Observation of the Fe^{II}–O_2 Stretching Raman Band for Cytochrome Oxidase Compound A at Ambient Temperature

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Cytochrome oxidase (cytochrome *c*:oxygen oxidoreductase; EC 1.9.3.1) reduces more than 90% of inhaled dioxygen to water, and the dioxygen reduction is coupled with transmembrane proton translocation, generating the electrochemical potential to be used for ATP synthesis.[2] The reduction mechanism of dioxygen by this enzyme has been investigated mainly with visible absorption[3a-e] and EPR spectroscopies,[4a,b] but it remains to be clarified due to the lack of structural information for intermediates. Resonance Raman (RR) spectroscopy enables us to reveal the heme vibrational spectra, which sensitively reflect the structure of the heme and its vicinity, and has indeed provided substantial information on the structure–function relationship of heme proteins.[5a-c] The transient RR technique was also applied to detect reaction intermediates of cytochrome oxidase,[6a-d] but the most serious problem in RR studies has been a possibility that the exciting laser light may alter the state of a sample via photodissociation or photoreduction. In order to circumvent this uncertainty, we constructed a novel device for Raman/absorption simultaneous measurements,[7] which allows us to monitor absorption spectra of the molecules in the laser beam. Furthermore, we designed an artificial cardiovascular system for accumulating the spectroscopic data of reaction intermediates of cytochrome oxidase for a long time with a limited amount of enzyme.[8] This device makes it possible to use a low laser power for the measurements of RR spectra and thus to avoid photoreactions. These two devices were successfully combined to detect the early intermediate of cytochrome oxidase. Here we report the observation of the Fe^{II}–O_2 stretching RR band for compound A at room temperature defined by the absorption spectrum.

Bovine heart cytochrome oxidase (150 mL, 50 μM), which was isolated according to Yoshikawa et al.[9] and dissolved in 50 mM phosphate buffer, pH 7.2, was circulated through the artificial cardiovascular system. Raman scattering was excited at 425 nm (18 mW/200-μm diameter) with an argon laser-pumped dye laser (stilbene 420) and detected with a diode array (PAR 1420) attached to a Spex 1877B triple polychromator. The Raman and absorption spectra were monitored simultaneously with the device

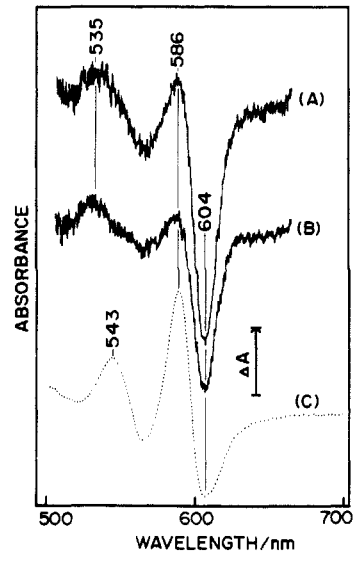

Figure 1. Transient absorption spectra of reaction intermediates of cytochrome oxidase represented as the difference spectra: (A) $^{16}O_2$ derivative minus CO-photodissociated form; (B) $^{18}O_2$ derivative minus CO-photodissociated form; (C) CO adduct minus fully reduced form. Spectra A and B were observed with the device for Raman/absorption simultaneous measurements[7] with a pathlength of 1 mm. Spectrum C was obtained with an ordinary spectrophotometer (Hitachi 220S) with a path length of 10 mm and an enzyme concentration of 13.4 μM. The ordinate scale of ΔA corresponds to 0.002 for spectra A and B and to 0.02 for spectrum C.

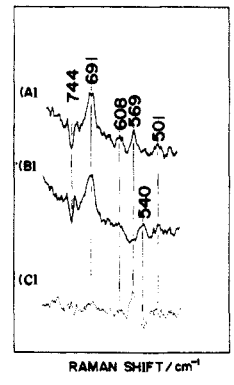

Figure 2. Transient resonance Raman spectra of the reaction intermediates of cytochrome oxidase whose absorption spectra are specified by Figure 1, spectra A and B, respectively: (A) $^{16}O_2$ derivative; (B) $^{18}O_2$ derivative; (C) difference spectrum [=(spectrum A) − (spectrum B)]. Spectra A and B are represented as the difference spectra that were obtained by subtracting the spectrum of CO-photodissociated species observed under the same condifions but in the absence of O_2 from the raw spectrum of the intermediate. The sample solution contained 50 μM bovine heart cytochrome oxidase, 8 μM cytochrome *c*, and 50 mM sodium ascorbate in 50 mM sodium phosphate buffer, pH 7.2. The sample temperature at the measuring point was 5 °C, and the resident time of a given molecule in the laser beam was at most 200 μs. The actual time resolution depends on the laser power. Experimental procedures have been described elsewhere,[8] but three separate gas exchangers were used at the same time for $^{16}O_2$, $^{18}O_2$, and N_2 (for CO-photodissociated form). Accordingly, the spectra of three species were acquired successively in turn, in which the accumulation time of the spectra for each species was 11 min, and the successive measurements of the three species were repeated six times (the total accumulation time is 66 min for each species). Raman shifts were calibrated under the same geometry of the cell with ethanol as the second standard, whose frequencies were separately calibrated with indene. The accuracy of Raman band positions was ±2 cm^{-1}.

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reported previously[7] but with some improvement on a linear photodiode array detection of absorption spectra. Details of experimental conditions are described in the figure captions.

Figure 1 shows the absorption spectra of the $^{16}O_2$ (A) and $^{18}O_2$ (B) derivatives, which are represented as difference spectra of the intermediates minus the CO-photodissociated species. The absolute spectra (not shown) indicated that 90% of the enzyme adopted the CO-photodissociated form in the laser beam in the absence of O_2. The two difference spectra in Figure 1 are very much alike and reproduce the previous observation for compound A.[8] Although the peak at 586 nm and the trough at 604 nm resemble those of the Fe^{II}-CO form drawn by a dotted line (C), the broad features of spectra A and B around 530-540 nm are distinct from those of spectrum C. The asymmetry between the 586-nm peak and the 604-nm trough might imply contamination of the subsequent intermediate, because they are more symmetric in the corresponding difference spectrum for the 20-μs transient species reported by Orii[3e] but are asymmetric in the subsequent intermediate. The intensity of the 586-nm band became weaker for lower concentrations of oxygen or for slower flow rates of sample.

Traces A and B in Figure 2 show the RR spectra observed simultaneously with absorption spectra A and B, respectively, shown in Figure 1. These RR spectra are represented as difference spectra, observed minus CO-photodissociated. Since the CO-photodissociated form was measured with the same instrumental conditions at the same time as for the intermediate except for the absence of O_2 in the solution (see caption of Figure 2), any possible contribution from the spectrum of the unphotodissociated form is subtracted in the difference spectra. A Raman band at 569 cm^{-1} in spectrum A is missing in spectrum B, where a new band appears at 540 cm^{-1}. The difference between spectra A and B, delineated by Figure 2C, clearly indicates that the 569-cm^{-1} band for the $^{16}O_2$ derivative is shifted to 540 cm^{-1} for the $^{18}O_2$ derivative. Note that this feature was reproduced for several different preparations of the enzyme and two different excitation wavelengths (425 and 418 nm). The observed isotopic shift (29 cm^{-1}) is slightly larger than the calculated shift for the Fe-O_2 two-body harmonic oscillator (21 cm^{-1}) but is close to the value (26 cm^{-1}) expected when the oxygen is vibrating against the combined body of the iron and its trans ligand (imidazole) and in agreement with the observed isotopic shifts for oxyhemoglobin (oxyHb) (27-28 cm^{-1}).[10a,b] Accordingly, the 569-cm^{-1} band is assigned to the Fe-O_2 stretching vibration [ν(Fe-O_2)]. It was unexpected that the ν(Fe-O_2) frequency of this enzyme would be remarkably close to those of oxyHb (567 cm^{-1}, pH 7.4 at 10 °C,[10a] and 572 cm^{-1}, pH 8.5 at 10 °C[10b]) and oxyMb (569 cm^{-1}, pH 6.8 at 15 °C)[11] and also to that of a model heme *a* imidazole complex (576 cm^{-1}).[12] This may imply that the Fe^{II}-O_2 heme of this enzyme adopts a structure similar to that in oxyHb and oxyMb.

The present ν(Fe-O_2) frequency is different from that (589 cm^{-1}) reported by Varotsis et al.,[6d] who used 10-ns laser pulses. The delay time in this experiment has appreciable distribution but is at most 200 μs, which is the resident time of a molecule in the laser beam. Although this might be a possible origin for the discrepancy, it is noted that the actual time resolution in this experiment strongly depended on the laser power. When we used higher laser power, we could not identify the present intermediate. Whatever the time resolution is, it is important that the species exhibiting the absorption spectrum of compound A gives the ν(Fe-O_2) RR band.

The 569-cm^{-1} RR band of this intermediate was observable upon excitations at 418, 425, and 430 nm but not at 406.7 and 441.6 nm. For oxyHb the ν(Fe-O_2) RR band was reported to be most resonance enhanced at the Soret maximum (413 nm).[13] If the mechanism of intensity enhancement is the same between Hb and this intermediate, the Soret maximum of the a_3^{II}-O_2 heme would be located around 420-430 nm.

Note Added in Proof. After submission of this paper, the reported ν(Fe-O_2) frequency by Varotsis et al.[6d] was corrected from 589 to 571 cm^{-1},[14a] in agreement with the present results. On the other hand, the ν(Fe-O_2) RR band for the mixed-valence cytochrome oxidase was found at 568 cm^{-1},[14b] which is also very close to the present observation.

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10-Methylacridine Dimer Acting as a Unique Two-Electron Donor in the One-Electron Reduction of Triphenylmethyl Cation

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Most organic reductants (DH) are known as two-electron donors, and the two-electron oxidation is normally accompanied by the removal of a proton (eq 1).[2,3] On the other hand, or-

$$DH \xrightarrow{-e} DH^{+}\cdot \xrightarrow{H^{+}} D\cdot \xrightarrow{-e} D^{+} \quad (1)$$

ganometallic compounds containing metal-metal bonds such as permethylated catenates of group 4B elements, Me_3M-$M'Me_3$ (M, M' = Sn, Ge, Sn), are known to act as pure two-electron donors by successive one-electron oxidation which involves cleavage of the metal-metal bond following the initial one-electron oxidation (eq 2).[4-6] However, no organic reductant acting as a pure

$$M\text{-}M' \xrightarrow{-e} M\text{-}M'^{+}\cdot \xrightarrow{M^{+}} M'\cdot \xrightarrow{-e} M'^{+} \quad (2)$$

two-electron donor without removal of a proton has so far been reported, since oxidative C-C bond cleavage is generally believed to be extremely difficult compared to that of C-H as well as metal-metal bonds.[7,8]

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