Rapid Formation of a Four-Helix Bundle. Cytochrome b_{562} Folding Triggered by Electron Transfer

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Understanding how the secondary and tertiary structures of proteins are formed from non-native conformations is a continuing challenge for both theory[1] and experiment.[2] This complex process involves dynamics on time scales that range from picoseconds to minutes. We have shown previously that the folding of redox-active proteins can be triggered by electron transfer (ET), thereby opening the way for investigations of early events in the folding process.[3] Since it is important that the redox-active cofactor remains bound to the unfolded protein (eliminating the possibility that the rate-limiting step will be bimolecular capture of the cofactor), we initially thought that only redox proteins with covalently attached cofactors would be amenable to study by this method. Indeed, thus far we have restricted our work on folding dynamics to ferrocytochrome *c*, a protein with a covalently attached heme.

Here we show that ET triggering can be employed to study the folding of a four-helix-bundle protein, cytochrome b_{562}. Although the porphyrin is not covalently attached to the protein, the heme iron is ligated axially by the side chains of Met7 and His102.[4] It is likely that one of these ligands remains attached to the heme in the unfolded state of the oxidized protein, since electron transfer to the ferric center leads to rapid formation of the native, four-helix-bundle structure of ferrocytochrome b_{562}.

Titrations with guanidine hydrochloride (GuHCl) confirm that reduced cytochrome b_{562} is more stable toward unfolding than the oxidized protein.[5] Monitoring the unfolding by circular dichroism (reporting on secondary structure) and Soret-band absorbance (reporting on heme environment) gave identical results, consistent with a two-state process. Soret absorption spectra of the unfolded proteins indicate that the heme iron is high-spin in both oxidation states.[6] Oxidized cytochrome b_{562} is fully denatured at 2 M GuHCl, whereas reduced cytochrome b_{562} does not unfold below 6 M GuHCl (Figure 1). The folding free energies for oxidized and reduced proteins depend linearly on [GuHCl] (Figure 1), and extrapolating to [GuHCl] = 0 gives values for the free energy of folding in aqueous solution.[7] The observed difference in folding free energies between oxidized and reduced cytochrome b_{562} ($\Delta\Delta G_f$ = 25(12) kJ/mol ≈0.25-(12) eV) is in reasonable agreement with the difference in reduction potentials (folded, 0.18;[8] unfolded, −0.15 V[3,9] vs NHE). Refolding experiments by dilution were performed for both oxidized and reduced proteins.[10] Reduced, unfolded cytochrome b_{562} does not refold upon dilution of GuHCl. By contrast, oxidized protein refolds at various GuHCl concentrations, and the refolding kinetics show no protein-concentration dependence, indicating that the heme is still associated with the protein in the unfolded state.

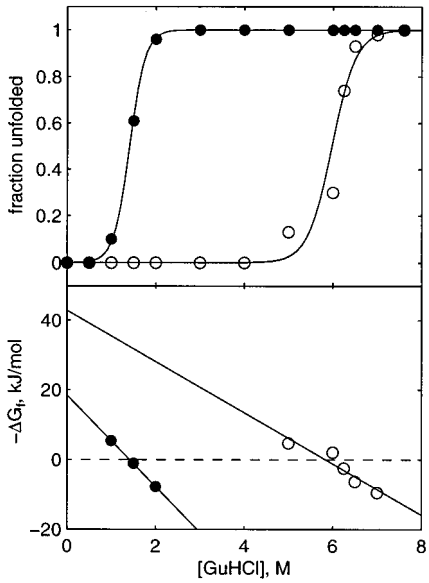

Figure 1. (upper) Folding titration curves for oxidized (Fe^{III}, closed circles) and reduced (Fe^{II}, open circles) cytochrome b_{562}, with GuHCl as a denaturant (pH 7.0, 20 °C). (lower) Dependence of folding free energies (ΔG_f) on [GuHCl] for oxidized and reduced cytochrome b_{562} (pH 7.0, 20 °C).

Oxidized cytochrome b_{562} is completely unfolded in GuHCl solutions between 2 and 6 M, whereas the reduced form is fully folded. Preliminary experiments in which dithionite was added to unfolded, oxidized cytochrome b_{562} showed that the protein does indeed fold upon reduction; however, the yield of folded protein is strongly dependent on [GuHCl].[11] Upon increasing [GuHCl], the amount of folded protein produced from unfolded, oxidized protein decreases, showing that heme dissociation

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(5) Plasmid (pNS207) containing the cytochrome b_{562} gene was provided by S. Sligar, University of Illinois. The plasmid was inserted in a BL21 strain of *Escherichia coli* by N. Farrow, Caltech. The protein was purified by anion exchange chromatography. GuHCl titrations were performed (5 mM phosphate buffer, pH 7.0, 20 °C) with oxidized and reduced cytochrome b_{562} by following both the far-UV circular dichroism signal (Jasco 600 instrument, 1 mm cell) and Soret absorbance (Hewlett-Packard 8452 diode array spectrophotometer). Slight excess of sodium dithionite was used to keep the protein reduced.

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(7) The folding free energies extrapolated to [GuHCl] = 0 M are the following: $-\Delta G^\circ_{ox} = 18 \pm 2$ kJ/mol; $-\Delta G^\circ_{red} = 43 \pm 10$ kJ/mol.

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(10) Protein (oxidized or reduced) was unfolded in GuHCl. The sample was subsequently diluted with buffer to a GuHCl concentration favoring folding, and the Soret absorption was studied with time.

(11) Adding dithionite to unfolded, oxidized protein in 2 M GuHCl yielded about 75% folded, reduced protein. The same experiment in 2.5 M GuHCl produced only ∼30% folded, reduced protein (measurements performed on a 10−20 min time scale). Similar results were obtained in photochemical electron-transfer triggering experiments with oxidized, unfolded protein at various GuHCl concentrations.

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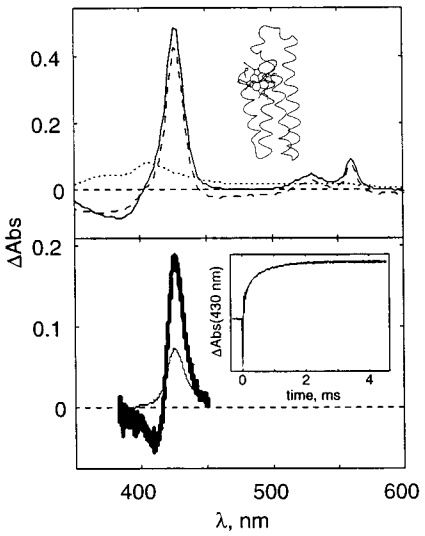

Figure 2. (upper) Difference absorption spectra (determined from equilibrium measurements of the various species) between reduced, unfolded protein and oxidized, unfolded protein (dotted line) and between reduced, folded protein and oxidized, unfolded protein (solid line). Difference absorption spectrum of cytochrome b_{562} measured before and after photochemical electron injection (dashed line). (inset) Backbone structure of cytochrome b_{562}. (lower) Transient absorption spectra recorded 200 μs (thin line) and 2 ms (thick line) after electron injection into a sample of unfolded, oxidized cytochrome b_{562} in 2.25 M GuHCl. Spectra taken after 2 ms (up to 400 ms) show no further change in absorption. (inset) Kinetics trace measured at 430 nm after electron injection (2.25 M GuHCl).

competes with the folding process. Between 2 and 3 M GuHCl, however, it is possible to obtain a significant amount of folded, reduced protein.

Laser excitation of NADH generates reductants that can inject electrons into oxidized cytochrome b_{562} in a few microseconds.[12,13] The difference spectrum obtained by absorbance measurements on samples before and after photochemical electron injection agrees closely with the calculated difference spectrum for conversion of unfolded, oxidized protein to folded, reduced protein (Figure 2). Far-UV CD spectra confirm that the reduced protein adopts its native fold. The transient difference spectrum measured 200 μs after laser excitation (Figure 2) is consistent with that of a high-spin Fe^{II} heme.[6] The spectrum measured 2 ms after excitation indicates the formation of a low-spin Fe^{II} heme and closely matches that expected for folded ferrocytochrome b_{562} (Figure 2). The ferrocytochrome b_{562} folding kinetics can be described by a dominant kinetic phase with a first-order rate constant of 800 $\pm$ 200 s^{-1} at a driving force of 25 kJ/mol (2.5 M GuHCl).[14] At a similar driving force, reduced cytochrome c folds much more slowly (10 s^{-1}).[3] On the other hand, the non-heme four-helix-bundle acyl-coenzyme A binding protein (ACBP) folding kinetics[15] are similar to those of ferrocytochrome b_{562}.

A key prediction of energy-landscape theory is that symmetrical structures will fold faster than nonsymmetrical structures, owing to smoother landscapes.[16] In cytochrome b_{562}, the tertiary fold can be approximated as a symmetric bundle of four cylinders. In oxidized, unfolded cytochrome b_{562}, the high-spin heme is probably attached to the protein through the His102 axial ligand.[17] Upon reduction of the heme, the polypeptide α-helices cluster around the heme, and the methionine sulfur bonds to iron, yielding the final low-spin complex. Our results demonstrate that ferrocytochrome b_{562} folding can compete with heme dissociation. It is not yet clear whether the folding process involves an initial collapse to a compact denatured state followed by rearrangement to the final folded form. On this point, it is of interest to note that the folding of ACBP proceeds on a comparable time scale (<5 ms at room temperature) and there is no evidence for the involvement of any intermediates on the folding pathway.[15]

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(13) The pseudo-first-order rate constant for reduction of fully folded cytochrome b_{562} was found to be (2.2 $\pm$ 0.5) $\times$ 10^4 s^{-1} ([protein] = 100 μM); reduction of unfolded protein is expected to be even faster. The transient absorption kinetics were measured using an apparatus described in Stowell, M. B. H.; Larsen, R. W.; Winkler, J. R.; Rees, D. C.; Chan, S. I. *J. Phys. Chem.* **1993**, *97*, 3054−3057. The samples were deoxygenated by repeated evacuation/fill cycles on a Schlenk line. We used 5 mM phosphate buffer, pH 7.0, 100 μM protein, 250 μM NADH, and an appropriate amount of GuHCl. Samples were excited with pulses from a Nd:YAG laser (355 nm, 10 ns). Folding kinetics were monitored at several wavelengths in the Soret region. Full transient absorption spectra were measured at time points between 35 μs and 400 μs after electron injection using a diode array detector.

(14) The observed folding rate does not depend on protein concentration in the range of 100−300 μM.

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(17) There is only one additional histidine in cytochrome b_{562}; we have measured the ET-triggered folding rate of a mutant lacking this histidine (His63Glu) and found that it is about the same as that of the native protein.