J|A|C|S COMMUNICATIONS

Published on Web 04/10/2009

Direct Observation of Ligand Dynamics in Cytochrome *c*

Megan C. Thielges, Jörg Zimmermann, and Floyd E. Romesberg*

Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received December 31, 2008; E-mail: floyd@scripps.edu

Horse heart cytochrome *c* (cyt *c*), which uses a covalently bound heme to shuttle electrons between cyt *c* oxidase and reductase, has emerged as a paradigm for the study of protein folding, in large part because its covalently bound heme facilitates characterization. Moreover, the reduced protein may be destabilized with denaturant such that it is unfolded in the presence of CO, which coordinates the heme iron along with His18, but folded in the absence of CO, where Met80 and His18 act as ligands.[1] Thus, under these conditions, changes in the heme absorption following CO photodissociation have been interpreted in terms of protein folding, which progresses until CO rebinding returns the protein to the unfolded state. Following a nanosecond light pulse, four transitions with time constants of approximately 1−5, 50−100, 200−500, and 1000−10000 μs have been resolved.[2] The two shorter time constants have been attributed to ligation at the heme center by Met65 or Met80 and His33 or His26,[2] respectively, and correspondingly are perturbed by modification of the corresponding residues and insensitive to the concentration of CO in solution.[2] These time constants have been used to estimate the interchain diffusion rates of the protein. The two longer time constants are sensitive to CO concentration and were originally attributed to the rebinding of CO to two different partially folded states of cyt *c*.[1,2b] However, Arcovito et al.[3] also observed the longer time scale processes after photodissociation of CO from *fully* unfolded cyt *c* as well as from free heme and imidazole. These authors convincingly argued that these time constants should be interpreted via the base elimination mechanism, in which CO dissociation results in the rapid dissociation of the proximal base (histidine or imidazole) and that the 200−500 and 1000−10000 μs time constants correspond to CO and base rebinding, respectively. Thus, the two longer time constants in fact do not report on protein folding but instead reflect the complexity of heme ligation. These experiments highlight the caution required when interpreting spectral changes associated with probes having inherently low structural resolution.

One ultimately high-resolution probe is the CO ligand itself. While it does not directly report on the structural changes of the protein, the time dependence of the CO absorption should clearly distinguish between the post-photolysis events that involve CO rebinding, and possibly subsequent changes at the proximal coordination site, from those involving the protein. Here, to unambiguously determine the post-photodissociation steps involving CO, we monitored the CO vibration following photodissociation with step-scan FT-IR spectroscopy.

We first characterized the steady-state FT-IR absorption spectrum of CO bound to reduced cyt *c* as a function of GdnHCl (Figure 1A). At low GdnHCl concentrations (≤2 M), where cyt *c* is fully folded, the absorption band was well-fit by a single Gaussian function at 1963 cm^{-1} with a line width of 24 cm^{-1}.[4] As the GdnHCl concentration was increased, the absorption band was red-shifted, and a high-frequency shoulder appeared.[4b] Correspondingly, two Gaussian functions were required in order to fit the spectra, one for the major absorption between 1958 and 1955 cm^{-1} with a line width of ∼24 cm^{-1} and the other for the minor absorption at ∼1975 cm^{-1} with a line width of ∼17 cm^{-1} [see the Supporting Information (SI)]. The observed transition midpoint of 3.0 M (see the SI) was lower than that monitored by UV−vis[1] because the folded protein is not saturated with CO whereas the unfolded protein is, resulting in an apparent shift of the transition. Importantly, the presence of two distinct CO absorptions reveals the presence of at least two unique CO microenvironments, perhaps differentiated by hydrogen-bonding interactions, as suggested previously.[4c]

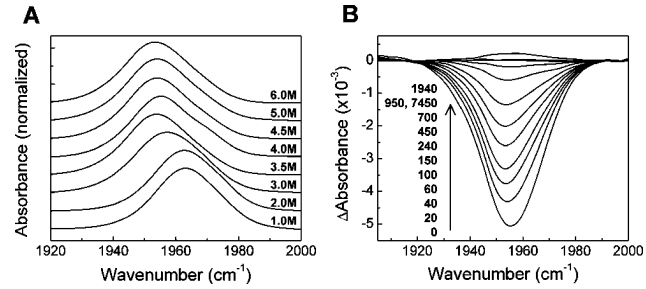

Figure 1. (A) IR absorption spectra of CO bound to cyt *c* at different GdnHCl concentrations. (B) Absorption changes for different delay times at 6 M GdnHCl (times in μs).

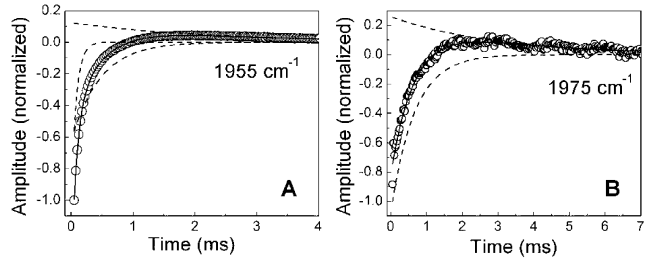

Figure 2. Time-dependent absorption decays at (A) 1955 cm^{-1} and (B) 1975 cm^{-1} at 6 M GdnHCl. Dashed lines represent the multiexponential fits.

We next used step-scan FT-IR spectroscopy to monitor the spectral changes of the CO ligand as a function of GdnHCl on the micro-to-millisecond time scale following photodissociation with a ∼10 ns pulse of the 532 nm second harmonic of a Nd:YAG laser (see the SI). Immediately following the laser pulse, a bleaching of the signal was clearly apparent for GdnHCl concentrations above 3.0 M (Figure 1B); no signal was observed at lower denaturant concentrations, likely because rebinding to the folded protein was slower than the repetition rate of the laser.[5] Interestingly, the bleached signal did not simply decay to zero but instead changed with increasing delay time to a positive transient absorption band, which reached a maximum at ∼2 ms and then fully decayed within ∼7 ms. At each time point, the observed transient absorption spectra were, within error, adequately fit by two Gaussians centered at 1955 and 1973 cm^{-1}, which represent the bleaching of the steady-state absorption spectrum. The time dependence

10.1021/ja810155s CCC: $40.75

Table 1. Parameters from Multiexponential Fits to the Decays of the 1955 and 1975 cm^{-1} Bands

	1955 cm^{-1}						1975 cm^{-1}			
[GdnHCl] (M)	A_1	T_1 (μs)	A_2	T_2 (μs)	A_3	T_3 (ms)	A_1	T_1 (μs)	A_2	T_2 (ms)
6.0	-0.50 ± 0.07	80 ± 14	-0.50 ± 0.07	460 ± 62	0.11 ± 0.05	2.5 ± 0.9	-1	760 ± 170	0.40 ± 0.18	3.1 ± 1.6
5.0	-0.49 ± 0.02	110 ± 21	-0.51 ± 0.02	570 ± 73	0.17 ± 0.03	2.1 ± 0.6	-1	980 ± 340	0.52 ± 0.11	2.9 ± 1.0
4.5	-0.49 ± 0.03	98 ± 9	-0.51 ± 0.03	530 ± 54	0.11 ± 0.02	2.8 ± 0.3	-1	610 ± 23	0.41 ± 0.03	5.4 ± 1.6
4.0	-0.45 ± 0.04	120 ± 13	-0.55 ± 0.04	640 ± 110	0.21 ± 0.13	2.1 ± 0.6	-1	590 ± 60	0.22 ± 0.09	4.9 ± 1.6
3.5	-0.46 ± 0.07	120 ± 37	-0.53 ± 0.07	530 ± 54	0.15 ± 0.05	2.3 ± 0.8	-1	510 ± 80	0.17 ± 0.07	3.8 ± 2.1
3.0	-0.24 ± 0.10	180 ± 32	-0.76 ± 0.10	670 ± 110	0.32 ± 0.14	1.6 ± 0.3	-1	640 ± 20	0.2 ± 0.07	3.2 ± 0.9

of the amplitudes of these two absorptions were fit to multiexponential decays (Figure 2 and Table 1), and three time components were found for the major absorption at ~1955 cm^{-1}: two decays with negative amplitudes (bleaching) and time constants of ~100 μs and ~500 μs and a decay with positive amplitude (induced absorption) and a time constant of ~2 ms. Only the ~500 μs and ~2 ms components were observed in the time dependence of the minor band at 1970 cm^{-1}. None of the time constants depend on denaturant concentration. Evaluation at different CO concentrations revealed that the longer time constants depend on [CO] while the 100 μs process did not (see the SI).

Observation of the 500 μs and 2 ms time scale components in the CO spectra is not surprising, as they have been assigned previously to CO rebinding and subsequent rebinding of His18, to which CO is sensitive.[3] In addition, the accumulation of a CO-bound intermediate, evident from the transient increase in positive amplitude, provides direct support for the proposed base elimination mechanism.[3] However, while the time resolution of the current studies was insufficient to observe the 2 μs component, the observation of the 100 μs component in the temporal changes of the CO vibration is inconsistent with its previous assignment to His26 and/or His33 misligation.[2] Whatever the structural origins of the 100 μs time scale transition, they must involve CO rebinding or changes that affect an already bound CO. Consequently, interpretation of this data in terms of interchain diffusion should be reevaluated.

Although cyt *c*−CO is not folded at high denaturant concentration, it is not necessarily a random coil either,[4b,6] and the protein may still provide a barrier that photodissociated CO must pass to escape to solvent. We propose that the three time constants observed in the cyt *c*−CO spectra result from two processes that are differentiated by whether or not the photodissociated CO escapes to solvent (Figure 3). In both cases, CO dissociation induces His18 dissociation via the base elimination mechanism. Some CO clearly does escape, and its rebinding followed by His18 rebinding gives rise to the CO-concentration-dependent time constants of ~500 μs and ~2 ms. Conversely, some CO is likely to undergo CO-concentration-independent geminate recombination, with its rebinding being too fast to be resolved in the current experiments.[7] However, His18 must also rebind following geminate recombination, and we suggest that this is a likely candidate for the 100 μs time scale process. The time scale of

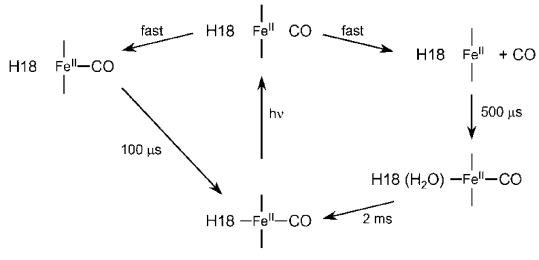

Figure 3. Proposed mechanism of CO dissociation and religation. The + sign indicates that CO has escaped to the solvent.

His18 rebinding after geminate recombination may be shorter than that after bimolecular CO recombination because of competition with water ligation.[3,8] The absence of the 100 μs time scale decay at 1975 cm^{-1} is consistent with a unique CO environment for the minor species that apparently favors CO escape relative to geminate recombination. In light of this data, it seems likely that mutation of His26 or His33 indirectly affects the amplitude of the 100 μs process, perhaps by favoring CO escape.

While intrinsic chromophores such as a heme facilitate the characterization of many proteins, it is clear that caution is required when interpreting the data because of their inherently low structural resolution. It would clearly be of great value to have probes with greater structural resolution (as provided in this case by the CO ligand) that probe the protein directly. Such probes may be provided by nitrile groups appended to an amino acid[9] or by isotopic substitution with carbon−deuterium bonds.[10] Both probes provide an IR absorption in an otherwise unobscured region of the protein's IR spectrum, and they promise to provide the same high-resolution, unambiguous data afforded by the CO ligand in the current study for the general characterization of protein dynamics. Efforts directed toward these goals are underway.

Acknowledgment. This material is based upon work supported by the National Science Foundation under MCB 034697.

Supporting Information Available: Experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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JA810155S