Transient Resonance Raman Evidence for Structural Reorganizational Dynamics during Electron Transfer in Ruthenated Yeast Cytochrome *c*

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Metalloporphyrin (MP)-mediated electron transfer (ET) reactions play major roles in the metabolism of virtually all living organisms. Biological ET generally occurs over long distances and through the nonuniform environment of donor and acceptor proteins. The influence of the heterogeneous protein medium on the dynamics of ET is an area of much current debate and investigation.

One strategy by which many of these issues have been addressed is to attach a photoactive redox center to the surface of an ET protein. Ruthenium polypyridine derivatives can be covalently attached to externally accessible amino acids including lysine, histidine, and cysteine.[1−3] Photoexcitation creates a long-lived Ru triplet state[4] that can donate or accept electrons directly from the heme.[1−3,5] ET between the Ru at the protein surface and the heme embedded in the protein can thus be photoinitiated in a well-defined manner. Studies of the distance and driving force dependence of ET have been carried out on Ru-modified proteins including cytochrome *c*, and the behavior of these systems has been found to be fairly consistent with traditional Marcus theory.[1−3,5,6] Here we describe the first use of transient resonance Raman spectroscopy (TR^2S) and time-resolved resonance Raman spectroscopy (TR^3S) to address the nature of the structural dynamics of the heme and surrounding protein in yeast cytochrome *c* that has been ruthenated at Cys-102 (RuC).

TR^2 and TR^3 spectra in the high-frequency region were obtained from RuC by using protocols described in detail elsewhere.[7−9] Spectral deconvolution was carried out as described in ref 7. Figure 1 summarizes the flux-dependent response of the MP oxidation state marker line, ν_4,[10] for oxidized RuC over a range of $<5 \times 10^7$ W/cm^2 to $\sim 5 \times 10^8$ W/cm^2.

Table 1. Summary of ν_4 Behavior during Photoinitiated ET

transient (10 ns)	position (cm^{-1}) ± 1 cm^{-1}		width (cm^{-1}) ± 2 cm^{-1}		area (%) ± 5%	
no neutral density	1358	1371	18	20	25	75
+0.3 OD neutral density filter	1359	1371	11	20	10	90
+0.6 OD neutral density filter	1360	1371	9	17	5	95
+0.9 OD neutral density filter		1372		20		100
equilibrium fully reduced	1362		13		100	

time-resolved	position (cm^{-1}) ± 2 cm^{-1}		width (cm^{-1}) ± 2 cm^{-1}		area (%) ± 10%	
probe only		1374		20		100
$\Delta t = 10-40$ ns	1364	1372	13	18	20	80

Excitation with low laser intensity produces a single band at 1371 cm^{-1}, demonstrating a completely ferric sample. An increase in the incident intensity leads to the appearance of a second peak at positions ranging from 1358 to 1362 cm^{-1}, indicative of ferrous heme. This behavior shows that ET to the heme occurs within the ~10 ns laser pulse width.[24] The relative area of the transient ν_4 band increases with increasing laser intensity. Furthermore, the position and line width of the transient newly reduced heme band depend on the incident laser flux. At highest flux, a broad (FWHM ≈ 18 cm^{-1}) transient ν_4 band appears at 1358 cm^{-1}. As the flux is decreased, this band shifts and narrows until it resembles ν_4 of the equilibrium chemically reduced species (1362 cm^{-1}, ~12 cm^{-1} FWHM). The TR^3 spectrum in Figure 1e shows that by $\Delta t = 40$ ns the ferrous ν_4 band has relaxed to a position and line width similar to those of the equilibrium reduced species. Taken together, these data provide evidence for rapid structural relaxation of the MP active site subsequent to ET.

The mechanisms by which protein structure modulates ET are largely unknown. The results of recent experimental and theoretical studies of ET in cytochromes have revealed the complexity of this problem.[1,6b,11] These studies suggest that, in some cases, the protein functions as a nonspecific dielectric solvent,[12] while in others, the specific identities of amino acids in the putative ET pathways can significantly influence the

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(24) Time-resolved luminescence studies by Millett and Durham (unpublished) have established an intramolecular ET rate constant of less than 1×10^{-6} s^{-1} for RuC. The ET kinetics observed in the present study are clearly much faster. In order to resolve this discrepancy, control experiments were conducted that revealed no observable ET occurring during 10 ns pulses in native yeast cytochrome *c* under solution and excitation conditions similar to those described in Figure 1. This ensures that the Ru−polypyridine moieties are in fact acting as the donors in the ET process apparent in the TR^2 and TR^3 spectra. We speculate that the higher concentrations of RuC required for TR^2 and TR^3 studies (~100 μM vs ~3 μM for luminescence studies) produce protein aggregates that exhibit rapid intermolecular ET, with the Ru moiety as the photoinduced donor.

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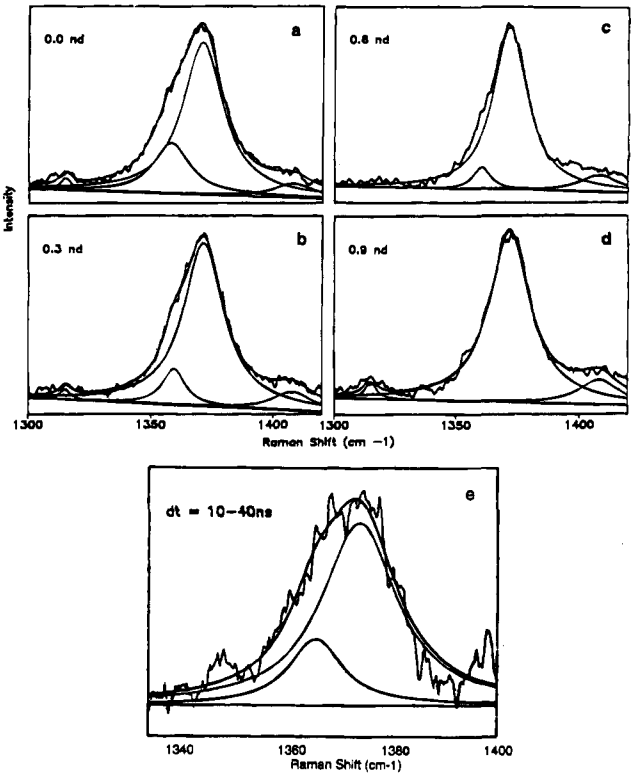

Figure 1. Transient and time-resolved resonance Raman spectra of RuC in the ν_4 region. The yeast cytochrome *c* labeled with $Ru(bpy)_2$-(methylbipyridine) ($Ru(bpy)_2(mbpy)$) at cysteine 102 (RuC) was synthesized and purified as described in ref 3. Samples were ~90 μM RuC in 10 mM Phosphate (pH 7.0) with ~2 μM bovine cytochrome *c* oxidase and 0.1% Brij (added to ensure that the RuC was completely reoxidized between excitation pulses). Panels a–d represent transient spectra obtained as a function of laser flux: (a) ~10^8 W/cm^2, (b) 50% intensity, (c) 25% maximum intensity, (d) 12.5% maximum intensity. TR^2 spectra were obtained via a single-pulse protocol which uses a train of ~10 ns pulses (406 nm, 15 Hz repetition rate) to both excite the sample and generate the TR^2 spectra. These spectra reflect the temporal evolution of all species (excited and ground state) during the 10 ns pulses. Panel e depicts a TR^3 spectrum obtained at the earliest possible time point (Δt = 25 ± 15 ns) after photoexcitation. This spectrum was obtained using 450 nm pump pulses (~10^8 W/cm^2, ~10 ns pulse width) to initiate ET and 404 nm probe pulses (~10^7 W/cm^2, ~10 ns pulse width) to generate the spectrum. Spectra were deconvoluted to extract the positions, line widths, and relative contributions of ferrous and ferric ν_4 bands to the net line shape using previously described methods. The Lorentzian line shapes yielding the best fits and their sums are displayed with the raw data. Line widths, positions, and intensities are summarized in Table 1.

observed kinetics.[13,14] The influence of the protein upon the heme can be explored by following the vibrational behavior of newly reduced heme centers with resonance Raman spectroscopy.

No Ru-label-induced changes in the equilibrium structures of the RuC acceptor (heme) are found. The positions, line widths, and relative intensities of resonance Raman bands in the high-frequency spectra of the equilibrium reduced and oxidized forms of RuC are quite similar to those of unlabeled forms (data not shown). These results are consistent with previous studies which found no evidence for alteration of heme redox properties upon cysteine ruthenation of yeast cytochrome *c*.[2,3]

Our data do, however, strongly suggest that structural dynamics occur rapidly (<40 ns) at the heme subsequent to ET. The behavior of ν_4 indicates an increase in the electron density of the heme π^* LUMO immediately (<10 ns) following ET. Similar behavior has been observed and carefully studied during the ligation dynamics of hemoglobins and other heme proteins.[15–23] Most hemoglobins exhibit 2–5 cm^{-1} downshifts in the ν_4 band in TR^2 spectra of photolytic transient species. These spectral shifts were interpreted as arising from a metastable geometry of the proximal heme pocket which altered the heme–histidine ligation geometry. This metastable configuration persists for 500 ns to 5 μs depending upon solution conditions. In view of dependence of relaxation kinetics upon solvent viscosity in Hb,[23] the structural rearrangement almost surely involves concerted motion of protein helices and can be related to global differences in the protein structures of the equilibrium species.

While the initial shift of ν_4 during ET in RuC is qualitatively similar to those of photolyzed HBs, its dynamics are quite different. The dependence of ν_4 position and line width upon laser flux in the transient experiments is consistent with relaxation processes occurring within the 10 ns pulse width.[25] Thus, the evolution of ν_4 to its equilibrium value is far too rapid to involve large-scale, concerted protein motions. It most likely reflects the structural response of the heme and its immediate environment to the change in iron oxidation state subsequent to ET. Crystal structures of equilibrium ferrous and ferric yeast iso-1 cytochrome *c* show no large-scale differences in protein structure but do reveal small differences in the heme local environment.[26,27] Likely candidates for the ET-induced heme response include His and Met axial ligation, thioether linkages to the protein, equilibrium between planar and nonplanar heme conformations, and nonbonded interactions of propionate side chains. Changes in these degrees of freedom would be expected to sterically and/or electronically influence heme π^* density and thereby lead to the observed behavior. Our data confirm that cytochrome *c* does not undergo intermediate global protein rearrangement during ET and demonstrate that local relaxation at the heme is quite rapid (<10 ns). Further investigations will concentration upon the low-frequency heme RR spectrum where the influences of axial-ligand bonding and heme planarity can be more directly assessed.

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(25) In these experiments, two photons are required to interact with a given molecule for a reduced ν_4 band to be observed: one to initiate the ET reaction and one to probe the intermediates and products via Raman scattering. It is known that the reoxidation of the heme occurs with a >10 ns lifetime.[2,3,5] Therefore, each molecule can only be photoreduced once in a given laser pulse. The incident laser flux determines the average time interval between the "pump" and "probe" photons within a given laser pulse. If the initial relaxation is rapid on the time scale of this incident laser pulse (i.e., <10 ns), then the distribution of electronic and structural states in the population that is probed by Raman spectroscopy is expected to be sensitive to the incident laser flux. At low flux, the molecules (which are excited throughout the duration of the pulse) have, on average, sufficient time to relax prior to being probed and thus yield vibrational bands indicative of the equilibrium reduced species. At higher laser fluxes, the relaxing molecules are, on average, probed before they can relax, and the net spectrum relfects the net contributions from hemes in various stages of relaxation. If the vibrational properties of the transient and relaxed species vary, the position and width of the net Raman bands observed within an ~10 ns pulse will be quite sensitive to small differences in the time between "pump" and "probe" photons (i.e., photon flux).

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