Heme orientation affects holo-myoglobin folding and unfolding kinetics[1]

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Abstract Native myoglobin (Mb) consists of two populations which differ in the orientation of the heme by 180° rotation (as verified by nuclear magnetic resonance) but have identical absorption spectra and equilibrium–thermodynamic stability. Here, we report that these two fractions of native oxidized Mb (from horse) both unfold and refold (chemical denaturant, pH 7, 20°C) in two parallel kinetic reactions with rate constants differing 10-fold. In accord, the oxidized heme remains coordinated to unfolded horse Mb in up to 4 M guanidine hydrochloride (pH 7, 20°C).

Key words: Protein folding; Myoglobin; Stopped-flow mixing; Heme protein

1. Introduction

Myoglobin (Mb) is one of the most thoroughly studied proteins with respect to folding, however, most investigations have been concerned with the apo-form [1–4]. Notably, Baldwin's group at Stanford has studied the dynamics of apo-Mb folding extensively. It has been demonstrated that apo-Mb unfolds through a compact intermediate upon lowering the pH [5,6]. This intermediate is also formed rapidly in the kinetic process of forming native apo-Mb at pH 6. The unfolding and refolding of the pH 4-stable intermediate (I) has been probed by stopped-flow experiments. This intermediate coexists in two distinguishable forms: I_a and I_b. Both are on-pathway intermediates in the refolding reaction of apo-Mb [7].

The globin fold is made up of eight helices that surround the heme group in a hydrophobic pocket-like structure; the heme iron is non-covalently linked to histidine 93. During protein synthesis, binding of the heme may proceed cotranslationally to the polypeptide still attached to the ribosome [8]. If this is the case for Mb, the heme group could affect its folding pathway in vivo. Folding studies of holo-Mb have however been limited [8,9]. For cyanomet holo-Mb, refolding was shown to occur by a first phase with a rate constant of ~ 1 s^{-1} where structure forms around the heme, followed by a slower ligand exchange process (suggested to be exchange of CN to the native histidine heme ligand) with a rate constant of $\sim 10^{-3}$ s^{-1} [8]. In addition, electron transfer-triggered folding experiments have shown reduced (deoxy) Mb to refold rapidly (10^2–10^3 s^{-1}) upon reduction of unfolded, oxidized (Met) Mb [9].

Nuclear magnetic resonance experiments have revealed that there are two holo-Mb forms in solution at equilibrium (their interconversion is extremely slow) which differ in the orientation of the heme by 180° rotation about the α-γ-meso axis [10–12]. The dominant (90% for sperm whale Mb) component has the same heme orientation as found in the Mb crystal structure [13]. This non-symmetrical arrangement of heme orientations in Mb at equilibrium was also probed by visible circular dichroism (CD) [11]. The amplitude of the positive CD signal in the Soret region was shown to correlate with the fraction of dominant heme orientation (as demonstrated for sperm whale Mb with heme ratio 9:1, and tuna Mb with ratio 3:2). Upon sperm whale Mb reconstitution (adding heme to folded apo-Mb), there is first (within ms) an initial 1:1 mixture of holo-Mb with heme in the two orientations, which then slowly (lifetime of hours to days) rearranges to the equilibrium 9:1 ratio [10–12]. Thus, at equilibrium, holo-Mb is heterogeneous albeit Soret absorption and far-UV CD spectra of the two forms are indistinguishable [12]. We here present results suggesting that these oxidized (Met) horse Mb populations (which differ in heme orientation) unfold and refold in two parallel, apparent two-state, reactions with a 10-fold difference in speed.

2. Materials and methods

Horse Mb was obtained from Sigma (sperm whale Mb was a gift from H.B. Gray). Gel electrophoresis showed the presence of a minor component (5–10%) in the horse Mb stock. Therefore, Mb was purified by gel filtration (and checked again by electrophoresis). We found no difference in folding behavior (equilibrium or kinetics) between the original stock and the purified Mb, indicating that our spectroscopic methods only probe Mb, and the data are not (if using the original stock) affected by the minor component. Guanidine hydrochloride (GuHCl) titrations were performed (5 mM phosphate, ±100 mM NaCl, pH 7.0, 20°C) with Met-Mb (5 μM) by following the far-UV CD signal (OLIS instrument, 200–300 nm), the Soret absorbance (Cary-50 spectrophotometer, 380–600 nm) and the tryptophan fluorescence (Shimadzu fluorometer, ex 280 nm, 295–450 nm). Kinetic measurements were made using an Applied Photophysics SX.18MV stopped-flow in either fluorescence (ex 280 nm, cut-off filter of 305 nm) or absorption (409 nm) mode. No amplitude changes occurred in the dead time (1.2–1.6 ms) of the instrument. Refolding was measured by (1:1, 1:5 and 1:10) dilutions of Mb (70 μM) in 2 or 2.5 M GuHCl with appropriate GuHCl/buffer solutions to give desired GuHCl concentrations (20°C). The averaged kinetic traces (minimum of eight) were fit to bi-exponentials using a non-linear least-squares algorithm. The kinetic traces could not be fit to single exponentials (except at [GuHCl] > 5 M, where more than 95% of the amplitude was found in one rate). Absorption and fluorescence-detected kinetics gave identical results, supporting that native Mb was not contaminated by apo-Mb.

The equilibrium unfolding data (Fig. 1A) were directly fit, according to a two-state model [14], to yield ΔG_U (unfolding free energy) and m (degree of hydrophobic exposure upon unfolding). The kinetic un-

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folding and refolding data were fit (Fig. 1B) assuming standard linear dependence of ln k_F and ln k_U on [GuHCl]:

$$\ln k = \ln[k_F(H_2O)\exp(m_F[\text{GuHCl}]/RT) + k_U(H_2O)\exp(m_U[\text{GuHCl}]/RT)]$$

where m_U is the slope of the unfolding branch and m_F is the slope of the folding branch. $k_F(H_2O)$ and $k_U(H_2O)$ are the folding and unfolding rate constants at 0 M GuHCl. The fits were only extended to 4 M GuHCl in the unfolding branch.

3. Results and discussion

Titrations of GuHCl, in 5 mM phosphate pH 7, show that the equilibrium unfolding of horse Met-Mb is consistent with a two-state reaction (Fig. 1A). Monitoring the equilibrium unfolding by far-UV CD, tryptophan fluorescence and Soret absorption all give identical results (midpoint at 1.5 M); the unfolding free energy that can be estimated from fits to the unfolding curves is 21(±2) kJ/mol (Table 1). This value is somewhat lower than earlier such estimates although the midpoints roughly synchronize [15]. The equilibrium unfolding transition for horse Met-Mb was not affected by inclusion of 100 mM NaCl (data not shown). The unfolding of Met-Mb is fully reversible (up to 5 M GuHCl) and no protein concentration-dependence was detected for the equilibrium unfolding transition, suggesting that the heme stays coordinated to the unfolded polypeptide, a conclusion also reached earlier [9,16]. In further agreement, gel filtration of unfolded Mb in up to 5 M GuHCl shows the heme to co-elute with the protein.

To study the kinetics of the Met-Mb folding and unfolding reactions, we employed stopped-flow mixing while monitoring absorption changes at 409 nm or fluorescence changes upon excitation at 280 nm. The same kinetic results were obtained independent of the detection method used. The kinetic unfolding data are best fit by a sum of two exponentials at each GuHCl concentration investigated; a faster rate corresponding to 20% of the amplitude change and a slower rate corresponding to 80% of the amplitude change. The logarithms of both the fast and the slow unfolding rate increase linearly when the GuHCl concentration is increased (Fig. 1B). In contrast, at a GuHCl concentration of 5 M or higher (inset Fig. 1B), the GuHCl-dependence decreases and 100% of the amplitude correlates with one fast rate (in 5–6 M GuHCl: rate constant of approximately 400 s^{-1}). This rate constant is similar to that determined for heme-His dissociation in unfolded cytochrome *c* [17] and, therefore, heme dissociation from His-93 in Mb is likely to occur under these strongly denaturing conditions. Gel filtration experiments of unfolded holo-Mb in 6 M GuHCl showed that unfolded polypeptide and heme eluted as two separate fractions, in support of heme dissociation at this high denaturant concentration.

The kinetic refolding data for Met-Mb (holo-Mb unfolded in GuHCl concentrations not dissociating the heme from the polypeptide) are also best fit by a sum of two exponential phases (Fig. 1B). The slower rate of the two corresponds to 80%, and the faster one to 20%, of the total absorption change, at all GuHCl concentrations investigated. There is no difference in refolding rates or amplitude changes when starting from Mb unfolded in 2.5 M or 2.0 M GuHCl and there is no protein concentration-dependence (5–35 μM investigated) in the refolding kinetics. Moreover, kinetic refolding data (rates and amplitude ratios) detected by tryptophan fluorescence and Soret absorption are identical. When unfolded Met-Mb is incubated for 1 h or less, we obtain 100% refolding of holo-protein. Extensive incubation (days), however, of unfolded Mb (in 2–2.5 M GuHCl) correlates with a decreased yield of refolded holo-Mb (also reported earlier in [18]). In contrast, when Mb refolding is initiated from 6 M GuHCl, no refolded holo-protein at all is detected using stopped-flow, again in accord with heme dissociation at very high denaturant concentrations.

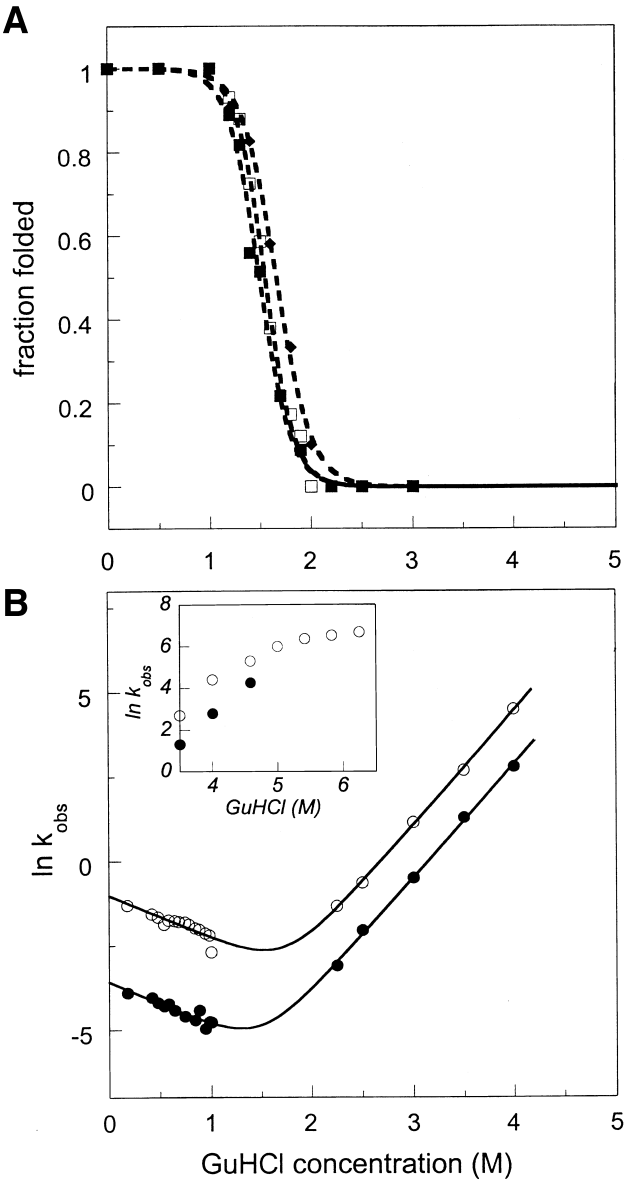

Fig. 1. A: Unfolding transition for horse Met-Mb as a function of GuHCl concentration (pH 7) (solid squares (far-UV CD), solid diamonds (tryptophan fluorescence) and open squares (Soret absorption)). B: Semi-logarithmic plot of horse Met-Mb folding and unfolding kinetics as a function of [GuHCl]. Open circles, fast rates (20% of amplitude), and filled circles, slow rates (80% of amplitude). The Chevron plot exhibits a midpoint around 1.5 M in agreement with that determined from equilibrium studies (A); each set of data shows the V-shape indicative of two-state folding. The results from fittings (solid lines) are summarized in Table 1. Inset: unfolding rates (open circles, fast; filled circles, slow rates) at high GuHCl concentrations.

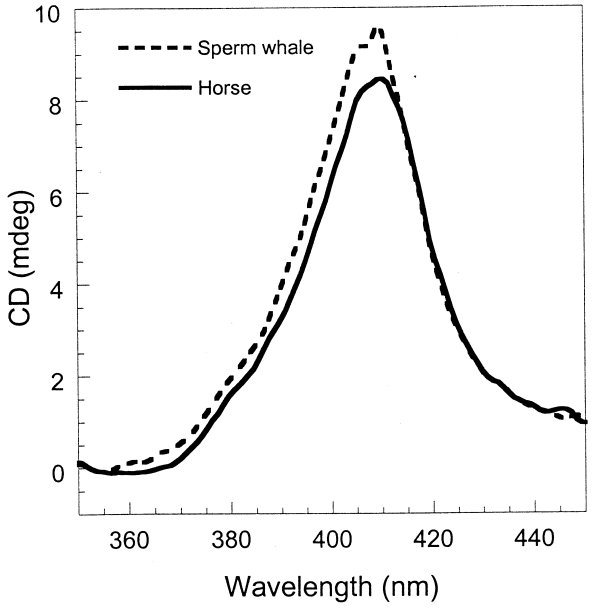

Fig. 2. CD spectra in the Soret region of horse (solid curve) and sperm whale (dashed curve) Met-Mb (10 μM protein; 1 cm pathlength). The magnitude of the CD signal at 409 nm reflects the ratio of heme in its two possible orientations in the native protein [11].

The equilibrium ratio of the two heme orientations in native horse Mb was probed by visible CD. Visible CD of sperm whale Mb, for which the heme orientation ratio has been determined earlier to be 9:1, was used as standard [10]. The signal for horse holo-Mb, at an identical concentration as sperm whale holo-Mb, has a slightly lower amplitude at 409 nm (9.5 mdeg versus 8.5 mdeg). This signal difference between horse and sperm whale samples, assuming only the dominant orientation of heme to contribute to the CD signal [11], therefore indicates an 8:2 ratio of heme orientations in native horse Mb (Fig. 2). Since the amplitude ratio for the unfolding kinetics (8:2) closely matches the equilibrium ratio of heme orientations (also 8:2) in native Mb, we suggest that these two populations of folded Mb unfold in two parallel, separate reactions. In accord, a related b-type heme protein, bovine cytochrome b_5, was also shown to unfold with biphasic kinetics. As for the Met-Mb unfolding results presented here, the cytochrome b_5 unfolding data were interpreted in terms of two protein fractions, ascribed to the different heme conformers in the native state, unfolding in two parallel reactions [19].

It has been shown that the iron–His-93 bond accounts for a major fraction of the heme-binding energy to folded Mb [20,21], suggesting that this interaction may prevail also in unfolded Met-Mb (at least under some conditions). Therefore, at GuHCl concentrations less than 5 M where no heme dissociation occurs, the heme is likely to remain coordinated to His-93 upon unfolding and a retention of the two different heme orientations is possible in the unfolded state of holo-Mb. The refolding kinetics (in addition to the unfolding kinetics) can thus also be affected by the heme orientation and may explain the observed biphasic refolding behavior. The same ratio of fast to slow rates (2:8) is observed in the refolding as in the unfolding experiments. The slow unfolding fraction (designated to Mb with heme in the dominant orientation) corresponds to the slow refolding fraction; the fast unfolding and refolding fractions (Mb with heme in the other orientation) belong together.

The finding of identical ratios of Soret absorption and tryptophan fluorescence amplitude changes for the two rates (both detection methods yield an amplitude ratio of 8:2 for slow to fast rates) is consistent with two protein populations unfolding and refolding in parallel. If instead an intermediate had been involved, the same alteration (relative to folded and unfolded Mb states) in the two different spectroscopic probes would not be expected. Further support for the link between heme orientation and kinetics emerges from refolding experiments performed after various lengths of incubation of unfolded holo-Mb samples. If unfolded holo-Mb (in 2.5 M GuHCl) is incubated for up to 1 h before refolding is initiated, 80% refolds by the slow rate and 20% by the fast rate. Upon extending this incubation time to 72 h, only 60% refolded in the slow phase and 40% in the fast. This result suggests that over time, the heme in unfolded Mb rearranges towards that of a 1:1 mixture of heme orientations. This is expected, since no structural constraints are present in the unfolded state (as is the case in native Mb) and a 1:1 ratio will be energetically favored. Prolonged incubation of unfolded holo-Mb also allows for some heme dissociation from histidine 93 since the total yield of refolding was found to decrease somewhat.

In Fig. 1B, the Met-Mb folding and unfolding rates are combined in a Chevron plot [22–24]. With respect to both folding and unfolding speed in water, the fast rates are approximately 10 times higher than the slow rates (Table 1). The apparent equilibrium stability, estimated from the ratio of the folding and unfolding rate constants, $RT\ln(k_f/k_u)$, in the case of both the fast and the slow data sets agrees closely with the value obtained from the equilibrium studies (Table 1). This observation strongly supports our conclusion of independent

Table 1
Summary of equilibrium and kinetic folding and unfolding data for horse Met-Mb

		$k(H_2O)$ (s^{-1})	ΔG_U (kJ/mol)	m (kJ/mol, M)
Equilibrium:				
	far-UV CD		22.8(±2.8)	14.1(±1.9)
	fluorescence		20.0(±3.9)	13.3(±2.6)
	Soret absorption		22.3(±2.4)	13.0(±1.4)
Kinetics:				
Fast phase (20%)	unfolding	0.00011(±0.00004)		8.1(±0.25)
	folding	0.36(±0.04)		−3.1(±0.4)
	sum[a]		19.7(±1.0)	11.2(±0.7)
Slow phase (80%)	unfolding	0.000025(±0.000007)		8.1(±0.21)
	folding	0.028(±0.003)		−3.2(±0.4)
	sum[a]		17.1(±1.0)	11.3(±0.6)

[a] $\Delta G_U = -RT\ln(k_u/k_f)$; $m_{eq} = m_u - m_f$.

two-state unfolding reactions for two Met-Mb populations; the data are not in agreement with a sequential mechanism including an intermediate. The relationship $m_{eq} = m_u - m_f$ is also satisfied for each set of data since the value $m_u - m_f$ (11.2 for fast and 11.3 kJ/mol,M for slow data) compares favorably with the equilibrium value (13–14 kJ/mol,M Table 1). The identical ratio (0.3) of kinetic and equilibrium m values (m_f/m_{eq} [25]; reporting on the transition state for folding) for both the fast and the slow folding holo-Mb populations indicates that these protein fractions still have very similar folding pathways.

In summary, our data are consistent (albeit not proven directly) with the two populations of native Met-Mb, which differ in their orientation of the heme, unfolding and refolding in parallel reactions. The unfolding and refolding rate constants differ by an approximate factor of 10 for the two protein fractions. This corresponds to an energy difference of about 6 kJ/mol, ascribed to a difference in transition state barriers since equilibrium measurements do not distinguish the two populations. Furthermore, our findings reported here emphasize the significance of specific interactions between a cofactor and its corresponding unfolded polypeptide despite the lack of covalent bonds; this has been noted only in a few other cases [26–29].

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