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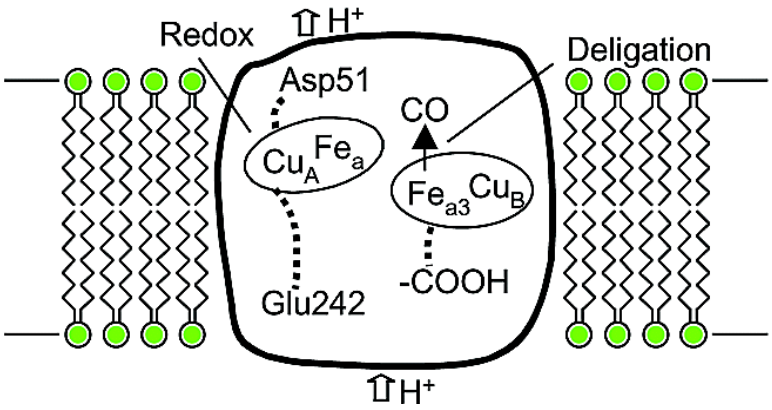

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FTIR Detection of Protonation/Deprotonation of Key Carboxyl Side Chains Caused by Redox Change of the Cu_A-Heme *a* Moiety and Ligand Dissociation from the Heme a_3-Cu_B Center of Bovine Heart Cytochrome *c* Oxidase

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Abstract: FTIR spectral changes of bovine cytochrome *c* oxidase (CcO) upon ligand dissociation from heme a_3 and redox change of the Cu_A-heme *a* moiety (Cu_AFe_a) were investigated. In a photosteady state under CW laser illumination at 590 nm to carbonmonoxy CcO (CcO−CO), the C−O stretching bands due to $Fe_{a3}^{2+}CO$ and $Cu_B^{1+}CO$ were identified at 1963 and 2063 cm^{-1}, respectively, for the fully reduced (FR) state [$(Cu_AFe_a)^{3+}Fe_{a3}^{2+}Cu_B^{1+}$] and at 1965 and 2061 cm^{-1} for the mixed valence (MV) state [$(Cu_AFe_a)^{5+}$-$Fe_{a3}^{2+}Cu_B^{1+}$] in H_2O as well as in D_2O. For the MV state, however, another band due to $Cu_B^{1+}CO$ was found at 2040 cm^{-1}, which was distinct from the α/β conformers in the spectral behaviors, and therefore was assigned to the $(Cu_AFe_a)^{4+}Fe_{a3}^{3+}Cu_B^{1+}CO$ generated by back electron transfer. The FR-minus-oxidized difference spectrum in the carboxyl stretching region provided two negative bands at 1749 and 1737 cm^{-1} in H_2O, which were apparently merged into a single band with a band center at 1741 cm^{-1} in D_2O. Comparison of these spectra with those of bacterial enzymes suggests that the 1749 and 1737 cm^{-1} bands are due to COOH groups of Glu242 and Asp51, respectively. A similar difference spectrum of the carboxyl stretching region was also obtained between $(Cu_AFe_a)^{3+}Fe_{a3}^{2+}Cu_B^{1+}CO$ and $(Cu_AFe_a)^{5+}Fe_{a3}^{2+}Cu_B^{1+}CO$. The results indicate that an oxidation state of the (Cu_AFe_a) moiety determines the carboxyl stretching spectra. On the other hand, CO-dissociated minus CO-bound difference spectra in the FR state gave rise to a positive and a negative peaks at 1749 and 1741 cm^{-1}, respectively, in H_2O, but mainly a negative peak at 1735 cm^{-1} in D_2O. It was confirmed that the absence of a positive peak is not caused by slow deuteration of protein. The corresponding difference spectrum in the MV state showed a significantly weaker positive peak at 1749 cm^{-1} and an intense negative peak at 1741 cm^{-1} (1737 cm^{-1} in D_2O). The spectral difference between the FR and MV states is explained satisfactorily by the spectral change induced by the electron back flow upon CO dissociation as described above. Thus, the changes of carboxyl stretching bands induced both by oxidation of (Cu_AFe_a) and dissociation of CO appear at similar frequencies (∼1749 cm^{-1}) but are ascribed to different carboxyl side chains.

Introduction

Terminal oxidases in respiration chains perform an active proton transport through energy transducing membranes during the catalytic reduction of dioxygen.[1−3] The enzyme for mammalian and bacterial cases is named cytochrome *c* oxidase (CcO) and generally contains four redox-active metal centers called Cu_A, heme *a* (Fe_a), heme a_3 (Fe_{a3}) and Cu_B, in the order of electron transfer. Although four electrons are transferred from cytochrome *c* to CcO to reduce one oxygen molecule bound to Fe_{a3} to water, four protons are translocated across mitochondrial inner membranes against a gradient of proton concentration. Although the number ratio of electrons to protons transferred is 1:1,[4] every electron transfer is not always accompanied by translocation of a proton. The timing of the proton release and a coupling mechanism between electron and proton transfers are under debate.[5−7] Because carboxyl side chains of proteins

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are most likely candidates for proton carriers, it is quite important to find the redox- or ligation-coupled protonation/deprotonation of carboxyl groups in mechanistic studies of proton pumping.

X-ray crystallographic analysis has been completed for CcOs from bovine heart,[8,9] *Paracoccus denitirificans*,[10] *Thermus thermophilus*,[11] and recently, a pathway of proton translocation is discussed from the structures.[12] Because an orientation of carboxyl side chain of Asp51 of the bovine enzyme is different between the fully reduced and oxidized states, this residue was thought to play a key role in proton pumping,[13] and the pathway through Asp51 is named 'H-channel'.[12] On the other hand, two other pathways are proposed from mutagenesis studies on *P. denitrificans* CcO;[14,15] One includes Lys354 → Thr351 → Tyr280 (K channel)[15] and the other includes Asp124 → Glu278 (D channel).[14] In all of these proposals, protonation/deprotonation of carboxyl side chains has been considered to serve as a possible gate for proton translocation.

IR spectroscopy is a powerful tool to investigate protonation/deprotonation of carboxyl groups and has been extensively applied to terminal oxidases.[16−32] Its combination with an IR-transparent electrode revealed the redox dependent frequency shifts of protonated carboxyl groups;[18−21] from 1748 (oxidized) to 1734 cm^{-1} (reduced) for *P. denitrificans* CcO, which was attributed to Glu278,[19] and from 1745 to 1735 cm^{-1} for *E. coli* cytochrome bo_3, which was attributed to Glu286.[25] For a bovine heart enzyme, two peaks were found at 1745 and 1737 cm^{-1} for the oxidized form.[20,29,31] Previously, we investigated the ligand-induced protonation/deprotonation of the bovine enzyme with IR spectroscopy for CO photolysis from Fe_{a3}, and pointed out the occurrence of deprotonation of a carboxyl side chain, which gives a band at 1737 cm^{-1} in D_2O at a room temperature.[17] In contrast, Rich et al.[24,30] measured a similar photolysis-difference spectrum for a bovine enzyme in H_2O solution and explained the observed frequency shift from 1749 to 1742 cm^{-1} in terms of a status change of a protonated carboxyl side chain. Mantele and co-workers,[27] who examined *P. denitrificans* CcO at 268 and 84 K, observed a single negative peak for the mode at 1746 cm^{-1} (1740 cm^{-1} in D_2O) upon CO photolysis, that may simply indicate deprotonation, and ascribed it to Glu278 on the basis of mutagenesis. Woodruff and co-workers[26,28] examined *E. coli* cytochrome bo_3 and found a shift of peak from 1731 to 1724 cm^{-1} in D_2O, which was assigned to Glu286. Heberle et al.[29] stressed different behaviors in the carboxyl stretching region of IR spectra between bacterial and mammalian enzymes. Thus, the observations reported are not always consistent. It is the aim of this study to finalyze the IR experimental part on the protonation/deptrotonation behaviors of bovine CcO caused by redox change of the Cu_A-heme *a* or ligand dissociation from heme a_3. Here occupations of electrons by Cu_A and heme *a* are not distinguished and accordingly, the reduced and oxidized forms of this moiety are denoted as $(Cu_A\text{-}Fe_a)^{3+}$ and $(Cu_AFe_a)^{5+}$, respectively.

Materials and Methods

CcO was purified from bovine heart as described previously,[33] and stored at −80 °C until use. The enzyme was dissolved in 50 mM potassium phosphate (Wako Pure Chemicals) buffer (pH 7.4) containing 0.2% (w/v) *n*-decyl-*β*-D-maltoside (Anatrace). To prepare the fully reduced CO-bound enzyme, CcO was reduced by addition of a small amount of dithionite (Wako Pure Chemicals) under N_2 or CO atmosphere. Mixed valence CO-bound form was prepared by overnight incubation of oxidized CcO with CO in the complete absence of O_2 at room temperature as reported.[34,35] Solvent exchange from the H_2O buffer to the D_2O buffer was performed through several repetitions of dilution by D_2O buffer followed by concentration of the solution.

For thorough deuteration, the enzyme was incubated in D_2O buffer for 7 days at 7 °C in one hand, and was subjected to enzymatic turnover in the D_2O buffer containing the detergent at pD 7.4 on the other. The enzymatic turnover experiment was performed in an Eppendorf tube containing CcO, horse cytochrome *c* (Sigma) and sodium ascorbate (Wako Pure Chemicals) with the concentrations of 100 μM, 200 μM, and 100 mM, respectively, for CO photodissociation measurements. In the case of photoreduction experiments, 100 μM phenazine methosulfate (Wako Pure Chemicals) and 100 mM NADH (Oriental yeast) were used instead of cytochrome *c* and ascorbate, respectively. Under this condition, CcO was reduced immediately. Accordingly, fresh air was introduced into the tube every 30 min while the tube was kept with a Vortex for 3 h at room temperature. After the reaction, CcO was washed with the same buffer three times using Centricon, and then subjected to IR measurements. Although this sample contained a small amount of remaining cytochrome *c*, the difference spectra between photodissociated and CO-bound forms were not affected by it. To see the IR spectral changes in the early stage of the deuteration process,

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the concentrated enzyme solution was diluted by 10-fold volume of the D_2O buffer and its IR spectra were measured without concentration using a high sensitivity measurement system with a specifically improved preamplifier.

IR spectra were measured with a Shimadzu FTIR 8100A or JASCO FT/IR-610 spectrometer with a MCT detector as reported previously.[17] To detect an absorbance change as small as 10^{-5}, we strove to reduce noise levels of the FT/IR-610 spectrometer through replacement of its preamplifier with an audio MC head amplifier (Nihon Audio, MC-20). This efforts appreciably raised S/N ratios and enabled us to measure a spectrum with S/N ratios as high as 20 000/1 ($\sim 2 \times 10^{-5}$ Abs) in 100 scans. The spectra presented are an average of 20 records and one record consists of 200 scans. In all measurements, the optical pathway was kept in a vacuum (inside the interferometer of FT/IR-610) or purged with N_2 gas and the sample temperature was maintained at 20 °C. The spectral resolution was 4 cm^{-1}.

The sample was introduced to the airtight IR cell, which consists of two CaF_2 windows with variable thickness between 6 and 80 μm, with a gastight syringe without contaminating air. The pump light for photodissociating CO from carbonmonoxy CcO (CcO−CO) was generated with an Ar^+ ion laser (Spectra Physics, model Stabilite 2017)-pumped dye laser (Spectra Physics, Model 375) operating with rhodamine 6G (Tokyo Chemical Industry) and its wavelength was adjusted to 590 nm. The laser output with ca. 100 mW was guided to the sample point with an optical fiber and focused to 5 mmϕ on the CaF_2 window except for the variation experiments of laser power, in which a laser power was reduced by placing a neutral density filter between the entrance of the optical fiber and the laser. Laser irradiation to a sample under this condition yielded a photosteady state in which about 80% of the enzyme were photodissociated. Although the absolute spectra were varied with every measurement due to changes of refractive index and water absorption, differences between CO-bound and -dissociated states were stably reproduced except for the 1600−1700 cm^{-1} region of H_2O solutions. Therefore, the results are represented in terms of the light (laser irradiated)-minus-dark (no irradiated) difference spectra; the positive and negative peaks reflect the CO-dissociated and -rebound states, respectively.

Since the difference spectrum in the 1600−1700 cm^{-1} region of H_2O solution was somewhat distorted due to heating[36] by laser irradiation (not for the D_2O solution due to the absence of solvent band), a very thin cell (thickness $\approx$ 6 μm) was used and the heating effect was corrected in the following way; the light-minus-dark difference spectrum was observed for CO-unbound CcO in the same IR cell and its absorbance at 590 nm was determined using the laser light from the optical fiber. This light-minus-dark difference spectrum is purely caused by laser heating and should be proportional to absorbance at 590 nm for individual samples. Accordingly, a factor to be multiplied was determined from the absorbance of individual samples at 590 nm and this difference spectrum multiplied by the multiplication factor was subtracted from the individual light-minus-dark difference spectra observed for the CO-bound form. When the difference spectrum thus obtained has an inclined baseline, it was corrected by subtraction of a polynomial function fitted to the experimental baseline curve.

To measure the redox difference spectra under an identical cell condition, we adopted the method of photoreduction using flavin and EDTA, in which flavins are photoreduced by laser irradiation in the presence of EDTA,[37] and the photoreduced flavins reduce CcO.[23] The concentrations of flavins (riboflavin was used) and EDTA were 20 μM and 23 mM, respectively. For D_2O solutions, the EDTA-containing enzyme solution was left standing for an overnight before laser irradiation. Regarding the mixed valence state, the EDTA-containing enzyme solution was incubated with CO for an overnight for both H_2O and D_2O solutions. The irradiation light at 441.6 nm, obtained from a

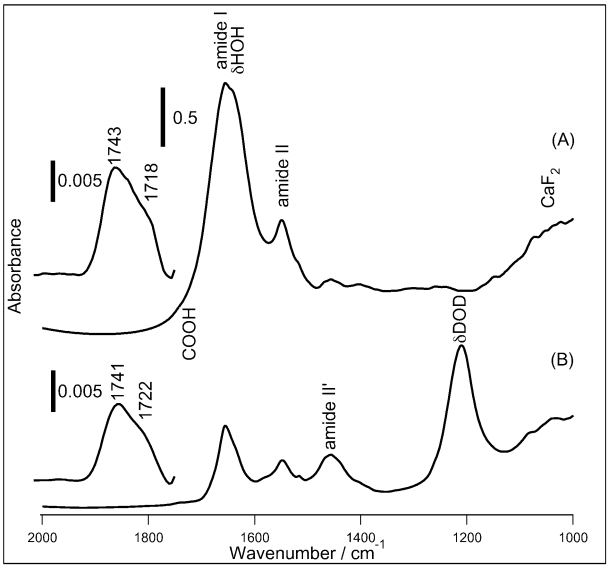

Figure 1. Absolute IR absorption spectra of fully reduced bovine cytochrome *c* oxidase at 20 °C. (A) H_2O solution at pH 7.4 measured with a cell of 18.0 μm thick; enzyme concentration, 0.98 mM. (B) D_2O solution at pD 7.4 measured with a cell of 17.2 μm thick; enzyme concentration, 0.72 mM. Both are an average of 5 spectra of 100 scans. Insets show the 100 fold expanded spectra in the 1700−1750 cm^{-1} region which were corrected by subtracting the eleventh order polynomial function fitted to the baseline.

He−Cd laser (Kinnmon Electrics, Model CD4805R), was lead with an optical fiber to the sample point, where the laser power was about 39 mW. Normally, the sample was irradiated for 30 s and then subjected to IR measurements. The duration time was determined by a trial and error method, and 30 s was the shortest duration sufficient to reduce the sample completely. Anaerobic laser irradiation yielded the fully reduced CcO and that under CO atmosphere yielded the fully reduced CO-bound form.

Results

Ligand Dissociation in the Fully Reduced State. Figure 1 shows the absolute IR absorption spectra in the 2000 to 1000 cm^{-1} region of CcO in H_2O (A) and D_2O (B). Inclined baselines at lower wavenumbers are due to absorption by CaF_2. Strong absorption bands are present around 1650 (amide I), 1550 (amide II), 1440 cm^{-1} (amide II′), 1640 (HOH bending, δ_{HOH}) and 1220 cm^{-1} (δ_{DOD}). The tail part of the amide I band was fitted with an eleventh order polynomial function and its subtraction from the observed spectrum revealed the presence of weak absorption around 1750−1700 cm^{-1} as demonstrated by the inset, which are provided by protonated carboxyl groups. The main peak is located at 1743 and 1741 cm^{-1} for H_2O and D_2O solutions, respectively, and their absorbances are roughly one hundred's of that of amide I. The presence of this band is easier to identify for the D_2O (B) than H_2O (A) solutions owing to the frequency shift of the water deformation mode. The integrated intensity ratio of this band to that of amide I suggests that eleven carboxyl groups per molecule are contained in this band, indicating that 7% of the total number of aspartates (Asp) and glutamates (Glu) are protonated at pH 7.4. The peak frequencies are higher than those of free aspartic and glutamic acids.[38,39]

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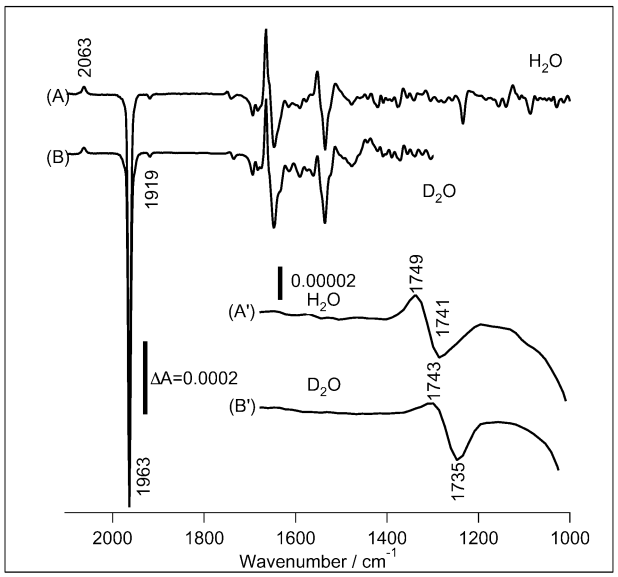

Figure 2. Light-minus-dark difference spectra of fully reduced CcO in H_2O (A) and D_2O buffer solutions. The H_2O and D_2O spectra were obtained with cells of 6 and 20 μm thick, respectively, whereas spectra are normalized with the 1963 cm^{-1} band of Fe-bound CO. The H_2O spectrum was corrected to remove the thermal artifact from the raw spectra as mentioned in Materials and Method. Inset; A′ and B′ represent the expansion of A and B in the designated frequency region.

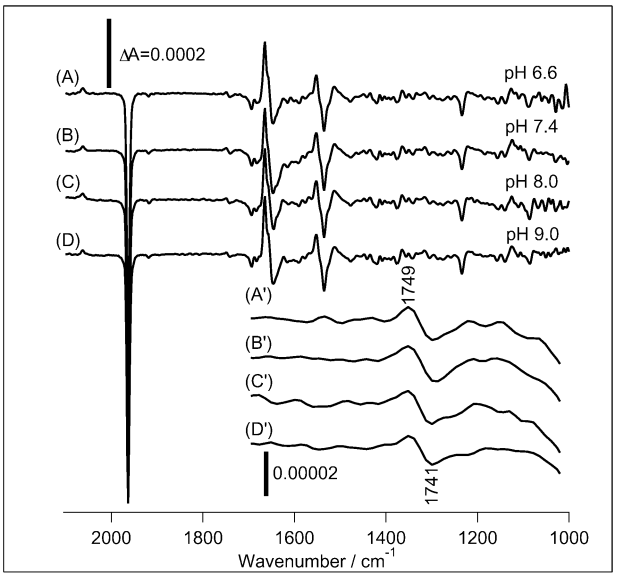

Figure 3. pH dependence of the light-minus-dark difference spectra of fully reduced CcO in H_2O solutions. The cell of 6 μm thick was used and thermal effects were corrected as mentioned in Materials and Method. The inset shows the expanded spectra of (A) through (D) in the carboxyl stretching region.

Figure 2 shows the light-minus-dark difference spectra of fully reduced CcO−CO in H_2O (A) and D_2O (B). A negative peak at 1963 cm^{-1} and a positive peak at 2063 cm^{-1} are due to the C−O stretching of $Fe_{a3}^{2+}CO$ and $Cu_B^{1+}CO$, respectively.[40] In our previous measurements,[17] the 2063 cm^{-1} band of Cu_BCO was identified only for D_2O solutions, but improved S/N ratios of the present experiment revealed the presence of this band in the photosteady state of H_2O solution, too. A small negative peak at 1919 cm^{-1} is due to Fe_{a3}-bound ^{13}CO of natural abundance. The spectra in the carboxyl stretching region are expanded by five times in the inset (A′ and B′). It is apparent that a positive and negative peaks appear at 1749 and 1741 cm^{-1}, respectively for the H_2O solution (A′) and at 1743 and 1735 cm^{-1} for the D_2O solution (B′). Note that the positive peak for the D_2O solution is significantly weak. The intensity of the negative peak is 20−40% less than that expected for one COOH group per CcO molecule, if the absorption coefficient of free Glu or Asp[38,39] is used for estimation.

The pH dependences of these spectra in the H_2O solution are displayed in Figure 3, where the spectral intensities are normalized to the negative peak at 1963 cm^{-1}. Single positive and negative peaks were observed at 2063 and 1963 cm^{-1}, respectively, in the pH range between 6.6 and 9.0. The absence of the pH dependent conformational change for the bovine enzyme is consistent with the observation by Einarsdottir et al..[41] Thus, a bovine CcO is distinct from a bacterial CcO which yielded the pH dependent two positive and negative bands for both $Fe_{a3}CO$ and Cu_BCO with apparent pK_a at 7.3.[42] It is also evident that the intensities and frequencies of the 1749/1741 cm^{-1} difference peaks do not depend on pH for H_2O solutions in the pH range between 6.6 and 9.0. Therefore, this carboxyl group must be placed under so hydrophobic environment that it can be protonated even at pH 9.0. In D_2O solutions, on the other hand, the behavior was complicated. The positive peak was shifted to a lower frequency with significantly reduced intensity, while the negative peak appeared clearly. This is the reason we reported only the negative peak for D_2O solutions in the previous study.[17] If deuteration of the carboxyl side chain in question is very slow, then the positive peak of the deuterated component may be canceled by the negative peak of remaining undeuterated component, and as a result the intensity of the positive peak in the deuterated spectrum might appear weaker than that expected from the negative peak of deuterated component. If so, then the spectrum in D_2O would depend on time.

However, the reduced intensity of the positive peak was not changed by prolonged incubation in D_2O and even after enzymatic turnover in D_2O as demonstrated in Figure S1 in the Supporting Information, where the spectra after simple incubation in D_2O for one through 7 days and that after turnovers in D_2O are depicted. The differential pattern of the 1749/1741 cm^{-1} bands for the H_2O solution can be interpreted by the idea that the environment around a protonated carboxyl group are changed by CO-dissociation. However, the asymmetric appearance of the 1743/1735 cm^{-1} difference pattern for D_2O cannot be explained by such a simple idea. Rich and Breton[30] also noted that the carboxyl group yielding the positive peak was hard to deuterate, although in their experiment deuteration appreciably progressed after turnovers in D_2O.

If the carboxyl group yielding the positive peak is hard to deuterate, the ratio of the positive to negative peaks would not be proportional to the H_2O/D_2O ratio of solvent. So same measurements were carried out for mixed solvents. As shown in Figure S2, the result from the mixing ratio of H_2O/D_2O = 1/1 was quite close to the spectrum calculated as one-half of sum of the spectra observed for the pure H_2O and D_2O solutions. The integrated intensity of the positive peak is smaller than that

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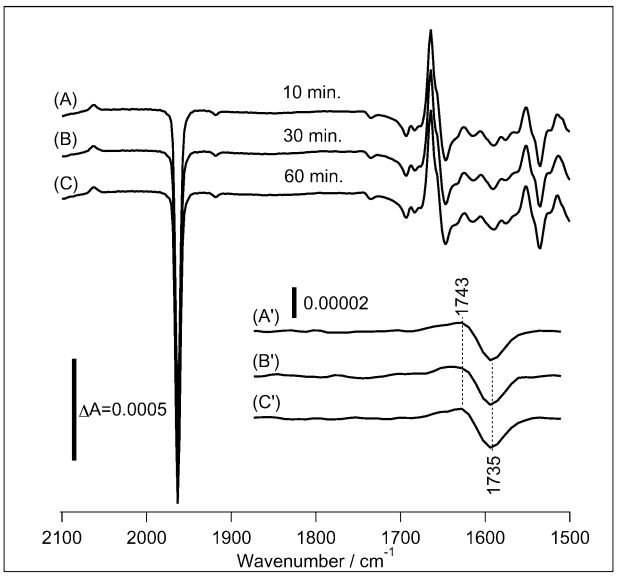

Figure 4. Light-minus-dark difference spectra in the early stage of deuteration. Each spectrum was measured at a designated time following dilution by 9-folds in volume of D_2O buffer. The baseline in the carboxyl stretching region was fitted with a sixth order polynomial function and it was subtracted from the observed spectra. They are expanded in the spectra shown in the inset: Cell thickness, 80 μm.

of the negative peak similarly in the observed and calculated spectra, suggesting that the deuteration is complete and already in equilibrium with solvent having a given molecular ratio of H_2O/D_2O.

To examine the kinetic difference between the positive and negative peaks in the early stage of deuteration, the H_2O sample was diluted with 9-fold volume of D_2O buffer and the diluted CcO was immediately incorporated into the IR cell. The light-minus-dark difference spectra at 10, 30, and 60 min after dilution are illustrated in Figure 4 (A−C). Because the spectra in the carboxyl stretching region are distorted, baselines were fitted with a 6th order polynomial, and the baseline-corrected spectra are displayed in the inset (spectra A′−C′). In spectrum A′ a negative peak is clearly seen at a frequency lower by 6 cm^{-1} than that for the H_2O solution but a positive peak is very weak.

Laser power dependence of IR spectral intensity was examined and the results are shown in Figure 5, where the light-minus-dark difference spectra for a variety of laser powers are depicted. The laser power is represented in terms of the fraction (%) of the maximum power, because the power was reduced by placing a neutral density filter. The difference spectrum for the maximum power is drawn with a contraction of the ordinate scale by a factor of 8 at the top, in which the peak at 1963 cm^{-1} is not saturated. The relative peak intensities of the $Fe_{a3}{}^{12}CO$ (1963 cm^{-1}) to the natural abundant $Fe_{a3}{}^{13}CO$ (1919 cm^{-1}) bands can be estimated by this spectrum. The negative peak of photodissociated $Fe_{a3}{}^{13}CO$ at 1919 cm^{-1} can be identified even with 2% of the maximum laser power but that of Cu_BCO at 2063 cm^{-1} is already missing with 5% of the maximum power. The difference peak at 1749/1741 cm^{-1} is clearly seen even with 2% of the maximum laser power. The intensities of the Cu_BCO, $Fe_{a3}{}^{12}CO$, and $Fe_{a3}{}^{13}CO$ bands and the valley to peak height of the 1749/1741 cm^{-1} bands are plotted against the laser power in the inset. The intensity behaviors of the 1749/1741 cm^{-1} bands seem to parallel roughly those of $Fe_{a3}CO$ but not those of Cu_BCO. The same results were

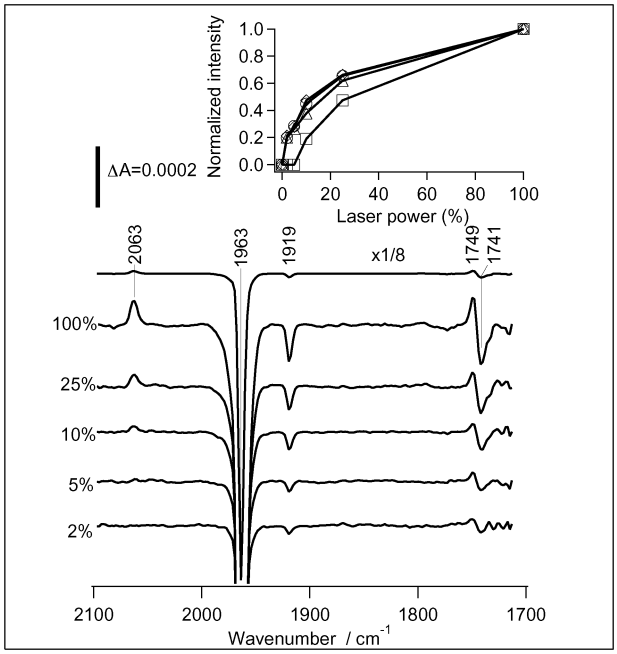

Figure 5. Laser power dependence of the light-minus-dark difference spectra of CcO−CO. The laser power is reduced by neutral density filters and accordingly the laser powers are represented in terms of a fraction of the maximum power (no filter). The top spectrum is the same as the 100% spectrum, but the ordinate scale is contracted by a factor of 8 to show the relative intensities of the $Fe^{12}CO$ (1963 cm^{-1}) and $Fe^{13}CO$ (1919 cm^{-1}) bands. The peak intensities of individual peaks are plotted against the laser power in the inset where the intensities are fraction of those of the maximum laser power; circle: $Fe^{12}CO$, square: Cu_BCO, triangle: COOH, diamond: $Fe^{13}CO$.

obtained with D_2O solutions (not shown). These observations suggest that it is the dissociation of CO from the Fe_{a3} atom which triggers the spectral change of the carboxyl group. This is contradictory to the conclusion from the time-resolved measurement by Heberle and co-workers,[29] who noted that the trigger is binding of CO to Cu_B.

Ligand Dissociation in the Mixed Valence State. It is quite important to determine whether ligand dissociation from Fe_{a3} causes environmental changes of a single protonated carboxyl group or simultaneous protonation and deprotonation of different carboxyl groups. So we examined the mixed valence (MV) state, which was generated by incubation of oxidized CcO with CO. The sample was transferred to the IR cell in the complete absence of oxygen, so that the CO-photodissociated enzyme could not react with a trace amount of contaminated oxygen during the measurement. On the other hand, repeated laser irradiation for the MV state $[(Cu_AFe_a)^{5+}Fe_{a3}{}^{2+}Cu_B{}^{1+}]$ may photoreduce the enzyme to the fully reduced (FR) state $[(Cu_AFe_a)^{3+}Fe_{a3}{}^{2+}Cu_B{}^{1+}]$, because CcO is photoreducible.[43,44] Because the C−O stretching band of $Fe_{a3}{}^{2+}CO$ is sharp and its frequency is slightly different between the FR (1963 cm^{-1}) and MV states (1965 cm^{-1}),[45,46] the difference spectrum between the CO-bound forms of the sample before and after the photodissociation experiments could reveal the extent of photoreduction, if any. If a trace amount of the 1963 cm^{-1} band is seen, the spectrum of the FR state was subtracted from the

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observed MV spectrum so that the intensity of the 1963 cm^{-1} shoulder of $Fe_{a3}^{2+}CO$ becomes zero. Thus, the light-minus-dark difference spectra shown below are expected to reflect the effect of CO dissociation from heme a_3 of the purely mixed valence state.

Figure 6(A) displays the light-minus-dark difference spectra of MV CcO in H_2O (b) and D_2O (c) in comparison with that of the FR CcO in H_2O (a) observed under the same experimental conditions. In the inset, the spectra in the C−O stretching region are expanded in the abscissa scale but contracted in the ordinate scale. It is evident that the C−O stretching band of $Fe_{a3}^{2+}CO$ is shifted to a higher frequency for the MV state by 2 cm^{-1} than that for the FR state, in agreement with the previous reports.[45,46]

As demonstrated in Figure 6B, the expansion of the Cu_B-bound C−O stretching spectra in the ordinate scale revealed the presence of two positive peaks at 2061 and 2040 cm^{-1} for the MV state (b′,c′), which were shifted to 2015 and 1994 cm^{-1}, respectively with ^{13}CO (not shown), while a single peak was observed at 2063 cm^{-1} for the FR state (a′). The 2061 cm^{-1} band of the MV state reflects a main component of $Cu_B^{1+}CO$, whose frequency is lower by 2 cm^{-1} than that of the FR state. This is in agreement with the results for *P. denitrificans* cytochrome aa_3 at 84 K, although the corresponding bands were absent at 268K for both the H_2O and D_2O solutions.[27]

The presence of a CO-isotope sensitive band around 2040 cm^{-1} was previously noticed for the FR state of *R. sphaeroides* CcO in the pH region below 7.0. Therefore, pH dependence of the Cu_BCO band was examined carefully and the results are summarized in Figure 7, parts A and B for the FR and MV states, respectively, where the light-minus-dark difference spectra at pD 6.4 (a), 7.4 (b), and 9.0 (c) are depicted. For the FR state (A), there is no trace of absorption around 2040 cm^{-1} at any of pD 6.4, 7.4, and 9.0, while its presence for the MV state (B) is evident. There was no other Fe-bound C−O stretching band except for the 1965 cm^{-1} band at these pH values. Thus, it became clear that the presence of the 2040 cm^{-1} band is specific to the MV state and distinct from the pH dependent appearance of the β confomer.[42] The frequency difference of 21 cm^{-1} means a fairly large perturbation at the binuclear center.

It is known that a part of electrons are back transferred from Fe_{a3}^{2+} to $(Cu_AFe_a)^{5+}$ upon dissociation of CO from Fe_{a3} in the MV state.[47−49] Because localization of an electron on the Cu_A and Fe_a atoms are not distinguished in this experiment, it is deduced that an appreciable amount of $(Cu_AFe_a)^{5+}$ would stay in the $(Cu_AFe_a)^{4+}$ state in a photosteady state of the CO-bound MV species under 590 nm irradiation. Then, $(Cu_AFe_a)^{4+}Fe_{a3}^{3+}$-$Cu_B^{1+}CO$ would be generated instead of the ordinary MV state, $(Cu_AFe_a)^{5+}Fe_{a3}^{2+}Cu_B^{1+}CO$. The C−O stretching frequency of Cu_BCO will be distinctly different between the two cases in which Fe_{a3}^{2+} or Fe_{a3}^{3+} is present near CO; the C−O bond would be weaker when Fe_{a3}^{3+} is located near the oxygen atom than the case in which Fe_{a3}^{2+} is present there, because the $C^{\delta+}$-$O^{\delta-}$ polar structure would be more stabilized for Fe_{a3}^{3+}. Therefore, it is reasonable that the 2040 cm^{-1} band arises from the electron back-transferred species. Thus, the electron back transfer should not be neglected in interpretation of the photosteady-state spectra of the CO-bound MV state as will be discussed below.

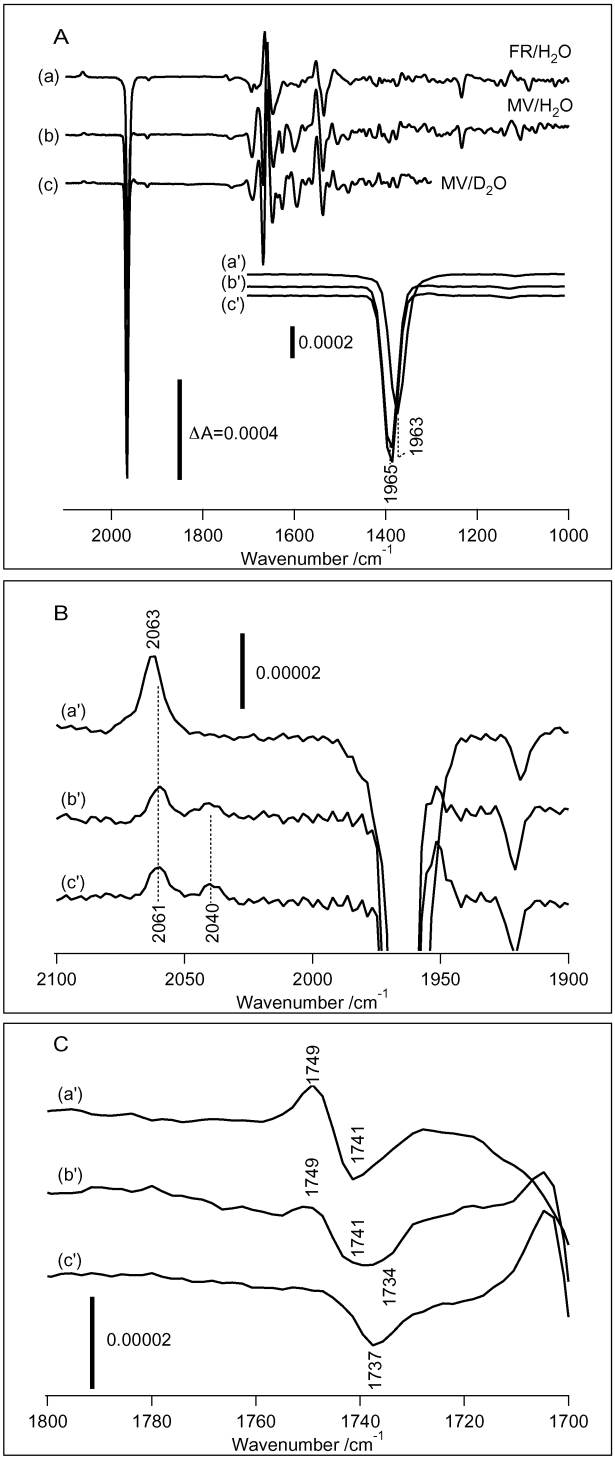

Figure 6. Light-minus-dark difference spectra of mixed valence CcO−CO in H_2O and D_2O solutions. (A) whole region together with the expansion in the Fe(a_3)-bound C−O stretching region. (B) Expansion of spectra in the Cu_B-bound C−O stretching region. (C) Expansion of spectra in the carboxyl stretching region. (a and a′) Fully reduced (FR) state in the H_2O solution (reference spectra). (b and b′) Mixed valence (MV) state in the H_2O solution. (c and c′) MV state in the D_2O solution. The spectra for the H_2O and D_2O solutions were obtained with the cells of 6 and 20 μm thick, respectively.

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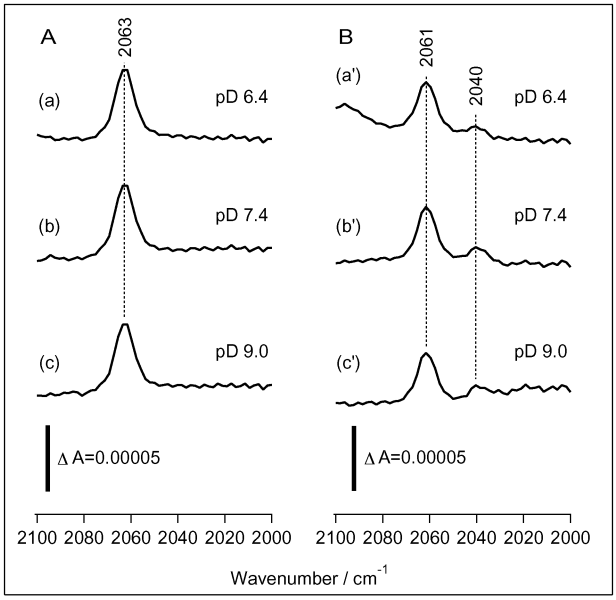

Figure 7. pD dependence of the C−O stretching spectra of Cu_B-bound CO for the fully reduced (A) and mixed valence (B) states of bovine CcO. These are a part of light-minus-dark difference spectra in which the ordinate scales are normalized with the 2063 (2061) cm^{-1} band. The cell path-length was 20 μm. Spectra (a,a′), (b,b′) and (c,c′) were observed for D_2O solutions at pD 6.4, 7.4, and 9.0, respectively.

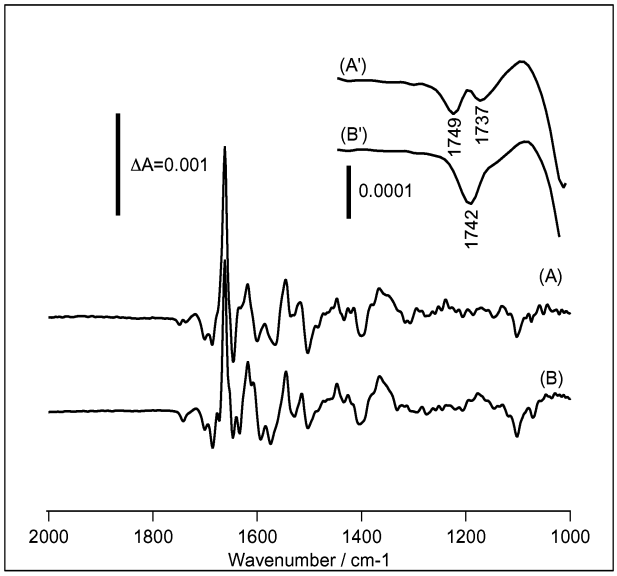

Figure 8. Fully reduced-minus-fully oxidized difference spectra of bovine CcO in H_2O (A) and D_2O (B). The oxidized enzyme was photoreduced in the presence of riboflavin (20 μM) and EDTA (23 mM) by laser illumination for 30 s at 441.6 nm (39 mW). Inset shows the expanded spectra in the carboxyl stretching region. Cell thickness, 6.75 μm for (A) and 9.09 μm for (B).

The accompanied IR spectral changes in the carboxyl stretching region are depicted in Figure 6C. In contrast with the presence of a positive and a negative peak for the FR CcO in H_2O (a′), mainly a negative peak was observed for the MV state at 1741 and 1737 cm^{-1} for the H_2O (b′) and D_2O (c′) solutions, respectively. While the negative peak for the H_2O solution is broad and another peak might be present around 1734 cm^{-1}, the peak frequencies of the negative peaks for the H_2O and D_2O solutions are close to those of the FR state.

These results were essentially the same in the pH range between 6.6 and 9.0 (results not shown). The early deuteration process was examined by 9-fold dilution of the MV CcO−CO with D_2O buffer as done for the FR state, and the results are displayed in Figure S3, where the light-minus-dark difference spectra at 5, 30, and 60 min after dilution are illustrated together with the expanded spectra in the inset. As evident in Figure S3, the negative peak was immediately shifted to a lower frequency by 6 cm^{-1} (A′), whereas a positive peak was absent. The appearance of only a negative peak for the MV state was reported previously for *P. denitrificans* cytochrome aa_3 in both H_2O and D_2O solutions.[27] The laser power dependence of IR spectral intensity was investigated for the MV state and the same results as that of the FR state (Figure 5) were obtained. This confirms that the spectral change of this carboxyl group is triggered by release of a ligand from Fe_{a3} but not by binding of a ligand to Cu_B (data not shown).

Redox Difference Spectra. Because X-ray crystallographic analysis pointed out a significant structural change of Asp51 between the oxidized and fully reduced states,[13] we tried to identify the spectral contribution of Asp51 to the carboxyl stretching region in IR spectra. It is extremely difficult to prepare independently the fully reduced and oxidized samples which have an identical experimental condition. Therefore, we adopted the photoreduction technique to prepare the reduced enzyme.[23] An IR spectrum is measured for the oxidized CcO solution containing flavin and EDTA first, and subsequently for the photoreduced one which was obtained by the 30 s laser irradiation to the oxidized sample. The reduced-minus-oxidized difference spectra thus obtained for H_2O (A) and D_2O solutions (B) are shown in Figure 8, where the carboxyl stretching region is expanded by 50-fold in the inset (spectra A′ and B′). It is apparent that two absorption bands are present at 1749 and 1737 cm^{-1} for the oxidized form in H_2O, which are apparently merged into a single band centered at 1742 cm^{-1} in D_2O.

The thoroughly deuterated enzyme was prepared as mentioned in the Experimental and its redox difference spectrum was examined independently. The thorough deuteration did not yield another band at around 1725 cm^{-1}. The redox difference spectrum for an H_2O solution is in agreement with that reported by Heitbrink et al.[29] who emphasized that the redox difference spectra in the carboxyl region are distinctly different between bovine and *R. sphaeroides* CcOs and much weaker for the former. The presence of two peaks for an H_2O solution also agree with the results of Hellwig et al.[20] although they found additional peak at 1726 cm^{-1} for a D_2O solution.

The same technique was applied to the mixed valence CO-bound form under CO atmosphere. In this case, photoreduction occurs to Cu_A-heme *a*, and accordingly, the resultant state was the fully reduced CO-bound form. The reduced-minus-oxidized difference spectrum is expected to reflect the difference between $(Cu_AFe_a)^{3+}$ and $(Cu_AFe_a)^{5+}$, whereas Fe_{a3} and Cu_B are retained in the CO-bound form. The difference IR spectra thus obtained for H_2O (A) and D_2O solutions (B) are illustrated in Figure 9. Because the C−O stretching frequency of $Fe_{a3}^{2+}CO$ differs between the FR and MV states, a sharp differential pattern appears in the C−O stretching region, in agreement with the results given above. The spectra in the carboxyl stretching region are expanded by 50 times in the inset (spectra A′ and B′). Again two negative peaks were observed at 1749 and 1737 cm^{-1} for the H_2O solution, which are apparently merged into a single band centered at 1741 cm^{-1} in D_2O, although its component

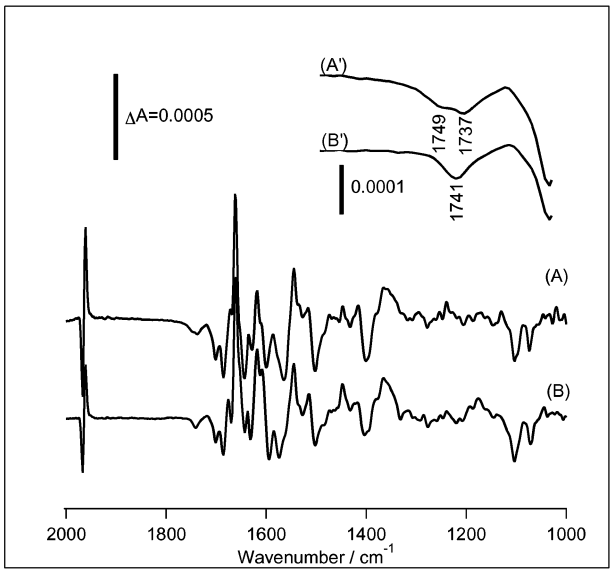

Figure 9. Fully reduced-minus-mixed valence difference spectra of bovine CcO−CO in H_2O (A) and D_2O (B). The mixed valence CcO−CO was prepared by incubation of oxidized CcO under dark and CO atmosphere in the presence of riboflavin (20 μM) and EDTA (23 mM). The fully reduced form was prepared by the 30 s illumination of laser light at 441.6 nm (39 mW) to the mixed valence sample. The cell thickness was 7.15 μm for (A) and 9.28 μm for (B).

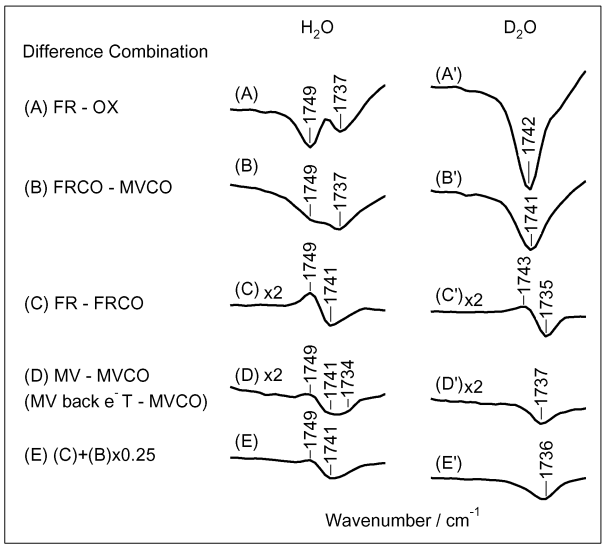

Figure 10. Comparison of the IR difference spectra in the carboxyl stretching region of bovine CcO in H_2O (left) and D_2O buffers (right). The ordinate intensities are normalized to the intensity of photodissociated C−O stretching peak around 1965−1963 cm^{-1} (CO is incorporated into the cell after this measurement in the case of A). The combinations for difference measurements are specified at the left side; FR: fully reduced state, OX: fully oxidized state, MV: mixed valence state, FRCO: CO bound form of fully reduced state. MVCO: CO bound form of mixed valence state, e^-T: electron transfer; A from Figure 8, B from Figure 9, C from Figure 2, D from Figure 6C. Electron back transfer is taken into consideration in a parenthesis. (E) Calculated spectra, $(E) = (C) + (B) \times 0.25$ and $(E') = (C') + (B') \times 0.25$.

bands might possibly be located at 1743 and 1739 cm^{-1} in consideration of the present spectral resolution. There was no peak around 1725 cm^{-1} for the D_2O solution even after extensive deuteration. Although the relative peak intensities of the 1749 to 1737 cm^{-1} bands in spectrum A′ are different from those in Figure 8(A′), the peak positions in Figure 9 are quite close to those in Figure 8, suggesting that the difference in the spectra of the carboxyl stretching region is associated mostly with the difference in the oxidation state of the Cu_AFe_a moiety irrespective of a state of heme a_3 and Cu_B.

Discussion

Absolute IR Spectra of CcO. The strong IR absorption due to the bending mode of water and the amide I vibration of protein makes it difficult to see directly a weak absorption due to protonated carboxyl acid side chains. However, curve fitting using an appropriate polynomial function for the tail part of the strong absorption enabled us to reveal an absorption due to the C═O stretching mode of protonated carboxyl groups at 1743 and 1720 cm^{-1} even in the raw spectra (Figure 1). This intensity suggested that ca. 7% (11 residues) of the total number of carboxylic side chains in a molecule are protonated at pH 7.4. When we assume that carboxyl side chains located inner side by one turn from both ends of the trans-membrane helices are protonated owing to hydrophobic environments, twelve residues including Asp144(I), Glu242(I), Glu474(I), Glu18(II), Glu19-(II), Asp25(II), Asp60(III), Glu90(III), Glu180(III), Glu236(III), Asp246(III), and Glu99(IV) (Greek characters denote a subunit number) could be candidates on the basis of the 3D structure of bovine CcO.[8,9] The spectral patterns in the 1700−1000 cm^{-1} region were little different between H_2O and D_2O solutions, suggesting that deuteration of protein including OH, and NH_x ($x = 1\sim3$) groups dose not proceed so much even in D_2O. The low intensity of the carboxyl peaks in the light-minus-dark difference spectra (compare the scale bars in Figure 1 with those of Figure 2) means that structural changes of carboxyl groups and their surroundings upon redox and ligation changes are very limited.

Redox Dependent Changes of Carboxyl Groups. Protonation/deprotonation of carboxylic acid side chains is important to discuss a mechanism of proton pumping by CcO, but the interpretation of results is not always straightforward. The spectra in the carboxyl stretching region observed in this study for H_2O and D_2O solutions are summarized in Figure 10. X-ray analysis revealed that location of Asp51 is different between the fully reduced and fully oxidized states;[13] Asp51 is directed toward inside and presumably protonated in the oxidized state but is exposed to solvent and deprotonated in the reduced state. This feature is specific to bovine CcO,[10,11,13] The spectral difference caused by this structural change should be contained in Figure 10A.

A likely interpretation for Figure 10A is to assign the 1737 cm^{-1} band to Asp51 as suggested by Hellwig et al.[20] who noted the absence of the corresponding band for a bacterial CcO. This band is expected from the X-ray structure[13] to disappear in the reduced state and in fact, this is satisfied. The low-frequency implies that the C═O group is hydrogen bonded. When a C═O group is hydrogen-bonded, its stretching IR absorption might be stronger than the case of nonhydrogen-bonding. One may argue against this assignment by noting that the band did not shift to a lower frequency in D_2O. It would be correct for an ordinary COOH group. However, the C═O group of Asp51 is strongly hydrogen bonded with the OH group of Ser205 and also with the peptide NH group.[13] If the C═O...H-X hydrogen bond is replaced by C═O...D−X without changing the C to X distance, the hydrogen bonding would be weaker for the deuterated form due to the zero-point energy of H(D)−X bond

and accordingly, the C═O stretching frequency would be higher in D_2O than in H_2O. Such a D_2O/H_2O difference in hydrogen bonding has been in fact observed for the O−O stretching mode of oxyhemoglobin.[50] Because the C═O stretching motion of COOH is usually accompanied by appreciable movement of the OH group, its contribution lowers the C═O stretching frequency in D_2O than that in H_2O, as seen for an ordinary carboxyl group. Whether the resultant shift of the hydrogen bonded C═O stretching frequency upon deuteration is toward an up- or down-frequency would depend on a condition. The upshift by deuteration is possible in the case of a strongly hydrogen-bonded carboxyl group.

Various bacterial CcOs show a trough near 1745 cm^{-1} and a peak near 1735 cm^{-1} in the reduced-minus-oxidized difference spectra.[18,25] The mutagenesis experiments[19,25] demonstrated that these bands are due to glutamates corresponding to Glu242 of the bovine enzyme. The peak is located close to the trough at 1737 cm^{-1} due to Asp51 in the reduced-minus-oxidized difference spectrum. Because the IR band of Asp51 would be stronger than that of Glu242 due to strong hydrogen bond, the absorption of Asp51 would prevail over that of Glu242 to give a negative peak in the reduced-minus-oxidized difference spectrum. Judging from these considerations, it seems reasonable to assign the 1749 cm^{-1} band of the oxidized bovine enzyme to Glu242, and it is shifted to ~1737 cm^{-1} in the reduced form but its intensity is canceled by the peak of Asp51 and as a result, does not appear as a positive peak.

Ligation Dependent Changes of Carboxyl Side Chains in the FR State. In the light-minus-dark difference spectrum of CcO−CO in the FR state (Figure 10C), a clear derivative-like curve was obtained with a positive and negative peak at 1749 and 1741 cm^{-1} for the H_2O solution, respectively. The spectral appearance was not altered by pH change between 6.6 and 9.0. The differential pattern could be interpreted in terms of an environmental change of a protonated carboxyl group. If the C═O group of COOH is hydrogen bonded in the ligand-bound form but released from it in the ligand free form, then the C═O stretching frequency would be lower in the ligand bound form and accordingly, the light-minus-dark difference spectrum would be just as depicted by Figure 10C. In fact, this is the interpretation for the spectral change of Glu286 of fully reduced cytochrome bo_3 upon CO dissociation,[28] and therefore, Glu242 is a likely candidate for bovine CcO.

On the basis of the results from various examinations for H_2O/D_2O exchange efficiency under the present experimental conditions as described in Results, we concluded that the asymmetric differential pattern for the D_2O solution was not due to incomplete deuteration and is therefore incompatible to the interpretation in terms of an environmental change of one protonated carboxyl group. To overcome this difficulty Rich and Breton[30] considered overlapping of more than two carboxyl groups, which give rise to IR absorption bands at a same frequency in a H_2O solution but behave differently in D_2O; they observed a light-minus-dark difference pattern similar to Figure 2A′ for the H_2O solution of CcO−CO of *P. denitrificans*, attributing it to frequency shifts of Glu242 and an unassigned residue, and noted that only one-third of the carboxyl band for H_2O solution exhibited the deuteration shift in D_2O. If more than two COOH bands are overlapped for H_2O solution and exhibit a similar amount of low frequency shifts upon ligand dissociation but only a part of them can be deuterated, then the resultant spectrum in D_2O solution would yield a very weak and broad positive peak and a clear negative peak. Although this band cannot be attributed to Glu242 in the case of FR state, which is expected to give a band around 1737 cm^{-1} for the reduced state (1749 cm^{-1} for the oxidized form), the interpretation assuming an overlap of more than two COOH groups seems most likely at the present stage.

The ligation sensitive carboxyl side chains may include the propionate side chain of heme a_3. However, Hellwig et al.[19] noted from the mutation experiments on the *P. nitrificans* enzyme that the redox dependent spectral changes in the COOH region purely arise from Glu278 but contain no contribution from heme propionates for a bacterial enzyme, at least. In fact, the ^{13}C labeled heme propionate of P. *denitrificans* cytochrome aa_3 did not yield a redox dependent difference peak in the 1700−1800 cm^{-1} region.[51] Nevertheless, we keep a possibility of heme propionates of heme a_3 as a candidate of a ligation sensitive carboxyl group for bovine CcO.

Ligation Dependent Changes of Carboxyl Side Chains in the MV State. The light-minus-dark difference spectra of mixed valence CcO−CO yielded mainly a negative peak at 1741 and 1735 cm^{-1} for H_2O and D_2O solutions, respectively, while a positive peak was extremely weak (Figure 10D). These frequencies are close to those observed as negative peaks for the fully reduced state. The results could be interpreted in a following way. The light-minus-dark difference spectra for the fully reduced and mixed valence states would be alike when no back electron transfer takes place. If the oxidation state of Fe_{a3} affects little protonation/deprotonation of carboxyl groups, then the effect of 100% back electron transfer from Fe_{a3}^{2+} to $(Cu_AFe_a)^{5+}$ can be approximated by Figure 10B for the H_2O and D_2O solutions. The amount of back electron flow from Fe_{a3}^{2+} to $(Cu_AFe_a)^{5+}$ is estimated to be ~25% by the intensity ratio of the 2040/2061 cm^{-1} bands in Figure 6B. The expected spectra in the carboxyl stretching region for the photosteady state of the mixed valence CO-bound form was calculated by the sum of Figure 10C and 25% of Figure 10B after normalization of their spectra with the C−O stretching band intensity of the CO-bound form. The simulated spectrum (Figure 10E) reproduces essential features of the experimentally obtained spectrum (Figure 10D), indicating that the apparent spectral difference between the fully reduced (Figure 10C) and mixed valence states (Figure 10D) is caused by a certain amount of electron back transfer from Fe_{a3}^{2+} to $(Cu_AFe_a)^{5+}$ and thus, protonation of another carboxyl groups needs not be postulated. It means that a redox state of the (Cu_AFe_a) moiety determines the protonation/deprotonation of particular carboxyl groups (Glu242 and Asp51 for bovine CcO). However, the Fe_{a3}-ligation sensitive carboxyl groups are different. Very recently, IR studies on the mixed valence state of cytochrome bo_3 was reported[16] and importance of a redox state of cytochrome *b* was stressed, in agreement with the present interpretation if cytochrome *b* is replaced by Cu_AFe_a.

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Conclusion

The IR band due to Cu_B-bound CO was found at 2063 cm^{-1} in photosteady state of FR CcO−CO under laser irradiation at 590 nm both in H_2O and D_2O. For the MV state, however, two CO stretching bands were observed at 2061 and 2040 cm^{-1}. The latter was ascribed to the electron back transferred species. IR bands arising from two protonated carboxyl groups with their C═O stretching modes at 1749 and 1737 cm^{-1} were detected in the redox differece spectrum. Both were shifted to 1742 ± 2 cm^{-1} in D_2O. The former and latter were assigned to Glu242 and Asp51, respectively. There are another carboxyl groups which are sensitive to ligation to heme a_3 and this may include the heme propionate. These are protonated in the reduced state, giving rise to an absorption at 1749 and 1741 cm^{-1} in the CO bound and dissociated forms in H_2O, respectively, but part of them could not be deuterated under the present experimental conditions. The corresponding changes upon ligand dissociation in the MV state were satisfactorily interpreted by consideration of back electron transfer from Fe_{a3}^{2+} to $(Cu_AFe_a)^{5+}$ under the assumption that the redox change of Fe_{a3} does not cause an IR spectral change in the carboxyl stretching region.

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Supporting Information Available: Time course of the light-minus-dark difference spectra of CcO−CO, light-minus-dark difference spectra of CcO−CO in the carboxyl stretching region in H_2O/D_2O mixed solvents, and light-minus-dark difference spectra of mixed valence CcO−CO in the early stage of dilution with D_2O buffer. This material is available free of charge via the Internet at http://pubs.acs.org.

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