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A resonance Raman band assignable to the O–O stretching mode in the resting oxidized state of bovine heart cytochrome *c* oxidase

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Abstract In the resting oxidized state (the fully oxidized "as-isolated" state) of cytochrome *c* oxidase (C*c*O) preparation, a resonance Raman band is observed at 755 cm^{-1} upon 647.1 nm excitation in resonance with an absorption band at 655 nm. Addition of cyanide eliminates the Raman band concomitant with loss of the absorption band at 655 nm. These results strongly suggest that the Raman band at 755 cm^{-1} originates from the O–O stretching mode of the bridging peroxide (Fe–O^-–O^-–Cu) in the O_2 reduction site of the fully oxidized "as-isolated" C*c*O. Although the peroxide bridged structure has been proposed on the basis of X-ray crystallography and reductive titration experiments, the present vibrational spectroscopic analyses reveal conclusively the chemical nature of the bridging ligand at the O_2 reduction site of the fully oxidized "as-isolated" bovine heart C*c*O.

Keywords Cytochrome *c* oxidase · Resting oxidized state · O—O stretching mode · Resonance Raman · Bridging peroxide · Proton pump · Oxygen activation

Abbreviations

C*c*O	cytochrome *c* oxidase
RR	resonance Raman

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Introduction

Cytochrome *c* oxidase (C*c*O) is located at the terminus of the respiratory chain in the inner mitochondrial membrane. It couples the dioxygen reduction reaction with the proton pumping process. The resultant concentration gradient across the membrane is utilized by ATP-synthetase to phosphorylate ADP. Thus, C*c*O has been acknowledged as the key membrane protein in mitochondrial energy conversion. The functional unit of bovine heart C*c*O has 13 different subunits and its molecular weight is 210 kD (Tsukihara et al. 1996). It has four redox-active metal centers, provided by two copper sites (Cu_A and Cu_B) and two heme A groups (heme *a* and heme a_3). The Cu_A site accepts electrons from cytochrome *c* and donates them to heme *a*. Heme *a* delivers electrons to the O_2 reduction site, which include both heme a_3 and Cu_B. The mechanisms of O_2 reduction have extensively been studied mainly by resonance Raman (RR) spectroscopy, since this technique provides a powerful method for determining the chemical structure of the ligand at the catalytic site for dioxygen reduction (Ferguson-Miller and Babcock 1996; Kitagawa and Ogura 1997; Han et al. 2000).

It was reported that the fully oxidized form of C*c*O generated under turnover conditions pumps protons sequentially upon receiving each of the four electron equivalents, while the resting oxidized form (the fully oxidized "as-isolated" form) of C*c*O does not (Bloch et al. 2004). The coordination geometry of the heme a_3 site of the fully oxidized form under turnover conditions has been identified as Fe_{a3}^{3+}-OH^- by RR analyses. However, the coordination geometry of the resting oxidized form (the fully oxidized "as-isolated" form) has not been well established. The presence of a bridging ligand has been postulated by antiferromagnetic coupling between heme a_3

and Cu_B (Van Gelder and Beinert 1969). The fully oxidized "as-isolated" form and the oxidized form observed under turnover conditions require 6 and 4 electron equivalents, respectively, for complete reduction of the enzyme (Mochizuki et al. 1999). These results suggest that a peroxide $\left(O_2^{2-}\right)$ bridges between heme a_3 and Cu_B in the fully oxidized "as-isolated" form. The recently reported X-ray structure on this same form shows that an area of electron density comparable to that of two oxygen atoms is detectable between Fe_{a3} and Cu_B in the O_2 reduction site (Aoyama et al. 2009). The O–O distance is refined to be 1.70 Å. While this O–O distance is significantly longer than those of many model compounds (Kim et al. 2004; Cambridge Structural Database), it is absolutely too short to represent a hydrogen bond. Therefore, the X-ray structure strongly suggests the existence of a chemical bond between the two oxygen atoms. Vibrational spectroscopic analysis is crucial for identification of the factors providing this discrepancy in the O–O distance in order to elucidate the chemical properties of the bridging ligand between Fe_{a3} and Cu_B. These chemical properties could provide various insights into the proton pumping mechanism, since the bridging ligand blocks the proton pumping process as described above (Bloch et al. 2004).

Materials and methods

CcO was purified from bovine heart muscle as reported (Yoshikawa et al. 1977). The preparations used for the present research were purified further by microcrystallizat-rion as the final purification step (Mochizuki et al. 1999). All the purification procedures were performed under aerobic conditions. The absorption bands at 655 nm and 424 nm and the fast cyanide binding indicate that the present preparation "as-isolated" is the "fast" form (Mochizuki et al. 1999). The concentration of C*c*O for Raman measurements was 437 μM in 55 mM sodium phosphate buffer, pH 6.8, containing 0.2% n-decyl-β-D-maltopyranoside. The cyanide adduct was prepared by addition of appropriate volume of 20 mM KCN in 100 mM sodium phosphate buffer, pH 6.8, containing 0.2% n-decyl-β-D-maltopyranoside, freshly prepared by dilution of 2 M KCN stock solution in 0.1 M KOH, to make a final concentration of 2 mM KCN. The formation of the KCN adduct was monitored by the loss of the absorption band at 655 nm as shown in the inset in Fig. 1. Incubation for 25 min was sufficient for essential elimination of the band at 655 nm as given in Spectrum B' of Fig. 1 inset. Absorption spectra were recorded for the samples after 30-fold dilution of the samples used for Raman measurements on a Hitachi U-3310 spectrophotometer. The absorption path length was 10 mm. All the experiments were performed at 20°C.

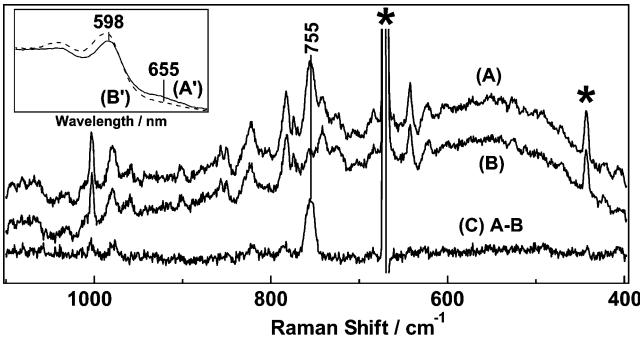

Fig. 1 Resonance Raman Spectra of Fully Oxidized "As-Isolated" Cytochrome *c* Oxidase (*A*) and its Cyanide Adduct (*B*) excited at 647.1 nm. Spectrum C is the difference spectrum obtained by subtracting spectrum B from spectrum A. The laser power at the sample was 11 mW. The accumulation period for spectra A and B was 90 min. Spectral features marked with an asterisk are due to emissions of the Kr^+ laser. The inset shows the visible part of the absorption spectra of fully oxidized "as-isolated" C*c*O (A') and its cyanide adduct (B') before the measurements of the Raman spectra.

Raman scattering was excited at 647.1 nm line from a Kr^+ laser (Spectra Physics, Model 2016), dispersed by a polychromator (Chromex, 500IS equipped with a 1,200 grooves/mm grating) and detected with a CCD detector (Roper Scientific, 400B/LN). A cylindrical spinning cell with an inner diameter of 3 mm was used to collect Raman scattering data in a 90° scattering geometry.

Results and discussion

Figure 1 depicts RR spectra of fully oxidized "as-isolated" (A), fully oxidized "as-isolated" and cyanide-bound C*c*O (B) and their difference spectrum $(C = A - B)$. The inset provides absorption spectra of fully oxidized "as-isolated" (A') and fully oxidized "as-isolated" and cyanide-bound C*c*O (B'). The single RR band at 755 cm^{-1} in Fig. 1c shows that the band at 755 cm^{-1} for the fully oxidized "as-isolated" C*c*O (Fig. 1a) is eliminated upon binding of cyanide (Fig. 1b). The enhancement of only one vibrational mode indicates that it arises from a simple molecule, most likely a diatomic molecule and the band position itself is fully consistent to that of the peroxide. The strong RR enhancement is induced by excitation at 647.1 nm near the 655 nm band of heme a_3, which has been suggested to be due to a ligand-to-metal charge transfer transition of the fully oxidized "as-isolated" form (Beinert et al. 1976; Mitchell et al. 1991) (Spectrum A' in the inset). Furthermore, addition of cyanide eliminates the band at 655 nm (Spectrum B' in the inset). These results strongly suggest that the RR band at 755 cm^{-1} observed upon 647.1 nm excitation originates from the O–O stretching vibration of the bridging peroxide between Fe_{a3} and Cu_B, thus corresponding to the enzyme form with the X-ray structure already mentioned (Aoyama et al. 2009).

The present laser illumination at 11 mW does not produce any detectable changes in the absorption spectra of fully oxidized "as-isolated" and cyanide-bound forms, while excitation of RR scattering at a much higher laser power (140 mW) decreases the intensity of the RR band at 755 cm^{-1}, relative to the intensities of other modes, due to photoreduction (Ogura et al. 1985). The RR band at 755 cm^{-1} has not been reported thus far in spite of many careful RR measurements for the fully oxidized "as-isolated" form. The photosensitivity of the bridging peroxide as well as the band position of the charge transfer band has contributed to the big delay in finding the RR band at 755 cm^{-1}. In fact, most of the RR analyses have been performed using laser illumination at the wavelength shorter than that used in the present study.

A linear relationship between the O–O stretching frequency (ν_{OO} / cm^{-1}) and the corresponding O–O bond length (r_{OO} / Å) ($\nu_{OO} = 5098.4 - 2963.3 \times r_{OO}$) is detectable for the O_2 species (Nakamoto 1997; Cramer et al. 2003) (O_2^+: 1,858 cm^{-1} and 1.12 Å, O_2: 1,555 cm^{-1} and 1.21 Å, O_2^-: 1,108 cm^{-1} and 1.28 Å, O_2^{2-}: 760 cm^{-1} and 1.49 Å, Nakamoto 1997). The relation suggests that the r_{OO} of the fully oxidized "as-isolated" CcO is 1.50 Å for the ν_{OO} frequency of 755 cm^{-1}. The reported r_{OO} of 1.70 Å determined by X-ray crystallography (Aoyama et al. 2009) is significantly larger than the estimated value. The crystals used for the above X-ray structural analysis were prepared from the present CcO preparation under aerobic conditions. The absorption band of the CcO crystals at 655 nm also indicates that CcO in the crystals is in the fully oxidized "as-isolated" form. It is impossible to exclude the possibility that the discrepancy between the Raman and X-ray results is due to the inaccuracy in the X-ray structural analysis even at 1.8 Å resolution (Aoyama et al. 2009). The $^{18}O/^{16}O$ isotope shift analysis for the ligand at the O_2 reduction site would provide further insights into the structure of the O_2 reduction site. Search for conditions is under way for preparing the fully oxidized "as-isolated" form from the fully reduced form under $^{18}O_2$ atmosphere.

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