# Presence of the Heme-oxo Intermediate in Oxygenation of Carbon Monoxide by Cytochrome *c* Oxidase Revealed by Resonance Raman Spectroscopy

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*Received July 28, 2000*
*Revised Manuscript Received November 3, 2000*

Since the first detection of oxygen-dependent oxidation of carbon monoxide in mammalian tissue,[1] the phenomenon has been confirmed by several groups.[2] Young and Caughey[3] demonstrated that bovine cytochrome *c* oxidase (CcO, EC 1.9.3.1), the terminal enzyme of the mitochondrial electron transport chain, catalyzes monooxygenation of CO to $CO_2$ when reductively activated in the presence of $O_2$, although the physiological function of CcO is reduction of $O_2$ to $H_2O$.[4] X-ray crystallographic analysis has been completed for bovine[5a] and bacterial CcOs,[5b] which contain in common two redox-active iron (heme *a* and heme $a_3$) and copper centers ($Cu_A$ and $Cu_B$). Among various oxidized heme proteins, CcO is most effectively reduced by CO to form the CO adduct,[6] presumably owing to the binuclear character of the active site. The reaction mechanism of CcO for $O_2$ reduction at the binuclear site has been studied extensively by absorption,[7] EPR,[8] and resonance Raman (RR) spectroscopy.[9−11]

The reaction of fully reduced CcO with $O_2$ is considered to proceed via the oxy, P, F, and O intermediates (in the order of appearance) which yield the oxygen-associated vibrations at 571/544, 804/768, 785/750, and 450/425 $cm^{-1}$ for $^{16}O_2/^{18}O_2$ derivatives, respectively, in our experiments.[11f] Only the last bands are shifted in $D_2O$ to 443/417 $cm^{-1}$, and assigned to the $Fe^{III}-OH$ species.[11g] Here the "P" intermediate corresponds to the so-called "peroxy" intermediate but has not been settled yet.[9c] Since the RR experiments with $^{16}O^{18}O$ have demonstrated that both 804/768 and 785/750 $cm^{-1}$ bands arise from iron-oxo species, we pointed out that the O−O bond had been cleaved in the peroxy state. However, there are arguments against it to claim that both 804/768 and 785/750 $cm^{-1}$ bands arise from the ferryl state with and without the hydrogen bond, respectively,[9b,10c] and that the P intermediate has Fe−O−O−Cu structure.[12] One of the reasons is that the 804/768 and 785/750 $cm^{-1}$ bands are not resolved in the RR spectra reported by two other groups.[9c,10c] To overcome this uncertainty and controversy, this study aims to prove that the 804/768 $cm^{-1}$ bands arise from the intermediate with the oxidation state different from what the 785/750 $cm^{-1}$ bands arise from, by showing that the reaction of oxidized CcO with CO in the presence of $^{16}O_2/^{18}O_2$ generates the 804/768 $cm^{-1}$ intermediate but not the 785/750 $cm^{-1}$ intermediate.

The enzyme was purified with Yoshikawa's method[13] with 0.2% *n*-decyl-β-D-maltoside as the detergent. For incubation with CO,[14] the enzyme was dissolved in 10 mM sodium phosphate buffer, pH 8.0,[15] where the concentration of CcO was ∼35 μM in terms of heme. Optical absorption spectra were measured with a Hitachi U-3210 spectrophotometer. Raman scattering was excited with CW lasers at 441.6 nm with a He−Cd laser (Kinmon Electrics, CD4805R), at 421.3 nm with a diode laser (Hidachi Metals, ICD-430), or at 590 and 607 nm with a tunable dye laser (Coherent, model 599) loading rhodamine 6G dye which was pumped by an Ar laser (Spectra Physics model 2017), and detected by a liquid nitrogen-cooled CCD detector (Princeton Instrument, CCD-1340/400) attached to a 100-cm single polychromator (Ritsu Oyo Kogaku, MC-100DG). The laser power was made as low as possible to avoid possible photoreactions. The slit width was 200 μm and the temperature of the sample was kept at 20 °C for all the measurements.

The absorption spectrum of this preparation in the oxidized form is depicted by trace a in Figure 1. When the enzyme was anaerobically incubated under CO for 3 h at room temperature, it gave the Soret and α bands at 430 and 591 nm, respectively, as shown by trace c in Figure 1. The characteristics are in agreement with those of mixed valence CcO−CO (MV−CcO−CO). RR spectra were examined under this condition with four CO isotopomers and the results are summarized in Table 1. A band for $^{12}C^{16}O$ at 517 $cm^{-1}$ was shifted to 512 $cm^{-1}$ for $^{13}C^{16}O$, to 508 $cm^{-1}$ for $^{12}C^{18}O$, and to 503 $cm^{-1}$ for $^{13}C^{18}O$. The monotonic decrease in frequency with an increase in the overall mass of CO is characteristic of a mode having primarily the Fe−C stretching [ν(Fe−C)] character of linear Fe−C−O. On the contrary, a band at 578 $cm^{-1}$ displayed a zigzag shifting pattern characteristic of the FeCO bending vibration [δ(FeCO)][16a] with

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(1) Fern, W. O. *Am. J. Physiol.* **1932**, *102*, 379−392.
(2) (a) Clark, R. T. *Am. J. Physiol.* **1950**, *162*, 560−564. (b) Brechenridge, B. *Am. J. Physiol.* **1953**, *173*, 61−69. (c) Luomanmaki, K.; Coburn, R. F. *Am. J. Physiol*. **1969**, *217*, 354−363.
(3) Young, L. J.; Caughey, W. S. *Biochemistry* **1986**, *25*, 152−161.
(4) (a) Wikstrom, M.; Krab, K.; Saraste, M. *Cytochrorme Oxidase; A Synthesis*; Academic: New York, 1981. (b) Malmstrom, B. G. *Chem. Rev.* **1990**, 1247−1260
(5) (a) Tsukihara, T.; Aoyama, H.; Yamashita, E.; Tomizaki, T.; Yamaguchi, H.; Shinzawa-Itoh, K.; Nakashima, R.; Yaono, R.; Yoshikawa, S. *Science* **1995**, *269*, 1069−1074. (b) Iwata, S.; Ostermeier, C.; Ludwig, B.; Michel, H. *Nature (London)* **1995**, *376*, 660−669.
(6) Bickar, D.; Bonaventura, C.; Bonaventura, J. *J. Biol. Chem.***1984**, *259*, 10777−10783.
(7) (a) Gibson, Q. H.; Greenwood, C. *Biochem. J.* **1963**, *86*, 541−554. (b) Orii, Y. *Ann. N.Y. Acad. Sci.* **1988**, *550*, 105−117. (c) Oliveberg, M.; Brzezinski, P.; Malmstrom, B. G. *Biochim. Biophys. Acta* **1989**, *977*, 322−328. (d) Sucheta, A.; Georgiadis, K. E.; Einarsdottir, O. *Biochemistry* **1997**, *36*, 554−565. (e) Chance, B.; Saronio, C.; Leigh, J. S., Jr. *J. Biol. Chem.* **1975**, *250*, 9226−9237.
(8) (a) Witt, S. N.; Chan, S. I. *J. Biol. Chem.* **1987**, *262*, 1446−1448. (b) Blair, D. F.; Witt, S. N.; Chan, S. I. *J. Am. Chem. Soc.* **1985**, *107*, 7389−7399.
(9) (a) Han, S.; Ching, Y.-C.; Rousseau, D. L. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 8408−8412. (b) Han, S.; Ching, Y.-C.; Rousseau, D. L. *Nature (London)* **1990**, *348*, 89−90. (c) Han, S.; Takahashi, S.; Rousseau, D. L. *J. Biol. Chem.* **2000**, *275*, 1910−1919.
(10) (a) Varotsis, C.; Woodruff, W. H.; Babcock, G. T. *J. Am. Chem. Soc.* **1990**, *111*, 6439−6440; **1990**, *112*, 1297. (b) Varotsis, C.; Woodruff, W. H.; Babcock, G. T. *J. Biol. Chem.* **1990**, *265*, 11131−11136. (c)Varotsis, C.; Zhang, Y.; Appelman, E. H.; Babcock, G. T. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 237−241.
(11) (a) Ogura, T.; Kitagawa, T. *J. Am. Chem. Soc.* **1987**, *109*, 2177−2179. (b) Ogura, T.; Yoshikawa, S.; Kitagawa, T. *Biochemistry* **1989**, *28*, 8022−8027. (c) Ogura, T.; Takahashi, S.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *J. Am. Chem. Soc.* **1990**, *112*, 5630−5631. (d) Ogura, T.; Takahashi, S.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *J. Biol. Chem.* **1990**, *265*, 14721−14723. (e) Ogura, T.; Hirota, S.; Proshlyakov, D. A.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *Bull. Chem. Soc. Jpn*. **1991**, *64*, 2901−2907. (f) Ogura, T.; Takahashi, S.; Hirota, S.; Shinzawa-Itoh, K.; Yoshikawa, S.; Appelman, E. H.; Kitagawa, T. *J. Am. Chem. Soc.* **1993**, *115*, 8527−8536. (g) Ogura, T.; Takahashi, S.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *J. Am. Chem. Soc.* **1996**, *118*, 5443−5449.
(12) Fabian, M.; Palmer, G. *Biochemistry* **1995**, *34*, 13802−13810.
(13) Yoshikawa, S.; Choc, M. G.; O'Toole, M. C.; Caughey, W. S. *J. Biol. Chem.* **1977**, *252*, 5498−5508.
(14) (a) Brzezinski, P.; Malmstrom, B. G. *FEBS Lett.* **1985**, *187*, 111−114. (b) Junemann, S.; Heathcote, P.; Rich, P. R. *Biochim. Biophys. Acta* **2000**, *1456*, 50−66.
(15) The results at pH 8.0 were best among pH 6.8, 7.4, and 8.0.
(16) (a) Tsubaki, M.; Srivastava, R. B.; Yu, N. T. *Biochemistry* **1982**, *21*, 1132−1140. (b) Yoshikawa, S.; Caughey, W. S. *J. Biol. Chem.* **1982**, *257*, 412−420. (c) Yu, N. T.; Kerr, E. A.; Ward, B.; Chang, C. K. *Biochemistry* **1983**, *22*, 4534−4540. (d) Nagai, M.; Yoneyama, Y.; Kitagawa, T. *Biochemistry* **1991**, *30*, 6495−6503.

10.1021/ja0027867 CCC: $20.00 
Published on Web 01/06/2001

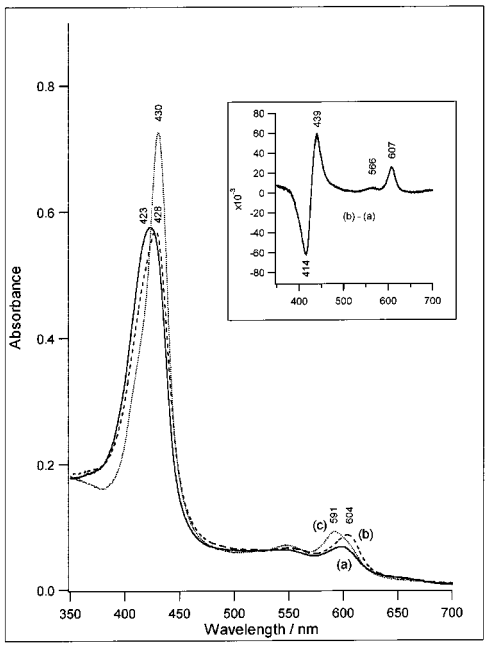


**Figure 1.** Optical absorption spectra of (a) oxidized CcO at pH 8.0, (b) CcO incubated with CO in the presence of $O_2$, and (c) CcO incubated with CO in the absence of $O_2$; incubation, 3 h at 20 °C. The inset represents the difference spectrum between traces b and a.

**Table 1.** Vibrational Frequencies of the FeCO Moiety of Bovine CcO−CO[a]

| | $^{12}C^{16}O$ | $^{13}C^{16}O$ | $^{12}C^{18}O$ | $^{13}C^{18}O$ |
|---|---|---|---|---|
| CO bound mixed valance CcO | | | | |
| $\nu$(Fe−C)[b] | 517 | 512 | 508 | 503 |
| $\delta$(Fe−C−O)[b] | 578 | 563 | 574 | 557 |
| $\nu$(C−O)[c] | 1965 | 1921 | | |
| CO bound fully reduced CcO | | | | |
| $\nu$(Fe−C)[d] | 520 | 516 | | |
| $\delta$(Fe−C−O)[d] | 578 | 564 | | |
| $\nu$(C−O)[e] | 1963 | 1919 | | |

[a] In $cm^{-1}$. [b] This work. [c] Okuno, D.; Iwase, T.; Kim, Y.; Kitagawa, T. To be submitted for publication. [d] Argade, P. V.; Ching, Y. C.; Rousseau, D. L. *Science* **1984**, *225*, 329−330. [e] Iwase, T.; Varotsis, C.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *J. Am. Chem. Soc.* **1999**, *121*, 1415−1416.

frequency shifts to 563, 574, and 557 $cm^{-1}$ for $^{13}C^{16}O$, $^{12}C^{18}O$, and $^{13}C^{18}O$, respectively. This means that ordinary heme $a_3$[2+]-CO with nearly linear geometry is formed from the oxidized enzyme. The $\nu$(Fe−C) and $\delta$(FeCO) frequencies observed for this preparation are compared with those of fully reduced CcO−CO in Table 1. The $\nu$(Fe−C) frequencies of this preparation are slightly lower than those of fully reduced CcO. This is compatible with the fact that $\nu$(C−O) of this preparation are higher than those of fully reduced CcO (unpublished results), being consistent with previous results for MV−CcO−CO,[16b] because generally $\nu$(Fe−C) and $\nu$(C−O) frequencies have a linear inverse correlation.[16c,d] This means that a small but definite structural change occurs at the distal side of heme $a_3$ upon the redox change of heme *a*.

When the oxidized CcO was aerobically incubated with CO, the absorption spectrum delineated by trace b in Figure 1 was obtained. The Soret and α peaks were shifted to 428 and 604 nm, respectively. An inset represents the difference spectrum, this species minus oxidized form, in agreement with the reported P-minus-O difference spectrum with maxima at 439, 566, and 607 nm and a minimum at 414 nm.[6] Figure 2 shows the RR

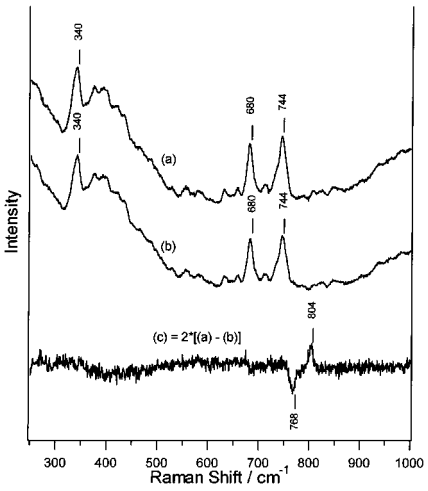


**Figure 2.** Resonance Raman spectra of the P form derived after 1-day incubation of oxidized CcO at pH 8.0 under CO with $^{16}O_2$ (a) and $^{18}O_2$ (b) and their difference spectrum [c = 2*(a−b)]. Experimental conditions: excitation 441.6 nm, 0.7 mW at the sample, temperature 20 °C, concentration 35 $\mu$M.

spectra of this species excited at 441.6 nm, where spectra a and b stand for this species derived from $^{16}O_2$ and $^{18}O_2$, respectively, and spectrum c represents their difference [c = 2*(a−b)]. While all porphyrin vibrations disappear in the difference spectrum, only one oxygen-isotope-sensitive mode was clearly observed at 804/768 $cm^{-1}$ for the $^{16}O_2/^{18}O_2$ derivatives, respectively. These frequencies agree with those observed for the P intermediate in the $O_2$ reduction process. The shape of the Raman difference spectrum as well as the absorption spectrum shown in the inset of Figure 1 were unaltered by variation of incubation times (30 min to 1 day), supporting the homogeneity of the sample made by this method. It is emphasized that the 785/750 $cm^{-1}$ bands observed for the $O_2$ reduction were not recognized even after prolonged incubation (24 h). The 804/768 $cm^{-1}$ bands were also detected upon excitation at 607 nm but not upon excitation at 413.1, 421.3, and 590 nm. This is consistent with the fact that the absorption maxima characteristic of the P intermediate are present near 439 and 607 nm (Figure 1).[17]

In the reaction of oxidized CcO with $H_2O_2$, it is known that the 607 nm absorbing form is generated first and it returns to the oxidized form through the 580 nm absorbing form. The RR study of this reaction[18a] has established that the 607 nm form has an oxo-iron heme with $\nu_{Fe=O}$ bands at 804/769 $cm^{-1}$ for $H_2{}^{16}O_2/H_2{}^{18}O_2$ derivatives. Similarities in the oxygen-associated vibrations of other intermediates[18] in the peroxide and dioxygen reactions have proved that the two reactions proceed via common intermediates. In addition, it has been conclusively demonstrated by this study that the incubation of oxidized CcO with CO in the presence of $O_2$ generates the P intermediate appearing in the physiological reaction of CcO, but it does not yield the F intemediate.

JA0027867

(17) The inset of Figure 1 locates the difference peaks at 439 and 607 nm, but these peak positions do not always indicate the absorption maxima of the P species, because they are affected by absorbances and wavelengths of the absorption maxima of the oxidized species. The Raman bands of the P species are expected to be intensity enhanced at the true absorption maximum.

(18) (a) Proshlyakov, D. A.; Ogura, T.; Shinzawa-Itoh, K.; Yoshikawa, S.; Appelman, E. H.; Kitagawa, T. *J. Biol. Chem.* **1994**, *269*, 29385−29388. (b) Proshlyakov, D. A.; Ogura, T.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *Biochemistry* **1996**, *35*, 8580−8586. (c) Proshlyakov, D. A.; Ogura, T.; Shinzawa-Itoh, K.; Yoshikawa, S.; Kitagawa, T. *Biochemistry* **1996**, *35*, 76−82