Review

Resonance Raman characterization of the P intermediate in the reaction of bovine cytochrome c oxidase

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Abstract

Reduced cytochrome c oxidase binds molecular oxygen, yielding an oxygenated intermediate first (Oxy) and then converts it to water via the reaction intermediates of P, F, and O in the order of appearance. We have determined the iron–oxygen stretching frequencies for all the intermediates by using time-resolved resonance Raman spectroscopy. The bound dioxygen in Oxy does not form a bridged structure with Cu_B and the rate of the reaction from Oxy to P (P_R) is slower at higher pH in the pH range between 6.8 and 8.0. It was established that the P intermediate has an oxo-heme and definitely not the Fe_{a_3}–O–O–Cu_B peroxy bridged structure. The Fe_{a_3}=O stretching ($\nu_{Fe=O}$) frequency of the P_R intermediate, 804/764 cm^{-1} for $^{16}O/^{18}O$, is distinctly higher than that of F intermediate, 785/750 cm^{-1}. The rate of reaction from P to F in D_2O solution is evidently slower than that in H_2O solution, implicating the coupling of the electron transfer with vector proton transfer in this process. The P intermediate (607-nm form) generated in the reaction of oxidized enzyme with H_2O_2 gave the $\nu_{Fe=O}$ band at 803/769 cm^{-1} for $H_2^{16}O_2/H_2^{18}O_2$ and the simultaneously measured absorption spectrum exhibited the difference peak at 607 nm. Reaction of the mixed valence CO adduct with O_2 provided the P intermediate (P_M) giving rise to an absorption peak at 607 nm and the $\nu_{Fe=O}$ bands at 804/768 cm^{-1}. Thus, three kinds of P intermediates are considered to have the same oxo-heme a_3 structure. The ν_4 and ν_2 modes of heme a_3 of the P intermediate were identified at 1377 and 1591 cm^{-1}, respectively. The Raman excitation profiles of the $\nu_{Fe=O}$ bands were different between P and F. These observations may mean the formation of a π cation radical of porphyrin macrocycle in P.
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1. Introduction

Cytochrome c oxidase (CcO) catalyzes reduction of molecular oxygen to water using electrons delivered by cytochrome c and functions as an electron-transfer-driven proton pump in aerobic organisms [1], generating the electrochemical potential to be used for ATP synthesis. CcO has four redox-active metal centers, i.e., two Cu centers (Cu_A and Cu_B) and two heme a groups (heme a and heme a_3). The Cu_A center contains two sulfur-bridged copper ions, receiving electrons from cytochrome c and giving them to heme a. Heme a, which is a six-coordinated low-spin heme, serves as an electron carrier from Cu_A to the heme a_3–Cu_B binuclear center, and its redox

may trigger protonation/deprotonation of key carboxylate groups working for the proton pump [2]. Heme a_3, which is a five-coordinated high-spin heme, binds dioxygen and converts it to water. While Cu_B is located close to Fe of heme a_3 (Fe_{a_3}) (500 pm apart) and therefore both Cu_B^{II} and Fe_{a3}^{III} are EPR silent due to antiferromagnetic coupling, a role of Cu_B in the dioxygen reduction is not clear. The fully reduced (FR) CcO ($Fe_a^{II}Cu_A^{I}Fe_{a3}^{II}Cu_B^{I}$) has four extra electrons and the mixed-valence (MV) CcO ($Fe_a^{III}Cu_A^{II}Fe_{a3}^{II}Cu_B^{I}$) has two extra electrons compared with the fully oxidized (FO) state ($Fe_a^{III}Cu_A^{II}Fe_{a3}^{III}Cu_B^{II}$). X-ray crystallographic structures have been reported for bovine [3–5], Paracoccus denitrificans [6,7], and Thermus thermophilus CcOs [8].

Resonance Raman (RR) spectroscopy has been acknowledged as a powerful tool to investigate the structure of the heme and its vicinity of hemoproteins [9]. The structures of reaction intermediates of CcO have extensively been studied with time-resolved RR (TR^3) spectroscopy

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[10–12]. The reaction starts from binding of dioxygen to the reduced heme a_3, yielding the oxygenated intermediate (Oxy). One electron reduction of Oxy provides the P intermediate. Accordingly, P has the same oxidation level as Compound I of horseradish peroxidase, which is higher than a ferric state by two equivalents. The location of the two extra oxidative equivalents has been a matter of chemical concern [13]. The structure of P has long been postulated to be $Fe_{a_3}^{III}-O^--O^--Cu_B^{II}$ without sound experimental basis [1], but it turned out from Raman experiments that dioxygen in Oxy has not a bridged peroxy structure[10–12] and that the O–O bond had been already cleaved in P [14].

As illustrated by (**1**)–(**5**) in Fig. 1, there are five possibilities for a structure of binuclear center of P. If the oxidative equivalents are shared by the heme and protein, it should yield a neutral $Fe^{IV}=O$ heme and an amino acid radical (**1**). When the extra oxidative equivalents are shared by iron and porphyrin macrocycle or concentrated on an iron atom, it should yield an $Fe^{IV}=O$ porphyrin π cation radical (**2**) or an $Fe^V=O$ heme (**3**), respectively. If Cu_B is responsible for one equivalent, $Cu^{III}=O^{2-}$(**4**) or $Cu^{II}-O^{.1-}$ (**5**) should be present together with a neutral $Fe^{IV}=O$ O heme. Location of the extra oxidative equivalents is a current subject of debate and also a subject of this paper. The reduction of P provides the F intermediate, which has an ordinary ferryloxo heme. Further reduction of F gives the $Fe^{III}-OH$ heme, which corresponds to the pulsed form of the FO–CcO. Here we derive the P intermediate with three different methods and compare its heme a_3 structures on the basis of their RR spectra.

2. P intermediate in the O_2 reduction by FR–CcO (P_R)

Fig. 2 shows the TR^3 spectra observed for the reaction of the FR–CcO with O_2 at 3 °C with the delay times (Δt) of 0.1 (A), 0.27 (B), 0.54 (C), 2.7 (D), and 5.4 ms (E) after the start of the reaction [14]. The spectra are represented as one-to-one differences between the $^{16}O_2$ and $^{18}O_2$ derivatives. Accordingly, positive and negative peaks indicate the vibrations associated with ^{16}O and ^{18}O, respectively, and the vibrations, which are not accompanied by movements of the oxygen atom, are canceled. Spectrum A gives rise to the Fe–O_2 stretching bands (ν_{Fe-O_2}) at 571/544 cm^{-1} for the $^{16}O_2/^{18}O_2$ derivatives. These frequencies and the isotope shift are very close to those seen for HbO_2 and MbO_2 [15] and, therefore, the $Fe_{a3}^{III}-O^--O^--Cu_B^{II}$ structure is ruled out. In the next stage ($\Delta t = 0.27$ ms), new bands appear at 804/764, 785/750, and 356/342 cm^{-1}. Spectrum D, which exhibits only the 785/750 cm^{-1} pair near 800 cm^{-1}, strongly suggests that the 804/764 cm^{-1} pair precedes the 785/750 cm^{-1} pair. Accordingly, the 804/764 and 785/750 cm^{-1} pairs are assigned to the P and F intermediates, respectively. The 356/342 cm^{-1} pair can be seen when the 804/764 cm^{-1} pair is present. A new pair appears at 450/425 cm^{-1} in spectrum D. The 450/425 cm^{-1} pair, which is shifted to 443/417 cm^{-1} in D_2O (data not shown) and therefore assigned to the Fe–OH stretching mode (ν_{Fe-OH}) of the $Fe_{a3}^{III}-OH$ heme, is present at $\Delta t = 2.7$ ms (spectrum D), but disappears at $\Delta t = 5.4$ ms (spectrum E) due to exchange of the bound $^{18}OH^-$ anion with $^{16}OH^-$ of bulk water. The ν_{Fe-OH} frequency is significantly lower than those of aquametHb (495 cm^{-1}) and aquametMb (490
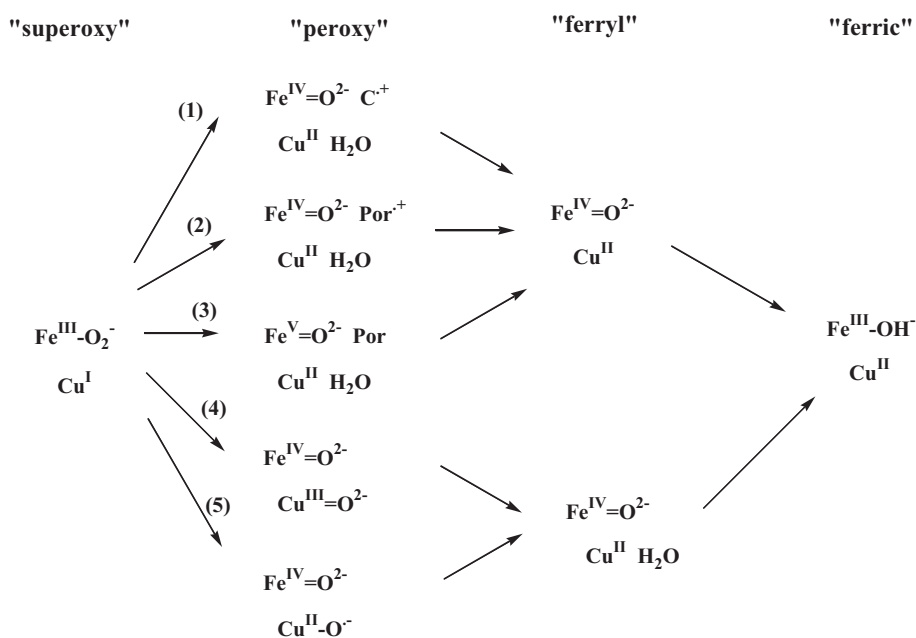

Fig. 1. Possible pathways for reduction of O_2 bound the heme a_3–Cu_B center of CcO. The oxidation state of the binuclear center is designated in terms of a journal oxidation level of Fe_{a_3}. The terms Por, Por$^+$, and C$^+$ mean a nonionized porphyrin, a prophyrin π cation radical, and an amino acid cation radical, respectively. (Reproduced from Ref. [14].)
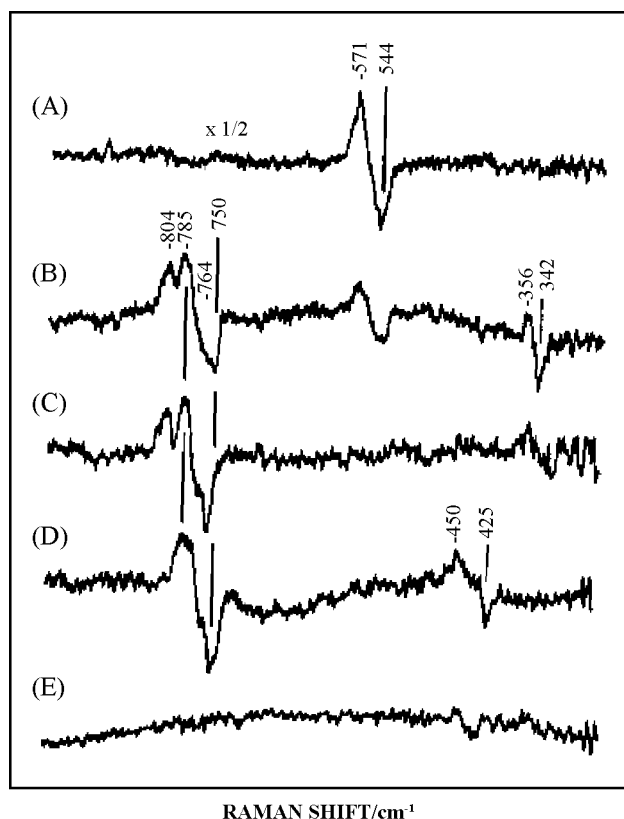
Fig. 2. The TR^3 difference spectra of reaction intermediates of FR-CcO in H$_2$O. The Raman difference spectrum obtained by subtracting the spectrum of the corresponding $^{18}O_2$ derivative from the spectrum of $^{16}O_2$ derivative at each delay time is depicted. Therefore, positive and negative peaks denote the contributions of $^{16}O_2$ and $^{18}O_2$ derivatives, respectively. Delay time after initiation of the reaction is 0.1 (A), 0.27 (B), 0.54 (C), 2.7 (D), and 5.4 ms (E). Excitation wavelength, 423 nm. Temperature, 3 °C. (Reproduced from Ref. [14].)

cm^{-1}) [16], presumably due to a strong interaction between the bound OH$^-$ group and Cu$_B$.

The assignment of the 804/764 and 785/750 cm^{-1} pairs holds the key for a structure of heme a_3. Two kinds of oxygen isotope sensitive bands are expected in this frequency region; one is the peroxy O$^-$–O$^-$ stretch and the other is an ironoxo Fe=O stretch. These two modes cannot be distinguished by the $^{16}O_2$ and $^{18}O_2$ isotopic frequency shifts. However, if $^{16}O^{18}O$ is used to produce such intermediates, the distinction would be possible. The peoxy intermediate for $^{16}O^{18}O$ is expected to give an additional ν_{O-O} band in the middle of those for the $^{16}O_2$ and $^{18}O_2$ derivatives. In contrast, the oxo intermediate for $^{16}O^{18}O$ is expected to yield only two bands at the same frequencies as those seen for the $^{16}O_2$ and $^{18}O_2$ derivatives but with one-half of their intensity.

Such a distinction was satisfactorily worked out with pure $^{16}O^{18}O$ on the 804/764 and 785/750 cm^{-1} bands [17]. The results indicated that the bands appear at the same frequencies as the case of $^{16}O_2$ and $^{18}O_2$ when $^{16}O^{18}O$ was used but with half intensities. This was demonstrated by the

difference calculations of the spectrum for $^{16}O^{18}O$ minus half of the sum of spectra for $^{16}O_2$ and $^{18}O_2$, which yielded no trace of remaining peaks [17]. The same features were also seen for the D$_2$O solution. These features definitely differed from that seen for the Oxy intermediate, which was turned out to be of an end-on type with ∼ 120° of the Fe–O–O angle [17]. These results indicate that only one atom of O$_2$ is primarily responsible for the two RR bands. In other words, neither of the bands at 804/764 nor 785/750 cm^{-1} is assignable to the O–O stretching mode. Although the two sets of RR bands arise from an Fe=O stretch, electronic properties of their hemes seem to be distinct. The 804/764 cm^{-1} bands were clearly identified upon excitation at 441.6 nm, but the 785/750 cm^{-1} bands could not be recognized. This means that the frequency difference between the two pairs cannot be interpreted simply by presence or absence of hydrogen bonding to the oxo-oxygen atom bound to ferryl-oxo heme a_3 having essentially an identical electronic structure, although both band pairs at 804/764 and 785/750 cm^{-1} were observed upon excitation at several wavelengths between 580 and 615 nm (T. Ogura and T. Kitagawa, unpublished observations).

The 356/342 cm^{-1} pair has the same frequencies for intermediates derived with $^{16}O^{18}O$. Its frequency is not sensitive to H$_2$O/D$_2$O exchange, indicating that this vibration is not associated with a hydroxy group having an exchangeable proton. Therefore, its assignment to Cu$_B$–OH stretch [18] is unlikely. This pair is missing in spectrum D, and indeed the relative intensities of the 356/342 cm^{-1} bands to the 804/764 cm^{-1} bands varied with Δt in repeated experiments, suggesting that these pairs arise from separate molecular species. Thus, it is most likely that another species is present between the P and F intermediates and that this putative species has the same oxidation state as that of P and would give both ∼ 805/ ∼ 765 and 356/342 cm^{-1} pairs.

The same experiments were carried out with D$_2$O solution [14]. The frequencies of all difference peaks were the same as those observed for the H$_2$O solution except for the 450/425 cm^{-1} pair. It is noted that the appearance of the 785/750 cm^{-1} pair was significantly delayed in D$_2$O compared with in H$_2$O. The 356/342 cm^{-1} pair was clearly seen in the time interval in which the 804/764 cm^{-1} pair was present. It is suggested from the frequency and isotope shift that the 356/342 cm^{-1} pair arises from the N(His)–Fe$_{a_3}$–O deformation vibration [14], and the assignment was indeed supported by recent theoretical calculations by Ghosh and Skanke [19]. This vibration should be Raman inactive for a linear N–Fe–O structure and has never been observed for compounds I and II of peroxidases. When N(His) is displaced from the upright position of the heme iron, it can be Raman active. Recalling that the rate of the P to F reaction is H$_2$O/D$_2$O sensitive, the phenomenon would be understandable if this process is coupled with proton pumping through deformation of proximal His of heme a_3. Consequently, it is deduced that the ∼ 805/ ∼ 765 and 356/

342 cm^{-1} pairs arise from an intermediate with the same oxidation state as P but with a deformed heme a_3 structure.

3. P intermediate in the O$_2$ reduction by MV-CcO (P$_M$)

Incubation of oxidized CcO under CO atmosphere overnight brings about the MV-CcO. Fig. 3 shows the time-resolved $^{16}O_2/^{18}O_2$-difference RR spectra of MV-CcO observed at pH 8.0 in the delay time region between 100 μs to 2 ms [20]. Spectrum A gives the ν_{Fe-O_2} band at 571/544 cm^{-1}. As it becomes weaker, another difference peaks appear at 804/768 and 356/342 cm^{-1}. However, even for the longest delay time (2 ms, spectrum F), the 785/750 cm^{-1} pair did not appear. This is compatible with the fact

Fig. 4. Temporal intensity change of the 571 (circle), 804 (triangle), and 356 (square) cm^{-1} bands relative to a porphyrin band at 683 cm^{-1} for the reaction of MV-CcO with O$_2$ at pH 6.8 (upper) and pH 8.0 (lower). Closed and open markers denote the values at pH 6.8 and 8.0, respectively, and solid lines represent smooth curves obtained with the least-square fitting method. (Reproduced from Ref. [20].)
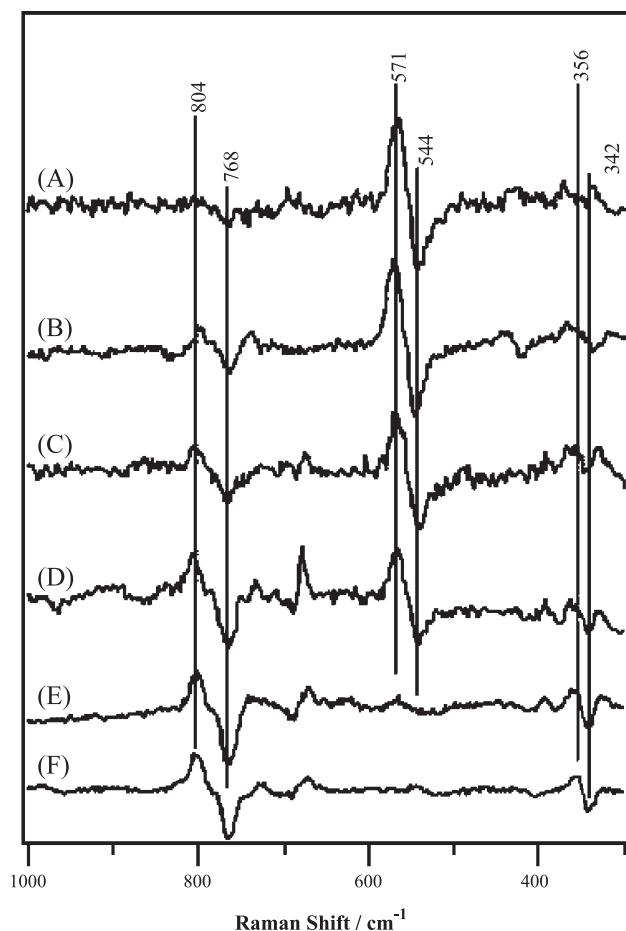

Fig. 3. The TR^3 difference spectra of reaction intermediate of MV-CcO at pH 8.0 in H$_2$O. The Raman difference spectrum obtained by subtracting the spectrum of the corresponding $^{18}O_2$ derivative from the spectrum of $^{16}O_2$ derivative at each delay time is depicted. Therefore, positive and negative peaks denote the contributions of $^{16}O_2$ and $^{18}O_2$ derivatives, respectively. These difference spectra are normalized with the ν_7 band at 683 cm^{-1} in the absolute spectra. Delay time after initiation of the reaction is 0.1 (A), 0.15 (B), 0.20 (C), 0.40 (D), 1.0 (E), and 2.0 ms (F). Excitation wavelength, 423 nm. Temperature, 20 °C.
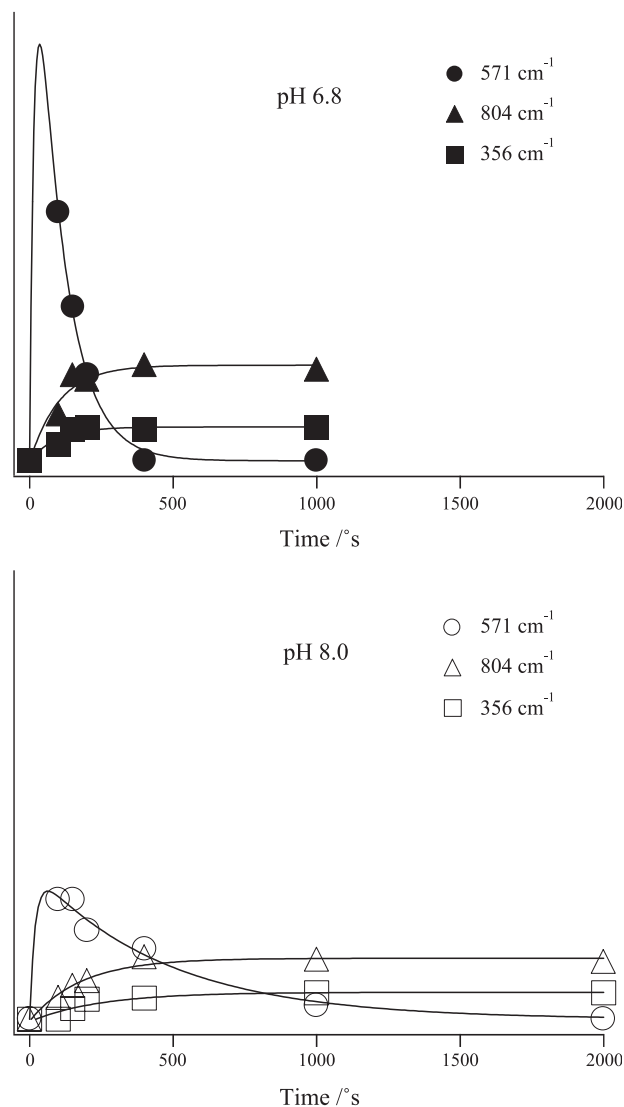
that MV-CcO has only two electrons and therefore the reaction cannot proceed to F.

Oda et al. [20] found for the reaction of MV-CcO with O$_2$ that the ν_{Fe-O_2} frequency remains unaltered at pH 6.8 but its decay is much faster than that in Fig. 3. The intensities of oxygen-isotope sensitive bands of the $^{16}O_2$ derivatives at 571, 804, and 356 cm^{-1} relative to a porphyrin band at 683 cm^{-1} in the raw spectra (I_{571}/I_{683}, I_{804}/I_{683}, and I_{356}/I_{683}, respectively) are plotted against delay time in Fig. 4, where upper and lower panels show the results at pH 6.8 and 8.0, respectively. The solid lines denote the least-square-fitted curves under the assumption that the 571 cm^{-1} band (ν_{Fe-O_2}) rises and decays exponentially and the 804 and 356 cm^{-1} bands rise to reach certain intensity without decay.
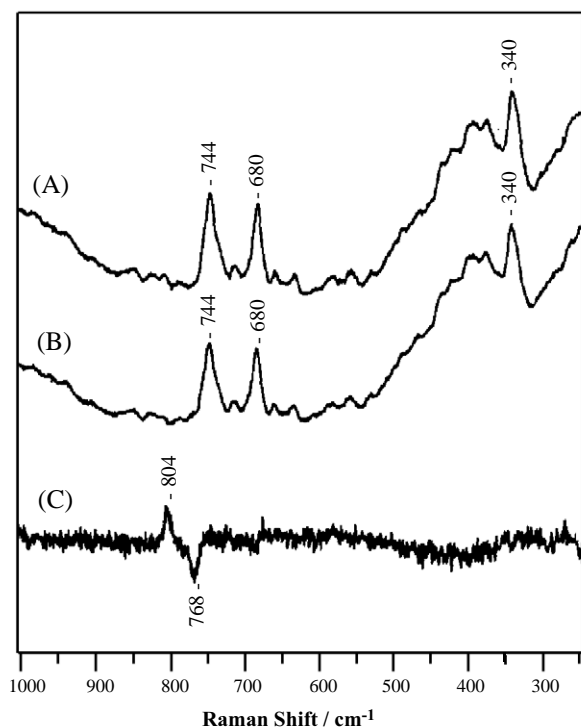
Fig. 5. The RR spectra of the P_M form derived after 1-day incubation of FO-CcO at pH 8.0 under CO with $^{16}O_2$ (A) and $^{18}O_2$ (B) and their difference spectrum [C = 2(A − B)]. Experimental conditions: excitation, 441.6 nm, 0.7 mW at the sample; temperature, 20 °C; concentration of enzyme, 35 μM. (Reproduced from Ref. [21].)

The pseudo-first-order rate constant for the rise of the band at 571 cm^{-1} was assumed to be 3.5×10^8 M^{-1} s^{-1} [11]. Since the concentration of oxygen in this experiment was 0.6 mM, the rise rate constant is 2.1×10^5 s^{-1}. Provided that k_1, k_2, and k_3 denote the decay rate constant of the 571

cm^{-1} band and the rise rate constants of the 804 and 356 cm^{-1} bands, respectively, the least square fitting yielded the values of $k_1 = 1.1(0.1) \times 10^4$, $k_2 = 9.7(2.8) \times 10^3$, $k_3 = 1.1(0.4) \times 10^4$ s^{-1} at pH 6.8 (a number in parenthesis denotes a standard deviation). These values became smaller at pH 8.0 and are $k_1 = 2.2(0.3) \times 10^3$, $k_2 = 5.6(1.0) \times 10^3$, $k_3 = 4.1(1.7) \times 10^3$ s^{-1}. Note that the decay of Oxy is much slower at pH 8.0 than that at pH 6.8, presumably due to partial deprotonation of Tyr-244 at pH 8.0, while the rise of the 804 cm^{-1} band seems to have a correlation with the decay of Oxy. Anyway, it is reasonable that the reaction stops at P for MV−CcO and that the reaction from Oxy to P is sensitive to pH since this process involves the O−O bond cleavage and internal proton transfer.

Addition of O_2 to CO adduct of MV−CcO provides a compound that exhibits a peak at 607 nm in the difference absorption spectrum with regard to FO−CcO. The RR spectra of this compound derived with $^{16}O_2$ and $^{18}O_2$, which were observed by Kim et al. [21] are displayed by Fig. 5A and B, respectively, and their difference by Fig. 5C. Since the excitation wavelength used was 441.6 nm, which is somewhat shifted to red compared with the absorption maximum of the P intermediate, Raman intensities of porphyrin modes are relatively weaker and as a result, the $\nu_{Fe=O}$ band is recognizable at 804 ($^{16}O_2$) and 768 ($^{18}O_2$) cm^{-1} in the raw spectra. Recent results by Oda et al. [20] using $^{16}O^{18}O$ gave the bands at 804 and 768 cm^{-1} each with half intensity of those for $^{16}O_2$ and $^{18}O_2$ similar to the case of FR-CcO with O_2 [17]. The 804 cm^{-1} band for the ^{16}O- and ^{18}O-derivatives showed frequency upshift by 2 cm^{-1} in D_2O, indicative of the presence of hydrogen bonding to the oxo oxygen atom.

In this spectrum, the 356 cm^{-1} band is absent and this feature was noted as a possible difference between the P_R
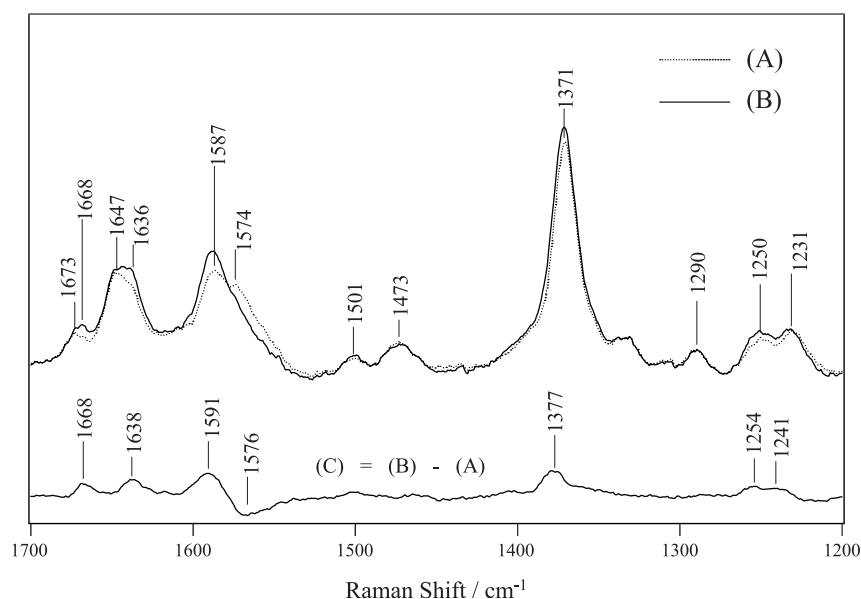

Fig. 6. The RR spectra in the high-frequency region of FO-CcO (A) and the reaction intermediate of MV-CcO with O_2 (B) and their difference (C = A − B). (Reproduced from Ref. [20].)

and P_M [21]. Recently, however, Raman excitation profiles of the 804 and 356 cm^{-1} bands were examined for the same P_M species and slight difference was found between the two modes, presumably due to overlapping of the linear P_M form only with the 804 cm^{-1} band. The 356 cm^{-1} band was in fact observed upon excitation at 427.1, 426.5, and 423.0 nm, but not at 441.6 nm [20]. The pH-dependent Raman and absorption changes of P_M were explored at pH values between 8.0 and 6.0 [20]. The absorbance at 607 nm was decreased by lowering pH and at the same time the intensities of the 804 and 356 cm^{-1} bands also decreased. These observations demonstrated that the 804 and 356 cm^{-1} bands as well as the 607-nm absorption arise from heme a_3 in the deformed P_M form, which is a stable P form, and the heme structure is essentially the same as that of P_R.

To see if heme a_3 of P_M is a porphyrin π cation radical or not, RR spectra in the high-frequency region, where RR bands due to porphyrin in-plane vibrations primarily appear, were examined [20]. Fig. 6 depicts raw RR spectra of FO–CcO (A) and the P_M intermediate (B) and their difference (C = B − A). Although the raw spectra contain the contributions from heme a^{3+} moiety, the difference spectrum is expected to reflect solely the contribution from the heme a_3 moiety, namely, the intermediate minus the resting a_3^{3+}. The difference spectrum indicates that the $ν_4$ and $ν_2$ modes of the intermediate lie at 1377 and 1591 cm^{-1}, respectively. The formyl CH=O stretching and $ν_{10}$ of the intermediate are seen at 1668 and 1638 cm^{-1}, respectively. The negative peak at 1576 cm^{-1} indicate the location of $ν_2$ of the ferric high-spin heme a_3. Previously, Oertling et al. [22] investigated RR spectral changes upon formation of π cation radical for several four-coordinate metalloporphyrins and concluded that the vibrations involving C_a–N and C_a–C_m stretches are shifted to lower frequencies and conversely the vibrations involving C_b–C_b stretch are shifted to higher frequencies irrespective of the a_{1u} or a_{2u} type. The high-frequency shift of $ν_2$ observed for P_M is consistent with the formation of π cation radical, although such an empirical rule might be changed for an oxoheme with a formyl side chain. Recent DFT calculations on the P intermediate of CcO [23] also support the formation of π cation radical. One may argue against this suggestion by noting that the $ν_4$ frequency is shifted to a higher frequency contradictory to the empirical rule mentioned above. We note that such frequencies should be compared between the same oxidation states of iron, because the $ν_4$ frequency is thought to be sensitive to the oxidation state of iron.

4. P intermediate in the reaction of FO–CcO with H_2O_2

It was pointed out by Vygodina and Konstantinov [24] that the reaction of FO–CcO with H_2O_2 yields first a species with a peak at 607 nm and second a species with a peak at 580 nm in the difference spectrum against that of FO–CcO. The EPR spectra of these intermediates were

examined by Fabian and Palmer [25]. Since the 607-nm absorption form is the first intermediate in the reaction with H_2O_2, it should correspond to compound I of peroxidases. The RR spectra of the '607 nm' form excited at 607 nm [26] are shown in Fig. 7, where the raw spectra observed for the reaction of FO–CcO with $H_2^{16}O^{16}O$ (A), $H_2^{18}O^{18}O$ (B), and $H_2^{16}O^{18}O$ (C) and differences, (D = A − B) and (E = C − A/2 − B/2) are depicted. The porphyrin modes are canceled in the difference spectra and accordingly, it is evident from spectrum D that the band at 803 cm^{-1} is shifted to 769 cm^{-1} when $H_2^{16}O^{16}O$ was replaced with $H_2^{18}O^{18}O$. Extension of the difference spectrum 7D is given in the inset. It is obvious that the 803/769 cm^{-1} pair is the only oxygen isotope sensitive band in the 950–500 cm^{-1} region.

A Raman band around 800 cm^{-1} with an isotopic frequency shift of 30–45 cm^{-1} can be assigned to either
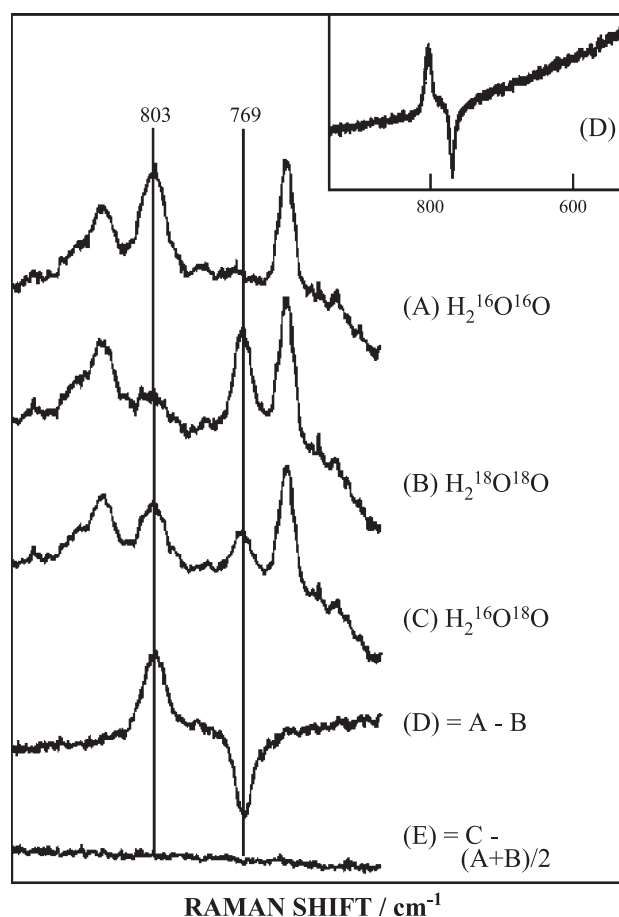

Fig. 7. The 607-nm excited steady state RR spectra in the 800 cm^{-1} region of the 607 nm form of CcO derived in the reaction of the oxidized enzyme with hydrogen peroxide. The hydrogen peroxide used are $H_2^{16}O_2$ (A), $H_2^{18}O_2$ (B), and $H_2^{16}O^{18}O$ (C). Spectra D and F show difference spectra; spectrum D = spectrum A − spectrum B and spectrum E = spectrum C − spectrum A/2 − spectrum B/2. The inset (spectrum D) is the same as spectrum D in the main frame but shows the full frequency range measured. Experimental conditions: cross section of flow cell, 0.6 × 0.6 mm^2; slit width, 4.2 cm^{-1}; laser 607 nm, 100 mW at the sample; total accumulation time, 78, 78, and 156 min for spectra A, B, and C, respectively, and 160 min for the resting enzyme; CcO, 50 μM, pH 7.45. (Reproduced from Ref. [26].)

a peroxo O–O stretch or an oxoiron Fe=O stretch. To distinguish between the two possibilities, asymmetrically labeled $H_2^{16}O^{18}O$ was synthesized and applied to this reaction. As shown by spectrum C (Fig. 7C), two bands are seen at 803 and 769 cm^{-1}, each with half intensity of the band in spectra 7A and 7B, when a porphyrin band is used as an internal intensity standard. This is more explicitly seen by spectrum 7E, in which no peak is present. If the 804 cm^{-1} band arose from the ν_{OO} mode, a positive peak would have appeared around 787 cm^{-1} in Fig. 7E. No such a feature exclusively leads us to conclude that the 803/769 cm^{-1} pair arises from the ν_{FeO} mode and that the O–O bond had already been cleaved in this stage. When the same experiments were carried out with D_2O, the 803 cm^{-1} band exhibited an upshift by 1–2 cm^{-1} [26]. This fact was interpreted by that the oxygen atom bound to Fe_{a_3} was hydrogen bonded. Since the Fe_{a_3} site lacks the so-called distal histidine, which is widely seen in oxygen carriers and peroxidases, the most probable candidate for the hydrogen donor to the bound oxygen would be Cu_B-OH.

Fig. 8 shows the simultaneously observed steady state RR and absorption spectra of reaction mixtures of FO–CcO and H_2O_2 [27]. The left side depicts Raman spectra, which are represented in terms of the difference of $H_2^{16}O_2$ derivative minus $H_2^{18}O_2$ derivative, while the right side depicts the absorption spectra, which are represented as the difference spectrum of the intermediate minus fully oxidized. Solution conditions were adjusted to yield predominantly the P or F intermediate in a steady state mixture of the flowing sample, and in fact, spectra 8A′ and 8B′ have

stronger absorption at 607 and 580 nm, respectively. In accord, the 804/769 and 785/750 cm^{-1} bands are stronger in spectra 8A and 8B, respectively. The 355/340 cm^{-1} bands are seen in both spectra but it is more intense in spectrum 8B, for which a positive peak seems to be present around 800 cm^{-1}. It is most reasonable that the '607 nm absorbing form' yields a single oxygen-isotope-sensitive band at 804/769 cm^{-1}, while the '580 absorbing form' contains two kinds of intermediate; one gives two pairs of oxygen isotope sensitive bands at 785/750 and 355/340 cm^{-1} and the other does at $\sim 800/\sim 770$ and 355/340 cm^{-1}. The 607 and 580 forms correspond to the P and F intermediates in the catalytic cycle and the presence of the 355/340 cm^{-1} bands implicates that the His–Fe_{a_3}=O is deformed from a linear structure irrespective of the oxidation state of iron. However, we cannot rule out the possibility that the bands at 355/340 cm^{-1} are not accompanied by the bands at 785/750 cm^{-1}. In this case, the band at 355/340 cm^{-1} is specific to the bent P intermediate.

5. Conclusion

Three kinds of P intermediates were examined with RR spectroscopy. All of them have the same oxoheme a_3 structure yielding the Fe=O stretching mode at 804/769 cm^{-1} for the $^{16}O/^{18}O$ derivatives and absorption peak at 607 nm. When the proximal histidine is deformed from the linear N–Fe=O structure, the heme a_3 gives the N–Fe=O deformation vibration at 356/342 cm^{-1} for the $^{16}O/^{18}O$
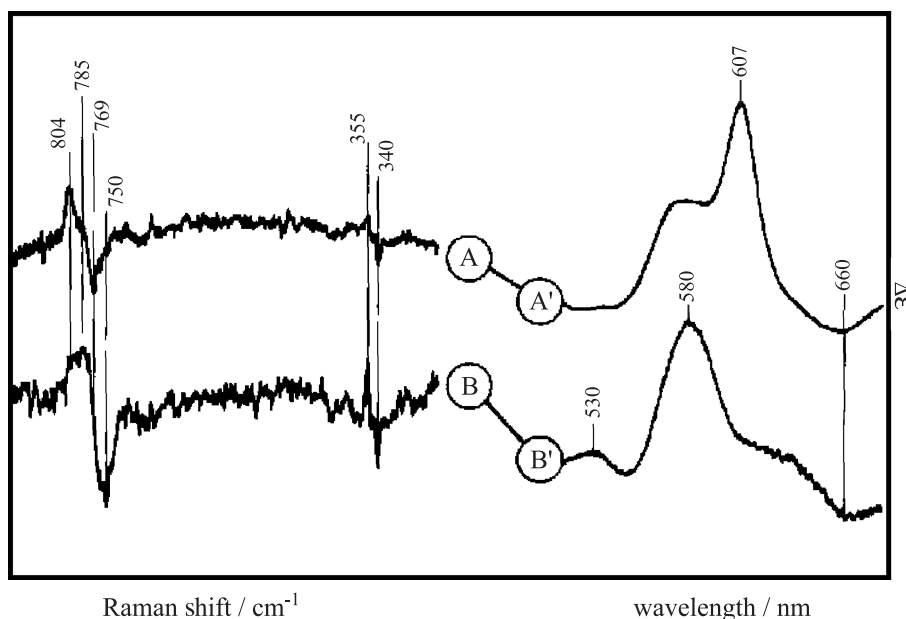

Fig. 8. The steady state Raman and absorption simultaneously observed spectra of the 607- and 580-nm forms of CcO at pH 8.5. Raman spectra A and B are shown as a difference of $H_2^{16}O_2$ compound minus $H_2^{18}O_2$ compound. Spectra A/A′: laser, 427 nm, 2.5 mW; accumulation time, 3×2400 s for each isotope; CcO, 50 μM; initial concentration of H_2O_2, 1 mM. Spectra B/B′: laser power, 7.5 mW; accumulation time, 3×800 s for each isotope; CcO, 10 μM; initial concentration of H_2O_2, 5 mM. Absorption spectra are represented as a difference with regard to the spectrum of the resting enzyme; ordinate full scale $\Delta\varepsilon = 11.7$ mM^{-1} cm^{-1}; pathlength, 0.6 mm. (Reproduced from Ref. [27].)

derivatives. The frequency and excitation profile of the Fe=O stretching mode of P is distinctly different from those of F, suggesting that the electronic state of heme a_3 is qualitatively different between them.

Acknowledgements

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