Scheidt, W. R., & Gouterman, M. (1983) in *Iron Porphyrins* (Lever, A. B. P., & Gray, H. B., Eds.) Part I, pp 89–139, Addison-Wesley, London.
Schejter, A., Glauser, S. C., George, P., & Morgoliash, E. (1963) *Biochim. Biophys. Acta 73*, 641–643.
Schmidt, J., Coudron, P., Thompson, A. W., Watters, K. L., & Mc Farland, J. T. (1983) *Biochemistry 22*, 76–84.
Schulz, G. E., Schirmer, R. H., & Pai, E. F. (1982) *J. Mol. Biol. 160*, 287–308.
Silvestrini, M. C., Brunori, M., Tegoni, M., Gervais, M., & Labeyrie, F. (1986) *Eur. J. Biochem. 161*, 465–472.
Spiro, T. G. (1985) *Adv. Protein Chem. 37*, 111–159.
Sugiyama, T., Nisimoto, Y., Mason, H. S., & Loehr, T. M. (1985) *Biochemistry 24*, 3012–3019.
Takano, T., & Dickerson, R. E. (1982) in *Electron Transport and Oxygen Utilization* (Ho, C., Ed.) pp 17–26, Macmillan Press, London.
Tegoni, M., Mozzarelli, A., Rossi, G. L., & Labeyrie, F. (1983) *J. Biol. Chem. 258*, 5424–5427.
Thomas, M.-A., Gervais, M., Favaudon, V., & Valat, P. (1983) *Eur. J. Biochem. 135*, 577–581.
Tsubaki, M., Nagai, K., & Kitagawa, T. (1980) *Biochemistry 19*, 379–385.
Vervoort, J., Müller, F., Le Gall, J., Bacher, A., & Sedlmaier, H. (1985) *Eur. J. Biochem. 151*, 49–57.
Vervoort, J., Müller, F., O'Kane, D. J., Lee, J., & Bacher, A. (1986) *Biochemistry 25*, 8067–8075.
Visser, A. J. W. G., Vervoort, J., O'Kane, D. J., Lee, J., & Carreira, L. A. (1983) *Eur. J. Biochem. 131*, 639–645.
Watari, H., Groudinsky, O., & Labeyrie, F. (1967) *Biochim. Biophys. Acta 131*, 592–594.
Watenpaugh, K. D., Sieker, L. C., & Jensen, L. H. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3857–3860.
Wierenga, R. K., de Jong, R. T., Kalk, K. H., Hol, W. G. J., & Drenth, J. (1979) *J. Mol. Biol. 131*, 55–73.
Xia, Z.-X., Shamala, N., Bethge, P. H., Lim, L. W., Bellamy, H. D., Xuong, N. H., Lederer, F., & Mathews, F. S. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 2629–2633.

Raman/Absorption Simultaneous Measurements for Cytochrome Oxidase Compound A at Room Temperature with a Novel Flow Apparatus†

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Received March 7, 1989; Revised Manuscript Received June 12, 1989

ABSTRACT: A novel flow apparatus for continuously producing reaction intermediates of cytochrome oxidase was constructed and applied successfully to observe the transient absorption and resonance Raman spectra in its reaction with oxygen. Time-resolved difference absorption spectra in 500–650-nm region clearly indicated the formation of compound A upon photolysis of the fully reduced CO-bound form at 5 °C, and at this stage electrons were not transferred from cytochrome *c* to cytochrome oxidase. However, at the stage of formation of compound B, cytochrome *c* was oxidized. Resonance Raman spectra of these intermediates measured simultaneously with the absorption spectra are also reported.

Cytochrome oxidase (EC 1.9.3.1) is the terminal enzyme in a respiratory chain of aerobic organisms. This enzyme catalyzes reduction of dioxygen coupled with vectorial proton translocation across the membrane (Wikström et al., 1981), and the resultant electrochemical potential is utilized by the H^+-ATPase to phosphorylate ADP. Mammalian cytochrome oxidases possess two heme A groups and two copper atoms per M_r = ~200 000, which are usually grouped into two functional units, namely, cytochromes *a* and a_3. Oxidized cytochrome *a* has a six-coordinated low-spin heme A and an EPR[1]-active copper atom, whereas cytochrome a_3 in the resting oxidized state has a high-spin heme A and an EPR-silent copper atom. Cytochrome *a* accepts electrons from cytochrome *c* and transfers them to cytochrome a_3. Cytochrome a_3 serves as the catalytic site for dioxygen reduction, but its mechanism still remains to be clarified. Since the reduced cytochrome oxidase reacts with O_2 very rapidly at physiological temperature, the conventional stopped-flow method with a few milliseconds of dead time after mixing is too slow to monitor the oxygenated reaction intermediates.

Cytochrome oxidase has a characteristic feature that its reduced form yields an adduct with carbonmonoxide (aa_3CO) which is photodissociable but otherwise stable. A lifetime of this adduct is about 30 s provided that it is mixed with O_2 in the dark. However, when the mixed solution is exposed to a flash of light, CO is photodissociated promptly and O_2 reacts with the photodissociated enzyme. The reaction kinetics involved were analyzed by monitoring the successive changes of absorption spectra after photolysis (Greenwood & Gibson, 1967; Orii, 1984; Blair et al., 1985). There have been arguments about whether the primary intermediate, compound A, which is characterized by absorption at 590 nm, is formed from the fully reduced enzyme at room temperature or not (Hill

† This work was supported by Grant-in-Aid for Scientific Research on Priority Areas 63617515 from the Ministry of Education, Science and Culture to T.K.

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[1] Abbreviations: RR, resonance Raman; EPR, electron paramagnetic resonance.

0006-2960/89/0428-8022$01.50/0

et al., 1986), although its formation at cryogenic temperatures has been established (Chance et al., 1975; Clore et al., 1980a,b).

Resonance Raman spectroscopy has been acknowledged as a powerful tool for studying structural details of biological pigments and has brought about important information about the iron-coordination environments of heme proteins. With regard to cytochrome oxidase, assignments of resonance Raman (RR) bands to each of cytochromes a and a_3 have been almost established (Callahan & Babcock, 1981; Babcock et al., 1981; Choi et al., 1983; Ogura et al., 1985; Ching et al., 1985). The iron–histidine stretching mode of cytochrome a_3 was assigned on the basis of ^{54}Fe isotopic frequency shift (Ogura et al., 1983). Protein dynamics after photolysis of carbonmonoxy cytochrome oxidase (Findsen & Ondrias, 1984; Sassaroli et al., 1988) and the redox-coupled proton pumping mechanism (Babcock & Callahan, 1983; Callahan & Babcock, 1983; Argade et al., 1986) were also discussed on the basis of RR spectra. In the next step this technique is expected to yield Raman bands associated with the bound oxygen of reaction intermediates involved in catalysis of cytochrome oxidase as has been seen for cytochrome P-450 (Bangcharoenpaurpong et al., 1986), horseradish peroxidase (Terner et al., 1988; Hashimoto et al., 1986), and model compounds (Wagner et al., 1988).

Babcock et al. (1984) reported the time-resolved RR spectra in the time region of 10 ns–1 ms after photolysis of cytochrome a_3CO and offered, although indirect, evidence for the presence of the O_2-bound form in the primary intermediates. Ogura et al. (1985) reported transient RR spectra for reaction intermediates generated within 450 μs after photolysis with a better signal to noise ratio and suggested that the heme ligand of the intermediate was an O_2 molecule or an O atom (ferryloxo form) on the basis of the frequency of the ν_4 mode [the mode numbers follow Abe et al. (1978)], which serves as a π-electron delocalization marker of the heme. These two transient RR studies failed to reveal a Raman band associated with a vibration of bound oxygen, which would serve as diagnosis for the structures of intermediates proposed so far. Furthermore, the species that gave the RR spectra could not be correlated with the intermediates defined in terms of the absorption spectra. To treat the oxygen-isotope shift of a Raman band, spectral improvement in the signal to noise ratio and thus longer accumulation of data were indispensable. The present study was undertaken to raise the signal to noise ratio of RR spectra of intermediates by using a limited amount of the enzyme and also to monitor resonance Raman and absorption spectra simultaneously. We constructed a novel apparatus and applied it successfully to the reaction intermediates of cytochrome oxidase. Identification of compound A at 5 °C will be particularly emphasized.

Materials and Methods

Cytochrome oxidase was isolated from bovine heart according to the method reported previously (Yoshikawa et al., 1977) and kept at 0 °C in 10 mM sodium phosphate buffer, pH 7.4, with a concentration higher than 300 μM (the enzyme concentration is represented in terms of heme A hereafter) until use. Since this preparation of cytochrome oxidase carries ca. 25% (w/v) of adherent Brij 35, the enzyme is apparently water soluble (Yoshikawa et al., 1977). All the measurements were carried out within 3 days after preparation. Horse cytochrome c (Sigma, type VI) was purchased and used without any further purification.

Figure 1 illustrates the "artificial cardiovascular system" apparatus for analyzing enzymic reactions. The solution

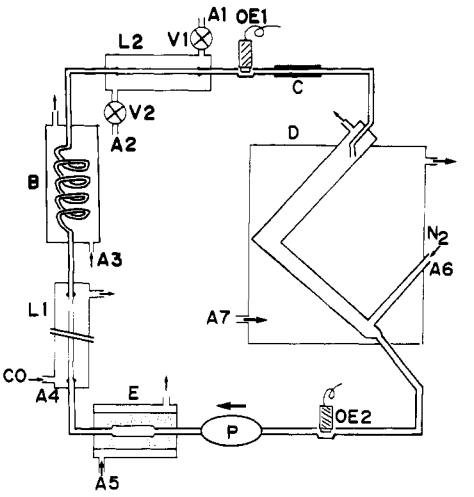

FIGURE 1: Schematic diagram of the artificial cardiovascular system for analyzing enzymic reactions: P, "heart" (pump); E, "kidney" (dialysis membrane); L1 and L2, "lungs"; B, flow cooler; C, flow cell; D, oxygen remover; OE1 and OE2, oxygen electrodes; V1 and V2, valves; A3, A5, and A7, inlets of temperature-controlled circulating water; A1 and A4, inlets for O_2 (or N_2) and CO gases, respectively. A2 is connected to a vacuum pump to change the atmosphere.

containing 50 μM enzyme, 8 μM cytochrome c, 50 mM ascorbate, and 50 mM potassium phosphate buffer, pH 7.4, was circulated through a Teflon tube (i.d., 2 mm; o.d., 4 mm) through the "heart" (P), which is a peristaltic pump (Cole Parmer, 7553-20). The solution pushed out by the pump comes to the "kidney" (E), which functions to maintain a constant concentration of the reductant and to remove the oxidized reductant. This consists of a tube of dialysis membrane (cellophane tube) immersed in 50 mM potassium phosphate buffer, pH 7.4, containing 50 mM ascorbate (total volume, 2 L). The cutoff molecular weight of the dialysis membrane (Spectrapor 1, Spectrum Medical Industries, Inc.) was chosen to be 8000 so as to prevent cytochrome c (M_r = 12400) from escaping. The outer solution of the "kidney" is kept always anaerobic by bubbling with nitrogen gas. Oxidized ascorbate comes out through the cellophane tube, and fresh ascorbate penetrates into the enzyme solution. It is noted that the main difference between the inner and outer solutions is the presence of the enzyme (1% w/v) and cytochrome c (0.01% w/v) in the inner solution, but the osmotic difference due to this is so small that no dilution of the inner solution was observed during the Raman/absorption measurements.

Next, the enzyme comes to two "lungs" (L1 and L2), which consist of a silicone tube (i.d., 2.0 mm; wall thickness, 0.2 mm; length, 100 mm for L2 and 2000 mm for L1) contained in a glass tube. The silicone membrane allows only small molecules to permeate through. The inside of the first "lung" (L1) is filled with carbon monoxide, and accordingly CO diffuses through the silicone wall into the enzyme solution to form a CO adduct. The inside of the second "lung" (L2) is filled with oxygen gas for the formation of reaction intermediates. O_2 molecules penetrate into the solution. However, for the measurements of the reduced form or its CO-bound form the inside of L2 is filled with N_2 gas.

At a flow cooler (B), the enzyme solution is cooled during passage through a coiled stainless steel tube (i.d., 2 mm; length, 1 m), which is immersed in a water bath kept at 0 °C. The flow cell (C) in this experiment is a quartz capillary (i.d., 1.6 mm), at which two laser beams cross the stream as was stated elsewhere (Ogura et al., 1987). The upstream beam (441.6 nm; diameter, 100 μm) is focused into the capillary and photolyzes CO. The photodissociated enzyme reacts with

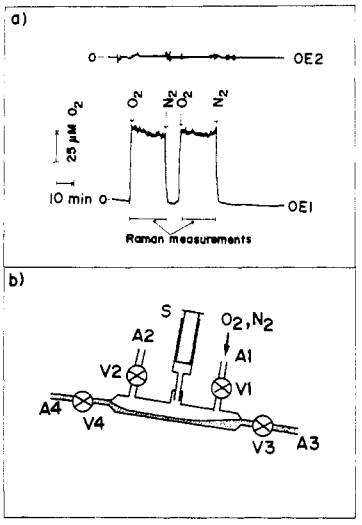

FIGURE 2: (a) Typical traces of the output from the oxygen electrodes (OE1 and OE2) during the Raman experiments. The timings at which O_2 or N_2 was introduced to the inside of L2 are indicated with arrows. (b) Illustration of a sealed gas exchanger placed instead of L2. Dotted part denotes the enzyme solution.

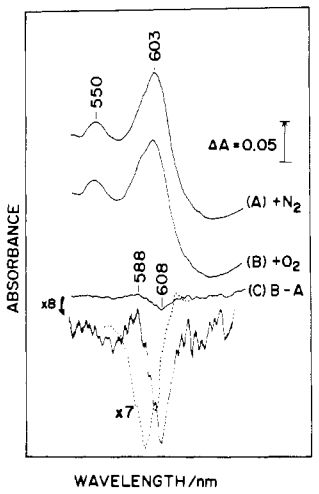

FIGURE 3: Absorption spectra of carbonmonoxy cytochrome oxidase in the absence (A) and presence (B) of oxygen under laser illumination (406.7 nm, 14 mW) and their difference spectrum (C) digitally obtained by subtracting spectrum A from spectrum B. The three spectra were obtained for the path length of 1.6 mm and are represented with a common absorbance scale designated in the figure. Dotted spectrum is a difference obtained by subtracting the spectrum of carbonmonoxy complex (not shown) from spectrum B. The flow rate was 82 mL/min, and the resident time of a given molecule in the laser and monitoring beams was 150 μs. The sealed gas exchanger was used for L2, and therefore oxygen concentration was 200 μM. Temperature was about 5 °C at the flow cell.

oxygen at once. The downstream beam (406.7 nm; diameter, 50 μm) excites Raman scattering. By varying the distance between the two laser beams, that is, by varying the time interval between the initiation of the reaction and observation of Raman scattering, we can obtain time-resolved RR spectra in the time region between 140 μs and 10 ms. This dual-beam apparatus can be replaced by the "device for simultaneous measurements of Raman and absorption spectra" (Ogura & Kitagawa, 1988), but in that case only a single laser beam can be used. Dry nitrogen is always flushed against the flow cell during the measurements to avoid condensation of atmospheric moisture. The oxygen remover (D) is designed for removing excess oxygen and allowing the enzyme to return to the fully reduced state. This part is made of a glass tube (i.d., 20 mm; length, 2 m) with water jackets to which thermostated water (0 °C) is circulated for maintaining the temperature of the sample solution.

OE1 and OE2 denote the Clark-type electrodes (Yellow Springs Instrument Co., Inc., 5331), which are connected to handmade control circuits and chart recorders. These electrodes monitor the oxygen concentration at the flow cell and at the exit of the oxygen remover, respectively. Figure 2a shows typical traces of the output of these electrodes. Note that the O_2 concentration at the exit of the oxygen remover can be kept always zero (upper trace) regardless of the O_2 concentration at the flow cell (lower trace). Briefly, the fully reduced enzyme yields a CO adduct at L1, and O_2 is introduced at L2. Reaction of the enzyme with O_2 starts at the flow cell, and during the trip through the oxygen remover the fully reduced state is restored and subjected to the next circulation.

L2 can be replaced by a sealed gas exchanger depicted in Figure 2b. This is a slightly tilted (5–10°) glass tube (i.d., 15 mm; length, 150 mm) to which a 50-mL airtight syringe is connected through a rubber septum. When the flow rate is raised, the surface level of the solution in the gas exchanger becomes higher and at the same time the concentration of O_2 becomes lower. However, when it occurs, the piston of the syringe is pushed down to maintain the constant surface level of the solution. Then the gas-phase pressure increases, and O_2 dissolves into the solution more quickly. In this way, the amount of O_2 that dissolves into the solution can be kept relatively constant (200 μM) regardless of the flow rate. The amount of the required sample depends on the flow rate, which will be discussed later. In this experiment 150 mL of 50 μM enzyme solution was circulated for a maximum of 5 h.

Raman scattering was excited with the 406.7-nm line of a Kr^+ laser (Spectra Physics, 164), dispersed with a double monochromator (SPEX 1404), and detected with a diode array detector with an image intensifier (PAR 1420). The data were processed with an OMA II system (PAR 1218). Absorption spectra were measured with the device for Raman/absorption simultaneous measurements (Ogura & Kitagawa, 1988), which enabled us to observe the absorption spectra of the enzyme present in the Raman-exciting laser beam (100 μm in diameter unless otherwise stated) by mixing the laser light and white light in an optical fiber.

RESULTS

Figure 3 shows time-resolved difference absorption spectra in the 500–650-nm region of the reduced form (A) and the reacted species (B) that were measured with the same apparatus by changing only the gas species in L2. Their difference is shown by a solid curve of spectrum C [=(B) − (A)]. The dotted line indicates the difference spectrum of the same intermediate with regard to the spectrum of the carbonmonoxy adduct (not shown). In this experiment, a single laser beam (406.7-nm line of a Kr^+ laser) was used, and a resident time of a given molecule in the laser beam was 150 μs. While a molecule stayed in the light beam, it was photolyzed by one photon and the Raman or absorption spectrum was measured by another photon. The difference spectrum (solid line) exhibits a positive peak at 588 nm and a trough at 608 nm, well reproducing the characteristic feature of compound A reported previously (Chance et al., 1975). The absorbance at 590 nm is considered to be slightly greater for the CO adduct than for the oxygenated intermediate. The absorption bands at 550 nm in spectra A and B arise from reduced cytochrome *c*. The flat feature at 550 nm in their difference spectrum indicates that the oxidation state of cytochrome *c* in the sample is not changed. In other words, electrons are not transferred from

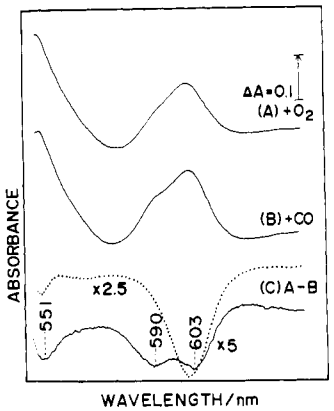

FIGURE 4: Absorption spectra of carbonmonoxy cytochrome oxidase in the presence of oxygen (A) with laser illumination (406.7 nm, 13 mW) and in the absence of oxygen without laser illumination (B) and their difference spectrum (C) obtained by subtracting spectrum B from spectrum A. The three spectra were obtained for the path length of 1.6 mm and are represented with a common absorbance scale designated in the figure. The flow rate was 60 mL/min. The beam diameter was 1 mm only for this experiment, and accordingly the resident time of a given molecule in the laser and monitoring beams was 1 ms. Dotted spectrum was obtained by subtracting the spectrum of fully reduced form (not shown) from spectrum A.

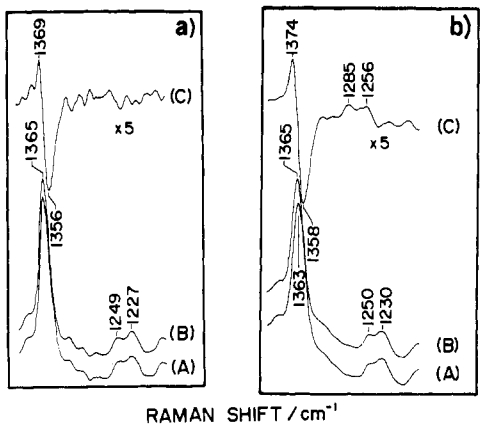

FIGURE 5: Resonance Raman spectra of the CO-bound cytochrome oxidase in the absence (spectra a-A and b-A) and presence (spectra a-B and b-B) of oxygen observed simultaneously with the absorption spectra shown in Figures 3 and 4. Raman difference spectra are represented as spectra C in each panel; spectrum a-C = spectrum a-B − spectrum a-A; spectrum b-C = spectrum b-B − spectrum b-A. Experimental conditions for (a) and (b) were identical with those for Figures 3 and 4, respectively.

cytochrome *c* to cytochrome *a* at this stage.

Figure 4 shows the spectra for later intermediates. In this experiment the inner diameter of the optical fiber for the source of spectroscopic measurements was made to be 1 mm, and accordingly the resident time of a given molecule in the laser and monitoring beams was 1 ms. Spectrum A was observed in the presence of O_2 under laser illumination, but spectrum B was observed in the absence of O_2 under no illumination of laser. Therefore, spectrum B represents the CO-bound form. The difference spectrum C [=(A) − (B)], which is distinct from the dotted curve of Figure 3C, coincides with those of compound B reported previously (Chance et al., 1975; Wikström et al., 1981). For comparison, the difference spectrum with regard to the reduced form is also depicted by a dotted line. The concentration of cytochrome *c* (8 μM) was the same as that for the experiment of Figure 3, but we note that a negative peak is appreciable at 550 nm in the difference spectrum, indicating that oxidation of cytochrome *c* took place to some extent.

Figure 5 shows resonance Raman spectra of cytochrome oxidase of the photodissociated carbonmonoxy form in the absence (a-A and b-A) and presence (a-B and b-B) of oxygen, where their Raman difference spectra [(C) = (B) − (A)] are also included. RR spectra of a-B and b-B of Figure 5 were obtained at the same time as the absorption spectra of Figures 3B and 4A, respectively, and thus they correspond to compound A and compound B. The frequency of the ν_4 band at 1365 cm^{-1} (Figure 5a-A) was apparently unshifted upon addition of oxygen (Figure 5a-B). This presumably implies that this band primarily comes from reduced cytochrome *c*. Subtracting the spectrum for the absence of oxygen (Figure 5a-A) from that for the presence of oxygen (Figure 5a-B) revealed an upwad shift of this band (Figure 5a-C).

The ν_4 frequency (1363 cm^{-1}) in Figure 5b-A obtained for the resident time of 1 ms was shifted slightly to a lower frequency than that of Figure 5a-A despite the fact that both spectra were observed in the absence of oxygen. We attribute this RR spectral difference to an increased amount of photodissociated cytochrome oxidase due to longer resident time in the measurements for compound B (Figure 5b-A). The Raman difference spectrum shown in Figure 5b-C clearly indicates an upward shift of frequency.

The results shown for the two kinds of intermediates were obtained in different experiments. However, we carried out another experiment with a completely different preparation of the enzyme and could reproduce the same difference spectra as shown in Figures 4C and 3C with a common apparatus by changing simply the flow speed. The difference spectrum of compound B was observed for the resident times of 200 and 160 μs, but the characteristic feature of compound A at 590 nm was noticed when the resident time was shortened to 140 μs, which was the highest limit within which the enzyme could be completely restored to the reduced form after one turn of circulation for this concentration of cytochrome *c*.

Discussion

The New Apparatus. About 1 g of purified cytochrome oxidase is obtained from 800 g of minced beef heart muscle in an overall elapsed time of 3 days. This amount of enzyme preparation can offer 200 mL of its 50 μM solution. If we use it in the mixed-flow type apparatus (Ogura et al., 1985) with time resolution of less than 100 μs (this requires a flow rate higher than 100 mL/min), we can accumulate the data for only 2 min. This is too short to obtain a spectrum of good quality. To repeat the experiment again, we have to concentrate the enzyme and remove oxidized reductant using a membrane filter. The handling of the enzyme takes several hours, at least, even for a 2-min extension of the accumulation time. This was the main reason why we could not go further using the previous apparatus (Ogura et al., 1985). The flow apparatus constructed in this study enabled us to accumulate the data for at least 5 h with the same amount of the sample.

Since the flow rate, length of the oxygen remover, volume of the sample solution, and concentration of cytochrome *c* were complicatedly interrelated with each other, some trials and errors were necessary to use the new flow apparatus most satisfactorily. The higher the flow rate is, the better the time resolution will be. However, if the flow rate were too high, O_2 would not be removed completely and the enzyme could not return to the CO-bound form in one cycle. O_2 might be consumed by the enzyme itself without the oxygen remover, but it is desirable to avoid extra catalytic turnovers of the enzyme, which are likely to induce denaturation of the enzyme. The longer the oxygen remover is, the more completely oxygen would be removed, but it would demand more enzyme. In practice, we adopted the length of 2 m for the flow rate of 70

mL/min, which could remove almost all (95%) of the oxygen dissolved in the solution.

Another problem with this apparatus lay in reduction of the enzyme. Since this enzyme does not receive electrons from ascorbate quickly, but does through cytochrome *c*, the rate of reduction depends on the concentration of cytochrome *c*. However, some RR bands of cytochrome *c* were strongly resonance enhanced upon excitation at 406 nm and were seriously overlapped with those of cytochrome oxidase. Excitation around 420–430 nm seemed to improve this difficulty, but we could not use any excitation lines between 406.7 and 441.6 nm in this laboratory. For attaining the high flow rate with the minimal amount of cytochrome *c*, we increased the amount of the enzyme solution as much as possible. Accordingly, we used 150 mL of the 50 μM enzyme solution for one measurement.

Compound A. There have been two approaches to follow the reaction kinetics of cytochrome oxidase with oxygen, cryogenic measurements and the flash photolysis at room temperature. The fully reduced CO-bound form kept at liquid nitrogen temperature was photolyzed in the presence of oxygen, and its absorption spectra were measured at various temperatures. At certain temperatures in a cryogenic region, Chance et al. (1975) found two distinct intermediates, which were called compound A and compound B in order of appearance. Malmström and co-workers (Clore et al., 1980a,b) measured the rapid-scan absorption spectra around 600 nm while the temperature of the photolyzed CO-bound enzyme was raised and pointed out that the first intermediate should have an absorption maximum at 590 nm similar to compound A. The spectrum with the characteristics of compound A was observed for photolysis of both cytochromes $a^{2+}a_3{}^{2+}$CO and $a^{3+}a_3{}^{2+}$CO.

The flash photolysis technique combined with the stopped-flow apparatus has been applied to study the room temperature kinetics of the enzyme catalysis (Greenwood & Gibson, 1967). Hill and Greenwood reported that compound A was formed only when the half-reduced enzyme ($a^{3+}a_3{}^{2+}$CO) was used as a starting material, and accordingly Hill et al. (1986) concluded it unlikely that compound A was formed from the fully reduced CO-bound form ($a^{2+}a_3{}^{2+}$CO). Orii (1984, 1988a) carried out the time-resolved absorption study of this enzyme at room temperature with the time resolution of 5 μs. Contribution of compound A, if any, should be noticed with this time resolution, and in fact appearance of transient absorption in the Soret region, which resembled the spectrum of the CO-bound form, was noted. However, the corresponding spectrum in the 590-nm region diagnostic for the formation of compound A was not measured in that experiment, and, therefore, its assignment to compound A was not necessarily established. In this sense there has been no report that indicates the formation of compound A at room temperature when the fully reduced form was used as a starting material.

The transient absorption spectrum shown in Figure 3C clearly exhibited the same characteristics as the spectrum of compound A obtained from the cryogenic measurements. This proved that compound A is indeed generated at 5 °C even when the fully reduced enzyme is used as starting material. Very recently, Orii (1988b) carried out the laser photolysis experiment to follow the absorption change around 600 nm and confirmed the formation of compound A at room temperature when the fully reduced CO-bound enzyme was photolyzed. The present difference spectrum of compound A is in practical agreement with the 20 μs − 5 μs difference spectrum reported by Orii (1988b). According to him its lifetime is considered to be shorter than 100 μs, and therefore the concentration of compound A at 150 μs would be significantly low. However, it should be kept in mind that the present method gives the cumulative spectrum from 0 to 150 μs after the start of reaction. The amount of compound B might be considerably larger than that of compound A at 150 μs, but the time-integrated absorbance for compound B would not be larger than that of compound A at this time. This would explain the reason why we could see the spectrum of an intermediate with a lifetime shorter than 100 μs in this experiment. We stress that the visible Soret spectra cannot bring about any direct evidence for the presence of dioxygen or an oxygen atom at the heme iron of cytochrome a_3.

Resonance Raman Spectra. RR spectra of cytochrome oxidase have been investigated by several groups (Babcock et al., 1978; Choi et al., 1983; Findsen & Ondrias, 1984; Ogura et al., 1985a; Rousseau, et al., 1988), and assignments of Raman bands are nearly established except for the formyl CH=O stretching mode of cytochrome *a*. Copeland et al. (1985) treated RR spectra of the pulsed form and noticed that the pulsed form is photoreduced during the Raman measurements similar to the resting form (Adar & Yonetani, 1978; Kitagawa & Orii, 1978; Salmeen et al., 1978). Babcock et al. (1984, 1985) applied the pulsed laser Raman technique to the oxygenated intermediates derived from the fully reduced and half-reduced forms, noting that the first intermediate generated in 10–50 μs was photolabile and therefore assignable to the Fe–O_2 type adduct. Ogura et al. (1985b) applied the CW laser Raman technique to the mixed-flow sample and reported a transient RR spectrum of intermediates formed within less than 450 μs after photolysis of the fully reduced CO-bound form in aerobic conditions. A clear frequency shift of the ν_4 band was observed for the intermediates. Unfortunately, however, in all these RR studies it was not clear what kind of intermediates gave the observed RR spectra. In the case of photolabile intermediates, it should be carefully examined whether the RR spectrum arises from the true intermediate or a photoproduct. Thus, it has been very desirable to establish the absorption spectrum of the species that yielded the RR spectra.

In this study we could observe RR spectra of compound A and compound B in the 1200–1400-cm^{-1} region, where the ν_4 band is expected. Transient absorption spectra indicated that the oxidation of cytochrome *c* was negligible for the experiment of Figure 3 but was evident for the experiment of Figure 4. Since the absorption maxima of the oxidized and reduced cytochrome *c* are located at 407 and 415 nm, respectively, resonance enhancement of Raman bands of cytochrome *c* upon excitation at 406.7 nm should be much greater for the oxidized form than for the reduced form. Although the concentration of cytochrome *c* (8 μM) was much lower than that of cytochrome oxidase (50 μM) in this experiment, oxidized cytochrome *c* is inferred to contribute significantly to Raman intensity for the 406.7-nm excitation. Consequently, it seems likely that the large difference peak of the ν_4 band in the RR spectrum of compound B (Figure 5b-C) is primarily due to the redox change of cytochrome *c*.

The redox state of cytochrome *c* was little altered within 150 μs after the initiation of reaction as seen in Figure 3. Therefore, the upward frequency shift of the ν_4 band in Figure 5a-C is attributed to cytochrome oxidase. For hemoglobin the ν_4 frequency is slightly higher for the oxygenated form than for the carbonmonoxy form (Rimai et al., 1975) and much higher than that of the reduced form. For horseradish peroxidase the ν_4 frequency of compound II with the ferryloxo heme is slightly higher than that of the oxidized form (Rakhit

et al., 1976; Felton et al., 1976; Teraoka et al., 1982). Accordingly, the upshift of the ν_4 frequency in compound A is qualitatively consistent with the formation of either the oxygenated form or the ferryloxo form. The latter is supposed to have an absorption maximum around 428 nm (Witt & Chan, 1987). However, the frequency of the positive peak in Figure 5a-C was not so high as that of the transient species (1376 cm^{-1}) of cytochrome oxidase observed previously (Ogura et al., 1985b). This might be due to the difference in excitation wavelength (416 nm in the previous one), which would cause resonance enhancement of Raman bands of different intermediate. Oxygen-isotope dependence of Raman spectra under improved time resolution is now in progress, but a method for making the next breakthrough with regard to structural elucidation of oxygenated intermediates was preliminarily established in this study.

ACKNOWLEDGMENTS

We thank Prof. Y. Orii of Kyoto University for sending us his paper (1988b) prior to publication.

REFERENCES

Abe, M., Kitagawa, T., & Kyogoku, Y. (1978) *J. Chem. Phys. 69*, 4526–4534.
Adar, F., & Yonetani, T. (1978) *Biochim. Biophys. Acta 502*, 80–86.
Argade, P. V., Ching, Y., Sassaroli, M., & Rousseau, D. L. (1986) *J. Biol. Chem. 261*, 5969–5973.
Babcock, G. T. (1988) *Biol. Appl. Raman Spectrosc. 3*, 293–346.
Babcock, G. T., & Callahan, P. M. (1983) *Biochemistry 22*, 2314–2319.
Babcock, G. T., Callahan, P. M., Ondrias, M. R., & Salmeen, I. (1981) *Biochemistry 20*, 959–966.
Babcock, G. T., Jean, J. M., Johnston, L. N., Palmer, G., & Woodruff, W. H. (1984) *J. Am. Chem. Soc. 106*, 8305–8306.
Babcock, G. T., Jean, J. M., Johnston, L. N., Woodruff, W. H., & Palmer, G. (1985) *Inorg. Biochem. 23*, 243–251.
Bangcharoenpaurpong, O., Rizos, A. K., Champion, P. M., Jollie, D., & Sligar, S. G. (1986) *J. Biol. Chem. 261*, 8089–8092.
Blair, D. F., Witt, S. N., & Chan, S. I. (1985) *J. Am. Chem. Soc. 107*, 7389–7399.
Callahan, P. M., & Babcock, G. T. (1981) *Biochemistry 20*, 952–958.
Callahan, P. M., & Babcock, G. T. (1983) *Biochemistry 22*, 452–461.
Chance, B., Saronio, C., & Leigh, J. S., Jr. (1975) *J. Biol. Chem. 250*, 9226–9237.
Ching, Y.-C., Argade, P. V., & Rousseau, D. L. (1985) *Biochemistry 24*, 4938–4946.
Choi, S., Lee, J. J., Wei, Y. H., & Spiro, T. G. (1983) *J. Am. Chem. Soc. 105*, 3692–3707.
Clore, G. M., Andreasson, L.-E., Karlsson, B., Aasa, R., & Malmström, B. G. (1980a) *Biochem. J. 185*, 139–154.
Clore, G. M., Andreasson, L.-E., Karlsson, B., Aasa, R., & Malmström, B. G. (1980b) *Biochem. J. 185*, 155–167.
Copeland, R. A., Naqui, A., Chance, B., & Spiro, T. G. (1985) *FEBS Lett. 182*, 375–379.
Felton, R. H., Romans, A. Y., Yu, N. T., & Schonbaum, G. R. (1976) *Biochim. Biophys. Acta 434*, 82–89.
Findsen, E. W., & Ondrias, M. R. (1984) *J. Am. Chem. Soc. 106*, 5736–5738.
Greenwood, C., & Gibson, Q. H. (1967) *J. Biol. Chem. 242*, 1782–1787.
Hashimoto, S., Tatsuno, Y., & Kitagawa, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2417–2421.
Hill, B. C., & Greenwood, C. (1983) *Biochem. J. 215*, 659–667.
Hill, B. C., Greenwood, C., & Nicholls, P. (1986) *Biochim. Biophys. Acta 853*, 91–113.
Kitagawa, T., & Orii, Y. (1978) *J. Biochem. (Tokyo) 84*, 1245–1252.
Ogura, M., Yamamoto, Y., & Ogura, Y. (1984) *Experientia 41*, 139–141.
Ogura, T., & Kitagawa, T. (1988) *Rev. Sci. Instrum. 59*, 1316–1320.
Ogura, T., Hon-nami, K., Oshima, T., Yoshikawa, S., & Kitagawa, T. (1983) *J. Am. Chem. Soc. 105*, 7781–7783.
Ogura, T., Yoshikawa, S., & Kitagawa, T. (1985a) *Biochemistry 24*, 7746–7752.
Ogura, T., Yoshikawa, S., & Kitagawa, T. (1985b) *Biochim. Biophys. Acta 832*, 220–223.
Ogura, T., Maeda, A., Nakagawa, M., & Kitagawa, T. (1987) in Primary Processes in Photobiology, *Springer Proc. Phys. 20*, 233–241.
Orii, Y. (1984) *J. Biol. Chem. 259*, 7187–7190.
Orii, Y. (1988a) *Chem. Scr. 28A*, 63–69.
Orii, Y. (1988b) *Ann. N.Y. Acad. Sci. 550*, 105–117.
Rakhit, G., Spiro, T. G., & Uyeda, M. (1976) *Biochem. Biophys. Res. Commun. 71*, 803–807.
Rimai, L., Salmeen, I., & Petering, D. H. (1975) *Biochemistry 14*, 378–381.
Rousseau, D. L., Singh, S., Ching, Y.-C., & Sassaroli, M. (1988) *J. Biol. Chem. 263*, 5681–5685.
Salmeen, I., Rimai, L., & Babcock, G. T. (1978) *Biochemistry 17*, 800–806.
Sassaroli, M., Ching, Y., Argade, P. V., & Rousseau, D. L. (1988) *Biochemistry 27*, 2496–2502.
Teraoka, J., Ogura, T., & Kitagawa, T. (1982) *J. Am. Chem. Soc. 104*, 7354–7356.
Terner, J., Sitter, A. J., & Reczek, C. M. (1985) *Biochim. Biophys. Acta 828*, 73–80.
Wagner, W.-D., Paeng, I. R., & Nakamoto, K. (1988) *J. Am. Chem. Soc. 110*, 5565–5567.
Wikström, M., Krab, K., & Saraste, M. (1981) in *Cytochrome Oxidase-A Synthesis*, Academic Press, New York.
Witt, S. N., & Chan, S. I. (1987) *J. Biol. Chem. 262*, 1446–1448.
Yoshikawa, S., Choc, M. G., O'Toole, M. C., & Caughey, W. S. (1977) *J. Biol. Chem. 252*, 5498–5508.