Communication

Observation of the Fe$^{4+}$=O Stretching Raman Band for Cytochrome Oxidase Compound B at Ambient Temperature*

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Resonance Raman and visible absorption spectra were simultaneously observed for cytochrome oxidase reaction intermediates at 5 °C by using the artificial cardiovascular system (Ogura, T., Yoshikawa, S., and Kitagawa, T. (1989) Biochemistry 28, 8022–8027) and a device for Raman/absorption simultaneous measurements (Ogura, T., and Kitagawa, T. (1988) Rev. Sci. Instrum. 59, 1316–1320). The Fe$^{4+}$=O stretching ($\nu_{\text{Fe}=0}$) Raman band was observed at 788 cm$^{-1}$ for compound B for the first time. This band showed the $^{18}$O/$^{16}$O isotopic frequency shift ($\Delta \nu_{\text{Fe}=0}$) by 40 cm$^{-1}$, in agreement with that for horseradish peroxidase compound II ($\nu_{\text{Fe}=0} = 787$ cm$^{-1}$ and $\Delta \nu_{\text{Fe}=0} = 34$ cm$^{-1}$). In the time region when the Fe$^{3+}$=O stretching band for compound A and the Fe$^{3+}$=O band for compound B were coexistent, a Raman band assignable to the Fe$^{3+}$=O=O-Cu$^{2+}$ linkage was not recognized.

Cytochrome oxidase (cytochrome c:oxygen oxidoreductase, EC 1.9.3.1), the terminal enzyme of the mitochondrial respiratory chain, catalyzes reduction of molecular oxygen to water and at the same time couples the reaction with proton translocation across the energy-transducing membrane (1, 2). The electrochemical potential thus generated is utilized to phosphorylate ADP. Mammalian cytochrome oxidases contain two heme $a$ groups called cytochromes $a$ and $a_3$, and two copper ions, namely Cu$\alpha$ and Cu$\beta$. Cytochrome c$\alpha$/Cu$\alpha$ moiety receives electrons from cytochrome $c$ and transfers them to the cytochrome $a_3$/Cu$\beta$ binuclear center. Dioxygen binds to the Fe(II) ion of cytochrome $a$, and is converted to water when four electrons are supplied. The mechanism of dioxygen reduction has been mainly investigated with visible absorption (3–14) and EPR (15–18) spectroscopies, but these methods hardly provide definite structural information on bound oxygen.

Resonance Raman (RR)$^1$ spectroscopy is a powerful technique for studying a structure of heme and its vicinity (19–21) and indeed provided important information on cytochrome oxidase (22–28). Particularly the RR spectra of transient species (25–28) seemed to bring about essential data on structures of intermediates, but observation of RR spectra with a much higher signal-to-noise ratio is required for examination of those labile intermediates. Since the reaction intermediates of cytochrome oxidase have been defined on the basis of visible absorption spectra, it is extremely desirous to correlate the observed RR spectra with visible absorption spectra. Accordingly, we constructed a device for Raman/absorption simultaneous measurements (29) and also a sample flow system (artificial cardiovascular system) (30), with which the reaction enzyme is automatically regenerated during circulation, in order to accumulate the RR spectra of intermediates for a long time with a limited amount of the enzyme.

Combination of these two apparatus enabled us to observe the Fe(II)=O stretching RR band for compound A (31) whose frequency was in agreement with the corrected value of Varotsis et al. (28) for a similar intermediate and also with that for mixed valence oxygenated cytochrome oxidase (32). In the extension of such experiments to subsequent intermediates, we succeeded in observing the Fe$^{4+}$=O stretching ($\nu_{\text{Fe}=0}$) RR band for compound B at ambient temperature for the first time. The $\nu_{\text{Fe}=0}$ frequency is markedly close to that of horseradish peroxidase compound II.

EXPERIMENTAL PROCEDURES

Bovine heart cytochrome oxidase was purified according to the method described elsewhere (33). About 150 ml of the 50 mM enzyme solution in 50 mM sodium phosphate buffer, pH 7.2, containing 8 mM equine cytochrome c (Sigma, Type VI) and 50 mM ascorbate was circulated through the artificial cardiovascular system. Details of the sample flowing procedures were explained previously (30), but the following points were revised in this experiment: three separate gas exchangers (lung-2 in Fig. 1 of Ref. 30) were used at the same time for $^{18}$O$_2$, $^{16}$O$_2$, and N$_2$ (for the CO-photodissociated form) so that the spectra of the corresponding three derivatives could be acquired successively in turn by simply switching the flow direction. A rectangular quartz cell ($2 \times 2 \text{ mm}^2$) was used in the cell part of the device for Raman/absorption simultaneous measurements. The flow rate of the sample through the cell was set to be 30 ml/min which makes the residence time of an arbitrary molecule in the coaxial laser and white light beam (diameter = 200 μm) 960 μs. Sample temperature at the cell was 5 °C. The accumulation time of the spectra of individual derivatives was 11 min, and the successive measurements of the three derivatives were repeated three times (total accumulation time was 33 min for each species). Raman scattering was excited at 418 nm by an argon laser-pumped dye laser with stilbene 420 and detected with a diode array (PAR 1420) attached to a Sper 1877B polychromator. Laser power at the sample point was 22 milliwatts. With regard to the photon density, the present laser power is lower by a factor of $10^6$ than the lower one used in Varotsis' time resolved RR experiments (28). Raman shift were calibrated under the same geometry of the cell by introducing ethanol into the cell as the second standard, and the Raman bands of ethanol were separately calibrated with indene. Accuracy of Raman band positions was ±2 cm$^{-1}$.

RESULTS AND DISCUSSION

Fig. 1 compares difference absorption spectra of the cytochrome oxidase intermediate for reactions with $^{18}$O$_2$ (A) and $^{16}$O$_2$ (B) with regard to the CO-photodissociated form ob-

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1 The abbreviation used is: RR, resonance Raman.
It is noted that when the laser power was raised for the same compound A was not seen. Thus, it became evident that both enzyme concentration might be slightly different, comparison of B, respectively, but since the path lengths are different and the 90% of carbon monoxide was photodissociated. These two spectra are quite alike and resemble the difference spectrum of compound B versus the fully reduced form (8). However, the fact that the difference spectrum shows no difference at 683 cm⁻¹ where the most intense porphyrin in-plane bands (ν∥) were observed makes the presence of the oxygen isotope-sensitive bands more reliable. The same difference spectra were repeatedly observed for independent preparations of the sample.

The Ramberg band at 569 cm⁻¹ for O₂ exhibits a downshift by 29 cm⁻¹ upon the ¹⁶O₂ replacement, in good agreement with...
the previous observation for the Fe(II)-O-O stretching vibration of compound A (31) and also with a very recent report for mixed-valence oxygenated cytochrome oxidase (32) and that of the corrected values of Varotis et al. (28). Accordingly, the 569/540 cm\(^{-1}\) bands for the \(^{16}\)O/\(^{18}\)O derivatives are assigned to compound A with no doubt. The other oxygen isotope-sensitive band at 788 cm\(^{-1}\) for hemeproteins (34-40) and around 800-850 cm\(^{-1}\) for Fe\(_0\) diatomic oscillator which is calculated to be 35 cm\(^{-1}\) in bulk water. However, the relative intensity of the '60/\(^{18}\)O frequency shift is close to the value expected for the FeO diatomic oscillator, which is calculated to be 35 cm\(^{-1}\) in bulk water. This may suggest that the oxygen atom of oxoferryl heme in compound B is closer to that of the alkaline form, and the oxygen was exchanged with water while the measurement was reacted in H\(_{16}\)O. The \(\nu_{\infty}\) frequency of cytochrome oxidase was 45 cm\(^{-1}\). So far the \(\nu_{\infty}\) bands have been observed around 760-800 cm\(^{-1}\) for heme proteins (34-40) and around 800-850 cm\(^{-1}\) for five- and six-coordinate oxoferryl-porphyrin complexes at low temperatures (41-43) as listed in Table I. Judging from these values and their \(^{16}\)O/\(^{18}\)O isotopic frequency shift, it is quite reasonable to assign the 788 cm\(^{-1}\) band of compound B to the Fe=O stretching vibration of the six-coordinate heme.

In conclusion, this study definitely demonstrated that compound B, a relatively stable intermediate with the absorption spectrum closely similar to that of the fully oxidized form, contains the oxoferryl ion at the dioxygen bonding site.

REFERENCES
1. Wikström, M., Krab, K., and Saraste, M. (1981) in Cytochrome Oxidase—A Synthesis, Academic Press, New York
2. Chan, S. I., and Li, P. M. (1990) Biochemistry 29, 1-12
3. Gibson, Q. H., and Greenwood, C. (1963) Biochem. J. 86, 541-544
4. Greenwood, C., and Gibson, Q. H. (1967) J. Biol. Chem. 242, 1752-1757
5. Hill, B., and Greenwood, C. (1984) Biochem. J. 218, 913-921
6. Hill, B., Greenwood, C., and Nichols, P. (1986) Biochim. Biophys. Acta 853, 91-113
7. Hill, B. C., and Greenwood, C. (1983) Biochem. J. 215, 659-667
8. Chance, B., Saranion, C., and Leigh, J. S. (1976) J. Biol. Chem. 250, 9220-9227
9. Clore, G. M., Andresson, L. E., Karlsson, B., Asa, R., and Malmström, B. G. (1980) Biochem. J. 185, 130-164
10. Clore, G. M., Andresson, L. E., Karlsson, B., Asa, R., and Malmström, B. G. (1980) Biochem. J. 185, 155-167
11. Denis, M. (1981) Biochim. Biophys. Acta 634, 30-40
12. Chance, B., Saranion, C., and Leigh, J. S. (1976) Proc. Natl. Acad. Sci. U.S.A. 72, 1635-1640
13. Orii, Y. (1984) J. Biol. Chem. 259, 7178-7190
14. Orii, Y. (1988) Ann. N. Y. Acad. Sci. 550, 106-117
15. Chan, S. I., Witt, S. N., and Blair, D. F. (1988) Chem. Soc. Rev. 26A, 51-56
16. Blair, D. F., Witt, S. N., and Chan, S. I. (1985) J. Am. Chem. Soc. 107, 7398-7399
17. Witt, S. N., Blair, D. F., and Chan, S. I. (1986) J. Biol. Chem. 261, 8104-8107
18. Witt, S. N., and Chan, S. I. (1987) J. Biol. Chem. 262, 1446-1448
19. Spiro, T. G. (ed.) (1988) in Biological Applications of Raman Spectroscopy, Vol. 3, John Wiley & Sons, New York
20. Kitagawa, T. (1986) Adv. Spectroscopy 13, 443-481
21. Kitagawa, T., and Asakura, Y. (1987) Struct. Bonding 64, 71-114
22. Babcock, G. T. (1988) in Biological Applications of Raman Spectroscopy (Spiro, T. G., ed.) Vol. 3, pp. 295-346, John Wiley & Sons, New York
23. Ogura, T., Hon-nami, K., Oshima, T., Yoshikawa, S., and Kitagawa, T. (1981) J. Am. Chem. Soc. 105, 7781-7783
24. Han, S., Ching, Y.-C., and Rousseau, D. L. (1989) J. Biol. Chem. 264, 6604-6607
25. Babcock, G. T., Jean, J. M., Johnston, L. N., Palmer, G., and Woodruff, W. H. (1984) J. Am. Chem. Soc. 106, 8506-8506
26. Babcock, G. T., Jean, J. M., Johnston, L. N., Woodruff, W. H. and Palmer, G. (1985) J. Inorg. Chem. 24, 242-251
27. Ogura, T., Yoshikawa, S., and Kitagawa, T. (1985) Biochim. Biophys. Acta 832, 220-223
28. Varotis, C., Woodruff, W. H., and Babcock, G. T. (1989) J. Am. Chem. Soc. 111, 6439-6440
29. Ogura, T., and Kitagawa, T. (1988) Rev. Sci. Instrum. 59, 1316-1320
30. Ogura, T., Yoshikawa, S., and Kitagawa, T. (1989) Biochemistry 28, 8022-8027
31. Ogura, T., Takahashi, S., Shinzawa-Itoh, K., Yoshikawa, S., and Kitagawa, T. (1990) J. Biol. Chem., in press
32. Han, S., Ching, V., and Rousseau, D. L. (1990) Biochemistry 29, 1380-1384
33. Yoshikawa, S., Choc, M. G., O'Tool, M. C., and Coughly, W. S. (1977) J. Biol. Chem. 252, 5488-5505
34. Terner, J., Sitter, A. J., and Reczek, C. M. (1985) Biochim. Biophys. Acta 828, 73-80
35. Sitter, A. J., Reczek, C. M., and Terner, J. (1985) Biochim. Biophys. Acta 828, 229-235
36. Sitter, A. J., Reczek, C. M., and Terner, J. (1985) J. Biol. Chem. 260, 7515-7522
37. Hashimoto, S., Tatsuno, Y., and Kitagawa, T. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 2417-2421
38. Hashimoto, S., Terao, J., Inubushi, T., Yonetsu, T., and Kitagawa, T. (1986) J. Biol. Chem. 261, 11110-11118
39. Oertling, W. A., Hoogland, H., Babcock, G. T., and Weber, R. (1988) Biochemistry 27, 5395-5400
40. Hashimoto, S., Tatsuno, Y., and Kitagawa, T. (1984) Proc. Jpn. Acad. Ser. B Phys. Biol. Sci. 60, 345-348
41. Proniewicz, L. M., Bajdor, K., and Nakamoto, K. (1986) J. Phys. Chem. 90, 1760-1766
42. Schappacher, M., Chottard, G., and Weiss, R. (1987) J. Biol. Chem. 52, 1448-1448
43. Koon, T. R., Oertling, W. A., and Babcock, G. T. (1987) J. Am. Chem. Soc. 110, 2185-2187
44. Mizutani, Y., Hashimoto, S., Tatsuno, Y., and Kitagawa, T. (1989) J. Am. Chem. Soc., in press