Time-Resolved Resonance Raman Investigation of Cytochrome Oxidase Catalysis: Observation of a New Oxygen-Isotope Sensitive Raman Band

Takashi OGURA, Satoshi TAKAHASHI, Kyoko SHINZAWA-ITOH,† Shinya YOSHIKAWA,† and Teizo KITAGAWA*

Institute for Molecular Science, Okazaki National Research Institutes/The Graduate University for Advanced Studies, Myodaiji, Okazaki 444

†Department of Life Science, Faculty of Science, Himeji Institute of Technology, 1479-1 Kanaji, Kamigoricho, Akogun, Hyogo, 678-12

(Received May 24, 1991)

The reduction of dioxygen by cytochrome oxidase has been investigated, in the time interval between 0.1 and 5.4 ms after initiation of the reaction, by using time-resolved resonance Raman spectroscopy combined with the Artificial Cardiovascular System [Ogura et al., *Biochemistry,* **28**, 8022 (1989)]. At 0.5 ms we observed a new oxygen-isotope sensitive Raman band at 356 cm^{-1} for $^{16}O_2$, which shifted down to 341 cm^{-1} for $^{18}O_2$. The temporal behavior of this band was found to be very close to that of another oxygen-isotope sensitive band at 788 cm^{-1} which had been assigned previously to the Fe(IV)=O stretching mode of the ferryloxo intermediate [Ogura et al., *J. Biol. Chem.,* **265**, 14721(1990)]. However, the fact that the 788 cm^{-1} band shifts to a higher frequency (802 cm^{-1}) in ^2H_2O, taken together with the present detection of two oxygen-isotope sensitive bands, prompts us to suggest an alternative assignment; namely, that these bands arise from the O^-–O^- (788 cm^{-1}) and Fe(III)–O^- (356 cm^{-1}) stretching modes of the Fe(III)–O^-–O^-–H intermediate. This conclusion forces a reconsideration of the currently accepted view of cytochrome oxidase catalysis.

Cytochrome oxidase is the terminal enzyme in the respiratory chain of aerobic organisms.[1,2] This enzyme is a membrane protein containing two heme A groups (Fe_a and Fe_{a3}) and two copper ions (Cu_A and Cu_B) as redox active metal centers and couples the dioxygen reduction reaction with proton translocation across the membrane. The electrochemical potential across the membrane thus generated is ultimately used to synthesize ATP. A functional unit containing the Fe_a and Cu_A ions serves as an electron transfer protein from cytochrome *c* to another functional unit containing the Fe_{a3}–Cu_B binuclear dioxygen reducing site. The Fe_{a3} and Cu_B ions are in close proximity so that they could be antiferromagnetically coupled in the oxidized state, while the Fe_a and Cu_A ions are separated and EPR active.

The reaction intermediates of this enzyme have been studied mainly with visible absorption[3–6] and EPR[7] spectroscopies. Recently, time-resolved resonance Raman spectroscopy added decisive structural information on the primary oxygenated derivative of the enzyme.[8–10] As to subsequent intermediates, a Raman band assignable to the Fe(IV)=O stretching mode of the ferryloxo heme was reported by ourselves and others.[11–13] On the basis of these results, a unified view of the catalytic cycle of cytochrome oxidase was proposed.[14] However, since the observation of the Fe(IV)=O stretching Raman band is crucial to this interpretation, if its assignment were not correct, the interpretation would lack experimental basis. In this paper we present new experimental results that are incompatible with the current interpretation of the resonance Raman spectra of the Fe(IV)=O intermediate and suggest an alternative assignment of this oxygen-isotope sensitive Raman band.

Material and Method

Cytochrome oxidase was isolated from fresh bovine hearts according to the method described elsewhere.[15] The sample solution contained cytochrome oxidase (100 μM in terms of aa_3 unit), horse heart cytochrome *c* (Sigma, 16.6 μM) and sodium ascorbate (Wako Chemicals, 50 mM) in 50 mM sodium phosphate buffer, pH 7.2. At relatively high salt concentration the cytochrome *c*-cytochrome oxidase complex formation is negligible. In order to continuously generate reaction intermediates in a flow-cell over the long time period (ca. 10 h) required for data accumulation, the "Artificial Cardiovascular System"[16] was used so as to limit the amount of enzyme needed. The quartz flow cell (cross section, 0.6×0.6 mm^2) was used in this experiment and the flow rate was held constant as 40 ml min^{-1}. Accordingly, the previously described dual-beam flow apparatus[17] was attached to the flow-cell in order to change the delay time (Δt_d) of the probe beam from the pump beam. The sample temperature in the flow-cell was maintained at 5 °C. The concentration of oxygen incorporated into the solution by the "Lung" was approximately 150 μM.

An argon ion laser (Spectra Physics, 164)-pumped dye laser (Spectra Physics, 375), employing Rhodamine 6G, was used to provide the pump beam. The output at 590 nm (100 mW), focused with a cylindrical lens to a 100 μm wide slit image, was used to photolyze carbonmonoxide from the CO-bound, fully-reduced enzyme. Photodissociation initiates the reaction of the enzyme with O_2, in the presence of O_2, but simply produces the deoxy state in the absence of O_2. Under these conditions, about 95% of the CO was photolyzed from the CO-bound enzyme, as judged from the intensity of the Fe(II)–CO stretching band. The probe beam was provided by a dye laser (Spectra Physics 375B), using stilbene 420, which was excited by another Ar^+ ion laser (Spectra Physics, 2045). The output at 423 nm (10 mW) was focused to a spot (20 μm in diameter) at various distances on the downstream side of the pump

beam. Under these conditions a given molecule is exposed to the pump and probe beams for 54 and 11 μs, respectively. The $^{16}O_2$ derivative, $^{18}O_2$ derivative and the deoxy state were measured in turn at every 5 min for the identical enzyme under identical illumination geometry. Therefore their difference spectra were very accurate. Each spectrum presented is a sum of six to nine of the individual (5 min) spectra.

The scattered light was dispersed with a double monochromator (Spex 1404) and detected with an intensified photodiode array (PAR 1421Q). Pixel-to-pixel variation of sensitivity of the photodiode array was corrected with white light; the observed Raman spectrum was divided by the spectrum of the white light. Raman shifts were calibrated with alcohol observed under the same flowing conditions and the accuracy of the peak frequency was ±2 and ±4 cm^{-1} for strong and weak Raman bands, respectively.

For a large molecule like cytochrome oxidase (M_r=200 000), the lowest frequency where any Raman band could be identified for the flowing sample was 600 cm^{-1} because of increased Rayleigh scattering, even when an optical filter (short cut filter) for removing it was placed before the monochromator. In order to lower this limit frequency, a special optical filter (referred to as a spatial filter) was designed and placed between the sample and the monochromator. This device is composed of a microscope objective lens, a rectangular slit, and a collection lens which is F-matched to the monochromator used. Although details of the device and its capability will be described separately,[18] we note here that the use of this device enabled us to measure the Raman spectrum until as low as 250 cm^{-1}.

Results

Figure 1 depicts time-resolved resonance Raman spectra of cytochrome oxidase in ^2H_2O for Δt_d=0.1 ms. Figs. 1A and 1B were observed for $^{16}O_2$ and $^{18}O_2$, respectively, and Fig. 1C shows their difference (A minus B). In both Figs. 1A and 1B porphyrin in-plane vibrational modes are seen at 750 (ν_{16}), 684 (ν_7), and 342 cm^{-1} (ν_8). Although the difference between the two spectra is small, a clear derivative-like curve is seen in Fig. 1C, indicating a frequency shift due to the oxygen isotope; a Raman band at 571 cm^{-1} for $^{16}O_2$ shifts down to 544 cm^{-1} for $^{18}O_2$. One can assign this Raman band to the Fe(II)–O_2 stretching vibration (ν_{Fe-O_2}) of the primary oxygen compound, compound A.[5] Since the intensity of the Raman band due to the heme-bound oxygen was fairly weak compared with those of porphyrin bands, hereafter we will present resonance Raman spectra of reaction intermediates as difference spectra ($^{16}O_2$ minus $^{18}O_2$).

Figures 2 and 3 display the time-resolved resonance Raman difference spectra of cytochrome oxidase in H_2O and ^2H_2O, respectively. The delay time was varied from 0.1 to 5.4 ms and is specified at the right side of each spectrum. The Raman band at 573 cm^{-1} for $^{16}O_2$ in Fig. 2A (Δt_d=0.1 ms) shows a downshift to 544 cm^{-1} for $^{18}O_2$, which well reproduced the previous observations.[8–10] This band exhibited no frequency shift in ^2H_2O within the experimental uncertainty (Fig. 3A), in agreement with the recent results by Han et al.[13]

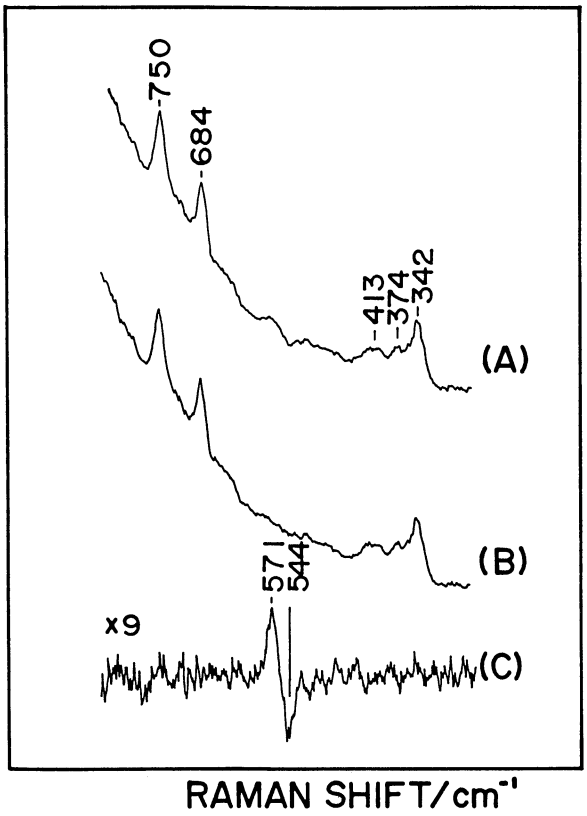


Fig. 1. Time-resolved resonance Raman spectra of cytochrome oxidase in ^2H_2O for Δt_d=0.1 ms. Spectra A and B were observed with $^{16}O_2$ and $^{18}O_2$, respectively, and trace C denotes the difference spectrum, A minus B. Accumulation time was 300 s.

In Fig. 2C, at Δt_d=0.5 ms, two new oxygen-isotope sensitive bands appeared at 788 and 356 cm^{-1} and the 573/544 cm^{-1} band apparently became weaker. The band for $^{16}O_2$ at 788 cm^{-1} exhibited a downshift to 749 cm^{-1} for $^{18}O_2$. Although it is not clear whether the 788 cm^{-1} band is actually composed of two bands or if the apparent doublet results from noise, the latter alternative appears more likely since in ^2H_2O this Raman band appeared as a singlet at 802 cm^{-1} for $^{16}O_2$ and at 766 cm^{-1} for $^{18}O_2$ as shown in Fig. 3B. The apparent upshift of the 788 cm^{-1} band is in agreement with the report by Han et al.[13] but not with that by Varotsis and Babcock.[12] This will be discussed later.

Development of the spatial filter in this study enabled us to detect another oxygen-isotope sensitive band at 356 cm^{-1} for $^{16}O_2$ which shifts to 342 cm^{-1} for $^{18}O_2$, as seen in Fig. 2C; i.e., the true positions of the 356 and 342 cm^{-1} bands might be slightly lower and higher, respectively, because those numbers were derived from the difference spectrum. This band first appears at Δt_d=0.4 ms (Fig. 2B) and persists until Δt_d=3.2 ms. In ^2H_2O the corresponding Raman band appeared at 358 and 343 cm^{-1} for $^{16}O_2$ and $^{18}O_2$, respectively. Thus, the vibrational frequency of the new band is insensitive to deuterium exchange within the experimental

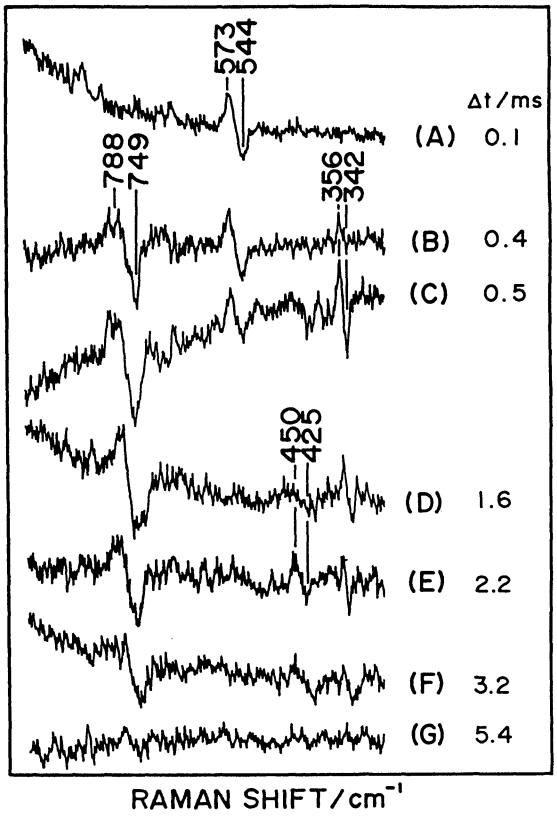


Fig. 2. Time-resolved Raman difference spectra of cytochrome oxidase in H_2O obtained by subtracting the $^{18}O_2$ spectrum from the corresponding $^{16}O_2$ spectrum. Δt_d values are specified at right side of each spectrum. The ordinate is arbitrarily scaled.

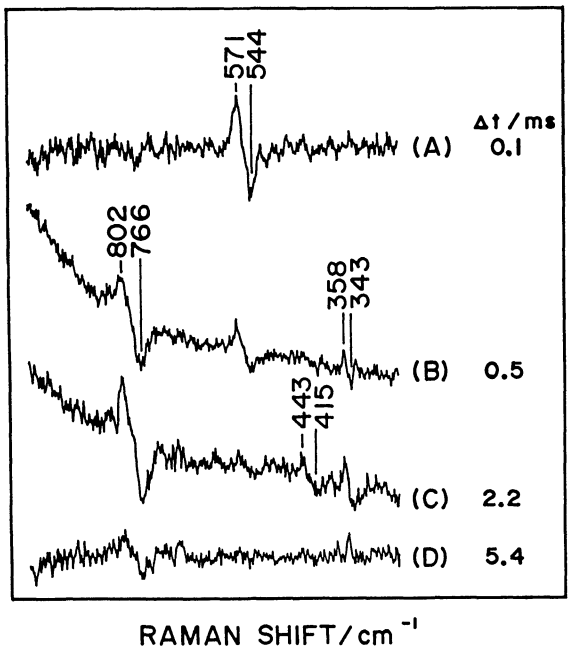


Fig. 3. Time-resolved Raman difference spectra of cytochrome oxidase in ^2H_2O obtained by subtracting the $^{18}O_2$ spectrum from the corresponding $^{16}O_2$ spectrum. Δt_d values are specified at right side of each spectrum. The ordinate is arbitrarily scaled.

uncertainty.

At Δt_d=1.6 ms the Fe(II)–O_2 stretching band at 573/544 cm^{-1} has disappeared while the other two oxygen-isotope sensitive bands remain. At Δt_d=2.2 ms (Fig. 2E) yet another oxygen-isotope sensitive Raman band is observed at 450 cm^{-1} for $^{16}O_2$, which experiences a downshift to 425 cm^{-1} for $^{18}O_2$. In ^2H_2O this Raman band appeared at 443 cm^{-1} for $^{16}O_2$ and at 415 cm^{-1} for $^{18}O_2$. This observation is consistent with the report by Han et al.[13] A simple diatomic approximation of the Fe–OH stretching mode, which is assumed to occur at 450 cm^{-1},[13] predicts a frequency shift of 19 cm^{-1} for Fe–^{18}OH and 10 cm^{-1} for Fe–O^{2}H. Accordingly, the 450 cm^{-1} band is assigned to the Fe(III)–OH stretching vibration in agreement with the reassignment by Han et al.[13] Finally, in Fig. 2G (with Δt_d=5.4 ms) no oxygen-isotope sensitive Raman band is observed, indicating that the Fe_{a3} center does not possess an oxygen ligand derived from O_2. If the Fe_{a3} had a ligand such as OH^- or H_2O group in the so-called "pulsed form",[1] the ligand

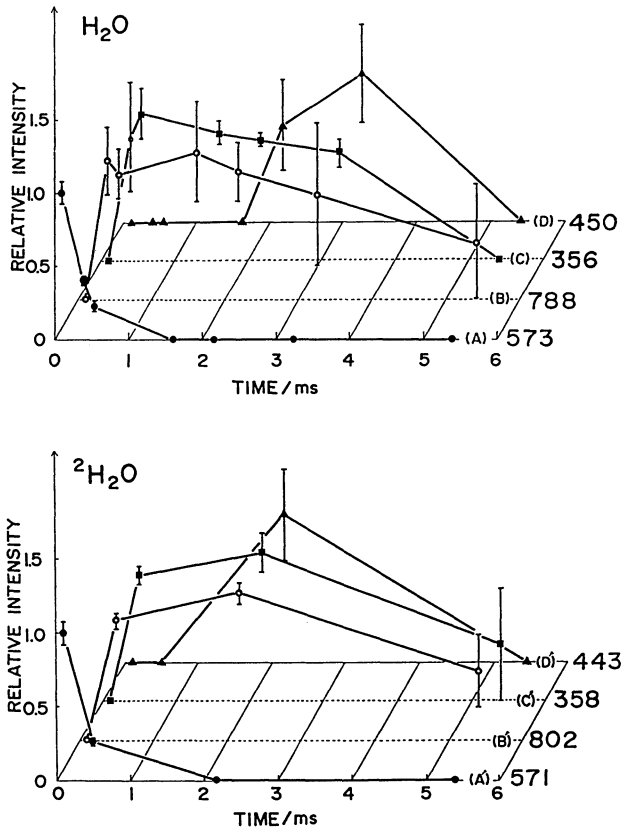
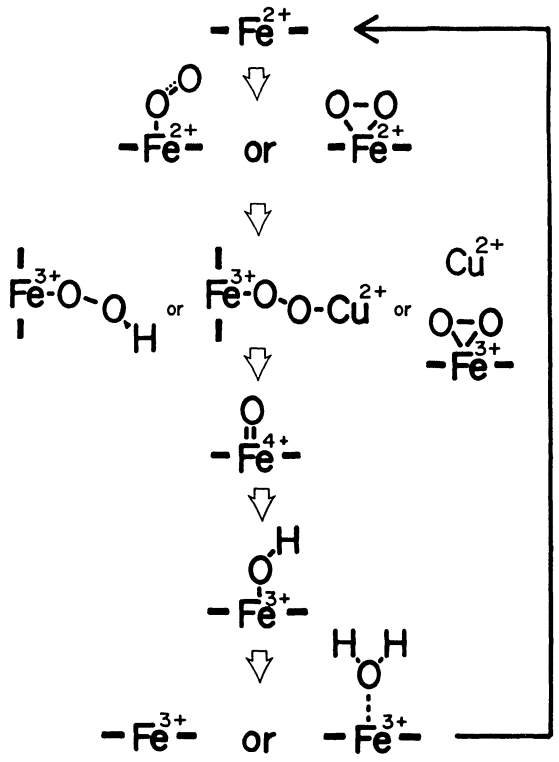


Fig. 4. Time-dependence of relatvie intensity of selected Raman bands at 573 (A, ●), 788 (B, ○), 356 (C, ■), and 450 cm^{-1} (D, ▲) for the H_2O solution and those at 571 (A′, ●), 802 (B′, ○), 358 (C′, ■) and 443 cm^{-1} (D′, ▲) in ^2H_2O solution. The trough-to-peak height of each spectrum was divided by the intensity of the porphyrin ν_7 band at 684 cm^{-1} of the original spectrum. In the plot, the highest value for individual bands is normalized to unity. Experimental uncertainty for the abscissa is smaller than the diameter of the circles.

must be exchanged with bulk water.

The peak height intensities in the difference spectra shown in Figs. 2 and 3 were evaluated with regard to the intensity of the ν_7 band (684 cm^{-1} in Fig. 1) in the corresponding raw spectra of intermediates. The relative intensities are plotted against Δt_d in Fig. 4, where the upper (A—D) and lower figures (A′—D′) present the data for the H_2O and ^2H_2O solutions, respectively. For individual curves the maximum value is normalized to unity. Plots using the ν_{16} band at 750 cm^{-1} as an intensity standard gave similar curves for all the bands (not shown). Although these two porphyrin bands experience slight intensity alterations for different Δt_d, the changes were sufficiently small that they still could serve as effective intensity standards. As expected, the 571 cm^{-1} band of the primary compound quickly loses its intensity ($t_{1/2}$<400 μs). The 788 and 356 cm^{-1} bands simultaneously increase in intensity, with a concomitant decrease in intensity of the 573 cm^{-1} band. Note that the temporal behavior of the 788 and 356 cm^{-1} bands seem to proceed parallel with respect to Δt_d throughout the entire range of Δt_d (between 0.1 and 5.4 ms). The 450 cm^{-1} band arises at Δt_d=2.2 ms and has disappeared at Δt_d=5.4 ms. These features are also retained in ^2H_2O, although the number of experimental points is smaller for ^2H_2O. It is noted that the decay of the 802 (B′) and 358 cm^{-1} (C′) bands observed for the ^2H_2O solution seem somewhat slower than those of the 788 (B) and 356 cm^{-1} (C) bands of the H_2O solution, indicating that this process involves protonation to oxygen and/or couples with the proton translocation.



Scheme 1. Possible reaction intermediates.

Discussion

Assignment of the 788 and 356 cm^{-1} Raman Bands. The mechanism of oxygen reduction reaction by cytochrome oxidase has not been fully understood mainly due to the lack of decisive structural information of the reaction intermediates. Scheme 1 illustrates possible reaction intermediates involved at the Fe_{a3} site where the oxidation and ligation states of Cu_B are not included except for two cases. Resonance Raman spectroscopy is particularly well suited to determine which intermediates are actually involved, since their iron–oxygen and oxygen–oxygen stretching frequencies are expected to be distinct. For the primary intermediate (Δt_d=0.1 ms), the end-on structure for the dioxygen bound form is established from the Fe(II)–O_2 stretching frequency.[8–10] This frequency and the size of the $^{18}O_2$ isotopic shift are remarkably close to those of oxymyoglobin (569 cm^{-1})[19] and oxyhemoglobin (567[20] and 572[21] cm^{-1}), for which the X-ray crystallographic analysis has been completed.

In our previous study we assigned the Raman band at 788 cm^{-1} (748 cm^{-1} for $^{18}O_2$) to the Fe(IV)=O stretching vibration of the ferryloxo heme of the next intermediate, mainly based on the frequency value and its shift upon $^{16}O/^{18}O$ isotopic substitution.[11] Similar observations have recently been reported by two other groups,[12,13] although there was a discrepancy about the deuteration effect on this band; Varotsis and Babcock[12] reported that the 790 cm^{-1} band in H_2O exhibited no freqeuency shift in ^2H_2O, while Han et al.[13] reported that the 786 cm^{-1} band in H_2O shifted up to 801 cm^{-1} in ^2H_2O. The present result agrees with the latter observation; the 788 cm^{-1} band exhibiting a clear upshift to 802 cm^{-1} in ^2H_2O [at Δt_d=0.5 and 2.2 ms (Fig. 3)].

All groups assigned this band to the Fe(IV)=O stretching mode. In order to explain its upshift in ^2H_2O, Han et al.[13] assumed that the Fe(IV)=O oxygen is hydrogen bonded in H_2O but not in ^2H_2O. This assumption is based on the fact that the Fe(IV)=O stretching Raman band of compound II of horseradish peroxidase is shifted to a higher frequency at alkaline pH where the participating histidyl imidazole residue is deprotonated and the hydrogen bond ceases to exist.[22,23] However, the validity of that interpretation is questionable inasmuch as the pK_a of a hydrogen-bond donor residue should not be largely different between H_2O and ^2H_2O. If the hydrogen bond were disrupted in ^2H_2O while the pK_a value of the donor residue remained unaltered, we would have to postulate a large structural change of the protein between the H_2O and ^2H_2O solutions. Judging from the spectral similarity and kinetic behavior of the enzymes in H_2O and ^2H_2O, shown in Figs. 2—4, there is no evidence for such a large structural difference between the two solutions.

Moreover, the detection of another oxygen-isotope sensitive band (at 356 cm^{-1} in Figs. 2B—2D) reported here argues against the previous assignment for the

following reasons. The kinetic behavior of the 788 and 356 cm^{-1} bands in H_2O strongly suggest that these two bands arise from an identical intermediate. The same conclusion can be attained from the 802 and 358 cm^{-1} bands in ^2H_2O. In the case of an Fe(IV)=O heme, we expect only one oxygen-isotope sensitive Raman band; that is, the Fe(IV)=O stretching mode. While it may be argued that the Fe(IV)=O bending mode is also oxygen-isotope sensitive, this mode belongs to the E species of the C_{4v} point group appropriate to the Fe(IV)=O heme, and is expected to be infrared active but resonance Raman inactive. Consequently, it is quite unlikely that the 788 cm^{-1} band arises from an Fe(IV)=O heme moiety.

We suggest here that the 788 and 356 cm^{-1} bands are most appropriately assigned to the O^-–O^- and Fe(III)–O^- stretching modes of an Fe(III)–O^-–O^-–H fragment. The reasons for this assignment are as follows; 1) A simple diatomic approximation for Fe(III)–O_2H (and also for Fe(III)–$O_2{}^2H$) predicts a downshift of 12—13 cm^{-1} for ^{18}O substitution, which is close to the observed values (14—15 cm^{-1}), but a negligible downshift for its O-deuteration (3 cm^{-1}). 2) If the Fe(III)–O stretching force constant of the Fe(III)–O_2H complex is the same as that of Fe(III)–OH whose stretching frequency is located at 450 cm^{-1}, the diatomic approximation for the Fe(III)–O_2H bond predicts its stretching frequency at 355 cm^{-1}. This is satisfactorily close to the new Raman band observed in the present study. 3) The O–O stretching frequency of $^2H_2O_2$ is higher than that of H_2O_2 (by 4 cm^{-1}).[24] 4) In the case of oxyhemerythrin, for which the Fe–O–OH structure has been proposed, the O–O stretching mode is observed at 845 cm^{-1} and shifts to a higher frequency (by 4 cm^{-1}) in ^2H_2O.[25] 5) The observed ^{18}O isotopic frequency shift for the 788 cm^{-1} band (−40 cm^{-1}) is reasonably consistent with its assignment to the O–O stretching mode; using even the simple harmonic oscillator approximation, the expected shift is −45 cm^{-1} for the O–O stretching and −35 cm^{-1} for the Fe(IV)=O stretching mode.

Although the magnitude of the shift in ^2H_2O (+14 cm^{-1}) is appreciably larger than in the case of hemerythrin, it is not unreasonable because the upshift is caused by vibrational coupling between the O–O stretching and O–^2H bending modes.[24] If the frequency difference between the O–^2H bending and O–O stretching modes of the Fe(III)–O–O–H heme of cytochrome oxidase is much smaller than that of oxyhemerythrin, a larger upshift would be expected. In fact, for a porphyrin iron(III)–hydroxy compound, the Fe(III)–^{16}OH stretching Raman band at 541 cm^{-1} was observed to shift up by 13 cm^{-1} to 554 cm^{-1} upon its deuteration as a result of its interaction with the O–^2H bending mode.[26] Taking all these facts into consideration, it is most reasonable at the present stage to assign the 788 and 356 cm^{-1} bands to the O^-–O^- and Fe(III)–O^- stretching modes of the Fe(III)–O–O–H complex formed soon after the Fe–O_2 complex. A reaction mechanism involving the hydroperoxo intermediate instead of a μ-peroxo intermediate is compatible with the suggestion[27] from the infrared study of cyanide binding to this enzyme.

Alternatively the 356 cm^{-1} band could be assigned to the Fe(III)–O–OH bending mode. The Fe(III)–O–O bending mode is reported for oxyphthalocyanine iron(II)[28] at 289 cm^{-1} but for all other oxy-iron porphyrins and oxy-hemeproteins the Fe(II)–O–O bending Raman band has not been identified. Such a band has not been observed for the primary intermediate of cytochrome oxidase shown in Fig. 2 (Δt_d=0.1 ms). The Fe–C–O bending Raman band for the CO-bound hemeproteins is usually much weaker than the Fe–CO stretching Raman band.[29,30] In contrast, the 356 cm^{-1} band of the intermediate in question has greater intensity than the Fe(II)–O_2 stretching mode. Consequently, the assignment of the 356 cm^{-1} band to the Fe(III)–O–O bending mode does not seem likely. However, definitive assignment of the 788 and 356 cm^{-1} bands will require experiments using $^{16}O^{18}O$ which will soon be undertaken in this laboratory.

On the basis of the present assignment, the 790 cm^{-1} Raman band reported by Varotsis and Babcock,[12] which showed no frequency shift upon deuteration, might be assigned to the Fe(IV)=O stretching mode of the ferryloxo complex. Since the peak power of the laser used by Varotsis and Babcock is higher by a factor of 10^5 than those used by the two other groups, it is certainly possible that the O–O bond of the Fe(III)–O–O–H complex suffered photocleavage and the Fe(IV)=O heme was generated. In fact, the O–O bond of Fe(II)–O–O–Fe(II) porphyrin complex is very photolabile even at low temperature and the photoreaction yields the Fe(IV)=O porphyrin.[31] If this is the case, the 790 cm^{-1} band should be regarded as an artifact and should not be assigned to a physiologically relevant intermediate, although this observation served as a basis for a unified view of the reaction scheme.[14]

Hydroperoxy and Ferryloxo Intermediates. The 788 and 356 cm^{-1} bands which apparently arise from a hydroperoxy intermediate, persisted (at 5 °C) at least up to 3.2 ms after initiation of the reaction (see Figs. 4B and 4C). The half-life is approximately 2.5 ms at 5 °C. The rate constant for the rate limiting step in the oxidation reaction of fully-reduced cytochrome oxidase with O_2, obtained from flash photolysis absorption studies, is ca. 1000 s^{-1} ($t_{1/2}$=700 μs) at 25 °C,[32] which is in good agreement with the value obtained in this study provided one takes into consideration the fact that the rate constant approximately doubles on every 10 °C temperature increase; $t_{1/2}$=700 μs at 25 °C should correspond to $t_{1/2}$=2.8 ms at 5 °C.

In Fig. 4 the hydroperoxy intermediate seems to be directly converted to the iron(III) hydroxide intermediate. However, this observation does not necessarily demand it. Involvement of a one electron reduced ferryloxo intermediate[7,33] in the process is possible,

although we could not clearly detect the Fe(IV)=O stretching Raman band, probably because of its short lifetime and/or weak enhancement of that intermediate. Different resonance Raman enhancement factors for two species may occur when the absorption maxima of the two are different. If the excitation wavelength of 423 nm in this study is accidentally much closer to the hydroperoxy intermediate rather than to that of ferryl-oxo intermediate, Raman bands of the hydroperoxy intermediate could dominate the resonance Raman spectra. Although the hydroperoxy-iron porphyrin[34] is generally much more reactive than the ferryloxo one, a shorter lifetime of the ferryloxo intermediate, compared with that of the hydroperoxy intermediate, is not unexpected, as was suggested by Oliveberg et al.[35]

It is useful to estimate the potential relative population of the ferryloxo intermediate during the cytochrome oxidase turnover. In order to gain an insight into this, we carried out a simple calculation for a chain reaction (A→B→C→D→E) in which each intermediate decays with rate constants of k_A, k_B, k_C, and k_D s^{-1}, respectively. The A, B, C, D, and E intermediates correspond to the Fe(II)–O_2, Fe(III)–O–O–H, Fe(IV)=O, Fe(III)–OH and the last Fe(III) species, respectively. As noted above, the final Fe(III) species might be a Fe(III)–OH compound but, if so, its axial ligand is exchangeable with bulk water. In this case, k_D represents the exchange rate. At time, t, after initiation of the reaction, the fractional population of each species can be represented by the following equations;

$$[A] = \exp(-k_A t),$$

$$[B] = k_A[\exp(-k_A t)/(k_B - k_A) + \exp(-k_B t)/(k_A - k_B)],$$

$$[C] = k_A k_B\{\exp(-k_A t)/[(k_B - k_A)(k_C - k_A)] + \exp(-k_B t)/[(k_A - k_B)(k_C - k_B)] + \exp(-k_C t)/[(k_A - k_C)(k_B - k_C)],$$

$$[D] = k_A k_B k_C\{\exp(-k_A t)/[(k_B - k_A)(k_C - k_A)(k_D - k_A)] + \exp(-k_B t)/[(k_A - k_B)(k_C - k_B)(k_D - k_B)] + \exp(-k_C t)/[(k_A - k_C)(k_B - k_C)(k_D - k_C)] + \exp(-k_D t)/[(k_A - k_D)(k_B - k_D)(k_C - k_D)],$$

$$[E] = 1 - ([A] + [B] + [C] + [D]).$$

Since the present experimental data are not sufficient for an accurate determination of rate constants, we tentatively used the following values for the rate constants; k_A=3000, k_B=250, k_C=1400, and k_D=1000. The calculated curves for the fractional population vs. time are depicted in Fig. 5. The curves satisfactorily reproduce the decay of the 571 cm^{-1} band, the rise and decay of the 788 and 356 cm^{-1} bands and the rise and decay of the 450 cm^{-1} band. We note that the mixmum population of the 450 cm^{-1} species takes place at t=3.5 ms. If k_C were smaller than k_B (for example, k_C=200), the increase in D [the Fe(III)–OH species] became noticeably slower and did not reproduce the observed curves. Consequently, it is likely that the population of the ferryloxo intermediate during turnover cannot be very large. Direct reduction of the hydroperoxy intermediate to Fe(III)–OH without forming a higher energy intermediate, that is, the Fe(IV)=O heme, is also possible for the cytochrome oxidase active site where two reductive equivalents are readily available through Fe_a and Cu_A as has been suggested by Yoshikawa and Caughey.[27] It is quite interesting to point out that if our assignment is correct, the decay rate of the hydroperoxy intermediate is close to the rate of proton release.[35]

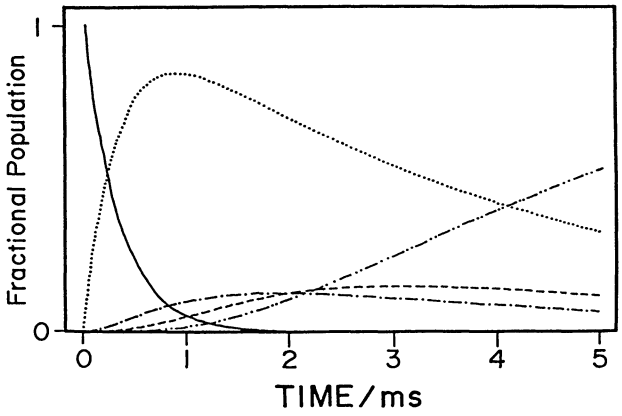


Fig. 5. Fractional population of the Fe(II)–O_2 (——), Fe(III) –O–O–H (·····), Fe(IV) =O (—·—), Fe(III) –OH (------), and the last Fe(III) compound (—··—). See text about the method of calculations, It is assumed that [Fe(II)–O_2] at time =0 is unity.

Previously Han et al.[36] reported the Fe(III)–OH stretching Raman band at 477 cm^{-1} for a compound produced under photoreduction of the resting enzyme in the presence of hexacyanoferrate(III). This compound, if correctly assigned, would be different from the Fe(III)–OH intermediate generated in the catalytic cycle. The present conclusion is consistent with another report by Han et al.[13] The iron–oxygen stretching frequency of the Fe(III)–OH compound of cytochrome oxidase is appreciably lower than those of aquametmyoglobin (490 cm^{-1}) and aquamethemoglobin (495 cm^{-1}).[37] In contrast, the iron–oxygen stretching frequency of the Fe(II)–O_2 complex of cytochrome oxidase (571 cm^{-1}) is noticeably close to those of oxymyoglobin (569 cm^{-1}) and oxyhemoglobin (567—572 cm^{-1}).[20,21] Based on these observations, it may be concluded that the distinguishing features of the cytochrome oxidase active site environment are most clearly reflected by their effects on the Fe(III)–OH stretching frequency.

The authors thank Dr. T. Egawa of IMS for his help in calculation of fractional population of intermediates and Professor Jim Kincaid of Marquett University for reading this manuscript. This study was partly supported by Grant-in-Aid for Encouragement of Young Scientists of the Ministry of Education, Science and Culture, to T. O. (02740250) and also Grant-in-Aid for Priority Areas (Bioinorganic Chemistry) to T. K. (03241105).

References

1) M. Wikstrom, K. Krab, and M. Saraste, in "Cytochrome Oxidase-A Synthesis," Academic Press, London (1981).
2) S. I. Chan and P. M. Li, *Biochemistry,* **29**, 1 (1990).
3) M. Wikstrom, *Proc. Natl. Acad. Sci. U.S.A.,* **78**, 4051 (1981).
4) Y. Orii, *Ann. N. Y. Acad. Sci.,* **550**, 105 (1988).
5) B. Chance, C. Saronio, and J. S. Leigh, Jr., *J. Biol. Chem.,* **250**, 9226 (1975).
6) G. M. Clore, L. E. Andreasson, B. Karlsson, R. Aasa, and B. G. Malmstrom, *Biochem. J.,* **185**, 139 (1980).
7) S. N. Witt and S. I. Chan, *J. Biol. Chem.,* **262**, 1446 (1987).
8) T. Ogura, S. Takahashi, K. Shinzawa-Itoh, S. Yoshikawa, and T. Kitagawa, *J. Am. Chem. Soc.,* **112**, 5630 (1990).
9) S. Han, Y. Ching, and D. L. Rousseau, *Proc. Natl. Acad. Sci.U.S.A.,* **87**, 2491 (1990).
10) C. Varotsis, W. H. Woodruff, and G. T. Babcock, *J. Am. Chem. Soc.,* **112**, 1297 (1990).
11) T. Ogura, S. Takahashi, K. Shinzawa-Itoh, S. Yoshikawa, and T. Kitagawa, *J. Biol. Chem.,* **265**, 14721 (1990).
12) C. Varotsis and G. T. Babcock, *Biochemistry,* **29**, 7357 (1990).
13) S. Han, C. Ching, and D. L. Rousseau, *Nature (London),* **348**, 89 (1990).
14) M. Wikstrom and G. T. Babcock, *Nature (London),* **348**, 16 (1990).
15) S. Yoshikawa, M. G. Choc, M. C. O' Toole, and W. S. Caughey, *J. Biol. Chem.,* **252**, 5498 (1977).
16) T. Ogura, S. Yoshikawa, and T. Kitagawa, *Biochemistry,* **28**, 8022 (1989).
17) T. Ogura, A. Maeda, M. Nakagawa, and T. Kitagawa, in "Primary Processes in Photobiology," ed by T. Kobayashi, Springer Proceedings in Physics, Vol. 20, pp. 233—241 (1987).
18) T. Ogura and T. Kitagawa, to be published.
19) H. E. Van Wart and J. Zimmer, *J. Biol. Chem.,* **260**, 8372 (1985).
20) H. Brunner, *Naturwissenshaften,* **61**, 129 (1974).
21) K. Nagai, T. Kitagawa, and H. Morimoto, *J. Mol. Biol.,* **136**, 271 (1980).
22) J. Terner, A. J. Sitter, and C. M. Reczek, *Biochim. Biophys. Acta,* **828**, 73 (1985).
23) S. Hashimoto, Y. Tatsuno, and T. Kitagawa, *Proc. Natl. Acad. Sci. U.S.A.,* **83**, 2417 (1986).
24) P. A. Giguere and T. K. K. Srinivasan, *J. Raman Spectrosc.,* **2**, 125 (1974).
25) A. K. Shiemke, T. M. Loehr, and J. Sanders-Loehr, *J. Am. Chem. Soc.,* **106**, 4951 (1984).
26) R. A. Reed, K. R. Rodgers, K. Kushmeider, T. G. Spiro, and Y. O. Su, *Inorg. Chem.,* **29**, 2881 (1990).
27) S. Yoshikawa and W. S. Caughey, *J. Biol. Chem.,* **265**, 7945 (1990).
28) K. Bajdor, H. Oshio, and K. Nakamoto, *J. Am. Chem. Soc.,* **106**, 7293 (1984).
29) N. T. Yu, E. A. Kerr, B. Ward, and C. K. Chang, *Biochemistry,* **22**, 4534 (1983).
30) M. Nagai, Y. Yoneyama, and T. Kitagawa, *Biochemistry,* **30**, 6495 (1991).
31) Y. Mizutani, S. Hashimoto, Y. Tatsuno, and T. Kitagawa, *J. Am. Chem. Soc.,* **112**, 6809 (1990).
32) Y. Orii, *Chem. Scr.,* **28A**, 63 (1988).
33) C. Kumar, A. Naqui, L. Powers, Y. Ching, and B. Chance, *J. Biol. Chem.,* **263**, 7159 (1988).
34) K. Tajima, M. Shigematsu, J. Jinno, K. Ishizu, and H. Ohya-Nishiguchi, *J. Chem. Soc., Chem. Commun.,* **1990**, 143.
35) M. Oliveberg, S. Hallen, and T. Nilsson, *Biochemistry,* **30**, 436 (1991).
36) S. Han, C. Ching, and D. L. Rousseau, *J. Biol. Chem.,* **264**, 6604 (1989).
37) S. A. Asher and T. M. Schuster, *Biochemistry,* **18**, 5377 (1979).