

Resonance Raman Spectra of Cytochrome *c* Oxidase in Whole Mitochondria

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Resonance Raman spectra of cytochrome *c* oxidase in intact whole mitochondria were selectively observed. The Raman bands observed at 576, 517, and 369 cm^{-1} for the carbon monoxide-bound state were assigned to the δ_{Fe-C-O}, ν_{Fe-CO}, and δ_{Fe-C-O} modes, respectively, of heme a_3-CO complex based on the $^{13}C^{18}O$ isotopic frequency shifts. These frequencies are essentially the same as those observed for solubilized cytochrome *c* oxidase, which implies that the environmental structure of the dioxygen reducing site in the solubilized state is not very much altered. It is likely that the dioxygen reducing reaction studied so far for solubilized enzyme is also valid for intact whole mitochondria.

Cytochrome *c* oxidase (CcO) is the terminal respiratory component in the mitochondrial electron transfer chain. The enzyme reduces dioxygen to water. This electron transfer reaction is coupled with proton translocation across the mitochondrial inner membrane.[1] This proton motive force based on pH difference and membrane potential is utilized to synthesize adenosine-3′-phosphate. X-ray crystallographic structures have been reported for bovine[2–4] and bacterial enzymes.[5,6] The proton pumping mechanism has been discussed based on the three dimensional structures. The enzyme has four redox-active metal centers: namely Cu_A, heme *a*, heme a_3, and Cu_B. Cu_A receives electrons from cytochrome *c* and gives them to heme *a*. Heme *a* is a six-coordinated, low-spin heme and works as an electron mediator between Cu_A and the heme a_3-Cu_B binuclear site. Heme a_3 is a five-coordinate, high-spin heme and serves as a dioxygen-reducing site. Resonance Raman (RR) spectroscopy has been acknowledged as a powerful tool for investigating the micro structure of the catalytic site of hemoproteins.[7] The mechanism of dioxygen reduction by CcO has been discussed based on the structures and dynamics of the reaction intermediates.[8,9] Time-resolved RR studies on CcO have been limited for solubilized enzyme. Since native CcO exists in phospholipid bilayers, the structure and dynamics in solubilized state might be different due to the environmental difference caused by the difference of the solution conditions and proton motive force. Thus, it is important to examine if the reaction intermediates and their dynamics observed for the solubilized enzyme are also the case for CcO in whole mitochondria. To approach this final goal, we have examined the structure at the catalytic site of CcO in intact whole mitochondria. For this purpose we employed carbon monoxide (CO) as a sensitive probe for the environments of the oxygen-binding site.

Experimental

Preparation of Intact Mitochondria. Mitochondria were isolated from fresh bovine heart and were suspended in 10 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose. The method to obtain fully reduced mitochondrial samples was as follows. Anaerobic mitochondrial samples were prepared by evacuating the gas phase of the sample to 0.01 hPa and introducing pure (99.999%) nitrogen gas. This procedure was repeated three times, followed by addition of $Na_2S_2O_4$ solution. The evacuation step often resulted in slightly different concentrations of the sample due to different amounts of evaporation of water. Fully-reduced and CO-bound form was prepared by addition of CO to the fully-reduced samples.

Measurement of Respiratory Control Ratio (RCR). The oxygen consumption rate was measured polarographically with a Clark-type oxygen electrode (Yellow Springs Instruments, Model 5331) driven by a home-made electronic circuit using succinate as a respiratory substrate. Intactness of mitochondrial samples is determined by RCR as a measure. The RCR is calculated by dividing the oxygen consumption rate in the presence of adenosine-2′-diphosphate (ADP) by that in the absence of ADP. If the membrane structure is destroyed, the RCR is 1 and mitochondria are more intact for a higher value. Samples with RCR values higher than or equal to 3 were used for Raman measurements.

Resonance Raman Measurements. RR scattering was excited at 427.0 nm from a frequency doubled $Cr:LiAlF_6$ laser (Hitachi Metals, ICD-430) and dispersed with a spectrograph (Chromex, 500IS) with a focal length of 0.5 m equipped with a 500 nm blazed, 1200 grooves/mm holographic grating. In order to remove the Rayleigh scattering, a holographic notch filter was used. Dispersed light was detected with a CCD camera (Princeton Applied Research, 1530-CUV) cooled at 140 K. A spinning cell with inner diameter of 3 mm was used to avoid sample heating and decomposition. A hemi disk magnet to stir the suspension was essential to prevent sedimentation of mitochondria on the cell wall.[10] Raman shifts were calibrated with indene and CCl_4 as frequency standards.

Results

Figure 1 depicts electronic absorption spectra of whole mitochondria in the region from 400 to 700 nm. Spectrum 1A for

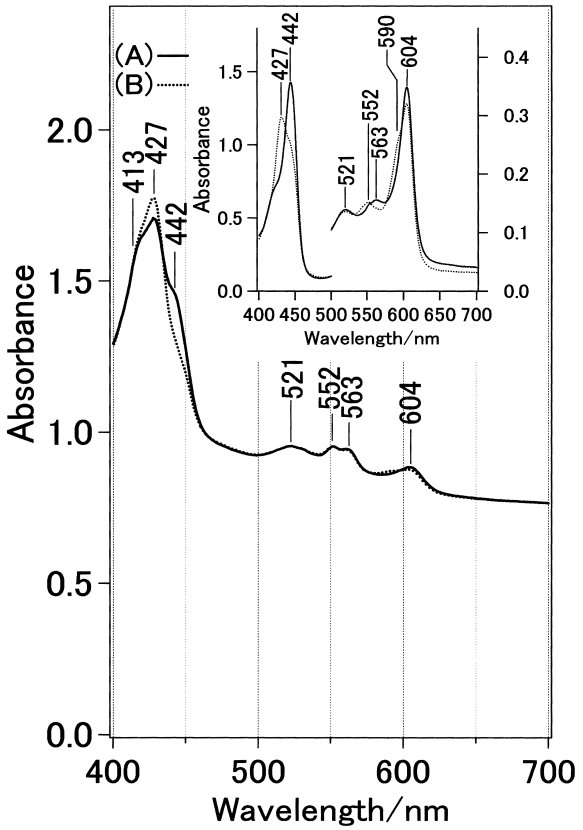

Fig. 1. Absorption Spectra of Intact Whole Mitochondria. Fully-reduced (A) and fully-reduced and CO-bound states (B). Concentration of CcO was approximately 19 μM (heme *a* basis) and path length was 2 mm. Inset depicts absorption spectra of isolated and solubilized CcO in its fully-reduced, and fully-reduced and CO-bound states. Vertical scale for wavelength region between 400 and 500 nm should be referred to the left hand axis and that for wavelength scale between 500 and 700 nm should be referred to the right hand axis, respectively.

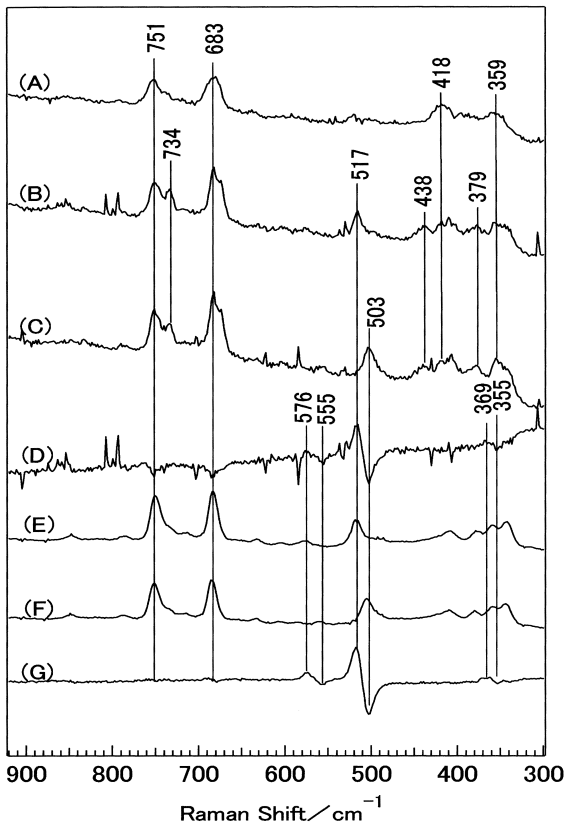

Fig. 2. Resonance Raman Spectra of Whole Mitochondria (Spectra A to D) and Solubilized CcO (Spectra E to G). Fully-reduced (A), fully-reduced and $^{12}C^{16}O$-bound (B), fully-reduced and $^{13}C^{18}O$-bound states (C), and the isotopic difference spectrum (Spectrum B − Spectrum C) (D) for mitochondria. Fully-reduced and $^{12}C^{16}O$-bound (E), fully-reduced and $^{13}C^{18}O$-bound states (F), and the isotopic difference spectrum (Spectrum 2E − Spectrum 2F) (G) for solubilized CcO.

fully reduced mitochondria gives absorption peaks at 427, 521, 552, 563, and 604 nm and shoulders at around 413 and 442 nm. These features are due to cytochromes *a*, *b*, *c*, and c_1. The assignments of the absorption bands will be discussed later. Spectrum 1B displays the absorption spectrum for fully reduced whole mitochondria in the presence of CO. The high background is due to light scattering by mitochondrial particles. The inset depicts absorption spectra of solubilized CcO in the fully reduced (solid line), and the fully reduced and CO bound states (dotted line) for comparison. Comparing Spectrum 1B with Spectrum 1A, one will note that the peaks at 427 and 552 nm increase, the shoulder at 442 nm decreases, the peaks at 563 and 604 nm decrease, and the shoulder at 590 nm increases in intensity upon CO binding. The inset shows the changes upon CO binding for the isolated and solubilized CcO, which reproduces the changes that occur for mitochondria upon CO binding; the intensities at 427, 552, and 590 nm increase and those at 442, 563, and 604 nm decrease upon CO binding. These results mean that the spectral changes that occur for mitochondria upon CO binding are due to changes that occur for CcO.

Figure 2 depicts RR spectra of intact whole mitochondria (Spectra A to D) and solubilized CcO (Spectra E to G) in the fully-reduced (Spectrum 2A), fully-reduced and $^{12}C^{16}O$-bound (Spectrum 2B), and fully-reduced and $^{13}C^{18}O$-bound states (Spectrum 2C), and the isotopic difference spectrum (Spectrum 2D = Spectrum 2B − Spectrum 2C) and fully-reduced and $^{12}C^{16}O$-bound solubilized CcO (Spectrum 2E), fully reduced and $^{13}C^{18}O$-bound solubilized CcO (Spectrum 2F), and the isotopic difference spectrum (Spectrum 2G = Spectrum 2E − Spectrum 2F), respectively. The sample conditions for Spectrum 2A of the fully-reduced state correspond to those of Spectrum 1A. The Raman bands at 751, 683, 418 and 359 cm^{-1} are due to porphyrin vibrations. Upon addition of CO, a new band at 517 cm^{-1} appears, as is seen in Spectrum 2B. In order to see if this band arises from the vibrations associated with heme-bound CO, we examined the effect of isotopic substitution of CO. Spectrum 2C for $^{13}C^{18}O$-form exhibits a new band at 503 cm^{-1}, meaning that the band at 517 cm^{-1} for $^{12}C^{16}O$ shows a downshift to 503 cm^{-1} for $^{13}C^{18}O$. The band at 517 cm^{-1} is accordingly assigned to the ν_{Fe-CO} mode. As is seen in Spectrum 2D, derivative-like difference features are evident at 576/555 cm^{-1} and 369/355 cm^{-1} for the $^{12}C^{16}O/^{13}C^{18}O$

combination in addition to the 517/503 cm^{-1} feature. Spectrum 2D exhibits slight intensity changes (negative peaks) at around 751 and 683 cm^{-1}, where porphyrin vibrational modes are located, and no negative features are expected upon isotopic substitution. The difference calculation was performed just by subtracting Spectrum 2C from Spectrum 2B. Since we use the same mitochondrial samples for Spectra 2B and 2C, the negative peaks might be caused by a slight concentration difference of mitochondria after gas exchange procedure (see Experimental). Spectrum 2E for the fully-reduced and CO-bound solubilized CcO gives a band at 517 cm^{-1}, as is seen in Spectrum 2B for the fully-reduced and CO-bound whole mitochondria. The band at 517 cm^{-1} shows a downshift to 503 cm^{-1} upon $^{13}C^{18}O$ isotopic substitution (Spectrum 2F). The isotopic difference spectrum (Spectrum 2G) gives difference features at 576/555, 517/503, and 369/355 cm^{-1} for the $^{12}C^{16}O/^{13}C^{18}O$ combination for the solubilized CcO as reported previously.[11] Comparing Spectra 2D and 2G, we note that the isotopic difference features of the three sets of frequency for mitochondria and solubilized CcO are very similar.

Discussion

Heme a_3 Environment of CcO in Whole Mitochondria. Adar and co-workers pioneered RR measurements of cytochromes in whole mitochondria.[12,13] They selectively observed vibrational modes of hemes in mitochondria and they discussed possible heme-heme interactions. A mitochondrion is a worm-shaped organelle and is typically one to several micrometers long. It has outer and inner membranes that consist of phospholipid bilayer and contains a large number of proteins as well as nucleic acids other than cytochromes. The cytochromes are localized in the inner membrane. The mitochondrial suspension is a strong light scatterer. Nevertheless, RR spectroscopy can preferentially reveal the vibrational spectra of hemes in cytochromes.

The absorption spectra of whole mitochondria in the visible region (Fig. 1) are ascribed to cytochromes.[14] Cytochrome aa_3 (CcO) exhibits absorption bands at 442 and 604 nm. Cytochrome b exhibits bands at 427 and 563 nm and cytochromes c and c_1 exhibit bands at 413, 521, and 552 nm.[14] X-ray crystallography[4] demonstrated that CO binds only at the heme a_3 site, which has the one vacant axial position in the fully reduced state and that heme a is six coordinated in the fully reduced state and inaccessible to external ligands. It is well known that cytochromes b, c_1, and c are all six coordinated and are inaccessible to external ligands.[14] Although the absorption peaks at 552 and 563 nm of cytochromes b, c_1, and c are insensitive to CO binding to cytochrome a_3, a small intensity increase at 552 nm and a small decrease at 563 nm upon CO binding are noted in Fig. 1. These changes are attributed to the changes of cytochrome a_3 upon CO binding, which are evident in the inset of Fig. 1. Taking these things into consideration, one may understand that only cytochrome a_3 in mitochondrial cytochromes binds CO and that this is the site to which dioxygen binds under physiological conditions. Other cytochromes play roles in the electron transfer.[14] Moreover, the vibrational modes associated with CO vibration are resonance-enhanced only when CO is bound to a heme-iron. The band at 517 cm^{-1} for CO-bound mitochondria is accordingly assigned solely to the ν_{Fe-CO} mode of cytochrome a_3. The band at 576 cm^{-1} that showed $^{13}C^{18}O$ isotopic frequency shift to 555 cm^{-1} is assigned to the δ_{Fe-C-O} mode. The band at 369 cm^{-1} for $^{12}C^{16}O$ shows a downshift to 355 cm^{-1} upon $^{13}C^{18}O$ substitution. Although this band is not very intense, it is also observed at around 365 cm^{-1} for other hemoproteins and is assignable to the δ_{Fe-C-O} mode.[11] It became clear that the frequencies of three Raman bands with CO-isotope sensitivity observed for heme a_3-CO in whole mitochondria are virtually identical to those of corresponding modes for solubilized CcO (Spectrum 2G).[11,15] The ν_{Fe-CO} mode of CO-bound hemoproteins has extensively been studied. The ν_{Fe-CO} frequency differs among different kinds of hemoproteins. For instance, the ν_{Fe-CO} modes are located at 507, 508, 530, and 517 cm^{-1} for hemoglobin,[16] myoglobin,[17] cytochrome c peroxidase,[18] and CcO,[11,19] respectively. The ν_{Fe-CO} is also different for different kinds of mutant of distal histidine,[17,20] even for the same protein, in which the histidine is replaced by glycine, methionine, leucine or others. The frequency differences are interpreted as a result of environmental difference of the heme. If the ν_{Fe-CO} changes, the δ_{Fe-C-O} usually changes. Thus the ν_{Fe-CO} and the δ_{Fe-C-O} modes have been used to examine the environmental difference of the heme pocket as a sensitive probe.[16,17,20] The results in this study mean that the environmental structures of heme a_3 in solubilized CcO and CcO in whole mitochondria are quite similar. Time-resolved RR studies of reaction intermediates of CcO have been done for the solubilized state so far,[8,9] and one may wonder if the structures of the reaction intermediates in mitochondria are the same or not. Present results demonstrate that, at least in the static state, the heme a_3 structure of the two kinds of CcO conditions are almost the same. It is stressed that only a single vibrational mode in whole mitochondria is selectively observed and it would be promising to detect the reaction intermediates of heme a_3 with dioxygen in a time-resolved fashion. This project is underway in this laboratory.

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