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Redox-Coupled Dynamics and Folding in Cytochrome *c*

Laura B. Sagle, Jörg Zimmermann, Shigeo Matsuda, Philip E. Dawson, and Floyd E. Romesberg*

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

Received February 4, 2006; E-mail: floyd@scripps.edu

Abstract: Cytochrome *c* functions as an electron carrier in the mitochondrial electron-transport chain using the Fe(II)−Fe(III) redox couple of a covalently attached heme prosthetic group, and it has served as a paradigm for both biological redox activity and protein folding. On the basis of a wide variety of biophysical techniques, it has been suggested that the protein is more flexible in the oxidized state than in the reduced state, which has led to speculation that it is the dynamics of the protein that has been evolved to control the cofactor's redox properties. To test this hypothesis, we incorporated carbon−deuterium bonds throughout cytochrome *c* and characterized their absorption frequencies and line widths using IR spectroscopy. The absorption frequencies of several residues on the proximal side of the heme show redox-dependent changes, but none show changes in line width, implying that the flexibility of the oxidized and reduced proteins is not different. However, the spectra demonstrate that folded protein is in equilibrium with a surprisingly large amount of locally unfolded protein, which increases with oxidation for residues localized to the proximal side of the heme. The data suggest that while the oxidized protein is not more flexible than the reduced protein, it is more locally unfolded. Local unfolding of cytochrome *c* might be one mechanism whereby the protein evolved to control electron transfer.

Introduction

Cytochrome *c* (cyt *c*) functions as an electron carrier in the mitochondrial electron-transport chain and has served as a paradigm for biological redox activity and protein folding.[1−6] The protein environment is thought to play an important role in controlling the redox state of the bound heme cofactor by selectively stabilizing the neutral, reduced species relative to the more polar, oxidized species.[7,8] While reduced and oxidized cyt *c* are biophysically distinct,[9−16] their solution structures are virtually identical.[17−19] However, it has been suggested that the oxidized protein is more flexible based on NMR,[20] radius of gyration,[21] adiabatic compressibility,[13] small-angle X-ray scattering,[10] amino acid reactivity,[11,14] and hydrogen exchange experiments.[12,22] More specifically, it has been suggested that the regions formed by residues His18 to Leu32 (loop A, see Figure 1), Asn70 to Gly84 (70's helix and loop D),[12,15,23] and the backbone atoms of the overlapping B/C loops[24] all undergo an oxidation-dependent increase in flexibility. This in turn has led to the suggestion that changes in flexibility affect the electron-transfer reorganization energy and that these dynamics may be important for controlling biological redox activity.

In principle, the most direct measure of protein dynamics would employ vibrational spectroscopy to characterize atomic motions. However, the spectral congestion inherent to proteins has limited the use of this technique. Previous studies have made use of isotopic labeling in conjunction with difference Fourier

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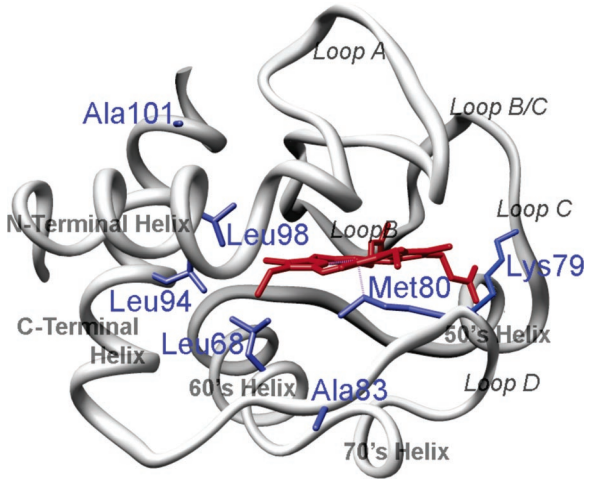

Figure 1. Structure of oxidized cyt *c*.[18]

transform infrared (FT IR) spectroscopy to alleviate some of the spectral complexity and thereby examine protein vibrations, such as those in the amide region of the IR spectrum.[25] While these studies have been informative, they remain limited by the significant protein absorption in this region of the IR spectrum and the inherent delocalization of these vibrations due to the presence of many other vibrations of commensurate frequency and the strong dipole−dipole interactions that couple them. As a result, line widths and frequencies of the observable absorptions are difficult to accurately determine and even more difficult to interpret in terms of specific protein motions. Interestingly, all proteins have a "transparent window" in their IR spectrum that is free of absorptions, between 1800 and 2500 cm^{-1}. Previous experiments have taken advantage of this window to observe protein-bound small molecules that have suitable IR absorption frequencies, such as small molecule heme ligands. Studies have probed carbon monoxide (∼1950−2150 cm^{-1}) in myoglobin[26−35] and hemoglobin,[36,37] nitric oxide in guanylate cyclase,[38] and azide (∼2050 cm^{-1}) in cytochrome bo_3.[39] In these studies, the absorption frequency and line width of the ligand absorption are used to characterize the ligand's environment, but the protein itself is not directly probed and thus has been more difficult to characterize.

Direct probes of proteins that also absorb in the "transparent window" of the protein IR spectrum would be desirable for the study of cyt *c* and protein dynamics in general. Consider, for example, the C−H bond, which has been a very useful probe of small molecules.[40−42] It is predominantly local-mode in character and thus, relative to amide absorptions, more straightforward to interpret in terms of its local environment.[43−48] However, proteins have many C−H bonds which precludes the observation of a specific bond of interest. The C−D bond possesses all of the spectroscopically desirable properties of a C−H bond, has also been used as a probe of small molecules,[49−59] may be uniquely incorporated into a protein, and most importantly, its stretching absorption is within the transparent window (∼2100 cm^{-1}).[60−62] Specific C−D absorptions may thus be observed; their frequency may be interpreted in terms of through space (increases in local polarity induce a red-shift) and through bond (decreases in bond strength induce a red-shift) effects; and their line widths may be interpreted in terms of inhomogeneous broadening and protein flexibility.[61,62]

In addition to providing bond-specific structural resolution, the C−D probe inherently provides high time resolution, which is critical for the detection and resolution of rapidly interconverting species. For example, using NMR spectroscopy, species that are separated by 100 Hz would not be resolved if they interconvert on a time scale faster than milliseconds. In contrast, intermediates separated by only a 1 cm^{-1} shift in the C−D stretching frequency would have to interconvert on the picosecond time scale in order not to be resolved. This high time resolution is critical to distinguish protein flexibility, which involves fluctuations about a single average structure, from discrete structural transitions, which involve fluctuations between two or more different structures. The former is manifest

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as broadened IR absorptions (from increased environmental heterogeneity), whereas the latter is manifest as multiple discrete absorptions (from the multiple species present).

To study cyt *c* we previously expressed or synthesized the protein site selectively labeled with C−D bonds. We have used the IR characterization of the resulting C−D absorptions to generate a residue-specific view of cyt *c* folding.[60] We have also examined the absorption frequency and line widths of C−D bonds incorporated at the protein-based heme ligand Met80 as a function of redox state.[61,62] In these studies, no evidence for redox-dependent flexibility changes was apparent. To more systematically examine the difference between oxidized and reduced cyt *c*, we now report the characterization of C−D bond absorptions at different positions throughout the protein. No residue shows evidence of redox-dependent changes in flexibility; however, several show redox-dependent local unfolding, suggesting that protein folding may be coupled to biological function.

Materials and Methods

Sample Preparation. Site-selectively deuterated cyt *c* was prepared using a semisynthetic approach, as described previously.[60,63] Briefly, the homoserine lactone peptide corresponding to residues 1−65 of horse heart cyt *c*, with a covalently bound heme, was generated using CNBr cleavage of commercially available cyt *c* (Sigma). The peptide was purified and then refolded at pH 7 in the presence of one equivalent of the chemically synthesized peptide corresponding to residues 66−104.[64] This peptide was either fully proteo or contained one specifically deuterated residue. Boc-protected, deuterated amino acids were purchased from Cambridge Isotopes (Boston, MA), except for lysine. N^{α}-*tert*-Butyloxycarbonyl-N^{ϵ}-(2-chlorobenzyloxycarbonyl)-d_8-L-lysine was prepared by protection of the free amino acid (Supporting Information). Fragment association and aminolysis of the homoserine lactone resulted in high yields of semisynthetic cyt *c* with the wild-type sequence, except at residue 65, which is a homoserine instead of a methionine.[65] The homoserine substitution has been shown to be inconsequential to the protein's biological function.[65] The final condensation product was purified from the fragments, oxidized with bis(dipicolinato)cobaltate-(III), and desalted with a Sephadex G25 column.[66] The samples were then washed with 10 mM sodium acetate buffer at pH 5, divided into single-use aliquots, lyophilized, and stored at −20 °C. Oxidized lyophilized protein samples were dissolved in 100 mM sodium acetate, pH 5, and allowed to equilibrate for approximately 10 min prior to IR characterization. Reduced samples were prepared from the oxidized lyophilized protein through the addition of a degassed solution of 100 mM sodium acetate buffer, pH 5, and 100 mM ascorbic acid. All proteins were characterized by analytical HPLC, ES MS, and UV/vis spectroscopy (Supporting Information).

FTIR Measurements. Samples were characterized using a Bruker Equinox 55 FT IR spectrometer equipped with a liquid nitrogen cooled MCT detector and a KBr beam splitter. The sample compartment was continuously flushed with dry nitrogen. Spectra were collected at both 2 and 4 cm^{-1} resolution and constructed from 8192 scans using the Blackman−Harris three-term apodization function, a 16-kHz low pass filter, and zero filling of 16. The liquid transmission cell consisted of 32-mm diameter CaF_2 disks and a 50-μm Teflon spacer. A spectrum of a proteo sample was taken under identical conditions directly after the measurement of every deuterated sample and subtracted using subtraction factors close to unity. Baseline drift was corrected by fitting a window of at least 150 cm^{-1} around the peaks of interest to a polynomial and subtracting the polynomial. For each set of experimental conditions, at least three pairs of deuterated and proteo samples were measured using at least two batches of independently synthesized protein to make sure that the unfolded protein was not present as a byproduct of synthesis. Standard deviations for the frequencies, line widths, and percent unfolded protein were calculated from at least three independent measurements.

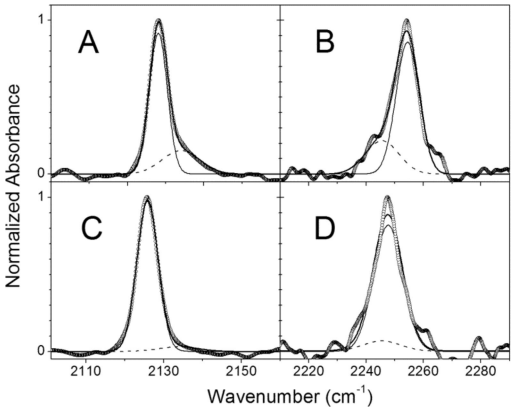

Figure 2. FT IR spectra for (d_3)Met80 cyt *c* deconvoluted into folded (solid line) and unfolded (dashed line) components. (A) Symmetric stretch, oxidized. (B) Overlapping asymmetric stretches, oxidized. (C) Symmetric stretch, reduced. (D) Overlapping asymmetric stretches, reduced.

Absorption bands were assigned by comparing the spectra of the deuterated cyt *c* protein with quantum mechanical calculations using Gaussian98[67] at the HF/LanL2DZ level of theory, with the exception of d_8-lysine, which was carried out with the HF/6-31G(d) basis set. Spectra corresponding to unfolded protein were assigned based on the spectra of the corresponding free deuterated amino acid under identical conditions. In all cases, an *F*-test was performed at the 99% confidence level to determine the number of Gaussians required to fit the spectra.[68] Spectra were fit using the Matlab program (Mathworks, Inc.) using the least-squares error method. The percent of unfolded protein was calculated from the relative amplitudes of the Gaussians weighted by the corresponding extinction coefficients.

Results

We first synthesized cyt *c* with (methyl-d_3) methionine at Met80 ((d_3)Met80). Met80 provides one of two protein-based heme ligands and is part of the D-loop that has been suggested to be more flexible in the oxidized state[19] (Figure 1). We observed a strong absorption at ∼2130 cm^{-1} in both oxidized and reduced (d_3)Met80 (Figure 2 and Table 1). On the basis of ab initio calculations (Supporting Information), we assigned this absorption to the symmetric stretch, in agreement with previous studies using recombinant protein.[61,62] In addition, we observed a previously unreported weak absorption at ∼2245 cm^{-1}, which we assigned on the basis of the calculations as overlapping asymmetric stretches. Upon oxidation, the asymmetric and symmetric (d_3)Met80 stretching absorptions of the reduced protein blue-shift (Table 1). This was previously interpreted in terms of through-bond interactions involving the adjacent sulfur atom.[61] The Met80 C−D absorptions do not show any consistent or significant redox-dependent changes in line width (Table 1). The absence of any significant line width changes at a residue so close to the cofactor implies that the Met80 ligand is held

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Table 1. Frequency, Line Width, and Percent Unfolded Values Measured for Seven Deuterated Residues throughout Cytochrome *c*

	frequency (cm^{-1})		line width (cm^{-1})				percent unfolded	
	oxidized	reduced	oxidized	reduced	$\Delta\nu$[a] (cm^{-1})	Δfwhm[a] (cm^{-1})	oxidized	reduced
				Leu68				
asym Cδ_2-A′/A″	2204.5 ± 0.2	2208.7 ± 0.6	14.9 ± 0.6	18.2 ± 2.0	−4.2 ± 0.6	−3.3 ± 2.1	7% ± 1%	2% ± 2%
asym Cδ_1-A′/A″	2216.8 ± 0.2	2217.1 ± 0.4	10.5 ± 0.5	7.5 ± 1.3	−0.3 ± 0.4	+3.0 ± 1.4		
sym Cδ_1/Cδ_2-A′	2060.7 ± 0.3	2061.2 ± 0.7	13.8 ± 0.9	13.1 ± 0.7	−0.5 ± 0.8	+0.7 ± 1.1	7% ± 2%	1% ± 2%
				Ala83				
asym A′	2231.2 ± 0.4	2231.4 ± 0.4	16.8 ± 1.2	14.3 ± 2.9	−0.2 ± 0.6	+2.5 ± 3.1	*b*	*b*
asym A″	2246.8 ± 1.1	2245.5 ± 0.6	18.8 ± 1.3	17.6 ± 2.5	+1.1 ± 1.3	−1.2 ± 2.8		
				Lys79[c]				
asym Cγ-A′	2237.2 ± 0.2	2237.4 ± 0.1	5.9 ± 0.7	6.4 ± 0.7	−0.2 ± 0.2	−0.5 ± 1.0	14% ± 2%	5% ± 1%
asym Cβ-A′	2223.7 ± 0.4	2223.1 ± 1.0	7.2 ± 1.4	8.2 ± 1.8	+0.6 ± 1.1	−1.0 ± 2.3		
asym Cδ-A′	2206.2 ± 0.6	2207.2 ± 1.9	6.4 ± 0.6	5.9 ± 0.9	−1.0 ± 2.0	+0.5 ± 1.1		
sym Cγ/Cβ-A′	2110.7 ± 0.4	2111.6 ± 0.6	15.5 ± 0.3	17.5 ± 1.1	−0.9 ± 0.7	−2.0 ± 1.1	14% ± 3%	4% ± 1%
sym Cδ-A	2121.6 ± 0.7	2122.2 ± 0.9	12.6 ± 0.3	12.1 ± 0.6	−0.6 ± 1.1	+0.5 ± 0.7		
				Met80				
asym A′/A″	2254.4 ± 0.4	2247.6 ± 0.2	9.5 ± 1.0	11.9 ± 2.6	+6.8 ± 0.4	−2.4 ± 2.8	14% ± 4%	4% ± 3%
sym A′	2128.0 ± 0.1	2125.8 ± 0.1	5.6 ± 0.2	6.4 ± 0.2	+2.2 ± 0.1	−0.8 ± 0.3	9% ± 3%	4% ± 0.4%
				Leu94				
asym Cδ_1-A′/A″	2207.3 ± 0.1	2207.6 ± 0.3	12.3 ± 1.4	10.6 ± 0.8	−0.3 ± 0.3	+1.7 ± 1.6	14% ± 3%	11% ± 3%
asym Cδ_2-A′/A″	2225.6 ± 0.2	2226.4 ± 0.3	14.9 ± 0.6	16.8 ± 1.0	−0.2 ± 0.4	−1.9 ± 1.2		
sym Cδ_1/Cδ_2-A′	2065.1 ± 0.3	2065.0 ± 0.1	10.5 ± 0.4	9.2 ± 0.4	+0.1 ± 0.3	+1.3 ± 0.6	12% ± 2%	14% ± 5%
				Leu98				
asym Cδ_2-A′/A″	2202.2 ± 0.2	2202.5 ± 0.1	13.5 ± 0.8	14.5 ± 1.4	−0.3 ± 0.2	−1.0 ± 1.6	11% ± 1%	13% ± 1%
asym Cδ_1-A′/A″	2217.0 ± 0.1	2218.5 ± 0.1	11.2 ± 0.3	10.7 ± 0.4	−1.5 ± 0.1	+0.5 ± 0.5		
sym Cδ_1/Cδ_2-A′	2060.0 ± 0.2	2060.6 ± 0.2	10.9 ± 1.1	11.7 ± 0.8	−0.6 ± 0.3	−0.8 ± 1.4	10% ± 1%	10% ± 1%
				Ala101				
asym A′/A″	2226.3 ± 0.2	2226.3 ± 0.1	8.4 ± 1.4	8.6 ± 1.2	0.0 ± 0.2	+0.2 ± 1.8	58% ± 6%	54% ± 4%

[a] $\Delta\nu$ corresponds to observed changes in frequency, and Δfwhm corresponds to observed changes in line width (full-width half-maximum). Positive values indicate oxidized state has a greater value, negative values indicate reduced state has a greater value. [b] Not determined due to spectral overlap, see text. [c] Both asymmetric and symmetric Cϵ absorptions of Lys79 were predicted by ab initio calculations to be very weak and were not observed.

rigidly and is not free to sample different environments, which would be expected to broaden the absorption.

Surprisingly, the spectra of the oxidized and reduced (d_3)-Met80 also show an absorption at 2134 cm^{-1}, which corresponds to a solvent-exposed ligand and suggests that a fraction of the protein is at least locally unfolded. Interestingly, the amplitude of this absorption depends on the redox state of the protein, indicating that 4% of cyt *c* at Met80 is unfolded in the reduced state, while ∼12% is unfolded in the oxidized state. Thus, oxidation induces rupture of the Met80−Fe bond and expulsion of the ligand into solvent.

To examine another region of the D-loop, we next investigated Ala83 (Figure 1). Two C−D absorptions were observed in reduced (d_3)Ala83 cyt *c*, one at 2245 cm^{-1} and one at 2231 cm^{-1} (Figure 3 and Table 1). On the basis of ab initio calculations these were assigned as the two asymmetric stretches (Supporting Information). The absorptions were generally less intense than those of (d_3)Met80, and we were unable to observe the symmetric stretch, predicted at 2072 cm^{-1}. Unlike Met80, oxidation induced no changes in frequency. In addition, neither of the absorptions exhibited a change in line width upon oxidation. These data suggest that there are no significant changes in the environment or dynamics at the Ala83 methyl group. This is perhaps not surprising, since the crystal structure shows that the methyl group is solvent exposed,[17,19] but it does suggest that either the environment and dynamics are not altered at this residue or that the methyl group absorptions are not sensitive to the changes. Local unfolding at this residue could not be determined due to the overlap of the folded and unfolded signals.

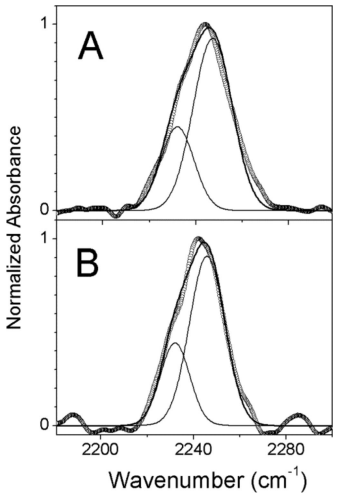

Figure 3. FT IR spectra for (d_3)Ala83 cyt *c* deconvoluted into folded (solid line) and unfolded (dashed line) components. (A**)** Asymmetric stretches, oxidized. (B) Asymmetric stretches, reduced. Due to its weak intensity the symmetric absorptions are not reported.

To determine if other D-loop residues showed evidence of redox-dependent changes in their environment or dynamics, we examined Lys79 (Figure 1). Cyt *c* with (Cβ,Cγ,Cδ,Cϵ-d_8) lysine at position 79 ((d_8)Lys79) was prepared and characterized. Two broad absorptions were observed in reduced (d_8)Lys79 cyt *c*, one strong band at ∼2220 cm^{-1} and a weaker one at ∼2110 cm^{-1} (Figure 4 and Table 1). On the basis of quantum mechanical calculations, the absorption at ∼2220 cm^{-1} was assigned as overlapping asymmetric stretches, and the one at 2110 cm^{-1}, as overlapping symmetric stretches (Supporting Information). Upon oxidation, there is a small red-shift in the

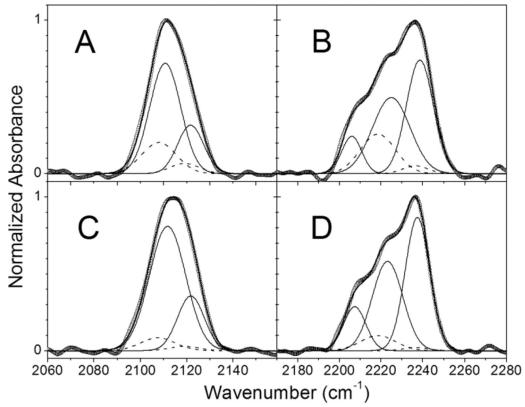

Figure 4. FT IR spectra for (d_8)Lys79 cyt *c* deconvoluted into folded (solid line) and unfolded (dashed line) components. (A) Overlapping symmetric stretches, oxidized. (B) Overlapping asymmetric stretches, oxidized. (C) Overlapping symmetric stretches, reduced. (D) Overlapping asymmetric stretches, reduced.

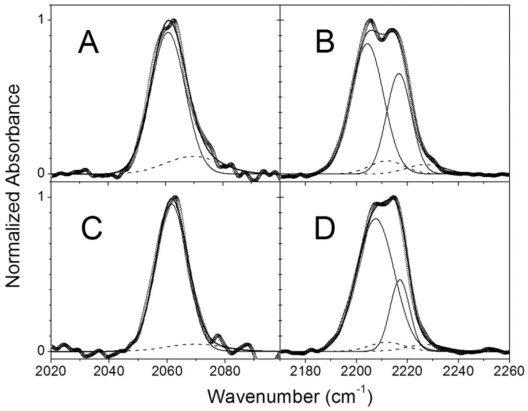

Figure 5. FT IR spectra for (d_3)Leu68 cyt *c* deconvoluted into folded (solid line) and unfolded (dashed line) components. (A) Symmetric stretch, oxidized. (B) Overlapping asymmetric stretches, oxidized. (C) Symmetric stretch, reduced. (D) Overlapping asymmetric stretches, reduced.

symmetric absorption, which on the basis of previously reported solvent studies,[60,62] suggests that the C−D bond experiences a less polar environment upon oxidation. No change in line width was observed for either absorption. Thus, similar to Met80 and Ala83, there is no indication of a redox-related change in flexibility at Lys79. However, as at Met80, the reduced protein contains only 4% unfolded protein, while the amount of unfolding increases to 14% upon oxidation. The identical amount of unfolded protein at Met80 and Lys79 strengthens the argument that at least this part of the D-loop is induced to unfold by oxidation.

To characterize an α-helix proximal to the D-loop, we examined Leu68. Leu68 is part of the 60's helix (Figure 1), which has been suggested to be more flexible in the oxidized protein, with the largest changes centered at Tyr67.[6] A 1:1 mixture of Cδ_1-d_3- and Cδ_2-d_3-labeled leucine isotopomers was incorporated at position 68. Reduced (Cδ-d_3)Leu68 showed two strong absorption bands at 2209 and 2217 cm^{-1}, and a weaker band at 2061 cm^{-1} (Figure 5 and Table 1), which, on the basis of ab initio calculations, were assigned as overlapping asymmetric absorptions of the diastereotopic Cδ_1 and Cδ_2 methyl groups and overlapping symmetric stretches, respectively (Supporting Information). Upon oxidation, the low-frequency asymmetric stretches red-shift by 4.4 cm^{-1}, which on the basis of previously reported solvent studies,[60] suggests that the oxidized

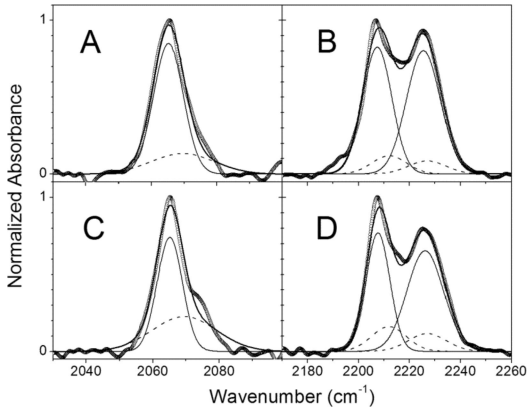

Figure 6. FT IR spectra for (d_3)Leu94 cyt *c* deconvoluted into folded (solid line) and unfolded (dashed line) components. (A) Overlapping symmetric stretches, oxidized. (B) Overlapping asymmetric stretches, oxidized. (C) Overlapping symmetric stretches, reduced. (D) Overlapping asymmetric stretches, reduced.

protein is less polar in the vicinity of Leu68. No changes in frequency are observed for the high-frequency asymmetric stretches. This disparate behavior of the absorptions assigned to the two methyl groups may be understood by examining the crystal structure,[18] which shows that one, the Cδ_2 methyl group, packs on the heme cofactor, which should make it sensitive to changes in the redox state of the cofactor, while the other, Cδ_1, is oriented away. Thus, the low-frequency asymmetric stretch absorption is assigned as Cδ_2. The absence of any consistent line width change suggests that, similar to Met80 and Ala83, there is no significant redox-dependent change in protein flexibility at Leu68. However, as at Met80 and Lys79, we did observe a significant change in the relative amplitudes of peaks corresponding to an unfolded and solvent-exposed Leu residue, increasing upon oxidation from 2% to 7%. When compared to the unfolding data at Met80 and Lys79, these data also demonstrate that different amounts of unfolding are induced in different parts of the protein.

To characterize the C-terminal helix we examined residues Leu94, Leu98, and Ala101. The C-terminal helix packs with the N-terminal helix and forms what is thought to be one of the most stable parts of the protein (Figure 1).[69,70] (Cδ-d_3)Leu incorporation at positions 94 and 98 resulted in two strong absorptions, at ∼2205 and ∼2220 cm^{-1}, and a weaker absorption at ∼2060 cm^{-1} (Figures 6 and 7 and Table 1). Again, on the basis of ab initio calculations, the two strong absorptions are assigned as overlapping asymmetric stretches of the two diasteriotopic methyl groups, and the weaker absorption is assigned as degenerate symmetric stretches (Supporting Information). Upon oxidation, the high-frequency asymmetric stretch of Leu98 showed a red-shift, indicating that, like Leu68, it experiences a less polar environment in the oxidized state. None of the Leu absorptions in the C-terminal helix showed significant changes in line width upon oxidation, again suggesting that there are no changes in flexibility. Despite the suggested stability of this region of the protein, absorptions corresponding to solvent-exposed Leu residues reveal that the C-terminal helix is unfolded in approximately l0% of the protein in both the oxidized and reduced states.

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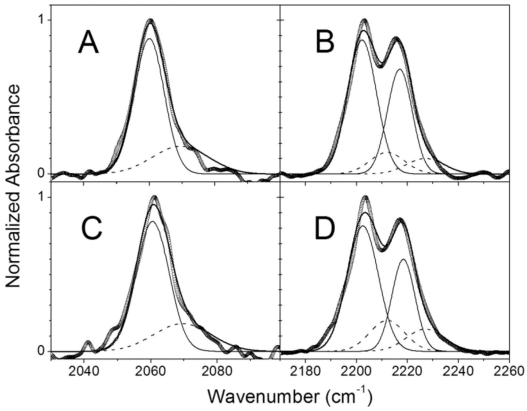

Figure 7. FT IR spectra for (d_3)Leu98 cyt *c* deconvoluted into folded (solid line) and unfolded (dashed line) components. (A) Overlapping symmetric stretches, oxidized. (B**)** Overlapping asymmetric stretches, oxidized. (C) Overlapping symmetric stretches, reduced. (D) Overlapping asymmetric stretches, reduced.

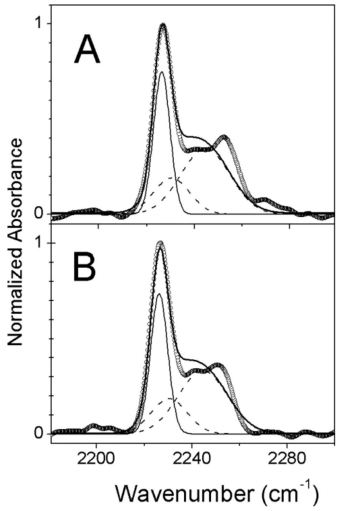

Figure 8. FT IR spectra for (d_3)Ala101 cyt *c* deconvoluted into folded (solid line) and unfolded (dashed line) components. (A) Asymmetric stretches, oxidized. (B) asymmetric stretches, reduced. Due to its weak intensity the symmetric absorptions are not reported.

Due to its location near the end of the C-terminal helix, Ala101 might be expected to be more sensitive to redox-dependent changes in environment, flexibility, or folding than residues located in the more stable core of the packed helices (Figure 1). Only one weak absorption was observed in (d_3)-Ala101 (Figure 8 and Table 1) which was assigned as overlapping asymmetric stretches on the basis of ab initio calculations (Supporting Information). The spectrum was virtually identical in the oxidized and reduced states, suggesting that there are no redox-dependent changes in the protein environment or dynamics at Ala101. Remarkably, the spectra demonstrate that more than half of the protein is unfolded at the C-terminus of cyt *c*, both in the oxidized and reduced proteins. While the experimental conditions employed for these studies were chosen on the basis of standard conditions where the protein was previously assumed to be well folded, we also determined the extent of Ala101 unfolding at pH 7 to ensure that the observations were not artifacts of the specific experimental conditions employed (Supporting Information). Again, a significant unfolding of the C-terminal helix is apparent, suggesting that the protein is significantly locally unfolded under these conditions as well.

Discussion

The potential contribution of dynamics to protein function is receiving increased attention.[71−74] Protein dynamics might contribute to function by controlling the fluctuations within one, or a set of related potential energy minima separated by low barriers. More flexible proteins should undergo larger amplitude fluctuations about the protein's average structure, while more rigid proteins should undergo smaller fluctuations. Alternatively, dynamics may be manifest as specific transitions between different minima, each corresponding to structures that are themselves either flexible or rigid. A significant body of results have been interpreted as evidence of a redox-dependent increase in cyt *c* flexibility, and such dynamics have been suggested to contribute to the proteins' biological redox properties.[6,23,24] However, these conclusions were largely based on indirect techniques (i.e., viscosity, radius of gyration, and adiabatic compressibility[6,13]) or techniques with either low spatial resolution (i.e., fluorescence[75,76] or absorption spectroscopy[77]) or low time resolution (i.e., NMR spectroscopy[78,79]).

The inherently high time resolution of IR spectroscopy allows the differentiation between changes in flexibility and rapid interconversion between multiple species, for example, the folded and unfolded protein. Increased flexibility will give rise to increased absorption line widths, as a given C−D bond experiences a greater diversity of environments, while interconversion between multiple species will give rise to multiple, distinct absorptions. No residue within cyt *c* is found to have a redox-dependent line width. The absence of a line width change at any single residue is difficult to interpret because the different environments may simply not alter the absorption of the C−D bond. However, the complete absence of redox-dependent line width changes for C−D bonds incorporated throughout the protein is more convincing. This is especially true, considering that C−D bonds at several positions within the protein do show significant redox-dependent shifts in absorption frequency, demonstrating that they are indeed sensitive to their environment. Sensitivity of the C−D absorption frequency to the bond's local environment is also supported by other experiments in cyt *c*,[60−62] as well as by the characterization of C−D bonds incorporated into a variety of small molecules.[49−59] Thus, the data suggest that the flexibilities of the oxidized and reduced states of cyt *c* are not significantly different.

The Met80, Lys79, and Leu68 data provide an alternative explanation of the results of previous studies that concluded that the oxidized state is more flexible—at least some parts of the protein are more unfolded in the oxidized state. Presumably, locally unfolded protein under similar conditions was not detected in previously reported experiments, likely due to a combination of low structural and temporal resolution; however, the results are consistent with other studies suggesting that the

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reduced protein is more stable than the oxidized protein.[79] The observation of a similar induction of unfolding at Met80, Lys79, and Leu68, but not at Leu94, Leu98, or Ala101, suggests that the unfolding is localized to the D-loop and perhaps other residues on the proximal side of the heme. Interestingly, in addition to oxidation-dependent unfolding, Met80 and Leu68 also showed the most significant redox-dependent frequency shifts, and they are also near a number of residues previously suggested to play a role in the regulation of the protein's redox activity.[80,81] This correlation between redox-dependent frequency shifts, unfolding, and contribution to redox activity suggests that this region of the protein may play a key role in mediating cyt *c* function. Perhaps these residues are more sensitive to the redox state of the heme, and the increased disorder associated with their local unfolding helps to regulate electron transfer.

The data also reveal a remarkable extent of unfolding in the C-terminal helix, especially at Ala101 near the end of the helix, where more than half of the protein is unfolded in both the reduced or oxidized proteins. The central part of the helix is more structured, with ∼12% of the protein unfolded, which again was not dependent on the redox state of the protein. In the reduced protein, the C-terminal helix is more unfolded than the D-loop or 60's helix, where only 2−5% of the protein is unfolded. These results contrast with previous studies, which suggested that, under these conditions, the protein is well folded[82] and that, in general, the C-terminal helix is one of the most stable parts of the protein.[69,70] However, because oxidation induces unfolding of the D-loop and 60's helix, all three regions of the protein are equally unfolded in the oxidized state (∼12%).

In all, the data suggest that cyt *c* is less stable than previously assumed and that parts of the protein exist in dynamic equilibrium with locally unfolded states and, at least at some residues, this unfolding is dependent on the protein's oxidation state. The folding of these regions may play a role in the regulation of biological function, as has been suggested for other proteins.[83] These studies highlight the utility of the IR-based approach with its inherently high spatial and temporal resolution. Further use of the C−D approach to test these ideas, both in steady-state and time-resolved formats, is currently underway.

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Supporting Information Available: Materials, methods, raw data, calculations, and complete ref 67. This material is available free of charge via the Internet at http://pubs.acs.org.

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