Resonance Raman Study on Photoreduction of Cytochrome *c* Oxidase: Distinction of Cytochromes *a* and a_3 in the Intermediate Oxidation States†

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ABSTRACT: Occurrence of photoreduction of bovine cytochrome *c* oxidase was confirmed with the difference absorption spectra and oxygen consumption measurements for the enzyme irradiated with laser light at 406.7, 441.6, and 590 nm. The resonance Raman spectra were obtained under the same experimental conditions as those adopted for the measurements of oxygen consumption and difference absorption spectra. The photoreduction was more effective upon irradiation at shorter wavelengths and was irreversible under anaerobic conditions. However, upon aeration into the cell, the original oxidized form was restored. It was found that aerobic laser irradiation produces a photo steady state of the catalytic dioxygen reduction and that the Raman scattering from this photo steady state probes cytochrome a^{2+} and cytochrome a_3^{3+} separately upon excitations at 441.6 and 406.7 nm, respectively. The enzyme was apparently protected from the photoreduction in the spinning cell with the spinning speed between 1 and 1500 rpm. These results were explained satisfactorily with the reported rate constant for the electron transfer from cytochrome *a* to cytochrome a_3 (0.58 s^{-1}) and a comparable photoreduction rate of cytochrome *a*. The anaerobic photoreduction did give Raman lines at 1666 and 214 cm^{-1}, which are characteristic of the ferrous high-spin cytochrome a_3^{2+}, but they were absent under aerobic photoreduction. The formyl CH=O stretching mode of the a_3 heme was observed at 1671 cm^{-1} for $a^{2+}a_3^{2+}CO$ but at 1664 cm^{-1} for $a^{2+}a_3^{2+}CN^-$, indicating that the CH=O stretching frequency reflects the π back-donation to the axial ligand similar to the oxidation state marker line (ν_4).

Cytochrome *c* oxidase (cytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) is a respiratory enzyme present generally in aerobic organisms and catalyzes reduction of dioxygen which is coupled with proton translocation through a membrane (Wikström, 1984). This enzyme is conveniently grouped into two functional units, namely, cytochrome *a* and cytochrome a_3, and is conveniently designated as $a^{n+}a_3^{m+}$ hereafter (*n* and *m* are 2+ or 3+ and denote the oxidation states of the heme iron; here the oxidation state of copper is neglected). The hemes of cytochrome a^{n+} and cytochrome a_3^{m+} are hereafter represented simply as the a^{n+} heme and a_3^{m+} heme, respectively, for convenience sake. Cytochrome *a* works as an electron-transfer protein between cytochrome *c* and cytochrome a_3, while cytochrome a_3 provides a catalytic site for dioxygen reduction. In the resting state cytochrome *a* contains a low-spin heme *a* and an electron paramagnetic resonance (EPR)[1] detectable copper, whereas cytochrome a_3 contains a high-spin heme *a* and an EPR silent copper [see Malmström (1979) for a review]. Spectroscopic distinction of the two functional units and the roles of the four metal ions have currently been investigated with various techniques including visible absorption (Blair et al., 1982), MCD (Babcock et al., 1976), EPR (Ohnishi et al., 1982; Boelens et al., 1984), infrared (Fiamingo et al., 1982; Yoshikawa & Caughey, 1982), Mössbauer (Kent et al., 1982, 1983), EXAFS (Scott et al., 1981; Powers et al., 1981; Naqui et al., 1984), and resonance Raman (RR) (Babcock et al., 1981; Choi et al., 1983; Argade et al., 1984; Ogura et al., 1984) spectroscopies.

It was noticed in previous RR studies that cytochrome *c* oxidase was photoreduced by laser irradiation (Adar & Yonetani, 1978; Kitagawa & Orii, 1978). On the other hand, Salmeen et al. (1978) pointed out that although the resting cytochrome *c* oxidase gave the RR spectrum similar to that of dithionite-reduced enzyme, it lacked the Raman lines at 1666 and 214 cm^{-1} which were characteristic of the high spin a_3^{2+} heme and were closely related to the activity of this enzyme (Ogura et al., 1984). Other RR studies by Bocian et al. (1979) and Woodruff et al. (1981) stressed that cytochrome *c* oxidase of their preparation was not photoreduced upon irradiation at ~600 and 406.7 nm, respectively. Thus, it seems as if the occurrence of photoreduction depends on the enzyme preparation. The present study was undertaken to resolve this controversial phenomenon.

We observed the oxygen consumption and difference absorption spectrum induced by laser irradiation and thus confirmed the occurrence of photoreduction upon laser irradiation at ~600 and 406.7 nm as well as at 441.6 nm. It is emphasized that the dominant species in the photo steady state under aerobic laser irradiation is a mixed valence state such as $a^{2+}a_3^{3+}$ and that the apparent RR spectrum depends on excitation wavelength; excitations at 406.7 and 441.6 nm enhance the RR spectra of the a_3^{3+} and a^{2+} hemes, respectively. Thus, this study will not only resolve the controversy but also bring a new idea to probe the *a* and a_3 hemes separately while the enzyme is turning over.

Materials and Methods

Cytochrome *c* oxidase was isolated from bovine heart ac-

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[1] Abbreviations: RR, resonance Raman; EPR, electron paramagnetic resonance; EXAFS, extended X-ray absorption fine structures; MCD, magnetic circular dichroism.

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cording to the methods described elsewhere (Yoshikawa et al., 1977) and was dissolved in 50 mM sodium phosphate buffer, pH 7.1, containing 5% (w/v) ammonium sulfate. The concentration of the enzyme was determined spectrophotometrically with the assumption of $\Delta\epsilon_{mM} = \epsilon_{605} - \epsilon_{630} = 33.0$ (in terms of two hemes *a*): it was 17 μM unless otherwise stated. To obtain the spectrum of $a^{2+}a_3{}^{2+}$, the enzyme was reduced by a slight excess amount of dithionite and kept under anaerobic conditions (0.01 mmHg) at 25 °C for 20 min before the measurements were started. Cyanide complexes of enzyme were prepared as follows: $a^{3+}a_3{}^{3+}CN^-$ was obtained by adding as much KCN solution (100 mM, pH 12) to the resting enzyme to make the final KCN concentration 5 mM and by standing for 1 h. With this concentration, the pH of the enzyme solution remained unaltered. The complex formation was confirmed with the disappearance of the high-spin marker line of the $a_3{}^{3+}$ heme at 1574 cm^{-1}, which is prominent upon excitation at 406.7 nm (see Figure 6). $a^{2+}a_3{}^{3+}CN^-$ was formed by adding a slight excess amount of dithionite to $a^{3+}a_3{}^{3+}CN^-$ $a^{2+}a_3{}^{2+}CN^-$ was obtained by adding the degassed KCN solution to $a^{2+}a_3{}^{2+}$ under anaerobic conditions so as to make the final KCN concentration 5 mM. The carbon monoxide complex ($a^{2+}a_3{}^{2+}CO$) was formed by bubbling CO gas through $a^{2+}a_3{}^{2+}$ for 2 min in the spinning cell, and then the cell was sealed under the CO atmosphere.

A variable speed spinning cell (diameter = 2 cm) that allows the measurements of Raman scattering under anaerobic conditions was designed for this study. Since laser light is focused to 0.1 mm and the effective height of the sample is 3 mm, the effective number of enzyme molecules in the laser beam is $1.4 \times 10^{10} c_0$ provided the sample concentration is c_0 (mM). When the cell is spun at n rpm, the time during which a given molecule is illuminated by laser light is $0.1/n$ seconds per one turn. Suppose that the laser power is p mW at 406.7 nm, the effective number of photons contained in the scattering volume is $(20p/n) \times 10^{13}$. Therefore, the ratio of the number of photons to the number of enzyme molecules is $14p/(nc_0)$ and is actually ca. 40 under standard conditions of $c_0 = 0.017$, $n = 10^3$, and $p = 60$. Note that the intensities of Raman lines of photoreduced and nonphotoreduced species are expected to be proportional to p^2 and p, respectively.

Raman scattering was excited at 441.6 nm with a He/Cd laser (Kinmon Electrics, CDR80MGH) or 406.7 nm with a Kr^+ laser (Spectra Physics, 165-03) and recorded on JEOL-400D Raman spectrometer equipped with a cooled RCA-31034a photomultiplier. Calibration of the Raman spectrometer was performed with indene (1200–1700 cm^{-1}) and carbon tetrachloride (150–500 cm^{-1}) as a standard. Temperature of the sample in the spinning cell was kept at 5 °C by flushing with cold nitrogen gas. For the stationary measurements, ca. 40 μL of the sample solution was put into a cylindrical cell which was held in a water-circulating cell holder.

Absorption spectra were obtained with a Hitachi 200S recording spectrophotometer. Difference spectra between the laser irradiated vs. nonirradiated samples were obtained as follows: (1) The enzyme solution in a cuvette was degassed and kept under anaerobic conditions for 10 min, and then the reference spectrum was recorded and memorized. (2) The cuvette was placed in front of the laser, and the solution was stirred under anaerobic conditions. (3) Laser light was made to pass through the solution under stirring for 10 min. (4) The cuvette was returned to the spectrophotometer, and the spectrum was measured. (5) The difference was calculated. Details of experimental conditions are described in the figure captions. For the irradiation at 590 nm, a dye laser (Spectra Physics, 375) with Rhodamine 6G was excited with an Ar^+ laser (NEC GLG3200).

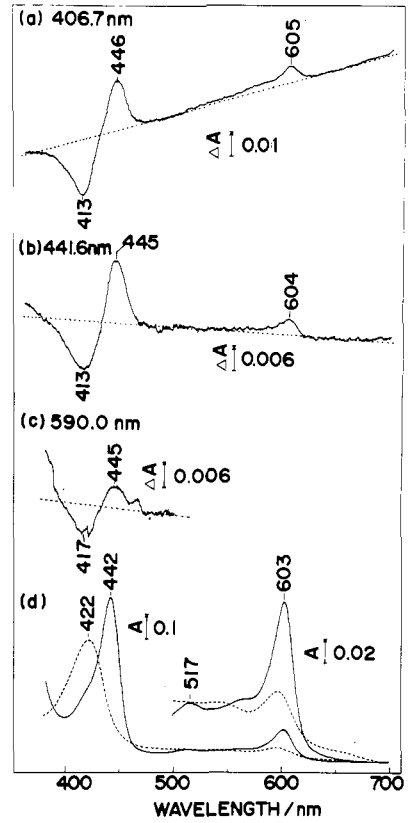

FIGURE 1: Difference absorption spectra between the laser-irradiated vs. nonirradiated cytochrome *c* oxidase and standard spectra of resting (···) and reduced (—) enzymes. Irradiation: (a) 406.7 (60 mW), (b) 441.6 (80 mW), and (c) 590.0 nm (200 mW). The size of the laser beam was ca. 1 mm. Three milliliters of the enzyme solution was put in a 1 cm × 1 cm × 3 cm cuvette and kept under 0.01 mmHg during the irradiation. Enzyme concentration was 5 μM for spectra a–c and 3.5 μM for spectrum d. The small positive bump around 460 nm in spectrum c is due to noise, because the control sample also exhibited the same bump.

Oxygen consumption was measured at 25 °C with a Clark type oxygen electrode (Gilson). To see the effect of laser irradiation, laser light was reflected into the reaction cell (2 mL) through the circulating water layer. The test solution with or without the enzyme was first stirred for 10 min in keeping the cap of the reaction cell open to attain an equilibrium with the atmosphere (O_2 concentration, 250 μM at 25 °C), then the cell was closed with the cap, and the measurements were started. For this experiment, more concentrated enzyme solution (50 μM) with 50 mM phosphate, pH 7.1, was used.

RESULTS

The effects of laser irradiation on the absorption spectra of enzyme placed under the anaerobic conditions are illustrated in Figure 1, where the difference spectra between the irradiated vs. nonirradiated samples (spectra a–c) and the standard spectra of the resting and reduced enzymes used in this study (spectrum d) are displayed. The difference spectra for laser irradiation at 406.7 (a), 441.6 (b), and 590 nm (c) are alike, giving rise to positive peaks at 445–446 and 604–605 nm and a negative trough at 413 nm, although the signal to noise ratio of spectrum c is not always satisfactory due to less photoreduction. The observed difference spectra resemble the difference spectra between the reduced vs. the oxidized cytochrome *a*; the intensity ratio of the positive peak at 445–446

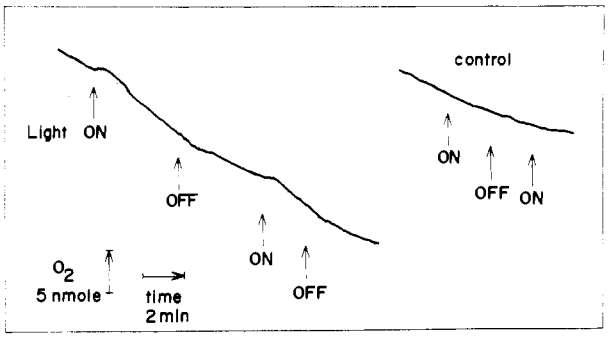

FIGURE 2: Oxygen uptake by resting cytochrome *c* oxidase upon laser irradiation at 441.6 nm. Left and right curves were obtained for solutions containing 50 μM cytochrome *c* oxidase and no enzyme, respectively. Accordingly, the right curves represent the background oxygen consumption of the cell used. "ON" and "OFF" in the chart indicate the starting and ending points of laser irradiation, respectively. Laser power was 80 mW. The sample solution was stirred during the measurements. The ordinate denotes the oxygen concentration, and its scale is represented in the figure.

nm to that at 604–605 nm (3.2–4.0) is coincident with that of cytochrome *a* (2.8–4.5) but distinct from that of cytochrome a_3 (23.3) (Yonetani, 1960; Vanneste, 1966).

The similar difference spectra were obtained even 1 h after the termination of laser irradiation. When air was introduced into the laser-irradiated sample, the spectral differences disappeared completely. The sample kept under the same anaerobic conditions without irradiating laser light did not show such a difference spectrum. Therefore, it became evident that cytochrome *a* of cytochrome *c* oxidase is photoreduced during the 10-min irradiation by laser light at any of the wavelengths used for photoreduction—406.7, 441.6, and 590 nm. However, the quantum yield of photoreduction seems to depend on the wavelength of light, because an amount of the photoreduced portion upon irradiation at 590 nm was much less than that at 406.7 nm despite the fact that the laser power used was higher at 590 nm (200 mW) than at 406.7 nm (60 mW).

It is interesting to see whether the photoreduced enzyme is a catalytically active one or not. To see it, the oxidase activity was examined during the laser irradiation. The effect of laser irradiation on oxygen consumption is illustrated in Figure 2, where the amount of oxygen present is plotted vs. the reaction time. Note that 2 mL of the solution under an equilibrium with air contains 500 nmol of molecular oxygen, and accordingly the ordinate scale of Figure 2 is ca. 10 times expanded. Under such an expanded scale, the background oxygen consumption due to the electrolysis and other unknown factors as well, which can be generally neglected in the measurements of mitochondrial respiration rate, becomes significant, although such effects were made as small as possible.

As shown in the left side of Figure 2, the rate of oxygen consumption decreased during the initial 40–50 s after irradiation of laser light, but thereafter the rate increased and kept a constant value. This was repeatedly confirmed. Although the origin of the first phase remains to be elucidated, it is evident that the rate of decrease of oxygen in the steady state is larger during the irradiation with laser light than during no irradiation. The right-side curve of Figure 2 was obtained under the same experimental conditions except that the reaction medium contained no enzyme. This shows the background oxygen consumption described above, and unexpectedly it is not linear with regard to time. It is stressed that in the absence of enzyme the rate of the background oxygen consumption was unaffected when the sample was irradiated with laser light. Quite the similar phenomenon was observed when the sample was irradiated at 406.7 nm, but it was less pronounced upon irradiation at 590 nm presumably due to a smaller quantum yield for photoreduction. At any rate, this result strongly suggests that the photoreduced enzyme was active in a catalytic reduction of dioxygen, being in a photo steady state as long as sufficient oxygen was supplied under laser irradiation.

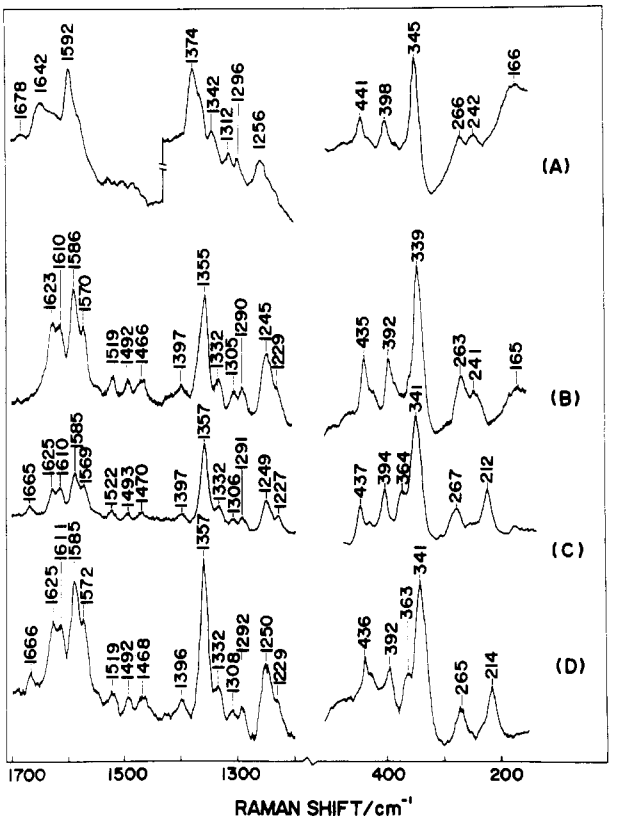

FIGURE 3: Resonance Raman spectra of cytochrome *c* oxidase with four variant cell conditions upon excitation at 441.6 nm. Spectra A–C were obtained for the resting enzyme, and spectrum D was obtained for dithionite-reduced enzyme. Spectrum A and spectra B–D were obtained with a spinning cell (1000 rpm) and a stationary cell, respectively. Spectra A and B were taken under aerobic conditions and spectra C and D under anaerobic conditions. Experimental conditions: enzyme concentration 17 μM; spectral slit width 8 cm^{-1}; time constant 8 s for (A) and 1.6 s for (B–D); scan speed 10 cm^{-1}/min for (A) and 25 cm^{-1}/min for (B–D); sensitivity 5000 counts/s; laser power 20 mW for (A) and 80 mW for (B–D).

The RR spectra of cytochrome *c* oxidase excited at 441.6 nm are shown in Figure 3, where spectra A–C were obtained for the resting enzyme but spectrum D was obtained for the dithionite-reduced enzyme. Only spectrum A was measured with a spinning cell (1000 rpm), but others were measured with a stationary cell. The cell inside was aerobic for spectra A and B but anaerobic for spectra C and D. It was noticed that the intensities of Raman lines of the peripheral formyl CH=O stretching mode around 1666–1678 cm^{-1} (Salmeen et al., 1973, 1978), the ν_4 mode at 1358–1374 cm^{-1} [numbering of the porphyrin modes follows Abe et al. (1978)], and the Fe^{2+}–histidine stretching mode at 214 cm^{-1} (Ogura et al., 1983) were varied with the four experimental conditions.

Spinning of the cell under aerobic conditions (A) gave the intense ν_4 line at 1374 cm^{-1}. When the spinning of the cell was stopped, the 1374-cm^{-1} line was replaced by a symmetric line at 1355 cm^{-1}. Since the ν_4 line is known to appear around 1355–1360 and 1370–1375 cm^{-1} for the ferrous and ferric hemes, respectively (Spiro & Stein, 1974; Kitagawa et al.,

1975), this observation also supports the occurrence of photoreduction upon laser illumination at 441.6 nm. Spinning of the cell apparently protected the enzyme from photoreduction. Actually, however, a small amount of photoreduction always took place even in the spinning cell, but the probability that the photoreduced enzymes encounter another photon to undergo Raman scattering is low when laser power is low. The pattern of spectrum A did not change noticeably upon a change of the spinning speed (1–1500 rpm). Implication of this observation will be discussed in detail later.

Spectrum B (Figure 3) was observed under aerobic conditions without spinning the cell. Therefore, the enzymes in the laser beam were probably turning over. The intense ν_4 line at 1355 cm^{-1} implies the presence of ferrous heme. Nevertheless, the Raman lines at 1666 and 214 cm^{-1} which are characteristic of the five-coordinate a_3^{2+} heme are not recognized. Therefore, it is most likely that the dominant species under turnover is an intermediate designated as $a^{2+}a_3^{3+}$, and the spectrum of the a^{2+} heme is mainly resonance enhanced upon excitation at 441.6 nm. This conclusion is consistent with Babcock et al. (1981), who noted that the resting enzyme in the presence of oxygen yielded the RR spectrum similar to that of $a^{2+}a_3^{3+}$HCOOH. The RR spectrum of resting enzyme reported by Carter et al. (1981) probably corresponds to the present photo steady state (spectrum B).

Spectrum C (Figure 3) was obtained under the same conditions that were used to measure the difference absorption spectra shown in Figure 1. This spectrum is essentially the same as spectrum D of dithionite-reduced enzyme. However, it is observed that the Fe–histidine stretching frequency of the a_3^{2+} heme is lower with the photoreduced enzyme than with the dithionite-reduced enzyme by 2 cm^{-1}, indicating slight conformational difference with regard to this bond. The spectral change from spectra B to C occurred during the initial 15 min, and the extent of the spectral change strongly depended on anaerobicity of the cell. When air was incorporated into the sample of spectrum C, spectrum B was reproduced. Thus, the enzyme did not suffer an irreversible denaturation. The results were unaltered by addition of equivalent amount of cytochrome *c*, although some interactions between cytochrome *c* and cytochrome *c* oxidase were suggested (Schroedl & Hartzell, 1977; Ferguson-Miller et al., 1976).

Figure 4 shows the RR spectra excited at 406.7 nm. The characters, A–D, denote the cell conditions identical with those specified for Figure 3. The ν_4 line of spectrum B is identified at the frequency of the ferric heme (1371 cm^{-1}) which is distinct from spectrum B shown in Figure 3. The Raman line at 1646 cm^{-1} and those in the low-frequency region (450–150 cm^{-1}) are stronger in spectrum A than in spectrum B, and they are absent in spectrum C. The formyl stretching mode is observed at 1671–1674 cm^{-1} for spectra A and B but at 1663–1665 cm^{-1} for spectra C and D. Spectrum C is practically identical with spectrum D.

To gain insight into the meaning of the RR spectra B in Figures 3 and 4, the RR spectra of the enzyme derivatives were measured and shown in Figures 5 and 6 for the 441.6- and 406.7-nm excitations, respectively, where the spectra of the resting and fully reduced enzymes are also included for comparison. When CN^- is bound to a_3^{3+}, the Raman line of $a^{3+}a_3^{3+}$ at 1678 cm^{-1} (spectrum A in Figure 5) disappears, but other parts are practically unaltered as shown by spectrum B. Therefore, the 1678-cm^{-1} line is assignable to the formyl CH=O stretching mode of the a_3^{3+} heme with the high-spin state, but most of the other lines are considered to arise from the a^{3+} heme or to be insensitive to the high- to low-spin conversion. When only the *a* heme of $a^{3+}a_3^{3+}CN^-$ was reduced, the Raman lines of $a^{3+}a_3^{3+}CN^-$ at 1644 and 1631 cm^{-1} were replaced by a single line at 1627 cm^{-1}, and the ν_4 line was shifted to 1358 cm^{-1} (spectrum C). These lines are therefore assignable to the *a* heme. Except for these changes, the RR spectra of the a^{3+} and a^{2+} hemes might be alike.

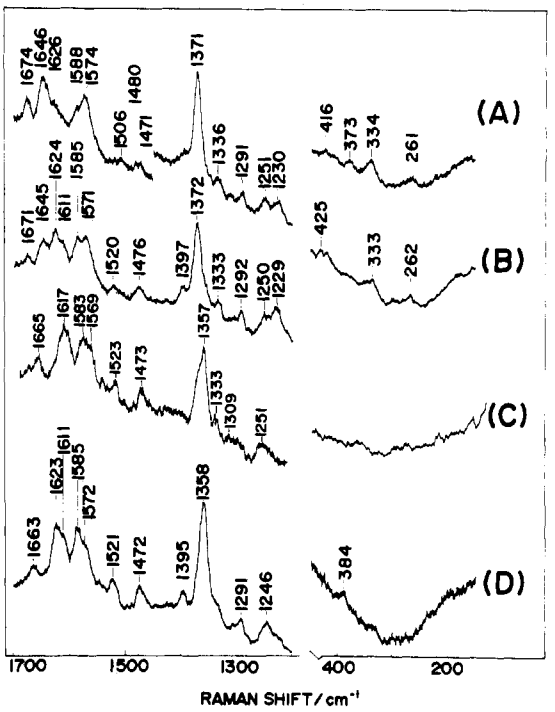

FIGURE 4: Resonance Raman spectra of cytochrome *c* oxidase with four variant cell conditions upon excitation at 406.7 nm. Each cell condition for spectra A–D is the same as those for Figure 3. Instrumental conditions were also the same as those in Figure 3 except for laser power (50 mW for all).

In the spectrum of $a^{2+}a_3^{2+}$ (spectrum D), additional RR lines compared with the spectrum of $a^{2+}a_3^{3+}CN^-$ (spectrum C) should be attributed to the a_3^{2+} heme. Accordingly, the lines at 1666, 1229, 363, and 214 cm^{-1} are assignable to the a_3^{2+} heme. These lines disappear or are decreased in intensity when CN^- (spectrum E) or CO (spectrum F) is bound to the a_3^{2+} heme. The Raman lines of $a^{2+}a_3^{2+}CN^-$ at 239 cm^{-1}, of $a^{2+}a_3^{2+}CO$ at 241 cm^{-1}, of $a^{2+}a_3^{3+}CN^-$ at 244 cm^{-1}, and of $a^{3+}a_3^{3+}CN^-$ at 240 cm^{-1} may arise from the six-coordinate a_3 heme, since $a^{2+}a_3^{2+}$ does not exhibit the corresponding line. Then the 242-cm^{-1} line of the resting enzyme ($a^{3+}a_3^{3+}$) may indicate that the a_3^{3+} heme adopts the six-coordinate structure. The RR spectra of $a^{2+}a_3^{3+}CN^-$, $a^{2+}a_3^{2+}CN^-$, and $a^{2+}a_3^{2+}CO$ excited at 441.6 nm are alike and are close to spectrum B of Figure 3. Presumably the spectral contribution from the a^{2+} heme is predominant as pointed out by Ching et al. (1985).

In the spectra excited at 406.7 nm shown in Figure 6, the Raman lines of $a^{3+}a_3^{3+}$ at 1574 and 1480 cm^{-1} (spectrum A) disappear upon binding of CN^- (spectrum B) and are accordingly assigned to the a_3^{3+} heme. The Raman lines of $a^{3+}a_3^{3+}CN^-$ at 1644 and 1503 cm^{-1} (spectrum B) diminished in intensity, and instead new lines appeared at 1624 and 1521 cm^{-1} upon reduction of its *a* heme (spectrum C). Therefore, the lines at 1644 and 1503 cm^{-1} and those at 1624 and 1521 cm^{-1} may be used as an indicator of the a^{3+} and a^{2+} hemes, respectively. The latter set is always seen for four complexes having the a^{2+} heme in Figure 6 (spectra C–F).

In comparison of the RR spectrum of $a^{2+}a_3^{3+}CN^-$ with that of $a^{2+}a_3^{2+}$ (spectrum D), a shift of the formyl CH=O stretching mode from 1673 to 1663 cm^{-1} is evident. This line can be used as a marker of the oxidation state of the a_3 heme

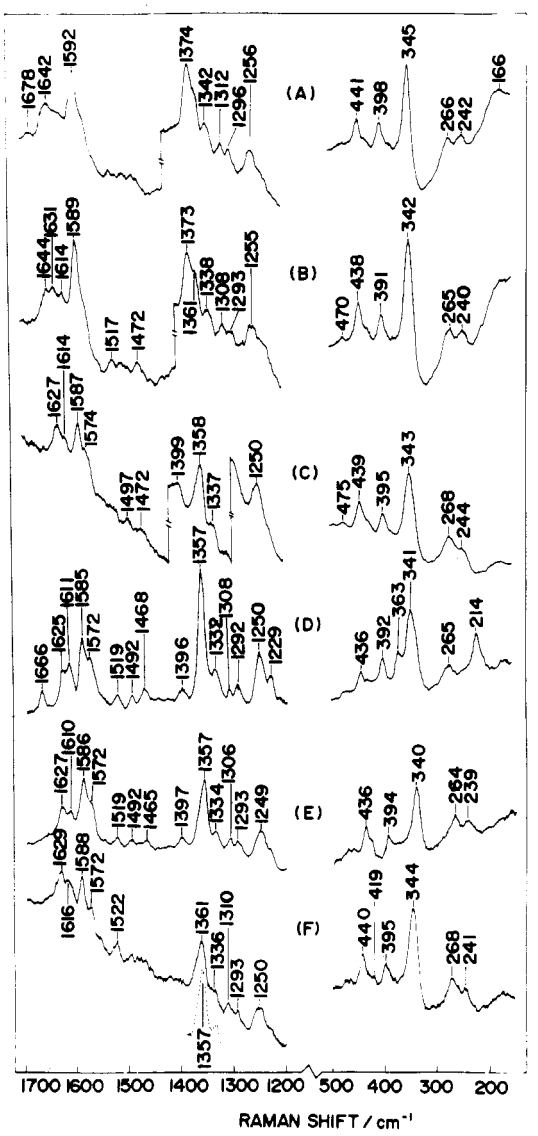

FIGURE 5: Resonance Raman spectra of $a^{3+}a_3{}^{3+}$ (A), $a^{3+}a_3{}^{3+}CN^-$ (B), $a^{2+}a_3{}^{3+}CN^-$ (C), $a^{2+}a_3{}^{2+}$ (D), $a^{2+}a_3{}^{2+}CN^-$ (E), and $a^{2+}a_3{}^{2+}CO$ forms (F) of cytochrome *c* oxidase upon excitation at 441.6 nm. Spectra A–C and F were obtained by using a spinning cell, while spectra D and E were obtained with a stationary cell. Experimental conditions: sensitivity 1000 counts/s for (A) and (B), 2500 counts/s for (C–E), and 250 counts/s for (F); time constant 16 s for (A) and (B) and the lower frequency part of (C), 0.8 s for higher frequency parts of (D) and (E), 1.6 s for the lower frequency part of (E), and 3.2 s for (F) and the lower frequency part of (D); scan speed 10 cm^{-1}/min for (A–C) and (F), 25 cm^{-1}/min for the lower frequency part of (D), and 50 cm^{-1}/min for (E) and the higher frequency part of (D); spectral slit width 9 cm^{-1}; output laser power 20 mW for (A–E) and 2.5 mW for (F). Dotted spectrum under (F) was obtained for $a^{2+}a_3{}^{2+}CO$ with a higher output laser power (5 mW) and shows the RR spectrum of the photodissociated form.

as previously suggested by Woodruff et al. (1981). Although this frequency does not depend on the spin state of the a_3 heme, it seems to be sensitive to delocalization of π electrons, because the CH=O stretching frequency of $a^{2+}a_3{}^{2+}CO$ is higher than that of $a^{2+}a_3{}^{2+}CN^-$ similar to the ν_4 frequency. On the basis of this marker line, the 1671-cm^{-1} line of Figure 4B suggests the presence of the $a_3{}^{3+}$ heme, and the 1665-cm^{-1} line in Figure 4C indicates the presence of the $a_3{}^{2+}$ heme.

Discussion

Distinction of the a and a_3 Hemes. Assignments of Raman lines of cytochrome *c* oxidase to the *a* or a_3 heme has been

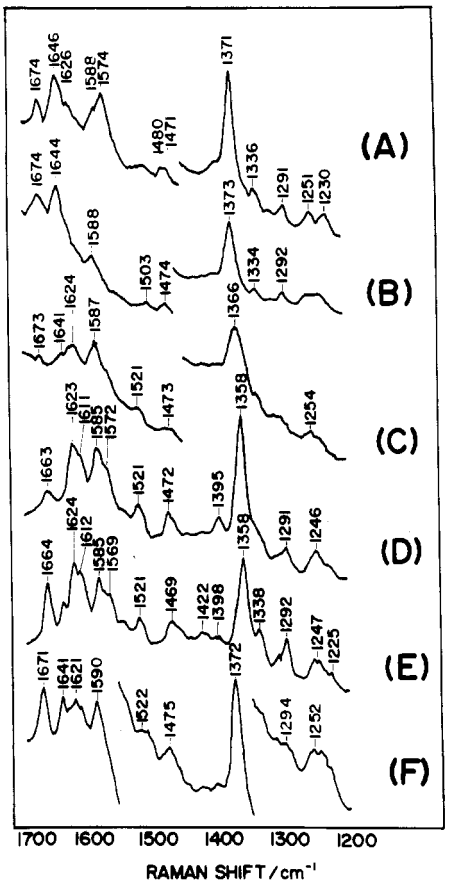

FIGURE 6: Resonance Raman spectra of $a^{3+}a_3{}^{3+}$ (A), $a^{3+}a_3{}^{3+}CN^-$ (B), $a^{2+}a_3{}^{3+}CN^-$ (C), $a^{2+}a_3{}^{2+}$ (D), $a^{2+}a_3{}^{2+}CN^-$ (E), and $a^{2+}a_3{}^{2+}CO$ forms (F) of cytochrome *c* oxidase upon excitation at 406.7 nm. Each sample is identical with the corresponding sample in Figure 5. The spectra in the lower frequency region were deleted due to the low intensities at this excitation wavelength. Spectra A–C and F were obtained with a spinning cell while spectra D and F were obtained with a stationary cell. Experimental conditions: sensitivity 5000 counts/s; scan speed 25 cm^{-1}/min for (A–D) and (F) and 50 cm^{-1}/min for (E); time constant 1.6 s for (A–D) and (F) and 0.8 s for (E); output laser power 65 mW.

a main subject in interpreting RR spectra of cytochrome *c* oxidase. This has been worked out on the basis of the RR spectra of heme *a* derivatives, and some marker lines have been assigned to the *a* and a_3 hemes satisfactorily (Babcock & Salmeen, 1979; Van Steelandt-Frentrup et al., 1981; Babcock et al., 1981; Choi et al., 1983; Kitagawa et al., 1977). However, since the frequencies of the model compounds are somewhat shifted from those of enzyme even when they are compared in the same spin and oxidation states, this approach does not always lead us to unequivocal assignments when the frequency difference between cytochrome *a* and cytochrome a_3 and also between their ferrous and ferric states is small. On the other hand, Ching et al. (1985) established the RR spectra of the $a_3{}^{2+}$ and a^{2+} hemes by using Raman difference spectroscopy. According to them, there are many overlapping bands between the spectra of the a^{2+} and $a_3{}^{2+}$ hemes. However, the Raman lines assigned to the $a_3{}^{2+}$ heme in this study are in agreement with Argade et al. except for the line at 239–244 cm^{-1} which is assigned to the six-coordinate a_3 heme in this study.

The formyl CH=O stretching frequency is appreciably different between the a^{3+} (1646 cm^{-1}) and $a_3{}^{3+}$ hemes (1674–1678 cm^{-1}). The frequency of this band in model compounds differs between an aqueous solution and an organic solution and accordingly is considered to be sensitive to hydrogen bonding with surroundings (Babcock & Salmeen, 1979;

Choi et al., 1983). The present study on $a^{2+}a_3{}^{2+}$CO revealed another feature about the CH=O stretching frequency of the a_3 heme. The ν_4 line of $a_3{}^{2+}$CO was observed at 1372 cm^{-1} close to 1371 cm^{-1} of $a^{3+}a_3{}^{3+}$ but distinctly higher than 1358 cm^{-1} of $a^{2+}a_3{}^{2+}$ (Figure 6). The CH=O stretching mode of $a_3{}^{2+}$CO was located at 1671 cm^{-1} which was again close to 1674 cm^{-1} of the $a_3{}^{3+}$ heme of the resting enzyme but clearly higher than 1663 cm^{-1} of the $a_3{}^{2+}$ heme of the fully reduced enzyme. On the other hand, this mode and ν_4 of $a^{2+}a_3{}^{2+}CN^-$ were observed at 1664 and 1358 cm^{-1}, respectively, similar to those of $a^{2+}a_3{}^{2+}$. The difference of the ν_4 frequencies between $a_3{}^{2+}CN^-$ and $a_3{}^{2+}$CO indicates that the former and the latter are the σ- and π-type ferrous low-spin complexes (Kitagawa et al., 1976), respectively. Since the latter complex assumes appreciable delocalization of iron d_π electrons to axial ligand and thus less delocalization of the d_π electrons to the porphyrin π^* orbital (Spiro & Strekas, 1974; Kitagawa et al., 1975), the CH=O stretching frequency of the a_3 heme is considered to reflect the delocalization of π electrons similar to the ν_4 frequency.

Photoreduction. Photoreduction of cytochrome *c* oxidase was first suspected to be caused by unknown impurity, but it took place even for very carefully purified enzymes (Adar & Yonetani, 1978). The photoreduction was also observed for the enzymes prepared independently from bovine heart mitochondria (Salmeen et al., 1978; Kitagawa & Orii, 1978) and from bacterium and yeast (Ogura et al., 1983, 1984) as well as for the membrane-bound enzymes in mitochondrial preparations (Adar & Erecińska, 1979). Bocian et al. (1979) and Babcock et al. (1981) attributed the photoreduction to a flavin impurity since it did not occur upon excitation at 600 nm. However, the difference absorption spectra shown in Figure 1 provided evidence for the occurrence of photoreduction with red light, although the rate was slow at longer wavelengths.

On the other hand, Woodruff et al. (1981) misunderstood the fact that the RR spectrum of the resting enzyme excited at 406.7 nm exhibited the characteristics of the ferric heme proteins, interpreting it as if the photoreduction did not take place. However, the results from absorption difference (Figure 1) and oxygen consumption experiments (Figure 2) demonstrated that the photoreduction definitely occurred upon irradiation at 406.7 nm, although the RR spectra observed with the 406.7-nm excitation under aerobic conditions were close to those of the ferric heme proteins as pointed out by Woodruff et al. (1981). Since the photoreduced enzymes are quickly reoxidized by oxygen present, the ordinary absorption spectra are poor criteria for examining the occurrence of photoreduction unless it is placed under strict anaerobic conditions. Furthermore, only a small volume of enzyme is illuminated by laser light, and hence, an overall change of absorption spectra is too small to be recognized. Difference absorption adopted in this study can sensitively monitor such a small change.

When the photoreduction takes place under aerobic conditions, the portion in the laser beam may contain several intermediates including $a^{2+}a_3{}^{2+}$, $a^{2+}a_3{}^{2+}O_2$, $a^{2+}a_3{}^{3+}$, and $a^{3+}a_3{}^{3+}$ (oxidation state of copper atoms is neglected here). The RR spectra of $a^{3+}a_3{}^{3+}$ and $a^{2+}a_3{}^{2+}$ are established by spectra A and D in Figures 3 and 4. Since the lifetime of $a^{2+}a_3{}^{2+}O_2$ is very short at room temperature (Greenwood & Gibson, 1967; Ludwig & Gibson, 1981; Hill & Greenwood, 1984; Orii, 1984) although it is relatively longer at −100 °C (Chance et al., 1978), its population in the photo steady state at room temperature is considered to be too small for its RR lines to be detected. Since it is generally accepted that the electron transfer from the a^{2+} heme to the $a_3{}^{3+}$ heme is the rate-limiting step of the enzymic reaction, the presence of $a^{3+}a_3{}^{2+}$ is unlikely. These considerations lead us to infer that $a^{2+}a_3{}^{3+}$ is the main species which gives spectra B in Figures 3 and 4. In fact, the similarity between spectra B and those of $a^{2+}a_3{}^{3+}CN^-$ (Figures 5 and 6) is noteworthy.

In the previous experiments on the photoreduction of mitochondria, Adar & Erecińska (1979) pointed out the appearance of the 214- and 1665-cm^{-1} lines upon excitation at 441.6 nm, although these lines were absent in the first three spectra. Under their experimental conditions, mitochondria were placed first under aerobic conditions, and accordingly the first few spectra reflected the mixed valence state of cytochrome *c* oxidase, that is, $a^{2+}a_3{}^{3+}$, as in spectrum B in Figure 3. After a while, however, oxygen in the capillary cell was presumably consumed by the photoreduced cytochrome *c* oxidase, and finally the surroundings around mitochondria became anaerobic. Then cytochrome *c* oxidase was photoreduced to the fully reduced state, which gave rise to the marker lines of the $a_3{}^{2+}$ heme at 214 and 1665 cm^{-1}, in agreement with spectrum C in Figure 3.

Rate of Electron Transfer. Spinning of the cell even with the rate of 1 rpm protected the resting enzyme from photoreduction upon excitation at 441.6 nm, but no spinning of the cell gave the RR spectrum of $a^{2+}a_3{}^{3+}$. Apparently this seems curious, but detailed analysis described here will demonstrate that this observation is quite consistent with the available kinetic data. Since the difference between spectra A and B in Figure 3 arises from cytochrome *a* but not from cytochrome a_3, we treat redox of cytochrome *a* here.

Provided that the rate constant of photoreduction of cytochrome *a* is k_1 (s^{-1}) and the rate constant of the electron transfer from cytochrome *a* to cytochrome a_3 is k_2 (s^{-1}), then the concentrations of the a^{2+} and a^{3+} hemes at t (s) after the initiation of laser irradiation are given by

$$[a^{2+}] = [c_0k_1/(k_1 + k_2)][1 - \exp(-k_1 - k_2)t] \quad (1)$$

$$[a^{3+}] = [c_0/(k_1 + k_2)][k_2 + k_1 \exp(-k_1 - k_2)t] \quad (2)$$

Here, c_0 is the concentration of the enzyme, and we assume the oxidation of cytochrome a_3 is very fast compared with its reduction. The period (t_0) of laser irradiation per one turn was altered between 0.1 s and 0.07 ms in accord with the spinning rates of 1 and 1500 rpm. Since the photoreduced enzyme is diluted into the bulk solution in one turn of the cell and reoxidized soon, the enzyme always adopts the oxidized form at the beginning of laser irradiation. The Raman lines of the a^{2+} and a^{3+} hemes are accumulated during the period of laser irradiation, and the Raman intensities of the a^{2+} and a^{3+} hemes, $I(a^{2+})$ and $I(a^{3+})$, respectively, are given by

$$I(a^{2+}) = A^{2+}\int_0^{t_0}[a^{2+}]\,dt \quad (3)$$

$$I(a^{3+}) = A^{3+}\int_0^{t_0}[a^{3+}]\,dt \quad (4)$$

where A^{2+} and A^{3+} are the molar intensities of a given Raman line of the a^{2+} and a^{3+} hemes upon excitation at 441.6 nm, respectively. Then the intensity ratio of the two forms is given by

$$y(t_0) = \frac{I(a^{3+})}{I(a^{2+})} = \frac{A^{3+}}{A^{2+}}\,\frac{(k_1 + k_2)k_2t_0 + k_1 - k_1\exp(-k_1 - k_2)t_0}{k_1[(k_1 + k_2)t_0 - 1 + \exp(-k_1 - k_2)t_0]} \quad (5)$$

When the spinning of the cell is stopped, the intensity ratio

is given by putting $t_0 \rightarrow \infty$ into eq 5, and it is

$$y(\infty) = (A^{3+}/A^{2+})(k_2/k_1) \quad (6)$$

This value should be smaller than 0.1, because Raman lines of the a^{2+} heme were dominant in spectrum B of Figure 3. Indeed judging from the differentiated resonance effects on the a^{3+} and a^{2+} hemes having the absorption maxima at 426 and 444 nm, respectively (Vanneste, 1966), we may expect that A^{3+}/A^{2+} is on the order of 10^{-1}–10^{-2}.

The half-time ($t_{1/2}$) for the electron transfer from the a^{2+} heme to the $a_3{}^{3+}$ heme is reported to be as long as 1.2 s (Gibson et al., 1965). This means $k_2 = (\ln 2)/t_{1/2} = 0.58\ s^{-1}$. If we assume $k_1 = k_2$, the intensity ratio becomes as follows: y(0.07 ms) = 49 000 (A^{3+}/A^{2+}) at 0.07 ms and y(0.1 s) = 35-(A^{3+}/A^{2+}) at 0.1 s. In this case the observed spectra would be effectively of the oxidized form even with 1 rpm. In the enzymic reaction, the electron transfer from cytochrome *c* to cytochrome *a* is much faster ($t_{1/2}$ = 5 ms), but if we assume k_1 is 10 times larger than k_2 with keeping $k_2 = 0.58\ s^{-1}$, then y(0.07 ms) = 4900(A^{3+}/A^{2+}) and y(0.1 s) = 3.2(A^{3+}/A^{2+}). Thus, the intensity ratio at 0.1 s (1 rpm) might not be interpreted satisfactorily. Therefore, $k_1 = k_2$ is the highest limit acceptable for the present data. If (A^{3+}/A^{2+}) were close to 10^{-2}, we would have to assume a still lower value for k_1. However, it is noted that the apparent value of k_1 contains a factor proportional to the number of photons. If we use the larger value for k_2 (k_2 = 750 s^{-1}) (Gibson & Greenwood, 1963), the Raman intensity ratio at 0.1 s can never be explained with any values of k_1. The slow rate of the electron transfer might be specific to the resting enzyme.

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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.

Adar, F., & Erecińska, M. (1979) *Biochemistry 18*, 1825–1829.

Argade, P. V., Ching, Y.-C., & Rousseau, D. L. (1984) *Science (Washington, D.C.) 225*, 329–331.

Babcock, G. T., & Salmeen, I. (1979) *Biochemistry 18*, 2493–2498.

Babcock, G. T., Vickery, L. E., & Palmer, G. (1976) *J. Biol. Chem. 257*, 7907–7919.

Babcock, G. T., Callahan, P. M., Ondrias, M. R., & Salmeen, I. (1981) *Biochemistry 20*, 959–966.

Blair, D. F., Bocian, D. F., Babcock, G. T., & Chan, S. I. (1982) *Biochemistry 21*, 6928–6935.

Bocian, D. F., Lemley, A. T., Peterson, N. O., Brudvig, G. W., & Chan, S. I. (1979) *Biochemistry 18*, 4396–4402.

Boelens, R., Rademaker, H., Wever, R., & Van Gelder, B. F. (1984) *Biochim. Biophys. Acta 765*, 196–209.

Callahan, P. M., & Babcock, G. T. (1983) *Biochemistry 22*, 452–462.

Carter, K. R., Antalis, T. M., Palmer, G., Ferris, N. S., & Woodruff, W. H. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1652–1655.

Chance, B., Saronio, C., Leigh, J. S., Jr., Ingledew, W. J., & King, T. E. (1978) *Biochem. J. 171*, 787–798.

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.

Ferguson-Miller, S., Brutigan, D. L., & Margoliash, E. (1976) *J. Biol. Chem. 251*, 1104–1115.

Fiamingo, F. G., Altschuld , R. A., Moh, P. P., & Alben, J. O. (1982) *J. Biol. Chem. 257*, 1639–1650.

Gibson, Q. H., & Greenwood, C. (1963) *Biochem. J. 86*, 541–563.

Gibson, Q. H., Greenwood, C., Wharton, D. C., & Palmer, G. (1965) *J. Biol. Chem. 240*, 888–894.

Greenwood, C., & Gibson, Q. H. (1967) *J. Biol. Chem. 242*, 1782–1787.

Hill, B. C., & Greenwood, C. (1984) *Biochem. J. 218*, 913–921.

Kent, T. A., Munck, E., Dunham, W. R., Wilter, W. F., Finding, K. L., Yoshida, T., & Fee, J. A. (1982) *J. Biol. Chem. 257*, 12489–12492.

Kent, T. A., Young, L. J., Palmer, G., Fee, J. A., & Munck, E. (1983) *J. Biol. Chem. 258*, 8543–8546.

Kitagawa, T., & Orii, Y. (1978) *J. Biochem. (Tokyo) 84*, 1245–1252.

Kitagawa, T., Iizuka, T., Ikeda-Saito, M., & Kyogoku, Y. (1975) *Chem. Lett.*, 849–852.

Kitagawa, T., Kyogoku, Y., Iizuka, T., & Ikeda-Saito, M. (1976) *J. Am. Chem. Soc. 98*, 5169–5173.

Kitagawa, T., Kyogoku, Y., & Orii, Y. (1977) *Arch. Biochem. Biophys. 181*, 228–235.

Ludwig, B., & Gibson, Q. H. (1981) *J. Biol. Chem. 256*, 10092–10098.

Malmström, B. G. (1979) *Biochim. Biophys. Acta 549*, 281–303.

Naqui, A., Kumar, C., Ching, Y.-C., Powers L., & Chance, B. (1984) *Biochemistry 23*, 6222–6227.

Ogura, T., Hon-nami, K., Ohshima, T., Yoshikawa, S., & Kitagawa, T. (1983) *J. Am. Chem. Soc. 105*, 7781–7783.

Ogura, T., Sone, N., Tagawa, K., & Kitagawa, T. (1984) *Biochemistry 23*, 2826–2831.

Ohnishi, T., LoBrutto, R., Salerno, J. C., Bruckner, R. C., & Frey, T. G. (1982) *J. Biol. Chem. 257*, 14821–14825.

Orii, Y. (1984) *J. Biol. Chem. 259*, 7187–7190.

Powers, L., Chance, B., Ching, Y., & Angiolillo, P. (1981) *Biophys. J. 34*, 465–498.

Salmeen, I., Rimai, L., Gill, D., Yamamoto, T., Palmer, G., Hartzell, C. R., & Beinert, H. (1973) *Biochem. Biophys. Res. Commun. 52*, 1100–1107.

Salmeen, I., Rimai, L., & Babcock, G. T. (1978) *Biochemistry 17*, 800–806.

Schroedl, N. A., & Hartzell, C. R. (1977) *Biochemistry 16*, 4966–4971.

Scott, R. A., Cramer, S. P., Shaw, R. W., Beinert, H., & Gray, H. B. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 664–667.

Spiro, T. G., & Strekas, T. C. (1974) *J. Am. Chem. Soc. 96*, 338–345.

Vanneste, W. H. (1966) *Biochemistry 5*, 838–847.

Van Steelandt-Frentrup, J., Salmeen, I., & Babcock, G. T. (1981) *J. Am. Chem. Soc. 103*, 5981–5982.

Wikström, M. (1984) *Nature (London) 308*, 558–560.

Woodruff, W. H., Dallinger, R. F., Antalis, T. M., & Palmer, G. (1981) *Biochemistry 20*, 1332–1338.

Yonetani, T. (1960) *J. Biol. Chem. 235*, 845–852.

Yoshikawa, S., & Caughey, W. S. (1982) *J. Biol. Chem. 257*, 412–420.

Yoshikawa, S., Choc, M. G., O'Toole, M. C., & Caughey, W. S. (1977) *J. Biol. Chem. 252*, 5498–5508.