Folding of Deoxymyoglobin Triggered by Electron Transfer

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The met and deoxy forms of sperm whale myoglobin (Mb) can be unfolded by guanidine hydrochloride (GuHCl). Electronic absorption and circular dichroism spectroscopic measurements show that folded deoxyMb is more stable than the folded met protein. Laser excitation of NADH generates species that rapidly reduce unfolded metMb, triggering the formation of folded deoxyMb in less than 10 ms (pH 7.0, 2.5 to 3 M GuHCl, 20 °C). At comparable reaction driving forces (∼10 kJ/mol), deoxyMb folds much faster than reduced cytochrome *c*.

Introduction

When the metal ion in a metalloprotein can exist in more than one oxidation state, the reduced and oxidized proteins are expected to have different stabilities. It may then be possible to find unfolding conditions in which one form is fully unfolded, whereas the other is folded. This opens the way for triggering a folding process by electron transfer (ET), as first shown with cytochrome *c*.[1,2] Although we thought initially that the method would be restricted to proteins with covalently attached cofactors, our subsequent work showed that the technique could be employed to study the folding of cytochrome b_{562},[3] a protein in which the heme has two axial ligands but the porphyrin itself is not covalently attached.

Here we demonstrate that ET triggering also can be employed to study the folding of sperm whale myoglobin (Mb), another heme protein with a noncovalently attached porphyrin. The heme in Mb is coordinated to a single axial histidine ligand, and both oxidized (ferric or met) and reduced (ferrous or deoxy) states have a high-spin electronic configuration.[4] As in the cases of cytochrome *c* and cytochrome b_{562}, the reduced form of the protein is more stable than the oxidized form.[5] The unfolding reactions of metMb and deoxyMb can be induced by guanidine hydrochloride (GuHCl) and monitored by electronic absorption and circular dichroism (CD) spectra. Between 2.5 and 3 M GuHCl, where metMb is fully unfolded, addition of dithionite leads to the formation of folded deoxyMb. Furthermore, laser excitation of NADH generates reductants[6,7] that rapidly convert unfolded metMb to the deoxy protein, thereby allowing examination of early events in the folding process. In contrast to apoMb,[8−10] very little is known about fast folding of the holoprotein.[11,12]

Materials and Methods

Sperm whale Mb, GuHCl, $Na_2S_2O_4$, and NADH were obtained from Sigma.

Spectra were measured on a Hewlett-Packard 8452 diode array spectrophotometer (absorption, 1-cm cell) and a Jasco 600 spectropolarimeter (CD, 1-mm cell). GuHCl titrations of oxidized and reduced Mb (∼5 μM) were performed in 5 mM phosphate buffer, pH 7.0, at 20 °C. The samples of reduced protein contained a slight excess of dithionite to prevent reoxidation. Refolding of metMb in solutions with high GuHCl concentrations was initiated by dilution with buffer. For experiments at pH 5 and 6, sodium acetate buffers were used.

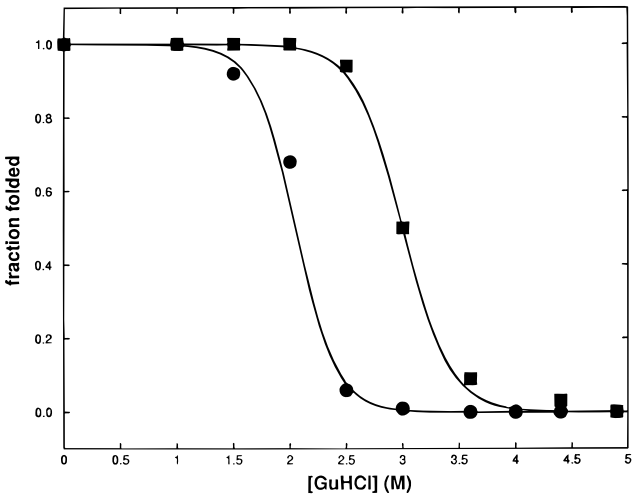

Figure 1. GuHCl-induced unfolding of metMb (●) and deoxyMb (■) at room temperature. Unfolding was monitored both by CD and Soret absorption (with virtually identical results).

Transient absorption kinetics were measured as described elsewhere.[13] Solutions (5 mM phosphate buffer, pH 7.0, 100 μM protein, 250 μM NADH, and an appropriate amount of GuHCl, 0 M or between 2.5 and 3 M) were deoxygenated by repeated cycles of evacuation and refilling with argon on a Schlenk line. Samples in a 1-mm anaerobic cell were excited with pulses from a Nd:YAG laser (355 nm, 10 ns, ∼10 mJ/pulse). Folding kinetics were monitored by absorption changes at 430, 415, and 400 nm. Transient difference spectra were recorded using a diode array detector (Princeton Instruments) with a xenon flashlamp probe (EG&G).

Results and Discussion

Unfolding curves for metMb and deoxyMb, with GuHCl as the unfolding agent, are shown in Figure 1. Identical results are obtained when following unfolding both by far-UV CD, monitoring secondary structure, and by Soret absorption,

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reflecting heme environment. MetMb unfolds reversibly and at lower GuHCl concentration than deoxyMb, which unfolds irreversibly. The folding free energy difference between the reduced and oxidized proteins ($\Delta\Delta G_f$) must correlate with the difference in reduction potentials of the folded and unfolded forms. In heme proteins, the reduction potential of the unfolded protein (~ -100 mV vs NHE) is largely determined by the exposure of the heme group to the solvent.[2] The finding that metMb unfolds more readily than deoxyMb is consistent with the 50-mV (vs NHE) potential[14] of the folded protein. Since the unfolding of deoxyMb is irreversible, we cannot obtain a reliable value of $\Delta\Delta G_f$ from the unfolding curves. The value of $\Delta\Delta G_f$ estimated from the Mb reduction potential ($\sim$15 kJ/mol) is lower than that found for cytochrome b_{562} ($\Delta\Delta G_f = 25$ kJ/mol, $E° = 180$ mV)[3] and cytochrome *c* ($\Delta\Delta G_f = 34$ kJ/mol, $E° = 260$ mV).[2] This trend may be related to the different degrees of hydrophobic encapsulation of the heme group in the three proteins.[15−17] A large $\Delta\Delta G_f$ (or high potential) corresponds to a more hydrophobic heme environment. In agreement, one axial ligand site in Mb is available to the solvent, and the heme edge is more exposed to solvent in cytochrome b_{562}[18] than in cytochrome *c*.[19] The axial protein ligands to the heme are also important in determining the potentials, myoglobin having only a His, whereas the other two proteins have a His and a Met.

Refolding of metMb was found on dilution of GuHCl solutions (from 3.5 to 1.5 M) at pH 6 and 7, but not at pH 5. At the higher pH values, it is likely that the heme is coordinated to the unfolded protein, probably via the axial histidine ligand. Above pH 6, the absorption spectrum of the unfolded protein has a Soret maximum at 400 nm; at pH 5, this peak shifts to 370 nm. The rate of refolding of metMb at pH 7 is independent of protein concentration (0.016 s^{-1} at 5, 10, and 15 μM metMb; 1.5 M GuHCl), which provides additional support for heme binding in the unfolded state. In contrast, upon dilution of a 5 M GuHCl solution of deoxyMb (to 1.5 M), folding was not observed; thus, the heme is probably not bound to the axial histidine of the unfolded deoxy protein. Between 2.5 and 3 M GuHCl, metMb, but not deoxyMb, is completely unfolded. Unfolded metMb in 2.5 M GuHCl gives folded deoxy protein upon reduction with dithionite (Figure 2A, solid line). We found 20−25% folded deoxyMb in 3 M GuHCl solution, much less than the maximum yield of 50% (Figure 1). Apparently, upon reduction of unfolded metMb, heme dissociation competes with the folding of the deoxy protein.

Laser excitation of NADH generates species[6,7] that reduce metMb; the pseudo first-order rate constant for this reduction was found to be 2.4(6) $\times 10^4$ s^{-1} (100 μM protein). Reduction of the unfolded protein is expected to be even faster, since the heme is relatively exposed. Reduction of unfolded metMb to the unfolded deoxy protein does not result in large absorption changes (Figure 2B, dashed line), whereas the difference spectrum associated with the conversion of unfolded to folded deoxyMb is quite distinctive (Figure 2A, solid line). The difference spectrum obtained from measurements on samples before and after 355-nm excitation of NADH agrees with the steady-state difference spectrum between unfolded metMb and folded deoxyMb (Figure 2A, dashed line), confirming that folding occurs.[20] Moreover, the difference spectrum recorded 10 ms after excitation demonstrates the formation of folded deoxyMb (Figure 2B, solid line). The kinetics of deoxyMb folding (2.5 M GuHCl) are independent of protein concentration; the rate constant is 5(2) $\times 10^3$ s^{-1}.

The millisecond time scale for deoxyMb folding contrasts with the much slower folding of reduced cytochrome *c* at a comparable driving force.[2] Heme misligation is believed to inhibit folding of the latter protein.[6] Reduced cytochrome b_{562} also folds rapidly ($\sim$ms), but at a much higher driving force ($\sim$25 kJ/mol).[3] Now that we have shown that deoxyMb folding can be triggered by electron transfer, more detailed studies of the steps leading to the formation of the folded protein can be made. Monitoring the folding by fluorescence and CD may reveal events that occur before or after the reaction we have followed by Soret absorption.

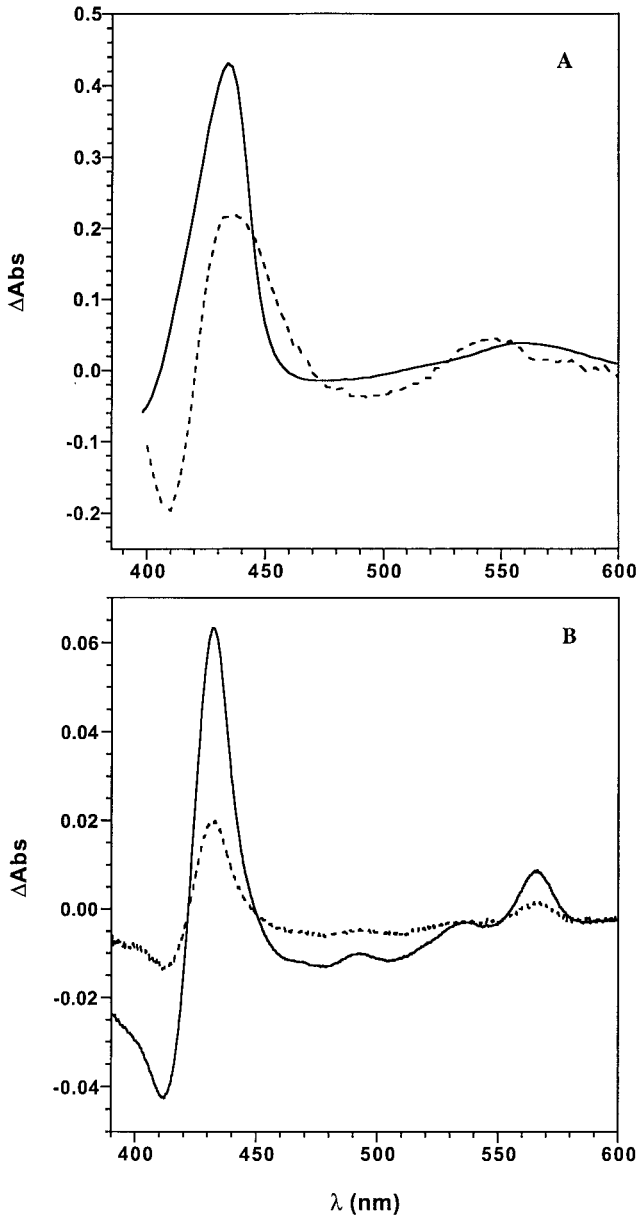

Figure 2. Difference absorption spectrum between folded deoxyMb and unfolded metMb determined from steady-state measurements (solid line), and differences in steady-state absorption spectra before and after photoinduced (355-nm excitation of NADH) electron injection into a sample of unfolded metMb in 2.5 M GuHCl (dashed line) (**A**). Difference spectra recorded 100 μs (dashed line) and 10 ms (solid line) after laser excitation; no further changes in absorption were observed after 10 ms (2.5 M GuHCl) (**B**).

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(20) Minor differences between steady-state and time-resolved spectra are due to the presence of dithionite used as a reductant, and to the fact that, upon photochemical reduction, only 20−25% of the protein refolds.