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Editor's Choice

An Intermediate Conformational State during Ligand Binding to Cytochrome *c* Oxidase Detected by Time-resolved Resonance Raman Analyses of Heme Peripheral Groups

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A time-resolved resonance Raman analysis shows that the vinyl stretching band (1629 cm^{-1}) of the O_2-binding heme of cytochrome *c* oxidase shifts to 1627 cm^{-1} instantaneously upon photolysis of CO and remains for at least 5 ms before reaching the static band (1626 cm^{-1}). Within the same time scale, an intermediate vinyl bending mode of another heme appears at 435 cm^{-1}. These results suggest that both hemes cooperatively control O_2 binding by forming an intermediate conformation for effective proton pumping.

Cytochrome *c* oxidase (CcO), the terminal enzyme of the respiratory chain in mitochondria, catalyzes the reduction of dioxygen to water with simultaneous translocation of protons across the mitochondrial inner membrane. The electrochemical potential thus generated is utilized to synthesize ATP. CcO has four redox-active metal centers, Cu_A, heme *a*, heme a_3, and Cu_B.[1] Cu_A accepts electrons from cytochrome *c* and transfers them via heme *a* to the heme a_3–Cu_B binuclear center where four-electron reduction of dioxygen to water takes place.

CO is a potent inhibitor of CcO and provides an excellent probe for investigating the function of the O_2 reduction site of CcO. Photocleavage of the Fe–CO bond in CcO occurs at a fast rate with a quantum yield of almost unity. By taking advantage of the phenomenon of photodissociation of CO, the process of dissociation of CO has been extensively studied by time-resolved vibrational spectroscopy.[2,3] The conditions for such experiments include a first light pulse for photodissociation of CO and a second light pulse to probe the resonance Raman (RR) scattering at a certain delay time after the first pulse.[3]

Findsen et al. reported that the Fe–His stretching ($\nu_{Fe\text{–}His}$) frequency exhibits biphasic relaxation after photodissociation of the CO-bound fully reduced form (FRCO).[3] The $\nu_{Fe\text{–}His}$ mode is located at 222 cm^{-1} between 10 and 200 ns after photodissociation of CO (first phase) and then exhibits a downshift to 215 cm^{-1}, the frequency of the fully reduced (FR) form. The second phase is completed within 50 μs. The first phase has been ascribed to a conformational change of the protein occurring at a location distant from the heme, and the second phase is initiated by relaxation of the heme pocket.[3] In this work, we conducted time-resolved RR measurements for photodissociated CcO, focusing on the dynamics of the heme peripheral groups, to obtain further insights into the structural relaxation of the heme.

In a comparison of the RR spectrum of FRCO with that of FR, we noticed small but significant frequency changes (1–3 cm^{-1}) of Raman bands assignable to the heme peripheral groups at 1668, 1629, and 435 cm^{-1}. The 1668 cm^{-1} band has been assigned to the C=O stretching band of the formyl group of heme a_3.[4] The 1629 cm^{-1} band has been assigned to the vinyl C=C stretch. However, it has not been determined which of the hemes provides the vinyl band.[4] The 435 cm^{-1} band has been assigned to ferrous heme *a* by a spectral isolation technique,[5] but no assignment to the specific heme structure has been given. On the other hand, the bands at 436 and 405 cm^{-1} of deoxy myoglobin have been assigned to the 2-vinyl and 4-vinyl bending vibrations, respectively, on the basis of reconstitution experiments with selectively deuterated hemes.[6] Thus, the 435 cm^{-1} band is assignable to the vinyl bending vibration of heme *a*. The C_β–CH=CH_2 bending and C_β–CH=O bending modes (where C_β denotes the carbon atom of pyrrole ring, to which the vinyl group is attached) are expected to have similar frequencies. Therefore, the band at 435 cm^{-1} in heme *a* is assignable either to the 4-vinyl or 8-formyl bending vibration of the porphyrin ring.

CcO was isolated from bovine heart muscle according to a previously published method.[7] A sample solution containing 80 μM CcO in 100 mM sodium phosphate buffer, pH 6.8, containing 0.2% *n*-decyl-β-D-maltopyranoside and 0.9 M $(NH_4)_2SO_4$ was used as a Raman internal intensity standard. A screw cap NMR tube (inner diameter 4.8 mm) was used for Raman measurements and was spun at a rate of 15 rpm. After anaerobic reduction by 10 mM $Na_2S_2O_4$, CO was introduced to make the final concentration of 96 μM to prepare the FRCO form. Time-resolved RR spectroscopy was performed using two pulsed lasers at 532.0 and 435.7 nm (stimulated Raman light obtained by focusing 532 nm pulse to H_2 gas) with a 7 ns pulse width operated at 30 Hz. The delay time (Δt) of the probe pulse (435.7 nm) from the pump pulse (532.0 nm) was adjusted to a value between 10 ns and 5 ms. Pulse energies of the pump and probe pulses were adjusted to 0.3 mJ and 32 μJ, respectively. The two pulses were focused on the sample with a cylindrical lens. A 135° back-scattering configuration was adopted to collect Raman scattering. The Raman scattered light was dispersed by a single polychromator (Horiba-SPEX-Jovon Yvon, 500M) and detected with a liquid nitrogen-cooled CCD detector (Princeton Instruments, LN/CCD-1340-40). Raman shifts were calibrated using indene and CCl_4 as frequency standards. The band frequencies were determined by fitting simulations assuming a Gaussian band shape as shown in Figures 1 and 2. All experiments were conducted at 23 °C. Since the energy of the pump pulse was sufficiently high for photodissociation of CO from CcO in all the orientations in the solution, no effect of the rotational relaxation of CcO molecule was observed. In fact, the pump pulse at parallel and perpendicular polarization

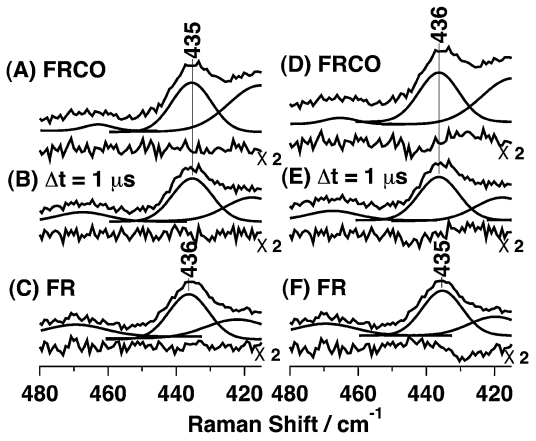

Figure 1. RR spectra of CcO in the lower frequency region. FRCO (A, D), transient form ($\Delta t = 1\,\mu s$ after CO photolysis) (B, E), and FR (C, F). From the top in each figure, the experimental spectrum, the assumed Gaussian bands, and the residual spectrum are shown. The residual spectrum was obtained by subtracting Gaussian bands from the experimental spectrum in each of A, B, C, D, E, and F. Fitting simulations were performed using a Gaussian band centered at 435 cm^{-1} for Figures A, B, and F and at 436 cm^{-1} for Figures C, D, and E.

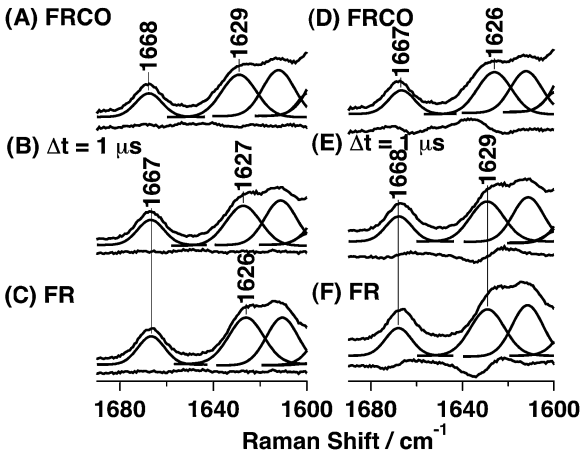

Figure 2. RR spectra of CcO in the higher frequency region. FRCO (A, D), transient form ($\Delta t = 1\,\mu s$ after CO photolysis) (B, E), and FR (C, F). From the top of each figure, the experimental spectrum, the assumed Gaussian bands, and the residual spectrum are shown. The residual spectrum was obtained by subtracting the Gaussian bands from the experimental spectrum in each of A, B, C, D, E, and F. Fitting simulations were performed using Gaussian bands centered at 1668 cm^{-1} for Figures A, E, and F and at 1667 cm^{-1} for Figures B, C, and D for the $\nu_{CH=O}$ mode (see text). Fitting simulations were performed using Gaussian bands centered at 1629 cm^{-1} for Figures A, E, and F, at 1627 cm^{-1} for Figure B and at 1626 cm^{-1} for Figures C and D for the $\nu_{C=C}$ mode (see text).

directions with respect to that of the probe pulse yielded identical time-resolved RR spectra.

Time-resolved RR measurements for photodissociated CcO were conducted for the three photosensitive bands at 1668, 1629,

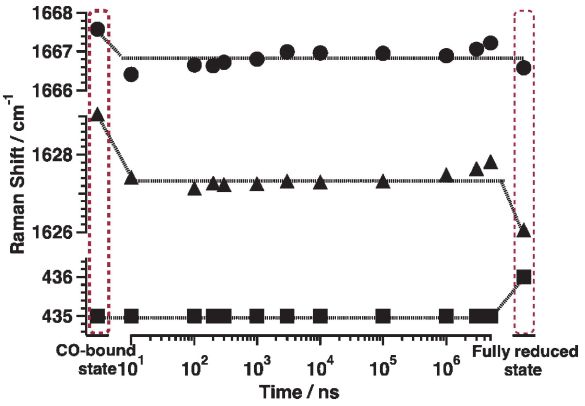

Figure 3. Plots of the frequencies of the three RR bands against a delay time; closed circles, 1668 cm^{-1}; closed triangles, 1629 cm^{-1}; closed rectangles, 435 cm^{-1}. The dotted rectangles on the left and right sides represent the FRCO state and the FR state, respectively. The dotted lines in the plot are drawn to facilitate the viewing of the change.

and 435 cm^{-1}, as described above. The time-resolved RR spectra obtained at $\Delta t = 10$, 100, 200, 300 ns; 1, 3, 10, 100 μs; 1, 3, 5 ms were measured in addition to the spectra of the FRCO and FR forms. No rebinding of CO was detectable under these experimental conditions. Figure 1 depicts selected RR spectra of the FRCO form (A), a transient form (B, $\Delta t = 1\,\mu s$), and the FR form (C) of CcO, in the lower frequency region. The flat residual spectra of FRCO (A), a transient form (B), and FR (C) show that these curve fittings are successful. In order to show the reliability of the band determination, the best fitting results fixing the band positions, shifted by 1 cm^{-1} from the band positions determined in A, B, and C are given in D, E, and F, respectively. The residual spectra in D, E, and F clearly show unsuccessful curve fitting. Thus, the resolution of the band determination under the present conditions is higher than 1 cm^{-1}. Figure 2 indicates that the reliability of the curve fitting in the higher frequency region is as high as that in the lower frequency region shown in Figure 1.

Figure 3 is a plot of the frequencies of the three Raman bands against the delay time. The frequencies of the $\nu_{CH=O}$ mode at 1668 cm^{-1} and the $\nu_{C=C}$ mode at 1629 cm^{-1} for the FRCO form both exhibit a downshift by 1 and 2 cm^{-1}, respectively, upon photodissociation of CO ($\Delta t = 10$ ns). This downshift is ascribed to the increase in the π^* electron density on the double bonds (CH=O or C=C) of the unligated transient form relative to those of the FRCO form. Indeed, the π^* electron densities on the porphyrin and conjugated CH=O and C=C bonds are somewhat decreased in the FRCO form relative to the FR form due to π-back donation of electrons to CO through Fe. The downshift of the 1629 cm^{-1} band upon photodissociation provides strong support for the assignment of the band to the $\nu_{C=C}$ mode of the 4-vinyl group of heme a_3.

The frequency of the $\nu_{CH=O}$ mode at 1667 cm^{-1} is constant in the time range between $\Delta t = 10$ ns and 5 ms and is identical to the value for the FR form. On the other hand, the frequency of the $\nu_{C=C}$ mode at 1627 cm^{-1} is constant in the time range between 10 ns and 5 ms but exhibits a downshift to 1626 cm^{-1} by 1 cm^{-1} beyond 5 ms. The frequency of the band at 435 cm^{-1} for FRCO is unchanged after photodissociation of CO up to

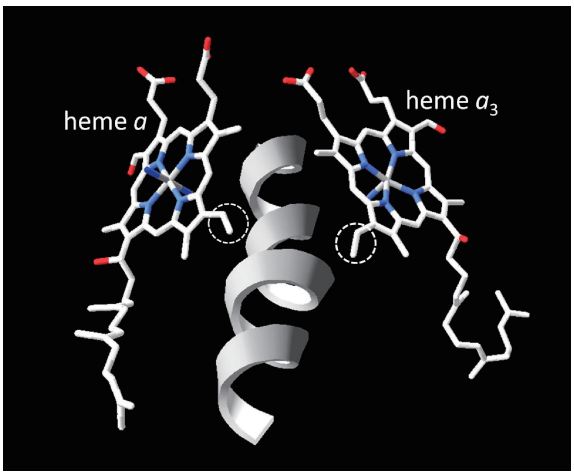

Figure 4. Crystal structure of the heme sites and helix X (see text). CcO is in the FR form. Coordinates are from 2EIJ (PDB). Broken circles indicate the vinyl groups.

5 ms, but it exhibits an upshift to 436 cm^{-1} by 1 cm^{-1} for FR. The time profile of the 435 cm^{-1} band after 10 ns is essentially identical to that of the $\nu_{C=C}$ mode of heme a_3.

As described above, the 435 cm^{-1} band is assignable either to the vinyl or to the formyl group of heme *a*. The time profile provides strong evidence that the conformational change of the group after 5 ms is tightly coupled with the conformational change in the vinyl group of heme a_3. Figure 4 depicts the crystal structure of heme *a*, heme a_3, and helix X in the FR form, showing the relative positions of the hemes and the helix. An X-ray structural analysis of bovine heart CcO shows that upon release of CO, a significant conformational change is induced in helix X, which is located between the two heme planes. The conformational change includes the bulge structural transition.[8] The two vinyl groups of the hemes are located near the bulge structures. Thus, the frequency shift of the 435 cm^{-1} band which occurs after 5 ms is expected to be triggered by the structural change of heme a_3 upon release of CO via the helix X to the vinyl group of heme *a*. In other words, it is more appropriate to assign the band to the vinyl group than the formyl group because the latter is located far from the bulge structure.[8] The upshift of the 4-vinyl bending mode is indicative of the increased interaction between the vinyl group and the protein surroundings and is ascribable to a geometric change of the vinyl group due to the decreased distance. Such an upshift of a bending mode ($\delta_{Fe\text{–}C\text{–}O}$) due to increased steric effects has been reported for a heme model complex.[9] The present study reveals that reduction of the distance takes place after 5 ms.

The previously reported dynamics of the Fe–His bond takes place in the microsecond time domain,[3] and the time domain of the dynamics found in the present study is quite different. We think that the difference is due to the different characters of the Fe–His bond and the vinyl group. The Fe–His bond is perpendicular with respect to the heme plane. On the other hand, the vinyl group is almost in parallel with the heme plane. The previous and present data suggest that the structural changes occurring at the Fe–His bond and the vinyl group are independent and that the time domains of these changes are quite different (50 μs and later than 5 ms).

The present study has been performed using CO as the external ligand to heme a_3. Although CO is not the physiological ligand (O_2), we believe that the mechanical response of heme a_3 upon binding of CO mimics that of O_2. The present results suggest that the O_2-binding process which occurs during normal physiological turnover includes a conformational change that produces the intermediate species which is also formed during the CO-photolysis process. The conformational change appears to activate the reactivity of Fe with respect to the external ligand (O_2). The conformational change could be controlled by various factors (for example, the protonation state of the proton-loading site of the proton pump system) in order to transfer O_2 to the Fe center with appropriate timing and conditions. It should be noted that both heme *a* and heme a_3, which drive the process of proton pumping and O_2 reduction, respectively, contribute to the triggering of this conformational change.

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