Infrared Evidence for Cu_B Ligation of Photodissociated CO of Cytochrome *c* Oxidase at Ambient Temperatures and Accompanied Deprotonation of a Carboxyl Side Chain of Protein

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Cytochrome *c* oxidase (CcO) catalyzes the reduction of dioxygen to water coupled with proton pumping.[1] The mammalian enzyme contains four redox active metal centers: Cu_A, heme *a*, and heme a_3-Cu_B binuclear center. The heme a_3 is the site of O_2 reduction, but CO also binds to it. The electron transfer between the heme $a-Cu_A$ and heme a_3-Cu_B centers after photolysis of heme a_3-CO has been probed by visible absorption[2] and resonance Raman (RR) spectroscopy.[3] Alben et al.[4] demonstrated with FTIR that photodissociated CO forms a stable complex with Cu_B below 140 K, and Dyer et al.[5] determined the lifetime of the transient Cu_B-CO at ambient temperatures to be ∼1.5 μs from transient IR spectroscopy. The heme pocket relaxation after CO photolysis was investigated with time-resolved RR (TR^3) spectroscopy by Babcock and co-workers,[6] who noted the absence of geminate recombination of photodissociated CO in a 10-ns time scale.

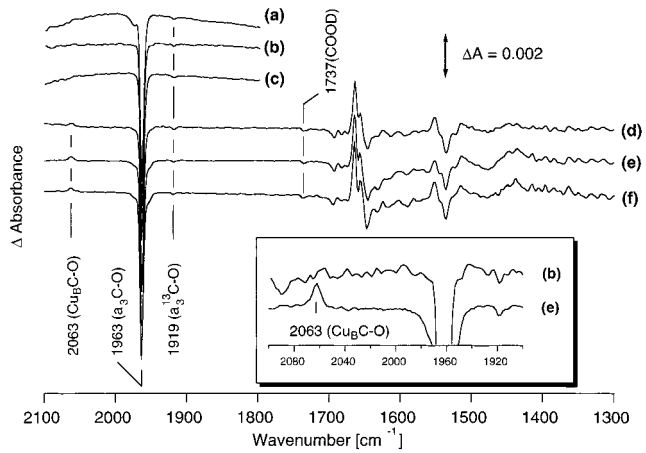

Figure 1. "Light" minus "dark" FTIR difference spectra in the photo-steady-state of CO-bound form of fully reduced bovine cytochrome *c* oxidase (CcO−CO) in H_2O and D_2O at 20 °C. (a) pH 6.8, (b) pH 7.7, (c) pH 9.0, (d) pD 6.8, (e) pD 7.4, (f) pD 9.2. "Light" means CcO under continuous illumination of 590 nm CW laser light (100 mW, focused to 3 mmϕ at the sample) generated by an Ar laser-pumped dye laser with rhodamine 6G. Uniform irradiation of laser light was attained with a step-indexed optical fiber. While one spectrum consists of 100 scans, the spectra for light and dark were measured alternately, and 16 to 24 spectra were averaged to improve the signal-to-noise ratio. Spectral resolution was 2 and 4 cm^{-1} for the H_2O and D_2O solutions, respectively. The ordinate of spectra are normalized with the ν_{CO} intensity of photodissociated heme a_3-CO. Sample concentrations, 1−2 mM in heme a_3 and path length, 20 or 50 μm. Inset shows the expanded spectra of (b) and (e). Spectral observation for the H_2O solution in the 1750 to 1300 cm^{-1} region was not successful due to strong absorption by H_2O.

To explore the role of Cu_B in the proton-pumping function of CcO, effects of H_2O/D_2O exchange on the transient binding of CO to Cu_B were examined by FTIR and a tunable IR diode laser spectrophotometer designed for time-resolved IR measurements.[7] Unexpectedly, the experimental results indicate that the lifetime of Cu_B-CO is much longer in D_2O than in H_2O and that migration of CO from heme a_3 to Cu_B provided a negative peak at 1737 cm^{-1} which is ascribed to deprotonation of a carboxyl group.

Figure 1 shows the FTIR "light" minus "dark" difference spectra observed at 20 °C. Under light conditions, the fully reduced CO-bound enzyme is in the photo-steady-state under continuous illumination of the 590-nm CW laser light in 1 atom of CO. The large negative peak at 1963 cm^{-1} arises from heme a_3-bound CO, and its frequency remained unchanged between H_2O and D_2O and also between pH 6.8 and 9.0, in agreement with Einarsdóttir et al.[8] A positive peak appears at 2063 cm^{-1} in D_2O (curves d−f), but not in H_2O (curves a−c). This band is

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(7) The apparatus is essentially the same as those reported in ref 5a and in *Spectrosc. Int.* **1990**, *2*, 29−35 by Mäntele, W.; Hienerwadel, R.; Lenz, F., Riedel, W. J.; Grisar, R.; Tacke, M. Briefly, IR probe light from a CW-tunable diode laser was introduced to the sample, and the transmitted light was passed through a monochromator and detected by a sensitive HgCdTe element ($D^* > 2 \times 10^{11}$ cm(Hz)$^{1/2}$ W^{-1}) with a narrow field-of-view (10°) cold stop. Temporal signal differences following photodissociation of CO−CcO by a nanosecond, 532-nm laser pulse operated at 10 Hz were digitized with a 12-bit AD converter and transferred to a computer. The recombination of photolyzed CO−CcO (τ = 15 ms) was assumed to be completed during the photolysis interval (100 ms). Typically the signals from 1024 or 4096 shots were averaged and converted to time-dependent absorbance data. The same measurements were repeated at different wavenumbers in a point-to-point manner. The highest spectral quality obtainable was attained by searching the best lasing mode of the diode laser with careful control of temperature and/or injection current. All of the apparatuses were controlled by a computer through a GPIB interface, which improved the quality of data significantly.

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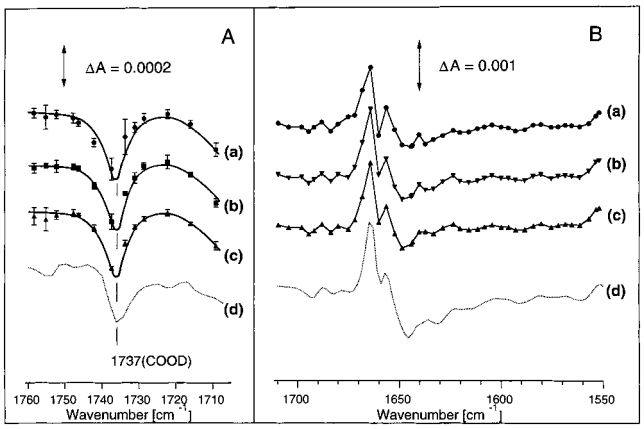

Figure 2. Time-resolved IR spectra of CcO−CO at 20 °C observed for the delay times of 10 μs (a), 100 μs (b), and 1 ms (c) after photolysis by a nanosecond laser. Spectrum (d) is reproduced from (e) in Figure 1. The 532-nm pulse from a Nd:YAG laser (10-ns width, 10 Hz) was used as a pump light (1 mJ/pulse, focused to 3 mmϕ), and its power was adjusted so as to keep the photolysis level of 60%. $^{13}C^{18}O$ was used to observe the ν_{CO} band of Fe−CO (1864 cm^{-1}) with the diode laser. Sample conditions; pD 7.4, concentration, 2.2 mM for (A) and 2.9 mM for (B). Cell thickness, 100 μm for (A) and 26 μm for (B). One point in a spectrum is an average of 1024 shots, and the error bars for (A) were obtained from six repeated measurements.

attributed to the C−O stretch (ν_{CO}) of Cu_BCO as found at low temperatures by Alben et al.[4] Noticeable spectral changes owing to CO photodissociation are observed in the amide I region in D_2O (spectra d−f), but this region for the H_2O solution was difficult to observe because of strong absorption by H_2O. In addition to these changes, a small but definite negative peak is observed at 1737 cm^{-1}.

Dependence of the intensity of this band on laser power was the same as that of the intensity reduction of the Fe_{a_3}-bound CO band. Free glutamate in D_2O, for example, showed absorption bands at 1710 and 1617 cm^{-1} at pD 3 and at 1614 and 1566 cm^{-1} at pD 7, suggesting that the deprotonation of a side-chain carboxyl group apparently causes a band shift from 1740−1710 to 1590−1560 cm^{-1}. The latter is assigned to the anti-sym CO_2^- stretch, which is stronger than the sym stretch around 1450−1400 cm^{-1}. A positive peak seems to exist around 1550−1600 cm^{-1} in spectra (d−f); however, many protein bands are overlapped there, and the present signal-to-noise ratios do not allow the assignment of the anti-sym CO_2^- band with confidence. The 1737 cm^{-1} band intensity relative to that of the Fe_{a3}-bound CO band in comparison with intensities of the corresponding bands of general R-COOD[9] suggests that the number of deprotonated COOD groups per heme a_3−CO would be less than one. The spectral changes detected in D_2O were not influenced by pD in the range examined (6.8−9.2), indicating that pK_a of this COOD is not in this range.

Figure 2 shows time-resolved IR difference spectra (A, 1760−1710 cm^{-1}; B, 1710−1560 cm^{-1}) of bovine fully reduced CcO−CO following photolysis by a nanosecond laser pulse (532 nm), which were measured with the time-resolved IR spectrophotometer.[7] The spectral features for the delay times of 10 μs (curve a) to 1 ms (curve c) are alike and almost the same as the FTIR spectrum of photo-steady-state (curve d). The 1737 cm^{-1} band due to deprotonation of a COOD group is already seen at 10 μs following the photolysis, but a positive peak was not recognized near 1737 cm^{-1}. On the basis of the recombination rate under the present conditions (τ = 15 ms, determined in this study), the average photodissociation level (70−80%) for heme a_3−CO, and the intrinsic IR intensity ratio of the bands of FeCO to CuCO (determined to be 10:1[4]), the observed intensity ratio of the 1963 to 2063 cm^{-1} bands (1:0.02) suggests that the lifetime of Cu_BCO in D_2O is ca. 1.5 ms, which is 10^3 times longer than that in H_2O.[5]

It is unlikely that the extremely long lifetime of Cu_B−CO in D_2O compared with that in H_2O is due to the stability of CO bound to Cu_B, since the ν_{CO} frequency is unaffected by H_2O/D_2O exchange. Presumably the size of an exit channel of CO from the binding site is smaller in D_2O due to stronger hydrogen bonds of the deuterated protein. In H_2O, it has been established that CO dissociation does not perturb the reaction of O_2 with the enzyme.[10] In D_2O, however, the long-lived Cu_B−CO could retard not only the initial binding of O_2 but also the electron transfer in the later steps.

The room-temperature FTIR experiments on *Paracoccus denitrificans* cytochrome *aa*$_3$ gave a positive and a negative peak at 1742 and 1730 cm^{-1}, respectively, in the redox-induced difference spectra, and they were ascribed to an environmental change of protonated Glu 278.[11] The redox difference spectrum can be distinct from the present ligation difference spectrum in a single redox state. On the other hand, the low-temperature FTIR experiment on *Escherichia coli* cytochrome *bo*$_3$ gave a positive and a negative peak at 1731 and 1724 cm^{-1}, respectively, in the light minus dark difference spectra, and both were ascribed to Glu 286.[12] However, as stated above, no positive peak was detected near 1737 cm^{-1} for bovine CcO. This discrepancy might be due to the difference in the temperature between the two experiments. It is likely in the low temperature that when CO is transferred from $Fe_{a_3}^{2+}$−CO to Cu_B^{1+}, the protein conformation changes near Glu 242 (Glu 286 in cytochrome *bo*$_3$) influence only hydrogen bonding to the COOH but are not large enough to deprotonate the group.

In all of the crystal structures of bovine heart cytochrome *c* oxidase so far obtained,[13] i.e., those for the fully oxidized, fully reduced CO-bound and fully oxidized azide-bound forms, Glu 242 is hydrogen-bonded to Met 71. The crystal structure of the fully reduced form with CO at Cu_B^{1+} and the practical trigger for deprotonation remain to be determined. Further studies are under progress in this laboratory.

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