Early events in the folding of four-helix-bundle heme proteins

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Topologically homologous four-helix-bundle heme proteins exhibit striking diversity in their refolding kinetics. Cytochrome b₅₆₂ has been reported to fold on a submillisecond time scale, whereas cytochrome c’ refolding requires 10 s or more to complete. Heme dissociation in cytochrome b₅₆₂ interferes with studies of folding kinetics, so a variant of cytochrome b₅₆₂ (cytochrome c-b₅₆₂) with a catalytic c-type linkage to the heme has been expressed in *Escherichia coli*. Early events in the electron transfer-triggered folding of Fe²⁺-cytochrome c-b₅₆₂, along with those of Fe²⁺-cytochrome c₅₅₆, have been examined by using time-resolved absorption spectroscopy. Coordination of S(Met) to Fe II occurs within 10 μs to seconds. Clearly, topology alone does not dictate folding kinetics, so a variant of cytochrome *b*-cytochrome (1). With the increasing number of structures available, it has become clear that polypeptide sequences with little or no homology can assume nearly identical three-dimensional backbone architectures. Theoretical models suggest (2, 4–7), and most experimental studies confirm (5–7), that a helical bundle is a fast folding structural motif. The presence of heme cofactors, however, can introduce new features into the helical bundle energy landscape that can greatly alter refolding pathways.

We have reported previously on the folding kinetics of two four-helix-bundle heme proteins, cytochrome b₅₆₂ (cyt b₅₆₂) and cytochrome c’ (cyt c’). Although the two cytochromes have nearly identical three-dimensional structures (3.4 Å rms deviation of backbone atoms), they have very low sequence identity (15%) and exhibit quite disparate folding kinetics (9–11). Fe⁶⁺-cyt b₅₆₂ folds in less than a millisecond, whereas Fe⁵⁺-cyt c’ folding is quite heterogeneous, spanning time scales from milliseconds to seconds. Clearly, topology alone does not dictate these refolding rates.

The folding of cyt b₅₆₂ is complicated by heme dissociation from the polypeptide, limiting the refolding yield (11). We suggested that heme dissociation could be responsible for the fast folding observed in cyt b₅₆₂ by selecting against slower-folding populations in the unfolded ensemble. Indeed, a recent investigation of cyt b₅₆₂ suggests that the heme in the denatured protein is bound to a native-like polypeptide conformation that is predisposed to fold rapidly (12).

To circumvent complications arising from heme dissociation, we have engineered a variant of *E. coli* cyt b₅₆₂ (cyt c-b₅₆₂) in which two thioether linkages bind the porphyrin to the polypeptide chain in the fashion of a c-type cytochrome (13–15). We also have investigated cytochrome c₅₅₆ from *Rhodopseudomonas palustris* (16), a protein with the same four-helix-bundle fold as cyt b₅₆₂ and cyt c’ (backbone atom rms deviations vs. cyt b₅₆₂, 3.3 Å; vs. cyt c’, 1.5 Å) (17–20) but with low sequence identity (cyt b₅₆₂, 21%; cyt c’, 34%). Here, we report early events in folding the Fe II forms of c-b₅₆₂ and c₅₅₆.

Materials and Methods

Guanidine hydrochloride (GuHCl, Sigma, ultrapure grade), tris(2,2’-bipyridine)ruthenium(II) chloride ([Ru(bpy)₃]Cl₂, Strem), and NADH (Sigma) were used as received.

*R. palustris* cyt c₅₅₆ was expressed and purified by published procedures with minor modifications (16). The N-terminal glutamine was fully cyclized by heating the protein at 50°C for 5 h in 0.5 M KH₂PO₄ (21). *E. coli* cyt c-b₅₆₂ was expressed by cotransforming the construct pETcb₅₆₂ (unpublished procedure) with pEC86 (22) into *E. coli* strain BL21 (DE3). Cyt c-b₅₆₂ was purified by ion exchange chromatography on CM Sepharose Fast Flow and followed by a second purification step on a Mono S column (FPLC, Amersham Pharmacia). Proteins were judged to be pure by SDS/PAGE (PhastSystem, Amersham Pharmacia) and electrospray ionization–MS analysis (Caltech Protein/Peptide Microanalytical Laboratory).

Circular dichroism (CD) spectra were recorded by using an Aviv 62ADS spectropolarimeter. Trp fluorescence spectra were recorded on a Jobin Yvon SPEX Fluorolog-3 spectrometer (∆λ₂₃ = 290 nm; ∆λ₃₄ = 300–500 nm). Steady-state absorption spectra were recorded on a Hewlett-Packard HP-8453 or HP-8452 diode array spectrometer. Protein (cyt c-b₅₆₂) concentrations were determined by using the extinction coefficients reported for cyt b₅₆₂ (23, 24).

Transient absorption kinetics measurements were made as described in refs. 11 and 25. Folding measurements were performed with solutions buffered to pH 7 with cyt c₅₅₆ and pH 5 with cyt c-b₅₆₂ to inhibit misligation of His-63. Samples for folding kinetics measurements [cyt c₅₅₆ or cyt c-b₅₆₂ (100 μM); Ru(bpy)₃]Cl₂ (50–150 μM) or NADH (200 μM); GuHCl (1–6 M)] were sealed in 1-mm cuvettes and deoxygenated by repeated evacuation/Ar or CO backfill cycles. GuHCl concentrations were determined after laser experiments from refractive index measurements (Abbe-3L refractometer, Millon Ray, Rochester, NY) (26).

Results and Discussion

Equilibrium Unfolding. Cyt c₅₅₆ and cyt c-b₅₆₂ are soluble proteins (molecular masses of 14.7 and 12.3 kDa, respectively). Each folded structure contains four antiparallel α-helices in a left-handed bundle with a heme group located in a hydrophobic pocket near the C and N termini; the porphyrin is covalently bound to the polypeptide chain through thioether linkages with two C-terminal cysteine residues, and the iron center is axially ligated by Met-12 and His-121 (cyt c₅₅₆) or Met-7 and His-102 (cyt c-b₅₆₂) (18). GuHCl titrations, monitored by absorbance, CD, and tryptophan fluorescence spectra, reveal cooperative

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**Abbreviation:** cyt, cytochrome.

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unfolding transitions (Fig. 1), and, as expected on the basis of their high reduction potentials, the Fe\textsuperscript{III} forms are less stable than the Fe\textsuperscript{II} proteins (27, 28). It is interesting to note that the introduction of two thioether linkages to the heme in cyt\textsubscript{c-b\textsubscript{562}} leads to a substantial stabilization of the folded protein (the denaturation midpoint, [GuHCl]\textsubscript{1/2}, increases by \approx 2 M).

The absorption spectra of native and denatured Fe\textsuperscript{III}/II-cyt\textsubscript{c\textsubscript{556}} and Fe\textsuperscript{III}/II-cyt\textsubscript{c-b\textsubscript{562}} (pH 5) are shown in Fig. 2. In both Fe\textsuperscript{III} and Fe\textsuperscript{II} oxidation states, the heme becomes exposed to the solvent upon unfolding, leading to distinct shifts in absorption spectra, including variations in extinction coefficients, which can be exploited to monitor folding kinetics. The blue shift in the Soret absorption upon denaturation of each Fe\textsuperscript{III} protein indicates that the heme has undergone a low- to high-spin transition, likely the result of replacement of the axial methionine ligand with a water molecule. In denatured Fe\textsuperscript{II} forms, however, both Soret and Q-band absorptions suggest that the heme remains low-spin. In contrast to the behavior of unfolded cytochrome c, it is not likely that nonnative His coordination accounts for the low-spin hemes in denatured Fe\textsuperscript{II}-cyt\textsubscript{c\textsubscript{556}} and Fe\textsuperscript{II}-cyt\textsubscript{c-b\textsubscript{562}}. Cyt\textsubscript{c\textsubscript{556}} has no available His residues, and denaturation of Fe\textsuperscript{II}-cyt\textsubscript{c-b\textsubscript{562}} was performed at pH 5 to inhibit His-63 misligation. As was suggested for unfolded Fe\textsuperscript{II}-cyt\textsubscript{c}, methionine residues apparently compete for the sixth Fe\textsuperscript{II} coordination site in the unfolded proteins. In Fe\textsuperscript{II}-cyt\textsubscript{c-b\textsubscript{562}}, both Met-7 and Met-58 are likely to be involved; Met-12, Met-19, and Met-20, and possibly Met-110, are the candidates in Fe\textsuperscript{II}-cyt\textsubscript{c\textsubscript{556}}. Analogous behavior was found in denatured \textit{R. palustris} Fe\textsuperscript{II}-cyt\textsubscript{c} where Met-15 and Met-25 are potential ligands (9, 10). Although the absorption spectra of denatured Fe\textsuperscript{II}-cyt\textsubscript{c\textsubscript{556}} and Fe\textsuperscript{II}-cyt\textsubscript{c-b\textsubscript{562}} resemble those of the folded proteins, CD spectra clearly indicate that the native helical secondary structures are substantially disrupted (12).

**Electron Transfer-Triggered Refolding.** At suitable denaturant concentrations (cyt\textsubscript{c\textsubscript{556}}, 1.5–2.5 M; cyt\textsubscript{c-b\textsubscript{562}} 3.8–4.8 M), electron injection into the denatured Fe\textsuperscript{III} protein will initiate folding of the Fe\textsuperscript{II} form (27). In prior work, we have demonstrated that Ru(bpy)\textsubscript{3}\textsuperscript{2+} and NADH are useful sensitizers for photochemical triggering of Fe\textsuperscript{II}-cytochrome refolding (27, 29). Electronically excited Ru(bpy)\textsubscript{3}\textsuperscript{2+} \left[ E^\circ(Ru^{3+/2+}) = -0.85 \text{ V vs. normal hydrogen electrode} \right] injects an electron into the unfolded protein within a few microseconds. Subsequent charge recombination regenerates the initial reagent in a few milliseconds, allowing extensive signal averaging but limiting the observable window for folding to \approx 1 ms. Two-photon excitation (355 nm) of NADH produces two reductants (\textit{e}_\text{aq} and NAD) that irreversibly reduce the heme group in \approx 100 \mu s (\approx 100 \mu M protein). Therefore, with NADH it is possible to expand the observation time window to seconds and longer.

We have used both Ru(bpy)\textsubscript{3}\textsuperscript{2+} and NADH to trigger Fe\textsuperscript{II}-cyt\textsubscript{c\textsubscript{556}} and Fe\textsuperscript{II}-cyt\textsubscript{c-b\textsubscript{562}} refolding. In experiments with \textit{Ru(bpy)\textsubscript{3}\textsuperscript{2+}} as the photoreductant, the observed transient absorption kinetics depend on denaturant concentration. These
data are adequately described by a biexponential function. The faster rate is independent of [GuHCl] and corresponds to decay of *Ru(bpy)₃*²⁺ (*k₁ = 1.6 × 10⁸ s⁻¹*) with parallel reduction of the Fe³⁺ protein. The rate constant for the slower phase varies with [GuHCl]; this reaction channel represents early events in the folding of the protein around the heme (Fe-cyt c₅₅₆, *k₂ = 4 × 10⁵ to 8 × 10⁵ s⁻¹*; [GuHCl] = 3–2 M; Fe-cyt c-b₅₆₂, *k₂ = 5 × 10⁸ to 2 × 10⁹ s⁻¹; [GuHCl] = 5.5–4.5 M). The transient spectra measured at the end of the slower phase (t = 100 μs) are consistent with the formation of reduced folded protein.

Biexponential kinetics also are observed when NADH is used as the photoreductant, with rate constants independent of [GuHCl] that are attributable to the reduction of the protein by *e₄₅ (4–5 × 10⁸ s⁻¹*) and NADH (*9 × 10⁸ s⁻¹*). The relative signal amplitudes observed after excitation of samples at low and high [GuHCl] (Fe-cyt c₅₅₆, 1.5 and 4 M; Fe-cyt c-b₅₆₂, 4 and 7 M) suggest that folded reduced protein is formed 300 μs after excitation (Fig. 3). No additional changes in absorption were detected on time scales as long as several seconds; the formation of folded reduced protein appears to be limited by the rate of reduction by NAD. Moreover, steady-state UV-visible and CD spectra recorded after laser excitation of NADH-containing samples confirm that the photochemically reduced proteins adopt native folds.

The transient absorption data suggest that Fe⁴⁺-c₅₅₆ and Fe⁴⁺-c-b₅₆₂ refolding rates are faster than 10⁴ s⁻¹. UV-visible spectra provide information about the immediate environment of the heme cofactor but do not directly report on the conformation of the polypeptide. To gain more insight into the submillisecond events, we examined the kinetics of CO rebinding to the heme after photoinitiated refolding experiments in the presence of CO. The deeply buried iron centers of native c-b₅₆₂ and c₅₅₆ are six-coordinate and therefore not available for CO binding. Under denaturing conditions, the ferroheme remains low-spin, likely due to Met ligation in the sixth coordination site. Nevertheless, CO will replace Met as an Fe²⁺ ligand in denatured c-b₅₆₂ and c₅₅₆. We have examined the kinetics of CO rebinding to the heme after photodissociation from denatured Fe²⁺-c₅₅₆ ([GuHCl] = 7 M) and Fe²⁺-c-b₅₆₂ ([GuHCl] = 4 M). Under 1 atm (1 atm = 101.3 kPa) of CO, the rate constant for CO rebinding to the Fe²⁺ heme is ~65 s⁻¹ and we have used CO ligation to the heme as a probe of the extent of heme protection afforded by the polypeptide during electron-transfer-triggered refolding. If the polypeptide wraps around the heme rapidly (>10⁵ s⁻¹), little CO should bind to the heme. On the other hand, if folding is slower than ~10² s⁻¹, substantial CO binding is expected. We find that at 7 M GuHCl, where formation of native, reduced c-b₅₆₂ is disfavored, there is a major change in absorbance consistent with CO binding after reduction of the Fe³⁺ protein. In contrast, very little CO binding is apparent under conditions favoring formation of the folded Fe⁴⁺ protein (4 M GuHCl) (Fig. 4).

Our electron transfer-triggered experiments reveal that early events (t < 100 μs) in Fe⁴⁺-c₅₅₆ and Fe⁴⁺-c-b₅₆₂ refolding involve formation of a low-spin heme and some degree of heme encapsulation by the polypeptide. A lower limit to the time required for the heme to ligate a Met residue can be estimated from studies of tertiary contact dynamics in unfolded proteins and peptides (31–33). Met ligation to the heme in Fe⁴⁺-c₅₅₆ will produce polypeptide loops comprised of 96, 97, and 104 residues, and loop sizes of 39 and 90 residues in Fe⁴⁺-c-b₅₆₂ are possible. Energy-transfer quenching studies in synthetic polypeptides suggest that tertiary contact rate constants (*k₉*) for 90- to 100-residue loops are 10⁴ to 10⁷ s⁻¹ (32). These values compare with our observed rates of 10⁴ to 10⁸ s⁻¹ for formation of native heme absorption spectra in electron transfer-triggered Fe⁴⁺-c₅₅₆ and Fe⁴⁺-c-b₅₆₂ refolding experiments. The variation of these rate constants with [GuHCl] concentration is likely due in part to the viscosity dependence of the intrachain diffusion dynamics that lead to tertiary contacts. In synthetic peptides, the logarithms of tertiary contact rates exhibit a linear dependence on [GuHCl] with *m₉* values (*m₉ = −RT ln(*k₉*))/[θ/GuHCl] ≈ 0.5 (kJ/mol)/M (34) that are substantially smaller than the *m₉* values (*m₉ = −RT ln(*k₉*))/[θ/GuHCl] found for the early kinetics phases of Fe⁴⁺-c₅₅₆ (~2.5 (kJ/mol)/M) and Fe⁴⁺-c-b₅₆₂ (~2.1 (kJ/mol)/M) refolding (Fig. 5). Fast-folding
These denaturant dependences observed for FeII-cyt suggest the early kinetics phases involve more than intrachain diffusion leading to Met-Fe ligation.

Rapid Met-Fe ligation could facilitate refolding of FeII-cyt by forming one or more strong native tertiary contacts that substantially reduce the size of the conformational space available to the polypeptide (35). Constraining the folding energy landscape in this manner could lead to a substantial reduction in the time required to find the native structure (36).

After reduction of the unfolded oxidized proteins, CO will bind to the ferroheme under solution conditions where formation of native structure is disfavored. The observed rate constant for CO binding to denatured FeII-cyt c556 and FeII-cyt c-b562 refolding suggests that the early kinetics phases are characterized by more intrachain diffusion leading to Met-Fe ligation. Rapid Met-Fe ligation could facilitate refolding of FeII-cyt c556 and FeII-cyt c-b562, because formation of one or more strong native tertiary contacts will substantially reduce the size of the conformational space available to the polypeptide (35). Constraining the folding energy landscape in this manner could lead to a substantial reduction in the time required to find the native structure (36).

Our finding that CO does not bind to the FeII-cyt c556 and FeII-cyt c-b562 hemes under GuHCl conditions favoring folding points to encapsulation of the heme by the polypeptide in <10 ms. The spectroscopic data do not reveal whether the polypeptide has developed secondary and tertiary structure by this time. Ultrafast mixing measurements on a F65W mutant of apo-cyt b562, which adopts a three-helix-bundle fold (19, 38), are consistent with a refolding rate constant of 2,600 s−1 in the absence of denaturant (12). This value represents a reasonable upper limit to the folding rate for the holoprotein and is in line with the lower limit indicated by our CO ligation measurements.

The refolding of FeII-cyt c556 and FeII-cyt c-b562 clearly begins from an extensively denatured state. This finding contrasts with recent results on FeII-cyt b562 (12) where, as we had suggested earlier (11), heme dissociation preselects fast-folding members of the denatured ensemble. The only events revealed by changes in heme absorption spectra in FeII-cyt c556 and FeII-cyt c-b562 occur on very early time scales (1–10 μs) and involve Met-Fe ligation processes. The absence of separate kinetics phases attributable to heme encapsulation by the polypeptide, and the observation that the heme is protected from CO binding, confirm that a substantial degree of refolding occurs on submillisecond time scales. This behavior contrasts sharply with results from FeII-cyt c’ experiments in which changes in heme spectra were observed on time scales from 10−6 s to 103 s after reduction of the unfolded oxidized protein. We suggest that formation of one persistent native contact in the early stages of FeII-cyt c556 and FeII-cyt c-b562 refolding puts each polypeptide on a fast track to its native structure (36). The slower and more complex refolding kinetics found for FeII-cyt c’ may be a consequence of weaker S(Met)-Fe bonding in the denatured protein.

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