

# Observation of the Fe–O<sub>2</sub> and Fe<sup>IV</sup>=O stretching Raman bands for dioxygen reduction intermediates of cytochrome *bo* isolated from *Escherichia coli*\*\*

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Received 20 July 1994; revised version received 8 August 1994

**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  $\mu$ s following photolysis of the enzyme–CO adduct in the presence of O<sub>2</sub>, we observed the Fe–O<sub>2</sub> stretching Raman band at 568 cm<sup>-1</sup> which shifted to 535 cm<sup>-1</sup> with the <sup>18</sup>O<sub>2</sub> derivative. These frequencies are remarkably close to those of other oxyhemoproteins including dioxygen-bound hemoglobin and *aa*<sub>3</sub>-type cytochrome *c* oxidase. In the later time range (20–40  $\mu$ s), other oxygen-isotope-sensitive Raman bands were observed at 788 and 361 cm<sup>-1</sup>. Since the 781 cm<sup>-1</sup> band exhibited a downshift by 37 cm<sup>-1</sup> upon <sup>18</sup>O<sub>2</sub> substitution, we assigned it to the Fe<sup>IV</sup>=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  $\mu$ s). The 361 cm<sup>-1</sup> band showed the <sup>16</sup>O/<sup>18</sup>O isotopic frequency shift of 14 cm<sup>-1</sup> 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 (see [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*<sub>3</sub>-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<sub>B</sub>) (see [3,12] for recent reviews). The high-spin heme and Cu<sub>B</sub> are antiferromagnetically coupled, forming an Fe–Cu<sub>B</sub> 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<sub>A</sub> 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<sub>2</sub>, although there are large differences between *bo*-type quinol oxidase and *aa*<sub>3</sub>-type cytochrome *c* oxidase, regarding electron donors, presence or absence of the Cu<sub>A</sub> 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<sub>2</sub> and Fe<sup>IV</sup>=O stretching modes at 568 and 788 cm<sup>-1</sup>, respectively, for the first time, suggesting that the dioxygen reduction mechanism of quinol oxidases is similar to that of *aa*<sub>3</sub>-type cytochrome *c* oxidases.

## 2. Materials and methods

Cytochrome *bo*-type quinol oxidase was purified from the cytochrome *bd*-deficient strain GO103 ( $\Delta$ *cyd*::Km<sup>r</sup> [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*<sup>\*</sup>) and was obtained from pHN3795 [26] as a clone that can support the aerobic growth of the  $\Delta$ *cyo*  $\Delta$ *cyd* 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  $\mu$ M ubiquinone-1 (a kind gift from Dr. S. Ohsono, Eisai Co. Ltd., Tsukuba) and 100 mM sodium ascorbate

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\*\*This is Paper XIII in the series 'Structure–function studies on the *E. coli* cytochrome *bo* complex'.

(pH 7.4). About 75 ml of the 50  $\mu\text{M}$  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  $\mu\text{M}$  using the artificial cardiovascular system. Details of this system are described elsewhere [23,24]. Raman scattering was excited at 406.7 nm by a  $\text{Kr}^+$  ion laser (Spectra Physics, Model 2016), and detected with a cooled ( $-20^\circ\text{C}$ ) diode array (PAR 1421HQ) attached to a single polychromator (Ritsu Oyo Kogaku, DG-1000), for which the slit width of 200  $\mu\text{m}$  and slit height of 10 mm were used. A single laser beam with a power of  $\sim 5$  mW was focused to 40  $\mu\text{m}$  on a flow cell with a cross section of  $0.6 \times 0.6$  mm<sup>2</sup> 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  $\mu\text{s}$  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  $\text{cm}^{-1}$  region for reaction intermediates of the *bo*-type quinol oxidase with  $^{16}\text{O}_2$  (A and C) and  $^{18}\text{O}_2$  (B and D). Spectra A and B represent the raw spectra of intermediates generated in the time range of 0–20  $\mu\text{s}$  following photolysis, while spectra C and D display those around 0–40  $\mu\text{s}$  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  $\text{cm}^{-1}$  was used as a marker for subtraction.

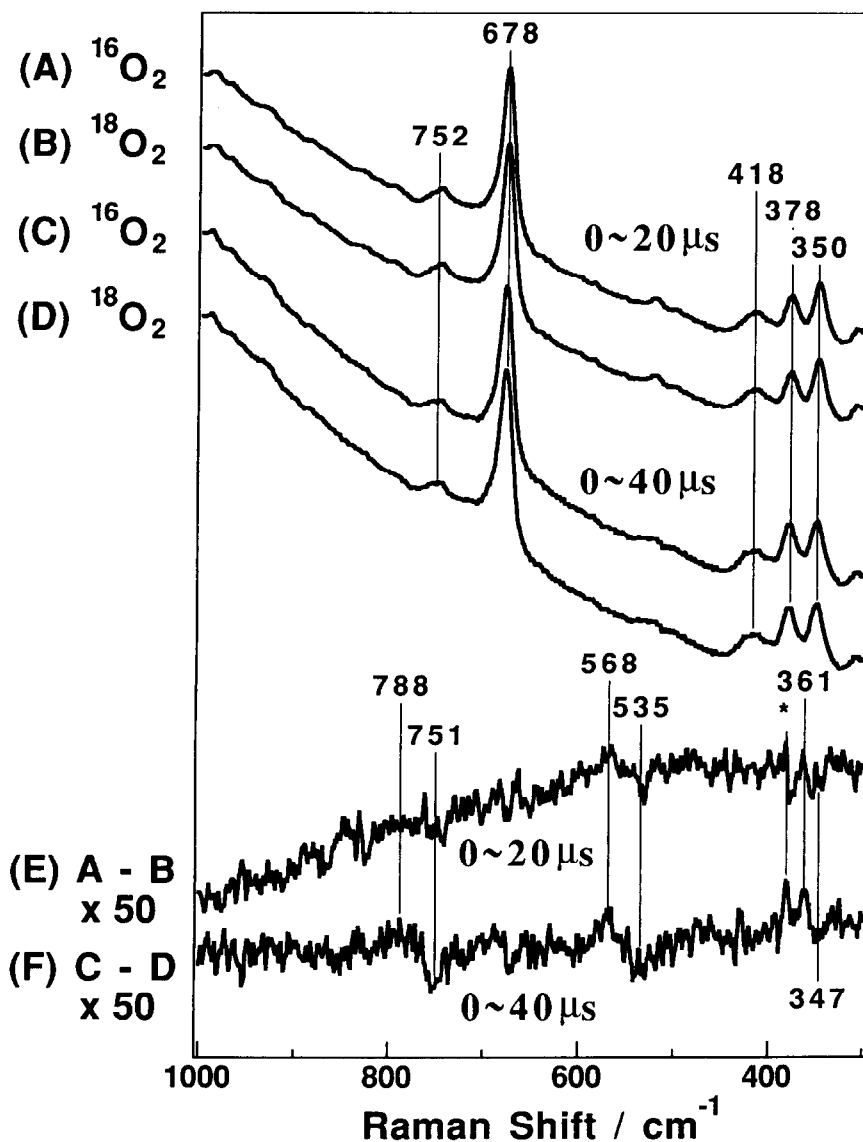


Fig. 1. Time-resolved RR spectra of the *E. coli bo*-type quinol oxidase in the 1000 to 300  $\text{cm}^{-1}$  region for  $^{16}\text{O}_2$  (A,C) and  $^{18}\text{O}_2$  (B,D) derivatives and their difference spectra (E,F). The ordinate scales of spectra (A), (B), (C) and (D) are normalized by the intensity of porphyrin bands. Traces E and F represent the differences; spectrum E = (spectrum A – spectrum B)  $\times 50$  and spectrum F = (spectrum C – spectrum D)  $\times 50$ . The bands marked with an asterisk denote a plasma line from a  $\text{Kr}^+$  ion laser. Transit time of a given molecule across the laser beam is 0–20  $\mu\text{s}$  for spectra A, B, and E and 0–40  $\mu\text{s}$  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  $568\text{ cm}^{-1}$  and a trough at  $535\text{ cm}^{-1}$  appeared in difference spectra E and F. The frequencies  $568\text{ cm}^{-1}$  for  $^{16}\text{O}_2$  and  $535\text{ cm}^{-1}$  for  $^{18}\text{O}_2$  are in reasonable agreement with the  $\text{Fe}-\text{O}_2$  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  $\text{Fe}-\text{O}_2$  stretching mode ( $\nu_{\text{Fe}-\text{O}_2}$ ) of the dioxygen adduct. Note that the  $\nu_{\text{Fe}-\text{O}_2}$  frequencies of the *bo*-type oxy-quinol oxidase ( $568/535\text{ cm}^{-1}$  for  $^{16}\text{O}_2/^{18}\text{O}_2$ ) and *aa*<sub>3</sub>-type oxy-cytochrome *c* oxidase ( $571/545\text{ cm}^{-1}$ ) are alike, suggesting similarity in their  $\text{Fe}-\text{O}-\text{O}$  geometry.

Spectrum F shows the presence of other oxygen-isotope-sensitive bands around  $785$  and  $361\text{ cm}^{-1}$  which must be arising from the species generated around  $20\text{--}40\text{ }\mu\text{s}$  following the start of the reaction. The  $785\text{ cm}^{-1}$  band is shifted to  $751\text{ cm}^{-1}$  with the  $^{18}\text{O}_2$  derivative. The frequency and its  $^{18}\text{O}/^{16}\text{O}$ -isotopic shift ( $37\text{ cm}^{-1}$ ) are close to those observed for the  $\text{Fe}^{\text{IV}}=\text{O}$  stretching mode ( $\nu_{\text{Fe}^{\text{IV}}=\text{O}}$ ) of the oxoferryl intermediate for the bovine cytochrome *c* oxidase [19–21], of horseradish peroxidase compound II [32–35], and of other oxoferryl heme protein species [36–39]. Therefore we assign this band to the  $\nu_{\text{Fe}^{\text{IV}}=\text{O}}$  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  $804\text{ cm}^{-1}$ ) [22,24], but it is not clear from this experiment whether the  $785\text{ cm}^{-1}$  feature in Fig. 1 is a single band or not. It should be noted that the rise time (ca.  $20\text{--}40\text{ }\mu\text{s}$  at  $5^\circ\text{C}$ ) of the oxoferryl intermediate of the *bo*-type quinol oxidase is significantly faster than that of bovine cytochrome *c* oxidase, in which the  $\nu_{\text{Fe}^{\text{IV}}=\text{O}}$  band was undetectable before  $100\text{ ms}$  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 ( $\text{Q}_\text{H}$ ) which exists in the proximity of both the quinol oxidation site ( $\text{Q}_\text{L}$ ) 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  $361\text{ cm}^{-1}$  showed an  $^{16}\text{O}/^{18}\text{O}$  isotopic shift of  $14\text{ cm}^{-1}$ . 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  $788\text{ cm}^{-1}$  during turnovers of the *E. coli bo*-type quinol oxidase for the first time, and assigned them to the  $\nu_{\text{Fe}-\text{O}_2}$  and  $\nu_{\text{Fe}^{\text{IV}}=\text{O}}$  of its oxy- and ferryl intermediates, respectively. These frequencies and their  $^{16}\text{O}/^{18}\text{O}$  isotopic shifts suggest that structures of these intermediates at the catalytic site are similar to the corresponding intermediates of *aa*<sub>3</sub>-type cytochrome *c* oxidases. However, the occurrence of the ferryl intermediate was evidently faster in the *bo*-type quinol oxidase than in the *aa*<sub>3</sub>-type cytochrome *c* oxidase.

**Acknowledgements:** We would like to thank Dr. Y. Orii of Kyoto University for invaluable comments. This work was supported in part by Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture (to T.M., T.O. and T.K.).

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