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Photodissociation of Heme Distal Methionine in Ferrous Cytochrome *c* Revealed by Subpicosecond Time-Resolved Resonance Raman Spectroscopy

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In heme proteins, binding and release of diatomic gaseous ligands (O_2, CO, NO) are controlled by the coordination and the redox state of the heme iron.[1] A number of proteins have been identified in which the displacement of a heme-liganded internal amino acid by an external diatomic gaseous ligand plays a physiological role. These include, among others, EcDOS (bacterial oxygen sensor),[2] CooA (bacterial carbon monoxide sensor),[3] and mammalian cytochrome *c* (cyt *c*). Indeed, mitochondrial cyt *c* has recently been shown to bind nitric oxide in apoptotic cells.[4] Here, since the heme is six-coordinated in the resting state, binding of a diatomic ligand requires the cleavage of one axial bond. The first step in understanding the activation mechanism of these enzymes is to structurally demonstrate the possibility of such a bond breaking and to identify its nature.

Generally, diatomic axial ligands can be photodissociated from the heme iron with high yield, providing a unique possibility to study the ultrafast heme structural dynamics after axial ligand bond breaking by femtosecond spectroscopy.[1] For cyt *c* in the resting state, the heme iron is coordinated by the two *endogenous* ligands, histidine (His-18) and methionine (Met-80). Previous photophysical studies on ferrous cyt *c* were based on femtosecond transient absorption (TA) spectroscopy with high-energy excitation in the Soret band of the heme.[5,6] These studies suggested the photolysis of either the Fe−His proximal bond[5] or the Fe−Met distal bond.[6] The latter conclusion was based on a similarity of the reconstructed photoproduct absorption spectrum with the stationary absorption spectrum of a model five-coordinated histidine-ligated complex, microperoxidase.[6] Also, femtosecond coherence spectroscopy study of cyt *c*,[6] performed along with TA, allowed to reconstruct the vibrational modes at ~40, ~80, and ~220 cm^{-1}, which were interpreted as Met-80 detachment.

However, TA spectroscopy monitors changes in the electronic system of the heme upon photoexcitation. For cyt *c*, the observed TA kinetics is extremely fast, with the longest time constant of ~6 ps interpreted as ligand geminate rebinding.[5,6] This is on the same time scale as electronic relaxation without ligand detachment, the effect that may complicate the photoproduct TA spectra. Time-resolved resonance Raman (TR^3) is known as the most direct structure-sensitive tool in studying the processes of axial ligand(s) binding and release.[3,7−9] In this study, we applied subpicosecond TR^3 (i) to directly assess whether the event of photodissociation of an internal axial ligand in ferrous cyt *c* takes place, (ii) to identify unequivocally which side chain is involved in bond cleavage, and (iii) to monitor heme structural changes during this process.

For this study, we have developed a novel subpicosecond Raman spectrometer, which will be described in detail elsewhere. Briefly, this device operates at 1 kHz repetition rate and consists of ~50-fs Ti:Sapphire oscillator−amplifier laser source and a spectrometer part based on optical parametric generator−amplifier systems in both pump and probe channels. Sample photolysis was performed by pump pulses with an energy of 2.0−2.5 μJ, centered at 550 nm, the maximum of the Q_{0-0} band, the lowest-lying electronic transition in cyt *c*. Thus, we minimized the effect of heme vibrational heating. Raman scattering was excited by 20−30 nJ probe pulses at 435 nm. This wavelength is near the photoproduct absorption maximum[6] and is considerably red-shifted with respect to the Soret band of the resting ferrous cyt *c* (λ_{max} = 416 nm). To achieve appropriate spectral resolution, the probe pulses were tailored by a specially designed narrow-band interference filter. The instrumental time response function was Gaussian with a half-width $T_G = 630 \pm 40$ fs, and the spectral resolution was 30 cm^{-1} (fwhm). Raman spectra were obtained in a 90° light-collection geometry using a 1-m single spectrograph with 1200 grooves/mm grating and a liquid nitrogen-cooled CCD.

Horse heart cyt *c* was purchased from Sigma. The ferrous sample, with concentration of 0.3 mM, was prepared by reducing the protein in 0.1 M Tris/HCl buffer (pH 7.4) using 1 mM sodium dithionite. A cyt *c* N-fragment (residues 1−56)[10,11] was obtained by limited proteolysis of the parental protein. Samples were placed under anaerobic conditions in a standard 1-cm quartz cuvette with a stirring bar. Ammonium sulfate (0.5 M) was added to the samples for Raman intensity calibration using the 980 cm^{-1} line of SO_4^-. Raman spectra normalization and reconstruction of TR^3 difference spectra representing pure photoproduct contribution have been described elsewhere.[9]

Figure 1 shows the results for ferrous cyt *c*. The high-resolution ground-state spectrum (Figure 1A) recorded with cw excitation at 441.6 nm is in agreement with literature data.[12] Apart from the spectral resolution, which illustrates the chosen spectral-temporal compromise, the ground-state spectrum (Figure 1B) recorded with subpicosecond excitation and negative time delay between pump and probe pulses ($\Delta t = -5$ ps) is identical to that in Figure 1A. This finding implies that (i) the probe pulse is weak enough not to induce noticeable photolysis and (ii) no long-lived (millisecond or longer) pump-induced spectral changes take place.

The distinctive feature in the 1-ps spectrum of the cyt *c* photoproduct (Figure 1D) is the appearance of a strong 216 cm^{-1} band, which is assigned to the Fe−His stretching (ν_{Fe-His}).[13] This band is not Raman-active in six-coordinated cyt *c*,[12] but is specifically enhanced in the case of five-coordinated ferrous high-spin domed heme structures.[13] Hence, instant rising of ν_{Fe-His} (Figure 1D) readily indicates the photodissociation of the internal axial ligand, namely Met-80. Indeed, although the Fe−Met stretch has not been identified for cyt *c*, it is expected[12] at much higher frequency, around 350 cm^{-1}. The observed Fe−His frequency is

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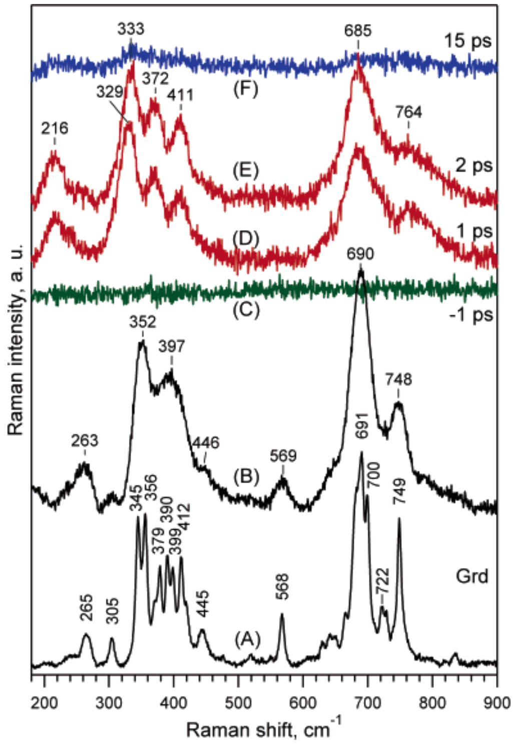

Figure 1. TR[3] spectra of ferrous cyt *c*. Accumulation time was 50 min for each spectrum. Spectra A and B are ground-state spectra recorded with cw excitation at 441.6 nm and subpicosecond excitation at 435 nm, respectively; spectrum B was recorded at $\Delta t = -5$ ps. Spectra C−F are difference spectra at various Δt: −1 (C), 1 (D), 2 (E), and 15 ps (F).

lower than that of the *b*-type hemes in deoxy Mb[3,13] or R-state HbA (220 cm^{-1}).[7] As it is equal to that of T-state HbA (216 cm^{-1})[7] and higher than that of photodissociated CooA (211 cm^{-1})[3], this frequency value is presumably indicative of the distortion, due to strain exerted by the protein, of the proximal His-18 side chain coupled to the domed cyt *c* heme. This effect, however, is less pronounced than in the sensor protein CooA[3].

Other prominent spectral changes in TR[3] spectra (Figure 1B versus Figure 1D,E) are (i) the transformation of the unresolved contour in ∼365−430 cm^{-1} range into two broad bands and (ii) disappearance of the band at 569 cm^{-1}. The former contour contains $\delta(C_\beta{-}C_a{-}C_b)$ and $\delta(C_\beta{-}C_a{-}S)$ bending modes of thioether substituents[12] participating in cyt *c*-specific interaction of the heme with cysteine side chains. The latter band is the out-of-plane pyrrole folding mode γ_{21}, which is activated because of protein-induced heme distortion; this mode is known to disappear when the heme geometry is relaxed.[12] Thus, the observed spectral changes indicate, for the cyt *c* five-coordinated transient heme structure, both an alteration in cysteine coupling and a relaxation of the protein-induced deformations of the heme macrocycle.

Concerning temporal evolution of the photoinduced changes, our TR[3] data are in a general accord with the reported TA kinetic measurements.[6] The instrument-limited rise of the ν_{Fe-His} (Figure 1C,D) implies axial ligand photolysis in the femtosecond time scale. The photoproduct spectra change modestly between $\Delta t = 1$ ps and $\Delta t = 2$ ps. The main difference is the red shift of the porphyrin ν_8* band (ground-state ν_8-mode[12] frequency is 345 cm^{-1} (Figure 1A)), from 329 to 333 cm^{-1} (Figure 1D,E). This shift can be associated with the heme cooling during the first few picoseconds, as deduced from TR[3] spectra in the high-frequency region of porphyrin marker bands (data not shown), where the effect is most pronounced. The reported recombination time of ∼6 ps[5,6] is fully supported here as the photoproduct TR[3] spectrum is almost

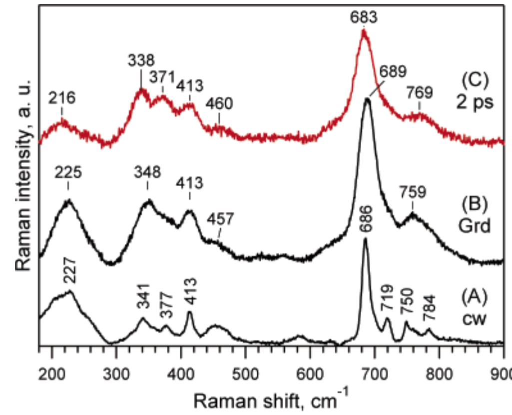

Figure 2. Same as in Figure 1 but for ferrous cyt *c* N-fragment (residues 1−56), in buffer pH 9. Spectrum C is a difference spectrum at $\Delta t = 2$ ps.

completely vanished at $\Delta t = 15$ ps (Figure 1F). Unfortunately, a quantitative Raman kinetic analysis is complicated by the changes in Raman excitation profiles due to spectral evolution of the photoproduct absorption bands during the first few picoseconds.[14]

Finally, to identify unequivocally the side chain that is photodissociated in native cyt *c*, a specially prepared[10] cyt *c* N-fragment has been studied (Figure 2). The ground-state spectrum (Figure 2A) recorded with cw excitation at 441.6 nm is in accord with the results of a recent study,[11] which demonstrates that, at pH 9, cyt *c* N-fragment exists predominantly as a six-coordinated bis-histidine complex, but with a small proportion of a five-coordinated monohistidine form. Thus, the ground-state N-fragment heme structure differs from that of the native protein, as reflected in the marked steady-state spectral differences (Figure 2A,B versus Figure 1A,B). By contrast, the photoproduct spectra, originated exclusively from the five-coordinated hemes, are almost identical (Figure 2C and Figure 1E). Since, for N-fragment, the five-coordinated species can only be a heme−His complex, the same structural state is obtained for native cyt *c*, thus confirming the photodissociation of Met-80 distal side chain.

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