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Observation of the Fe–O_2 and Fe^{IV}=O stretching Raman bands for dioxygen reduction intermediates of cytochrome *bo* isolated from *Escherichia coli***

Shun Hirota[a], Tatsushi Mogi[b], Takashi Ogura[a], Tomoyasu Hirano[b], Yasuhiro Anraku[b], Teizo Kitagawa[a,*]

[a]*Graduate University for Advanced Studies and Institute for Molecular Science, Okazaki National Research Institutes, Okazaki 444, Japan*
[b]*Department of Plant Sciences, Graduate School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan*

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Abstract Reaction intermediates in dioxygen reduction by the *E. coli* cytochrome *bo*-type ubiquinol oxidase were studied by time-resolved resonance Raman spectroscopy using the artificial cardiovascular system. At 0–20 μs following photolysis of the enzyme–CO adduct in the presence of O_2, we observed the Fe–O_2 stretching Raman band at 568 cm^{-1} which shifted to 535 cm^{-1} with the $^{18}O_2$ derivative. These frequencies are remarkably close to those of other oxyhemoproteins including dioxygen-bound hemoglobin and aa_3-type cytochrome *c* oxidase. In the later time range (20–40 μs), other oxygen-isotope-sensitive Raman bands were observed at 788 and 361 cm^{-1}. Since the 781 cm^{-1} band exhibited a downshift by 37 cm^{-1} upon $^{18}O_2$ substitution, we assigned it to the Fe^{IV}=O stretching mode. This band is considered to arise from the ferryl intermediate, but its appearance was much earlier than the corresponding intermediate of bovine cytochrome *c* oxidase (>100 μs). The 361 cm^{-1} band showed the $^{16}O/^{18}O$ isotopic frequency shift of 14 cm^{-1} similar to the case of bovine cytochrome *c* oxidase reaction.

Key words: *bo*-Type quinol oxidase; Oxy intermediate; Ferryl intermediate; Iron–dioxygen stretching mode; Ferryl–oxo stretching mode; Time-resolved resonance Raman spectroscopy

1. Introduction

Cytochrome *bo*-type ubiquinol oxidase is a terminal oxidase of the aerobic respiratory chain of *Escherichia coli* (*E. coli*) and is predominantly expressed under highly-aerated growth conditions (se [1] for a review). It catalyzes the four-electron reduction of molecular oxygen at the cytoplasmic side of membranes, which is coupled with the two-electron oxidation of two ubiquinol-8 molecules at the periplasmic side. Redox reactions at different surfaces of the membrane can create a difference in electrochemical potential across the cytoplasmic membrane. In addition to the protolytic scalar reactions, this enzyme is known to function as an electron transfer-linked proton-pump [2] like bovine aa_3-type cytochrome *c* oxidase. Recent molecular biological [3–7] and physicochemical [8–10] studies demonstrated that the *bo*-type quinol oxidase belongs to the heme-copper oxidase superfamily and shares a common molecular mechanism for the redox-coupled proton pumping [11].

Subunit I of the *E. coli bo*-type quinol oxidase contains a *hexa*-coordinated low-spin heme B, a *penta*-coordinated high-spin heme O and one copper ion (Cu_B) (see [3,12] for recent reviews). The high-spin heme and Cu_B are antiferromagnetically coupled, forming an Fe–Cu_B binuclear center where dioxygen reduction takes place. Subunit II provides the oxidation site for a lipophilic two-electron donor, ubiquinol-8 [13], but does not contain the Cu_A center which accepts electrons from ferrous cytochrome *c* for mammalian cytochrome *c* oxidases. Thus, electrons are transferred from the quinol bound to subunit II to the binuclear center of subunit I via the low-spin heme.

Resonance Raman (RR) spectroscopy can bring about structural information on hemes and their vicinities [14,15], and is especially powerful for studies of reaction intermediates. In fact, for the reaction of reduced bovine cytochrome *c* oxidase with dioxygen, the oxy [16–18], ferryl [19–21], and hydroxy [20,22] intermediates have been identified by this technique. It is extremely interesting to examine similar intermediates for the reaction of cytochrome *bo* with O_2, although there are large differences between *bo*-type quinol oxidase and aa_3-type cytochrome *c* oxidase, regarding electron donors, presence or absence of the Cu_A center, and heme species at the dioxygen reduction site. Therefore, we applied time-resolved RR spectroscopy using the artificial cardiovascular system [23,24] successfully to characterize intermediates involved in dioxygen reduction by *bo*-type quinol oxidase. We detected the Fe–O_2 and Fe^{IV}=O stretching modes at 568 and 788 cm^{-1}, respectively, for the first time, suggesting that the dioxygen reduction mechanism of quinol oxidases is similar to that of aa_3-type cytochrome *c* oxidases.

2. Materials and methods

Cytochrome *bo*-type quinol oxidase was purified from the cytochrome *bd*-deficient strain GO103 (*Δcyd*::Km^r [25]) harboring pHN3795–1 (H. Nakamura, unpublished results), as described previously [8]. pHN3795–1 is a derivative of pBR322 which carries the cytochrome *bo* operon (*cyoABCDE*$^+$) and was obtained from pHN3795 [26] as a clone that can support the aerobic growth of the *Δcyo Δcyd* strain on a nonfermentable carbon source.

The enzyme was dissolved in 100 mM Tris-HCl buffer (pH 7.4) containing 1.0% sucrose monolaurate SM-1200 (Mitsubishi-Kasei Food Co. Ltd., Tokyo), 200 μM ubiquinone-1 (a kind gift from Dr. S. Ohsono, Eisai Co. Ltd., Tsukuba) and 100 mM sodium ascorbate

*Corresponding author. Fax: (81) (564) 55 4639.

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(pH 7.4). About 75 ml of the 50 μM enzyme solution in the fully reduced CO-bound form was circulated at 5°C at a flow rate of 20 or 40 ml/min and brought under oxygen concentration of 150 μM using the artificial cardiovascular system. Details of this system are described elsewhere [23,24]. Raman scattering was excited at 406.7 nm by a Kr^+ ion laser (Spectra Physics, Model 2016), and detected with a cooled (−20 °C) diode array (PAR 1421HQ) attached to a single polychromator (Ritsu Oyo Kogaku, DG-1000), for which the slit width of 200 μm and slit height of 10 mm were used. A single laser beam with a power of ~5 mW was focused to 40 μm on a flow cell with a cross section of 0.6 × 0.6 mm^2 and was used to photolyze CO for initiation of the reaction and also to excite RR scattering. The transit time of a given molecule across the laser beam was 40 or 20 μs according to the flow rate used. Raman shifts were calibrated with ethanol as a standard under the same illumination geometry.

3. Results and discussion

Fig. 1 shows time-resolved RR spectra in the 1000 to 300 cm^{-1} region for reaction intermediates of the *bo*-type quinol oxidase with $^{16}O_2$ (A and C) and $^{18}O_2$ (B and D). Spectra A and B represent the raw spectra of intermediates generated in the time range of 0–20 μs following photolysis, while spectra C and D display those around 0–40 μs following photolysis. Spectra E and F illustrate the differences between spectra A and B, and between spectra C and D, respectively, in which an intense porphyrin band at 678 cm^{-1} was used as a marker for subtraction.

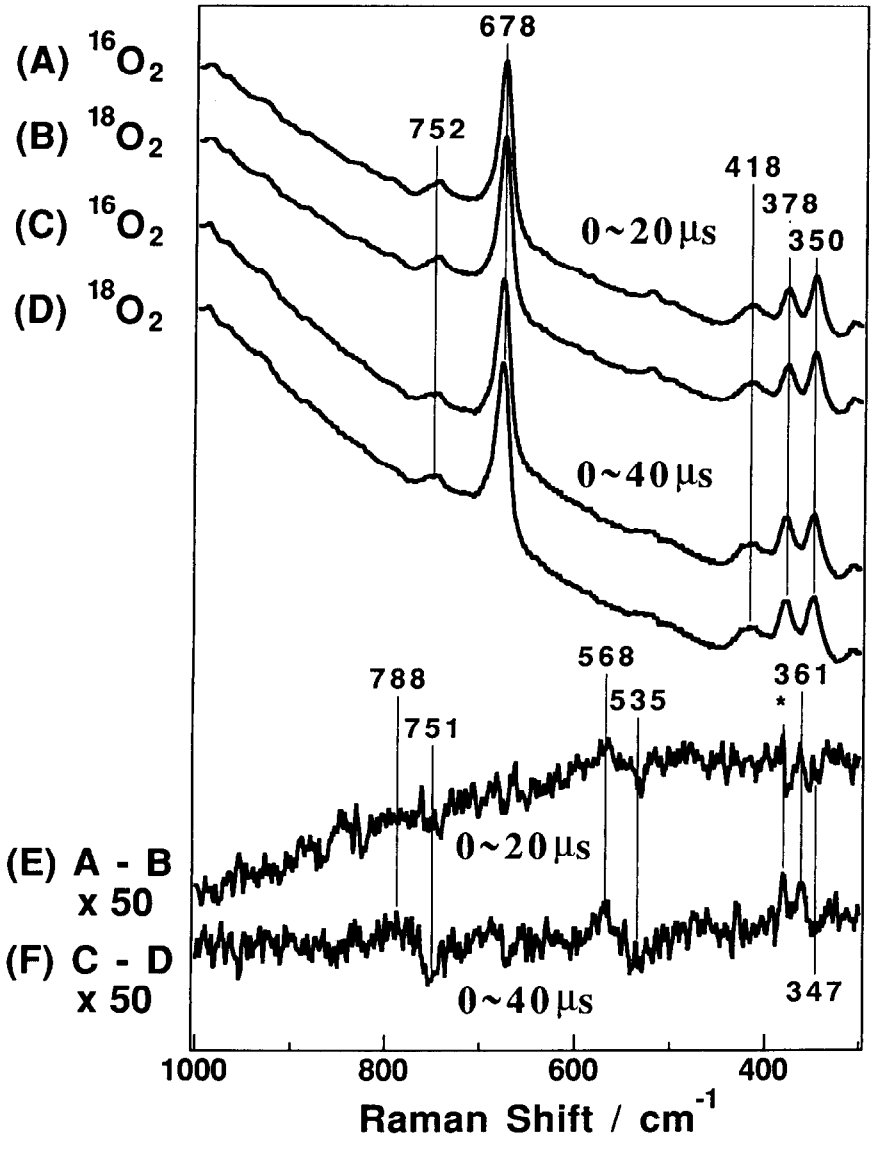

Fig. 1. Time-resolved RR spectra of the *E. coli bo*-type quinol oxidase in the 1000 to 300 cm^{-1} region for $^{16}O_2$ (A,C) and $^{18}O_2$ (B,D) derivatives and their difference spectra (E,F). The ordinate scales of spectra (A), (B), (C) and (D) are normalized by the intensity of porphyrin bands. Traces E and F represent the differences; spectrum E = (spectrum A−spectrum B) × 50 and spectrum F = (spectrum C−spectrum D) × 50. The bands marked with an asterisk denote a plasma line from a Kr^+ ion laser. Transit time of a given molecule across the laser beam is 0–20 μs for spectra A, B, and E and 0–40 μs for spectra C, D, and F. Experimental conditions: excitation, 406.7 nm, 5 mW (at the sample) for spectra A and B, and 6 mW (at the sample) for spectra C and D; accumulation time, 3840 s for spectra A and B, and 6080 s for spectra C and D.

A differential pattern exhibiting a peak at 568 cm^{-1} and a trough at 535 cm^{-1} appeared in difference spectra E and F. The frequencies 568 cm^{-1} for $^{16}O_2$ and 535 cm^{-1} for $^{18}O_2$ are in reasonable agreement with the Fe–O_2 stretching frequencies reported for oxyhemoglobin [27,28], oxymyoglobin [29,30], and dioxygen-bound bovine cytochrome *c* oxidase [16–18,24]. Cryogenic flash/trap absorption experiments on the CO adduct of this enzyme in the presence of oxygen suggested the formation of an oxygenated heme at low temperatures [31]. Therefore we assign this band to the Fe–O_2 stretching mode (ν_{Fe}–O_2) of the dioxygen adduct. Note that the ν_{Fe}–O_2 frequencies of the *bo*-type oxy-quinol oxidase (568/535 $cm^{-1}$for $^{16}O_2/^{18}O_2$) and aa_3-type oxy-cytochrome *c* oxidase (571/545 cm^{-1}) are alike, suggesting similarity in their Fe–O–O geometry.

Spectrum F shows the presence of other oxygen-isotope-sensitive bands around 785 and 361 cm^{-1} which must be arising from the species generated around 20–40 μs following the start of the reaction. The 785 cm^{-1} band is shifted to 751 cm^{-1} with the $^{18}O_2$ derivative. The frequency and its $^{18}O/^{16}O$-isotopic shift (37 cm^{-1}) are close to those observed for the Fe^{IV}=O stretching mode ($\nu_{Fe}{}^{IV}$=O) of the oxoferryl intermediate for the bovine cytochrome *c* oxidase [19–21], of horseradish peroxidase compound II [32–35], and of other oxoferryl hemeprotein species [36–39]. Therefore we assign this band to the $\nu_{Fe}{}^{IV}$=O mode of the oxoferryl intermediate. In the case of bovine cytochrome *c* oxidase, there are two oxygen-isotope-sensitive bands in this frequency region (786 and 804 cm^{-1}) [22,24], but it is not clear from this experiment whether the 785 cm^{-1} feature in Fig. 1 is a single band or not. It should be noted that the rise time (ca. 20–40 μs at 5°C) of the oxoferryl intermediate of the *bo*-type quinol oxidase is significantly faster than that of bovine cytochrome *c* oxidase, in which the $\nu_{Fe}{}^{IV}$=O band was undetectable before 100 ms under similar experimental conditions except for removal of detergents for bovine cytochrome *c* oxidase [24]. Recently, we found that the *E. coli bo*-type quinol oxidase contains a tightly-bound ubiquinone-8 (Q_H) which exists in the proximity of both the quinol oxidation site (Q_L) and the low-spin heme and may serve as a pathway for an electron transfer between these two centers [40]. The faster decay of the oxy intermediate to the oxoferryl intermediate in the *bo*-type quinol oxidase may be due to the unique electron transfer pathway. The other oxygen-isotope-sensitive band at 361 cm^{-1} showed an $^{16}O/^{18}O$ isotopic shift of 14 cm^{-1}. A similar band is also reported for an intermediate in dioxygen reduction by bovine cytochrome *c* oxidase [21,24,41], although the nature of the species giving this band has not been characterized yet.

In conclusion, we observed oxygen-isotope-sensitive Raman bands at 568 and 788 cm^{-1} during turnovers of the *E. coli bo*-type quinol oxidase for the first time, and assigned them to the ν_{Fe}–O_2 and $\nu_{Fe}{}^{IV}$=O of its oxy- and ferryl intermediates, respectively. These frequencies and their $^{16}O/^{18}O$ isotopic shifts suggest that structures of these intermediates at the catalytic site are similar to the corresponding intermediates of aa_3-type cytochrome *c* oxidases. However, the occurrence of the ferryl intermediate was evidently faster in the *bo*-type quinol oxidase than in the aa_3-type cytochrome *c* oxidase.

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