyheme under the same buffer conditions in 6.5 M GdmCl.

unfolding reaction is given by

$$\mathrm{Mb} \overset{K_{\mathrm{Mb,U}}}{\rightleftharpoons} \mathrm{U} + \mathrm{H} \quad (2)$$

In this case the fraction of holomyoglobin, Y_{Mb}, is described by

$$Y_{\mathrm{mb}} = \frac{2 + \frac{K_{\mathrm{Mb,U}}}{P_0} - \left[\left(2 + \frac{K_{\mathrm{Mb,U}}}{P_0}\right)^2 - 4\right]^{1/2}}{2} \quad (3)$$

where Mb represents holomyoglobin; U, the completely unfolded state; H, free monomeric heme; $K_{\mathrm{Mb,U}}$, the unfolding equilibrium constant; and P_0, the total protein (or heme) concentration. Most unfolding reactions involve only isomerization. However, holoprotein denaturation results in two products and should show dependence on protein concentration. Figure 2A shows denaturation curves simulated using eq 3 with values of 6, 60, and 160 μM for the total protein concentration, P_0. It is clear from these curves that a reaction described by eq 3 should exhibit a noticeable dependence on protein or heme concentration. The experimental denaturation curves in Figure 2B do not show a large dependence on protein concentration, suggesting that some mechanism

acts to diminish heme-induced refolding at high protein concentrations.

The dependence of unfolding on heme concentration can be diminished by taking into account nonspecific heme binding to unfolded myoglobin. The simplest two-step mechanism is shown in eq 4; a more rigorous treatment is given in the Appendix. The dependence of the fractions of Mb and the nonspecific heme−unfolded globin complex, UH, on [GdmCl] are given by eqs 5 and 6, respectively.

$$\mathrm{Mb} \overset{K_{\mathrm{Mb,U}}}{\rightleftharpoons} \mathrm{U} + \mathrm{H} \overset{K_{-\mathrm{NS}}}{\rightleftharpoons} \mathrm{UH_{NS}} \tag{4}$$

$$Y_{\mathrm{Mb}} = \frac{\left(2 + \frac{2K_{\mathrm{Mb,U}}}{K_{\text{-NS}}} + \frac{K_{\mathrm{Mb,U}}}{\mathrm{P_0}}\right) - \sqrt{\left(2 + \frac{2K_{\mathrm{Mb,U}}}{K_{\text{-NS}}} + \frac{K_{\mathrm{Mb,U}}}{P_0}\right)^2 - 4\left(\frac{K_{\mathrm{Mb,U}}}{K_{\text{-NS}}}\right)^2}}{2\left(1 + \frac{K_{\mathrm{Mb,U}}}{K_{\text{-NS}}}\right)^2} \tag{5}$$

$$Y_{\mathrm{UH}} = \left(\frac{K_{\mathrm{Mb,U}}}{K_{-\mathrm{NS}}}\right) Y_{\mathrm{Mb}} \tag{6}$$

The subscript NS refers to nonspecific binding, and $K_{-\mathrm{NS}}$ is the equilibrium constant for GdmCl-induced dissociation of nonspecifically bound heme in the $\mathrm{UH_{NS}}$ intermediate. $K_{\mathrm{Mb,U}}(\mathrm{x}) = K_{\mathrm{Mb,U}} \exp(m_{\mathrm{Mb,D}}\mathrm{X})$ and $K_{-\mathrm{NS(X)}} = K_{-\mathrm{NS}} \exp(m_{-\mathrm{NS}}\mathrm{X})$, where X = [GdmCl]. Similar mathematical expressions could be derived for the aggregation of free heme and its dissociation at high [GdmCl]. In previous work, we showed that heme binds nonspecifically to both intact holoprotein and unfolded apoprotein (Hargrove *et al*., 1996a). As a result, eq 4 was chosen as the mechanism for explaining the lack of dependence on protein concentration.

Denaturation curves simulated using eqs 5 and 6 are shown in Figure 2C. This figure shows that nonspecific heme binding during myoglobin unfolding prevents observation of a large dependence on protein or heme concentration. The mechanism described in eq 4 also accounts for the second absorbance transition seen at high [GdmCl] in the presence of high concentrations (60 and 160 μM) of protein. This transition appears to involve GdmCl-induced dissociation of nonspecifically bound heme. This phenomenon is seen in the simulations shown in Figure 2C and corresponds with the increase in Y_{UH} which is observed at moderate [GdmCl].

Ideally, experimental data at varying protein concentrations could be fitted to the sum of eqs 5 and 6 to yield values of $K_{\mathrm{Mb,U}}$ and $K_{-\mathrm{NS}}$. However, it is very difficult to obtain data with enough precision and resolution to assign individual equilibrium constants. Since little dependence on protein concentration is observed and most experiments were carried out at 6 μM heme, we have fitted the observed denaturation curves to a simple two-state unfolding reaction, Mb ⇌ D, with a single equilibrium constant, $K_{\mathrm{Mb,D}}$. The fraction of holoprotein is assumed to be

$$Y_{\mathrm{Mb}} = \frac{1}{1 + K_{\mathrm{Mb,D}}} \tag{7}$$

where $K_{\mathrm{Mb,D}}(\mathrm{X}) = K_{\mathrm{Mb,D}} \exp(m_{\mathrm{Mb,D}}[\mathrm{GdmCl}])$ and $K_{\mathrm{Mb,D}}$ is the isomerization constant for denaturation of the holoprotein in buffer. Good fits of the observed data to the simple

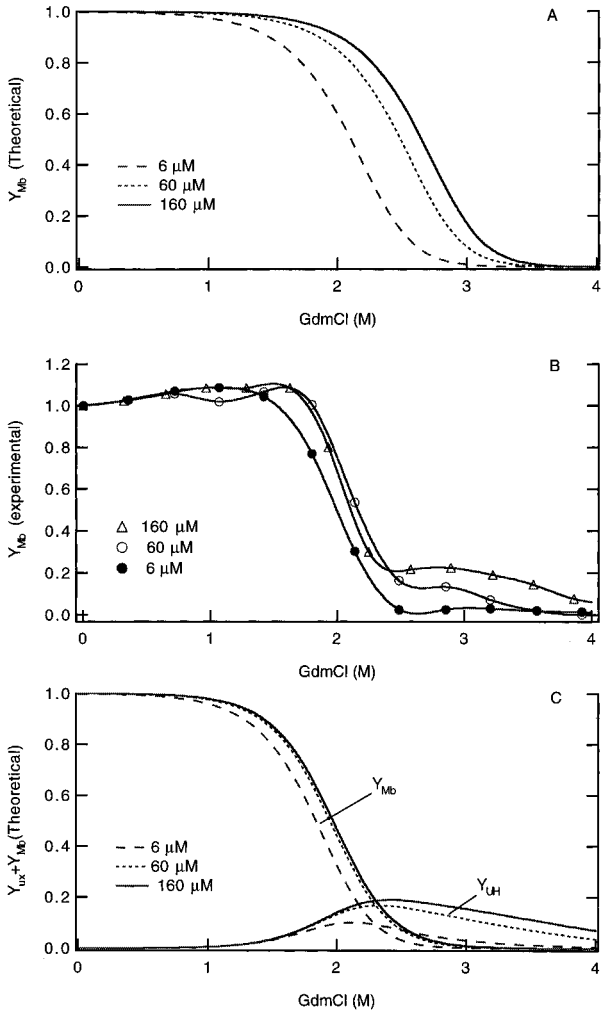

FIGURE 2: GdmCl titration of metmyoglobin at different protein concentrations. (A) These curves were generated in Matlab using eq 3 with $K_{\mathrm{Mb,U}} = 1 \times 10^{-11}$ M, and $P_0 = 6$, 60, and 160 μM. The m value for the transition was fixed at 6 $\mathrm{M^{-1}}$. (B) Plots of experimental data under the same conditions. The smooth lines were generated using a cubic spline routine in IGOR-Pro. (C) These curves are simulations of Y_{Mb} and Y_{UH} as a function of GdmCl concentration using eqs 5 and 6. The sum of these curves approximates the data in Figure 2B.

expression in eq 7 were obtained. As shown in the appendix, the fitted value of $K_{\mathrm{Mb,D}}$ is approximately equal to $K_{-\mathrm{H}}/K_{-\mathrm{NS}}$. This ratio of specific to nonspecific equilibrium dissociation constants for heme binding is expected to be unitless and should be significantly greater (10^4-10^7-fold) than the independently measured value of $K_{-\mathrm{H}}$.

Effects of Different Heme Ligands on Myoglobin Stability. A comparison of the melting curves for wild-type met-, cyanomet-, and deoxymyoglobins is shown in Figure 3. The fitted values for the single transition, $K_{\mathrm{Mb,D}}$, are listed in Table 1. The relative stabilities ($1/K_{\mathrm{Mb,D}}$) of the different liganded states of wild-type myoglobin are deoxyMb ≥ cyanometMb ≫ metMb. Increased resistance to denaturation caused by coordination of cyanide has been observed previously (*e.g*., Lin *et al*., 1993). Cyanide has greater ability to accept π electrons from the heme iron than coordinated water, and this interaction results in *trans*-stabilization of the $\mathrm{Fe^{3+}}-\mathrm{His^{93}}$ bond (Momenteau & Reed, 1994). The proximal $\mathrm{Fe^{2+}}-\mathrm{His^{93}}$ linkage in deoxymyoglobin is also known to be stronger than the more ionic bond with $\mathrm{Fe^{3+}}$ (Bunn & Jandel, 1968; Allis & Steinhardt, 1970).

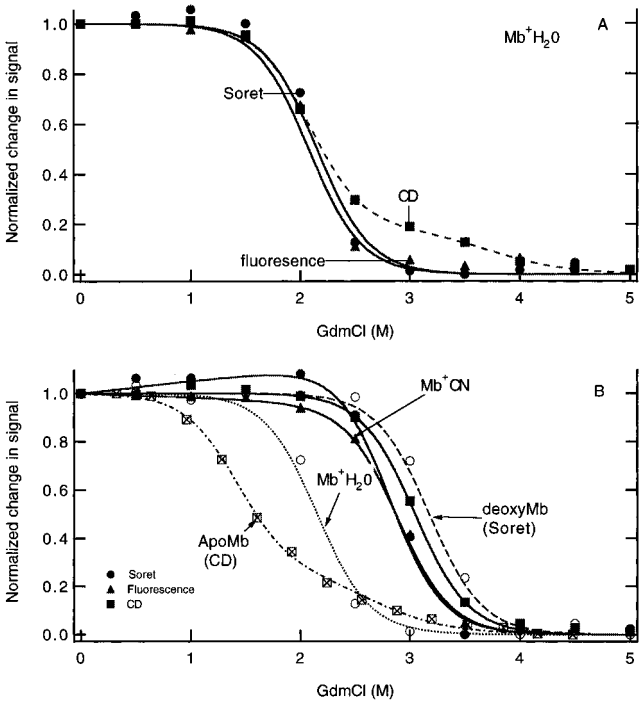

FIGURE 3: Comparison of Soret absorbance, fluorescence, and CD changes resulting from the unfolding of wild-type myoglobin. A) GdmCl-induced aquometmyoglobin unfolding monitored by Soret absorbance, fluorescence, and circular dichroism. B) Apo-, aquomet-, cyanomet-, and deoxymyoglobin unfolding induced by GdmCl. Unfolding of apomyoglobin was monitored by changes in circular dichroism; met- and cyanometmyoglobin unfolding was measured by the decrease in Soret absorbance and increase in fluorescence accompanying denaturation and heme dissociation. Unfolding of deoxymyoglobin was monitored by measuring the decrease in the Soret absorbance signal. The fluorescence and Soret melting curves for met- and aquometmyoglobin were coincident. The coordination and oxidation states of the heme greatly influence the observed stability of the holoprotein.

Table 1: Equilibrium Constants for GdmCl-Induced Denaturation of Wild-Type, V68T, and H97D Myoglobins[a]

	$K_{Mb,D}$ for aquometMb	$K_{Mb,D}$ for cyanometMb	$K_{Mb,D}$ for deoxyMb
	Wild-Type		
Soret absorbance	140×10^{-6}	6.2×10^{-6}	1.9×10^{-6}
fluorescence	180×10^{-6}	8.0×10^{-6}	
CD (222 nm)	140×10^{-6}	3.8×10^{-6}	
	V68T		
Soret absorbance	13×10^{-6}	23×10^{-6}	5.2×10^{-6}
fluorescence	14×10^{-6}	20×10^{-6}	
CD (222 nm)	25×10^{-6}	14×10^{-6}	
	H97D		
Soret absorbance	6000×10^{-6}	120×10^{-6}	130×10^{-6}
fluorescence	4200×10^{-6}	140×10^{-6}	
CD (222 nm)	4800×10^{-6}	170×10^{-6}	

[a] In most cases, the denaturation equilibrium constant $K_{Mb,D}$ was determined from fits to a single unfolding event (eq 1). The circular dichroism data for wild-type and H97D metmyoglobins were analyzed in terms of a three-state transition, and the fitted value of $K_{Mb,I}$ for the first transition is reported as $K_{Mb,D}$.

The fluorescence, Soret absorbance, and CD signals change simultaneously when cyanometmyoglobin is titrated with GdmCl, and these transitions fit reasonably well to a two-state model (Figure 3B). The unfolding of deoxymyoglobin can also be fitted to a simple, two-state model. In contrast, the CD changes accompanying metmyoglobin denaturation show the presence of an intermediate much like

Table 2: Equilibrium Constants for GdmCl-Induced Unfolding of Wild-Type, V68T, and H97D Apomyoglobins[a]

	apoMb unfolding		
	$K_{N,I}$	$K_{I,U}$	$K_{N,I}K_{I,U}$
	Wild-Type		
fluorescence	0.011	0.002	20×10^{-6}
CD (222 nm)	0.003	0.0006	2×10^{-6}
	V68T		
fluorescence	0.068	0.002	135×10^{-6}
CD (222 nm)	0.025	0.0018	45×10^{-6}
	H97D		
fluorescence	0.009	0.002	18×10^{-6}
CD (222 nm)	0.003	0.0004	1×10^{-6}

[a] Unfolding reactions were carried out in 0.2 M phosphate, pH 7.0 at 25 °C. Values of $K_{N,I}$ and $K_{I,U}$ were obtained from fits to eq 1 of Hargrove *et al.* (1994b).

that seen for the denaturation of apomyoglobin (Figure 3A). Goto and Fink (1994) observed a similar intermediate during the denaturation of native myoglobin with acid. These results suggest a three-state process for the denaturation of metmyoglobin. The first transition involves hemin loss and formation of the partially folded I state of apoglobin which has lost ~70% of the CD_{220nm} signal of the original holoprotein. Higher [GdmCl] is required to unfold this intermediate completely to the U state of apomyoglobin. The value of $K_{I,U}$ measured starting from holometmyoglobin is on the same order as that measured by CD for $K_{I,U}$ starting from apoprotein (Figure 3B, Table 2). The reason the I to U transition is not seen when denaturing cyanometmyoglobin and deoxymyoglobin is that these holoproteins are much more resistant to heme loss. The higher [GdmCl] required to cause cyano- or deoxyheme loss (~3 M) is also sufficient to unfold the intermediate, causing the N → I and I → U processes to occur simultaneously.

Apoglobin Stabilities and Hemin Dissociation Equilibrium Constants. The unfolding transitions of wild-type, V68T, and H97D apomyoglobins are shown in Figure 4A,B. Time courses for hemin dissociation from the corresponding aquometmyoglobin derivatives are shown in Figure 4C. When apomyoglobin unfolding is monitored by fluorescence, an initial increase in total emission intensity is observed for the native apoglobin (N) to intermediate (I) transition, followed by a decrease as the protein becomes completely unfolded (Hargrove *et al.*, 1994b). The peak corresponds to the formation of the intermediate state, which is thought to contain intact A, G, and H helices and retain 30−40% of the CD of the starting apoprotein (Hughson *et al.*, 1990). The corresponding CD changes also show a two-step unfolding process. There is an initial 50−60% decrease with a midpoint at ~1.0 M GdmCl and a second transition centered around 2.4 M GdmCl. The data in Figure 4A,B show that the stabilities of wild-type and H97D apomyoglobins are identical, as measured by either fluorescence or CD, whereas V68T apomyoglobin is considerably less stable and unfolds at significantly lower [GdmCl].

Although the fluorescence and CD changes give qualitatively the same results, the fitted values for $K_{N,I}$ and $K_{I,U}$ differ (Table 2). In general, the parameters obtained from CD data are 3−5 times smaller than those obtained from the fluorescence data. The unfolding data are obtained on a logarithmic x-axis scale since $K_{unfolding}$ is proportional to exp(m[GdmCl]), where $m \approx 4.1$ M^{-1}. The errors in the x-axis

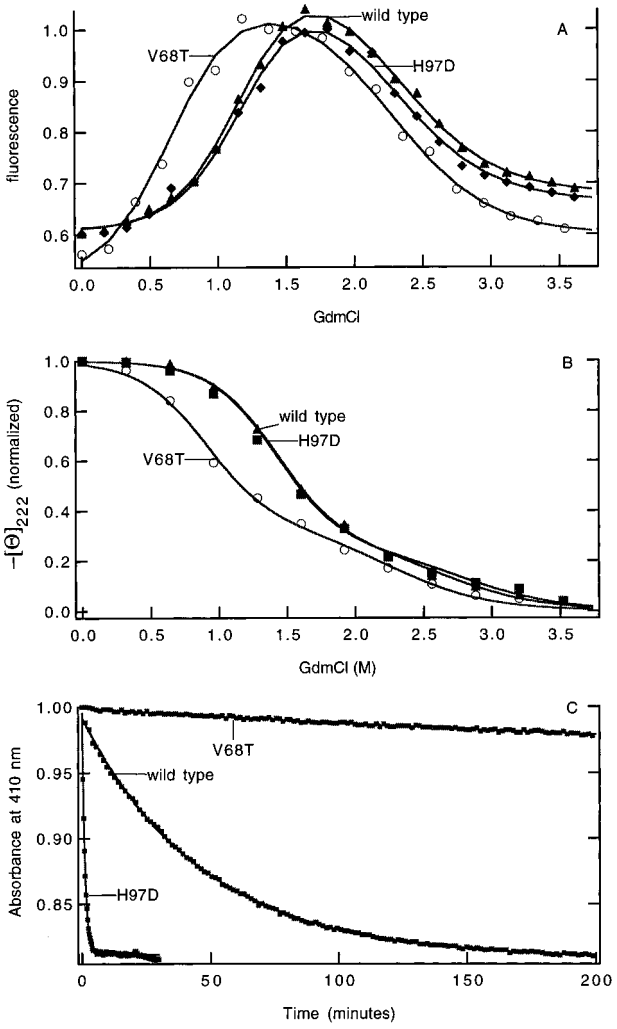

FIGURE 4: Stabilities and rates of hemin loss from wild-type, V68T, and H97D myoglobins. (A) Fluorescence and (B) CD changes observed during apoglobin unfolding. V68T apomyoglobin is clearly less stable than the wild-type and H97D apoproteins, which are similar in stability. Fitted equilibrium constants for these transitions are given in Table 2. (C) Time courses for hemin loss from the mutants and wild-type metmyoglobin at pH 5.0. V68T metmyoglobin has a much lower rate of hemin loss than wild-type protein, whereas the H97D mutant has a much higher rate (Table 3). Thus, apomyoglobin stability is not correlated with hemin affinity.

position are $\approx\pm 0.1$ M, which lead to errors in the unfolding constants of factors of ~ 2. Thus, although still apparent when superimposing the curves, the discrepancies between the CD and fluorescence data are small on a free energy scale. The fluorescence changes are due to tryptophans 7 and 14 and may result from conformational transitions which differ somewhat from those involving large changes in helical content which are monitored by CD (Fink, 1995). Resolution of these transitions will require combined CD/fluorescence

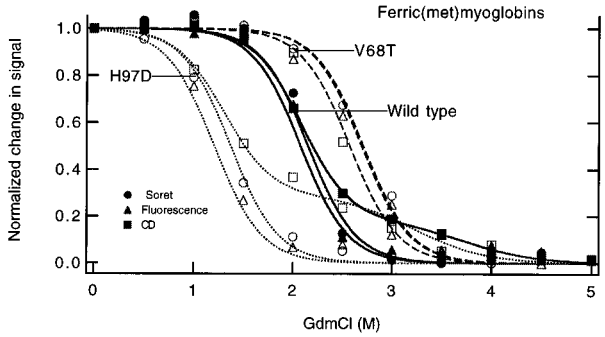

FIGURE 5: Comparison of the stabilities of wild-type, V68T, and H97D metmyoglobins. The V68T mutant is the most stable of the three holoproteins, and H97D metmyoglobin is the least stable. The I to U transitions of apomyoglobin can still be observed in the unfolding curves for wild-type and H97D metmyoglobins as measured by CD, but they are not observed by Soret absorbance or fluorescence emission.

titrations using a wider variety of myoglobins. However, the main conclusion is clear. The N state of V68T apomyoglobin is much less stable than the corresponding state for either wild-type or H97D apomyoglobin (Table 2, second and fourth columns).

In contrast to its relatively low apoglobin stability, V68T metmyoglobin shows the greatest resistance to hemin loss (Figure 4C and Table 3). The rate of hemin dissociation from this mutant is 25-fold less than that for wild-type metmyoglobin at pH 5 and too slow to be measured at pH 8.0. We have shown that the bimolecular association rate constants for monomeric hemin, CO-heme, and cyanohemin binding to apoglobins are $\sim 1 \times 10^8$ M^{-1} s^{-1} at 20 °C and roughly independent of protein structure, pH, and state of ligation (Hargrove *et al.*, 1996a). Thus, the equilibrium dissociation constants (K_{-H}) for hemin binding can be estimated as $k_{-H}/(1 \times 10^8$ M^{-1} $s^{-1})$ and are given in Table 3 for wild-type, V68T, and H97D metmyoglobins.

The hemin dissociation equilibrium constants, K_{-H}, differ significantly in the order V68T < wild-type $\ll$ H97D aquometmyoglobin at both pH values. This order corresponds with that observed for the holoprotein denaturation constants ($K_{Mb,D}$ in Table 1) but not with that for the apoglobin unfolding constants ($K_{N,I}$ or $K_{I,U}$ in Table 2).

Stability of Mutant Holoproteins. Unfolding curves for wild-type, V68T, and H97D metmyoglobins are shown in Figure 5. The fitted values of $K_{Mb,D}$ are listed in Table 1. The order of stability of the aquometmyoglobins is V68T > wild-type > H97D myoglobin. As was the case for wild-type protein, the CD data for H97D metmyoglobin required fitting to a three-state process with $K_{Mb,I}$ and $K_{I,U}$ being $\sim 5 \times 10^{-3}$ and $\sim 1 \times 10^{-4}$, respectively. The biphasic character of the H97D metmyoglobin curve is more pronounced than that of wild-type myoglobin due to dissociation of hemin

Table 3: Rate and Equilibrium Constants for Hemin Dissociation from Wild-Type, V68T, and H97D Metmyoglobins[a]

	hemin dissociation			
protein	k_{-H} at pH 5 (h^{-1})	K_{-H} at pH 5 (M)	k_{-H} at pH 8 (h^{-1})	K_{-H} at pH 8 (M)
wild-type Mb	1.0 ± 0.5	$2.8 \pm 5 \times 10^{-12}$	0.01	$2.8 \pm 5 \times 10^{-14}$
V68T Mb	0.04	0.1×10^{-12}	<0.005	$<0.05 \times 10^{-14}$
H97D Mb	38	110×10^{-12}	8	2200×10^{-14}

[a] Hemin loss reactions at pH 5 were carried out in 0.2 M sodium acetate and 0.45 M sucrose, at 37 °C. Reactions at pH 8.0 were in 50 mM Tris and 50 mM NaCl, at 37 °C. Values of the equilibrium constant for hemin dissociation, K_{-H}, were calculated using 1×10^{-8} M^{-1} s^{-1} for the association rate constant at both pH 5 and 8 (Hargrove *et al.*, 1996a) and the dissociation rate constants, k_{-H}, shown in the table.

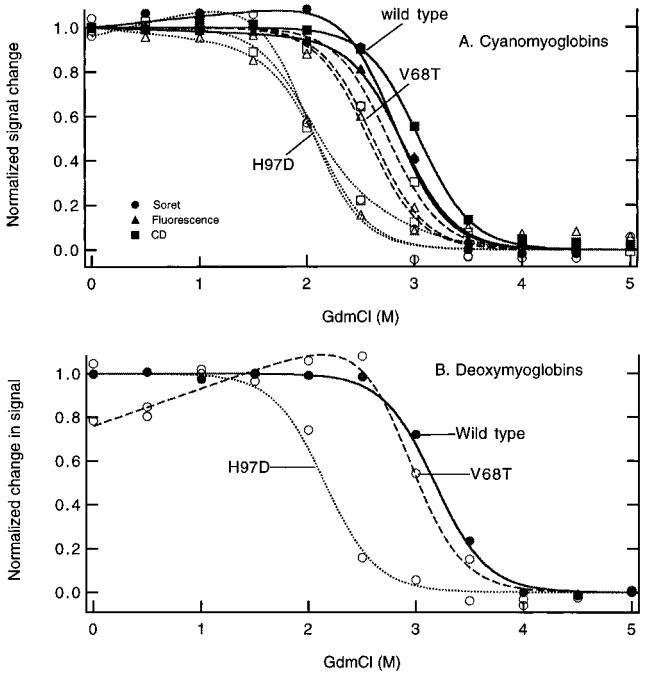

FIGURE 6: Comparison of the stabilities of wild-type, V68T, and H97D cyanomet- and deoxymyoglobins. (A) Denaturation of cyanometmyoglobins. (B) Denaturation of deoxymyoglobins.

from the mutant at lower [GdmCl]. The I to U transition occurs at the same [GdmCl] for both proteins (∼2.5−3 M). In contrast, the absorbance and CD data for V68T metmyoglobin were readily fitted with a single unfolding constant, $K_{Mb,D} \approx 2 \times 10^{-5}$.

In the case of the cyanomet derivatives, the observed stabilities are wild-type ≥ V68T > H97D myoglobin, and simple two-state transitions are observed for all three proteins (Figure 6A, Table 1). The change in order of wild-type and V68T myoglobin is due to unfavorable electrostatic interactions between the negative portion of the threonine hydroxyl and bound cyanide (Brancaccio *et al*., 1994; Dou *et al*., 1996). In the case of the deoxy forms, wild type and V68T myoglobins are equally stable, and the H97D mutant again denatures at much lower [GdmCl] (Figure 6B). We attempted to unfold the ferrous CO forms of these myoglobins. Unfortunately, the time required for the CO samples to reach equilibrium was so long (hours) that oxidation occurred. However, CO binding does clearly result in even greater stability than that observed for five-coordinated deoxymyoglobin. GdmCl concentrations on the order of 5 M were required to see significant (∼50%) absorbance changes due to CO-heme loss.

DISCUSSION

To a first approximation, the denaturation of holomyoglobin can be interpreted in terms of the following three-step scheme:

$$\mathrm{Mb} \overset{K_{-H}}{\rightleftharpoons} \mathrm{N} + \mathrm{H} \overset{K_{N,I}}{\rightleftharpoons} \mathrm{I} \overset{K_{I,U}}{\rightleftharpoons} \mathrm{U} \qquad (8)$$

Each equilibrium constant has an exponential dependence on [GdmCl]. Most apoproteins show a two-step denaturation curve both in fluorescence and CD experiments (Figure 4). However, the number of discrete transitions seen for denaturation of holoprotein is variable and depends on the oxidation state, iron coordination, and protein structure, all of which regulate hemin affinity. In the case of wild-type apomyoglobin, the concentrations of GdmCl required for $K_{N,I}$ and $K_{I,U}$ to be 1.0 are ∼1 and 2.5 M, respectively, whereas the concentration of GdmCl required for 50% dissociation of hemin, cyanohemin, and deoxyheme from the holoprotein are ∼2, ∼2.9, and ∼3.2 M, respectively. Two transitions are observed for the denaturation of wild-type metmyoglobin: Mb → H + I and I → U, since the concentration of GdmCl causing hemin loss (2−2.5 M) is insufficient to unfold completely the I apoprotein intermediate. In contrast, only one transition is observed for cyanomet- and deoxymyoglobin unfolding since the [GdmCl] required for heme dissociation in these cases (3−3.5 M) is greater than that required for complete unfolding of apomyoglobin (2.5−3 M). $K_{Mb,D}$, $K_{N,I}$, and K_{-H} values at pH 8 for wild-type, V68T, and H97D sperm whale metmyoglobins are given in Table 4.

Table 4: Summary of the Denaturation and Unfolding Constants of Met- and Apomyoglobins with the Corresponding Equilibrium Constants for Hemin Dissociation[a]

protein	$K_{Mb,D}$ for holo-Mb	$K_{N,I}$ for apo-Mb	K_{-H} (M)
wild-type Mb	150×10^{-6}	0.011	2.8×10^{-14}
V68T Mb	14×10^{-6}	0.068	$\leq 0.05 \times 10^{-14}$
H97D Mb	5000×10^{-6}	0.009	2200×10^{-14}

[a] $K_{Mb,D}$ and $K_{N,I}$ were determined as described in Tables 1 and 2, respectively. $K_{N,I}$ was based on fluorescence measurements only. $K_{Mb,D}$ is the average of the values obtained by absorbance, fluorescence, and CD measurements. K_{-H} was determined as described in Table 3.

Heme Affinity Is the Primary Determinant of Holomyoglobin Stability. The results in Tables 1−4 indicate that the factors governing the stability of holomyoglobin are the same as those which determine heme affinity. The strength of the His^{93}(F8)−iron bond decreases in the order ferrous pentacoordinate ≥ cyanomet > aquomet, which is the order of the stabilities of the corresponding myoglobin derivatives based on our GdmCl-induced unfolding studies (Table 1). As shown in Table 4, there is a direct correlation between the first equilibrium unfolding constant of aquometmyoglobin, $K_{Mb,D}$, and the equilibrium dissociation constant for hemin binding, K_{-H}. The V68T mutant shows lower values for both constants due to an "extra" hydrogen bond between the Thr^{68} hydroxyl and coordinated water. The H97D mutant shows higher values for both constants due to disruption of the hydrogen-bonding lattice between His^{97}, Ser^{92}, and the heme-7-propionate. In contrast, there is no correlation between the denaturation constants ($K_{Mb,D}$) of the holoproteins and the first unfolding constants for the corresponding apoglobins ($K_{N,I}$). V68T apomyoglobin is the least stable apoprotein, and wild-type and H97D apoglobins show almost identical stabilities (Table 4, Figure 4).

Discrepancies between Soret Absorbance and Far-UV CD Changes. When the unfolding of holomyoglobin is monitored by fluorescence or Soret absorbance, the observed changes reflect only the release of heme from the protein. In contrast, CD changes at 222 nm measure primarily alterations in globin secondary structure. In the case of cyanomet and ferrous myoglobin derivatives, heme dissociation occurs at GdmCl concentrations high enough to fully denature the corresponding apoglobins. As a result the

CD_{222nm} changes associated with loss of myoglobin secondary structure follow closely the absorbance and fluorescence changes associated with heme dissociation (Figure 6). However, if heme dissociation occurs at GdmCl concentrations which are insufficient to fully denature the apoprotein, residual CD_{222nm} changes will occur at higher [GdmCl] even though the fluorescence and absorbance changes associated with hemin loss are complete. The discrepancy between CD, absorbance, and fluorescence changes is most pronounced for the aquomet form of the H97D mutant, which has the poorest affinity for hemin of the proteins studied (Figure 4)

Interpretations of Heme Protein Stability Studies. Heme binding clearly stabilizes the globin tertiary structure with respect to both the intermediate and unfolded states (*e.g.*, $K_{Mb,D}$ vs $K_{N,I}$ in Table 4). The physiologically relevant parameter is the stability constant for the holoprotein, which is determined primarily by hemin affinity. Pinker *et al.* (1993) and Lin *et al.* (1993) showed that measurements of the unfolding of cyanometmyoglobin do not allow straightforward interpretations of the effects of mutagenesis on protein backbone stability but offered no explanation for this dilemma. For protein folding studies, the stability of apomyoglobin must be measured directly. It is certainly possible that apomyoglobin stability could affect heme affinity and *vice versa*, but a direct relationship is not required.

Mammalian holomyoglobins show only small variations in their resistances to acid-, heat-, urea-, and GdmCl-induced denaturation (Hapner *et al*., 1968; Harzell *et al*., 1968; Shen & Hermans, 1972a; Puett, 1973; Flanagan *et al*., 1983; Garcia Moreno *et al*., 1985). This result can now be interpreted more rigorously in terms of heme affinity. There is strong selective pressure in all mammals to prevent hemin loss and subsequent oxidative damage to surrounding muscle tissue. As a result, the rate and equilibrium constants for hemin dissociation from these proteins are all about the same, $\sim$0.01 h^{-1} and $\sim 1 \times 10^{-14}$ M^{-1} at pH 7 (Hargrove *et al*., 1996a,b). In contrast, the overall stabilities ($1/K_{N,I}K_{I,U}$) of the corresponding apoproteins vary widely, from $\sim$100 000 for sperm whale apoMb to $\sim$1000 for pig apoMb (Hargrove *et al*., 1994b).

Constitutive expression of myoglobin in bacteria is governed by both apomyoglobin stability and heme affinity. Apomyoglobin must be able to fold properly to prevent aggregation and precipitation from competing with heme binding. As a result, mutants with moderately poor heme affinities but very stable apoglobin structures can express as well as or better than those with high heme affinities (Hargrove *et al.*, 1994b). However, the holoproteins of mutants with high rates of hemin dissociation are much less stable to long-term storage at room temperature, repeated freezing and thawing, and other conditions that promote denaturation.

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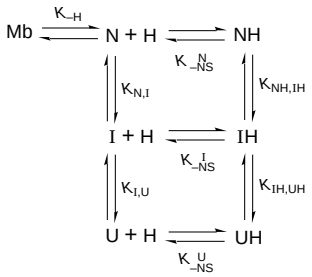
APPENDIX: GENERAL HOLOPROTEIN UNFOLDING REACTION

A complete description of holomyoglobin denaturation requires the following scheme:



(A1)

which takes into account nonspecific heme binding to the N, I, and U apoglobin intermediates. K_{-H} is the equilibrium constant for heme dissociation from intact myoglobin, and K^{N}_{-NS}, K^{I}_{-NS}, and K^{U}_{-NS} are the equilibrium constants for the dissociation of heme nonspecifically bound to the native (N), "molten globule" intermediate (I), and unfolded (U) states of apomyoglobin. $K_{N,I}$, $K_{I,U}$, $K_{NH,IH}$, and $K_{IH,UH}$ are the unfolding constants for apoprotein in the absence and presence of nonspecifically bound heme, respectively. Equation A1 is itself an approximation since multiple nonspecific heme binding processes occur when excess heme is added to either holo- or apomyoglobin (Hargrove *et al*., 1996a). However, because denaturation studies are normally carried out adding "extra" heme, only 1 equiv/protein is available, and eq A1 is a reasonable approximation.

The fractional amount of holoprotein is defined as

$$Y_{Mb} = \frac{[Mb]}{[Mb] + [N] + [NH] + [I] + [IH] + [U] + [UH]} \quad \text{(A2)}$$

which in terms of the equilibrium constants in eq A1 is

$$Y_{Mb} = \frac{1}{1 + \frac{K_{-H}}{[H]} + \frac{K_{-H}}{K^{N}_{-NS}} + \frac{K_{N,I}K_{-H}}{[H]} + \frac{K_{N,I}K_{-H}}{K^{I}_{-NS}} + \frac{K_{N,I}K_{I,U}K_{-H}}{[H]} + \frac{K_{N,I}K_{I,U}K_{-H}}{K^{U}_{-NS}}} \quad \text{(A3)}$$

The free concentration of heme is always small and given by $[H] = P_0(1 - Y_{Mb} - Y_{NS})$ where P_0 is the total protein concentration and Y_{NS} is the fraction of nonspecifically bound hemin. Substitution of this expression for [H] into eq A3 leads to a complex quadratic equation with a solution of the same form as eq 5 in the main text.

If the equilibrium constants for nonspecific heme binding are assumed to be the same for all three apoglobin forms, eq A3 reduces to

$$Y_{Mb} = \frac{1}{1 + \left(\frac{K_{-H}}{P_0(1 - Y_{Mb} - Y_{NS})} + \frac{K_{-H}}{K_{-NS}}\right)\left(1 + K_{N,I} + K_{N,I}K_{I,U}\right)} \quad \text{(A4)}$$

This expression can be simplified further by assuming that $K_{-H}/K_{-NS} > K_{-H}/P_0(1 - Y_{Mb} - Y_{NS})$ after some denaturation and heme dissociation have occurred. Under these conditions, the dependence on protein concentration can be neglected and

$$Y_{Mb} \approx \frac{1}{1 + \frac{K_{-H}}{K_{-NS}}(1 + K_{N,I} + K_{N,I}K_{I,U})} \quad \text{(A5)}$$

Comparison with eq 2 shows that $K_{Mb,D} \approx (K_{-H}/K_{-NS})(1 + K_{N,I} + K_{N,I}K_{I,U})$. For the mutants studied in this work, $K_{N,I} \approx 10^{-2}$ and $K_{I,U} \approx 10^{-3}$ in the absence of GdmCl, and thus $K_{Mb,D} \approx K_{-H}/K_{-NS}$ for these proteins.

Finally, it is important to remember that eq 7, $Y_{Mb} = 1/(1 + K_{Mb,D})$, is used to analyze Soret absorbance changes which reflect primarily the amount of native holoprotein present. Large fluorescence changes also accompany the loss of heme, and the fractional increase in fluorescence is approximately proportionally to $(1 - Y_{Mb})$. In contrast, the fractional CD changes reflect the extent of α-helical structure and are a measure of the relative amounts of unfolded states, primarily I and U. Thus, the CD signals are not always proportional to Y_{Mb}.

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