Room temperature characterization of the dioxygen intermediates of cytochromec oxidase by resonance Raman spectroscopy.Biochemistry 29: 10135-10140

ARTICLE *in* BIOCHEMISTRY · NOVEMBER 1990

Impact Factor: 3.02 · DOI: 10.1021/bi00495a018 · Source: PubMed

Wong, P. Y.-K., Westlund, P., Hamberg, M., Granström, E., Chao, P. H.-W., & Samuelsson, B. (1984) *J. Biol. Chem. 259*, 2683-2686.
Woollard, P. M. (1986) *Biochem. Biophys. Res. Commun. 136*, 169-176.
Yamamoto, S., Ueda, N., Yokoyama, S., Kaneko, F., Shinjo, F., Yoshimoto, T., Oates, J. A., Brash, A. R., Fitzsimmons, B. J., & Rokach, J. (1987) *Adv. Prostaglandin, Thromboxane, Leukotriene Res. 17*, 55-59.
Yokoyama, C., Shinjo, F., Yoshimoto, T., Yamamoto, S., Oates, J. A., & Brash, A. R. (1986) *J. Biol. Chem. 261*, 16714-16721.
Yoshimoto, T., Miyamoto, Y., Ochi, K., & Yamamoto, S. (1982) *Biochim. Biophys. Acta 713*, 638-646.

Room Temperature Characterization of the Dioxygen Intermediates of Cytochrome *c* Oxidase by Resonance Raman Spectroscopy†

Randy W. Larsen,‡ Wei Li,‡ Robert A. Copeland,‡,§ Stephan N. Witt,‡,⊥ Bih-Show Lou,‖ Sunney I. Chan,‡ and Mark R. Ondrias*,‖

Department of Chemistry, University of New Mexico, Albuquerque, New Mexico 87131, and Arthur Amos Noyes Laboratory of Chemical Physics, 127-72, California Institute of Technology, Pasadena, California 91125

Received February 23, 1990; Revised Manuscript Received July 30, 1990

ABSTRACT: Resonance Raman spectroscopy was employed to investigate the heme structures of catalytic intermediates of cytochrome *c* oxidase at room temperature. The high-frequency resonance Raman spectra were obtained for compound C (the two-electron-reduced dioxygen intermediate), ferryl (the three-electron-reduced dioxygen intermediate), and the fully oxidized enzyme. Compound C was formed by photolyzing CO mixed-valence enzyme in the presence of O_2. The ferryl intermediate was formed by reoxidation of the fully reduced enzyme by an excess of H_2O_2. Two forms of the oxidized enzyme were prepared by reoxidizing the fully reduced enzyme with O_2. Our data indicate that, in compound C, cyt a_3 is either intermediate or low spin and is nonphotolabile and its oxidation state marker band, ν_4, appears at a higher frequency than that of the resting form of the enzyme. The ferryl intermediate also displays a low-spin cyt a_3, which is nonphotolabile, and an even higher frequency for the oxidation state marker band, ν_4. The reoxidized form of cytochrome *c* oxidase with a Soret absorption maximum at 420 nm has an oxidation state marker band (ν_4) in a position similar to that of the resting form, while the spin-state region resembles that of compound C. This species subsequently decays to a second oxidized form of the enzyme, which displays a high-frequency resonance Raman spectrum identical with that of the original resting enzyme.

Cytochrome *c* oxidase is a multisubunit, membrane-bound protein that catalyzes the four-electron reduction of dioxygen in mitochondria. The oxygen reduction activity of the enzyme is coupled to proton translocation across the inner mitochondrial membrane during respiration. The enzyme utilizes four redox-active metal centers to perform its catalytic function. These centers include two heme A chromophores and two Cu ions. The dioxygen reduction site consists of a binuclear heme A/Cu cluster (designated cytochrome a_3, Cu_B). The two remaining metal centers (designated cyt a and Cu_A) mediate the electron transfer from ferrocytochrome *c* to cytochrome a_3, Cu_B (Wikstrom et al., 1977, 1981; Palmer et al., 1979). Although the dioxygen reduction kinetics have been studied extensively by a variety of spectroscopic techniques, much less is known about the structure of the intermediates formed during the turnover of the enzyme by O_2 [see Hill et al. (1986) and Chan et al. (1988) for reviews]. Because the turnover rate of the enzyme can be as high as 400 electrons transferred per second, spectroscopic studies of the intermediates have been difficult.

Low-temperature transient absorption studies of the fully reduced CO photolyzed enzyme in the presence of O_2 revealed the existence of at least three distinct species during turnover (Chance et al., 1975). Recent room temperature resonance Raman and transient absorption spectra indicate the possibility of four intermediates (Babcock et al., 1985; Hill & Greenwood, 1984). The first intermediate is believed to be an O_2 adduct bound to the ferrous heme a_3 similar to oxyhemoglobin (compound A) (Babcock et al., 1985; Han et al., 1990; Hill & Greenwood, 1984). Electron transfers from Cu_B and heme a_3 to the bound O_2 produce the second intermediate, generally assumed to be a peroxo heme a_3–Cu_B bridged species (compound C) (Hill & Greenwood, 1984). Blair et al. (1985), using EPR spectroscopy in conjunction with the low-temperature triple trapping technique pioneered by Chance et al. (1975), reported evidence for two distinct intermediates at the three-electron level of dioxygen reduction. It has been proposed that the first of these species is a cupric hydroperoxide coordinated to a ferrous heme a_3 in the binuclear cluster, while the second is an Fe(IV) heme a_3 species resulting from heterolytic cleavage of the O–O bond (Blair et al., 1984).

† Research supported in part by Grants GM 22432 (to S.I.C.) and GM 33330 (to M.R.O.) from the National Institute of General Medical Sciences, U.S. Public Health Service, Grant RR 08139 (to M.R.O.) from the Division of Research Resources, National Institutes of Health, and the donors of the Petroleum Research Fund, administered by the American Chemical Society (to S.I.C.). Contribution No. 8103 from Division of Chemistry and Chemical Engineering, California Institute of Technology.

* To whom correspondence should be addressed.
‡ California Institute of Technology.
§ Current address: Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637.
⊥ Current address: Department of Chemistry, Stanford University, Stanford, CA 94305.
‖ University of New Mexico.

0006-2960/90/0429-10135$02.50/0

Reoxidation of the fully reduced enzyme results in an unstable, fully oxidized form (generally referred to as "pulsed" cytochrome oxidase), which is spectroscopically distinct from the resting enzyme (Kumar et al., 1984).

Previous studies of the dioxygen chemistry of cytochrome *c* oxidase have been carried out under conditions that may not produce physiological forms of these species. Structural and kinetic studies that involve photolyzing the fully reduced CO-bound enzyme in the presence of O_2 may be complicated by CO binding to Cu_B at both low temperatures and room temperature. Fourier-transform infrared (FTIR) studies by Fiamingo et al. (1982) have demonstrated that photolysis of the CO-bound cytochrome a_3 complex at low temperatures (10 K) produces a species with CO bound to Cu_B. Recent studies by Dyer et al. (1989) have shown that CO binding to Cu_B subsequent to photolysis from cytochrome a_3 also occurs at room temperature on a microsecond time scale. In addition, low-temperature studies involve the use of solvents (such as glycerol) that may influence the structures of the dioxygen intermediates.

In order to characterize the electronic structures of several of these species under more physiological conditions and at room temperature, we have prepared several "static" derivatives of the enzyme with spectroscopic properties similar to those of the transient intermediates and characterized them by room temperature resonance Raman spectroscopy. When the CO mixed-valence enzyme (two-electron reduced) is mixed with O_2 in the presence of light, a species is formed in which the O_2 bound to cytochrome a_3 is at the two-electron level of reduction (compound C) (Babcock et al., 1984). The addition of excess H_2O_2 to the fully reduced enzyme produces a species with an optical signature and chemical reactivity similar to that of the oxyferryl transient (Witt & Chan, 1987). Resonance Raman spectroscopy has proven to be a powerful tool for probing the structure of the heme sites in heme proteins. This form of spectroscopy has already been used to characterize the equilibrium structures and several transient dioxygen intermediates, particularly at low temperature, of the enzyme [see Babcock (1988) for a general review]. In the present study, we show that the Raman spectrum of the two-electron-reduced O_2 derivative formed from the CO mixed-valence enzyme at room temperature is similar to the low-temperature spectra previously reported for compound C. The spectrum of the reoxidized form of the enzyme is similar to that of compound C in the spin-state-sensitive region. The oxidation state, however, is identical with that of the resting enzyme. Upon incubation of the reoxidized enzyme for >12 h, a species is formed with a distinct optical spectrum and vibrational bands that are identical with those of the resting form. Finally, the vibrational spectrum of the Fe(IV) derivative is very similar to that compound ES of cytochrome *c* peroxidase with an upshifted oxidation state marker and a low-spin heme a_3 center.

Materials and Methods

Bovine heart cytochrome *c* oxidase was prepared by the method of Hartzell and Beinert (1974) with some modifications. The enzyme was dissolved in 50 mM HEPES containing 0.5% Brij 35 at pH 7.4 and stored at 77 K until needed.

Two types of flow cells were employed in this study. In the double-mixing flow cell, the sample (cytochrome *c* oxidase) and O_2-saturated buffer are pumped from two reservoirs (one of which is anaerobic) through 0.5-mm-i.d. tygon tubing by using a Manostat peristaltic pump. The tubes from both reservoirs converge at a small capillary (2 mm × 1.5 mm), which acts as a "mixer". The sample/buffer mixture is then passed into a 5 mm × 0.5 mm capillary, which acts as the optical cell for the Raman measurements. The triple-mixing flow cell consists of three reservoirs. Two of these reservoirs form a double-mixing flow cell, as described above. The reactants from the third reservoir combine with the sample/buffer mixture just after the mixer capillary. An additional mixer allows for input from a third reservoir. The mixture is then flowed into a 5 mm × 0.5 mm capillary from which the Raman measurements are taken. The maximum flow rate for both flow cells is 2 mL/h.

Various species of oxidase were prepared as follows: (1) The 420-nm reoxidized enzyme. Enzyme, 5 mL, 150 μM (per aa_3), was degassed by five cycles of vacuum/N_2 in an anaerobic optical reservoir connected to a double-mixing flow cell. Solid sodium dithionite (Aldrich) was added under positive N_2 pressure until no further changes were observed in the 605-nm region of the optical spectrum. The fully reduced enzyme was then flowed together with O_2-saturated buffer. This protocol results in a slight excess of sodium dithionite, which produces small concentrations of H_2O_2 in the presence of O_2. The 420-nm complex may therefore contain small subpopulations of compound C. The Raman spectrum was collected several millimeters downstream from the initial mixing point (~1 min after mixing). (2) Compound C. This intermediate was formed by mixing the CO mixed-valence enzyme with O_2-saturated buffer and photolyzing the mixture with a laser pulse from a Nd:YAG pumped dye laser (QuantaRay DCR-II/DLII). The Raman spectrum was collected several millimeters downstream from the initiation point (~1 min subsequent to CO photolysis). The CO mixed-valence derivative was prepared by incubating 5 mL of degassed resting enzyme with CO (60 mmHg) for ≈48 h at 10 °C. Complete formation of the derivative was ensured by monitoring the 588-nm band in the CO mixed-valence minus resting difference spectrum. (3) The oxyferryl species. This intermediate was prepared by mixing 150 μM (per aa_3) fully reduced enzyme with 16 mM H_2O_2 in a triple-mixing flow cell (final H_2O_2 concentration was 8 mM after mixing). Approximately 60 s after mixing, catalase in HEPES/Brij 35 was added to remove excess H_2O_2. The Raman spectrum was obtained several millimeters downstream from the final mixing point (~1 min after mixing). (4) The 415-nm reoxidized species. The 415-nm reoxidized form of the enzyme was prepared by reoxidizing ~500 μL of reduced enzyme with O_2 and incubating the sample for several hours at 4 °C. The Raman spectrum was obtained by using a continuous-loop flow cell.

The absolute absorption spectra were obtained by using the following protocols: (1) The 420-nm reoxidized enzyme. Enzyme, 200 μL, 100 μM (per aa_3), was degassed with five cycles of vacuum/N_2 in an anaerobic optical cell and reduced with solid sodium dithionite. The samples were reoxidized with O_2 for ~1 min. (2) Compound C. This species was formed by mixing 200 μL of 75 μM (per aa_3) CO mixed-valence enzyme with ~10 μL of O_2-saturated buffer. The sample was then exposed to 532-nm radiation from a Nd:YAG pumped dye laser for ~1 min. (3) Oxyferryl form. The oxyferryl species was prepared by reoxidizing ~200 μL of 75 μM (per aa_3) dithionite-reduced enzyme with excess hydrogen peroxide. Final H_2O_2 concentration was 8 mM. Trace amounts of catalase were added ~1 min after addition of H_2O_2. (4) The 415-nm reoxidized enzyme. The reoxidized enzyme was obtained by incubating the 420-nm form of the reoxidized enzyme sample for 12 h at 4 °C. All absorption spectra were obtained by using a 2-mm path length quartz optical cell on

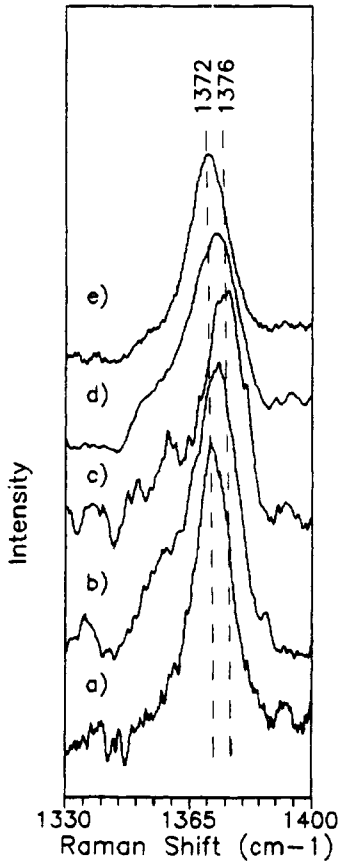

FIGURE 1: Resonance Raman spectra in the ν_4 region: (a) resting oxidase, (b) compound C, (c) oxyferryl, (d) 420-nm reoxidized, and (e) 415-nm reoxidized oxidase (see text for details of sample preparation). Excitation frequencies used were 428 nm (0.2 mJ/pulse) for the oxyferryl and compound C intermediates and 420 nm (0.1 mJ/pulse) for the resting, 420-nm reoxidized, and 415-nm reoxidized forms of the enzyme. The spectra are the average of three unsmoothed scans recorded at 1 cm^{-1}/s, with a spectral band-pass of ~ 10 cm^{-1}.

a Hewlett-Packard HP8452 diode array UV/vis spectrometer.

The Raman spectrometer consists of an N_2 pumped dye laser (Molectron UV24/DL-14), a SPEX 1402 double monochrometer, and a SPEX DM3000R controller [see Findsen (1986) for more details]. The Raman spectra were obtained by using a backscattering geometry. This protocol proved to be sufficient to obtain high-frequency spectra. However, attempts to obtain low-frequency spectra (200–1000 cm^{-1}) were unsuccessful due to low enzyme concentration. Low-frequency studies with higher enzyme concentrations are currently under way. Both compound C and the oxyferryl species were excited at 428 nm, while the reoxidized forms of the enzyme were excited at 420 nm.

Results

Figure 1 displays the high-frequency resonance Raman spectrum in the ν_4 region (oxidation-state marker band) for resting (a), compound C (b), ferryl (c), 420-nm reoxidized (d), and 415-nm reoxidized (e) forms of the enzyme. The resting, 420-nm reoxidized, and 415-nm reoxidized forms of the enzyme exhibit oxidation-state marker bands similar to those found in ferric heme A model compounds ($\nu_4 \approx 1372$ cm^{-1}) (Babcock, 1988). In compound C, ν_4 is up-shifted slightly from that of either the 420-nm or the resting forms of the enzyme ($\approx$1373–1374 cm^{-1}). The largest change in ν_4 is observed (1376 cm^{-1}) in the oxyferryl species.

Figure 2 shows the resonance Raman spectrum in the 1500–1700-cm^{-1} region for resting, compound C, oxyferryl, 420-nm reoxidized, and 415-nm reoxidized forms of cytochrome *c* oxidase. The 415-nm reoxidized form of the enzyme exhibits spectral characteristics similar to those of the resting enzyme. Previous studies have characterized vibrational modes at $\sim$1572 cm^{-1} (ν_2 for high-spin heme a_3), 1588 cm^{-1} (ν_2 for the low-spin heme a), 1650 cm^{-1} ($\nu_{C=O}$ for heme *a*), and 1676 cm^{-1} ($\nu_{C=O}$ for heme a_3) for the resting enzyme [see Babcock (1988)]. The region between 1560 and 1600 cm^{-1} potentially contains contributions from other depolarized modes such as ν_{35} and ν_{37}. However, the depolarization ratio (data not shown) for scattering in this region indicates that the polarized mode, ν_2, significantly dominates the intensity of the prominent band at $\sim$1580 cm^{-1} in the resting enzyme. The high-frequency spectra of the compound C, oxyferryl, and 420-nm reoxidized species are quite distinct from those of the resting and 415-nm reoxidized forms. In particular, in the spin-state sensitive region, there is a considerable decrease in the relative intensity of the high-spin marker band at 1572 cm^{-1} for all the species. The data displayed in Figures 1 and 2 show no significant evidence for either heme photoreduction or photodissociation within the 10-ns pulses. These spectra were obtained under "high-power" conditions ($\sim 1 \times 10^9$ W/cm^2) that have previously been shown to completely dissociate carbon monoxide bound to either the fully or partially reduced enzyme (Findsen et al., 1987).

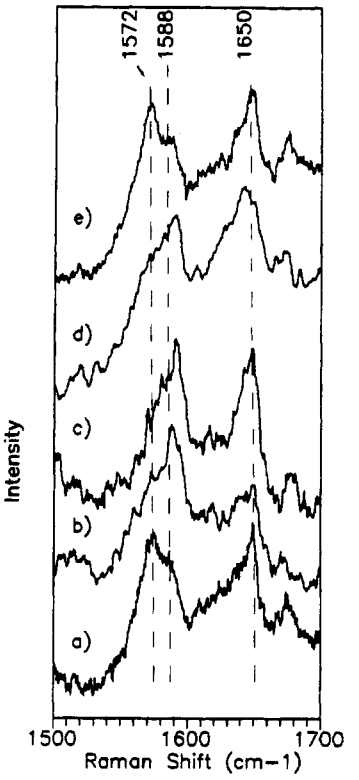

FIGURE 2: Resonance Raman spectra of cytochrome *c* oxidase in the 1500–1700-cm^{-1} region: (a) resting oxidase, (b) compound C, (c) the oxyferryl intermediate, (d) 420-nm reoxidized, and (e) 415 nm reoxidized forms of the enzyme. Spectral conditions are the same as in Figure 1.

Figure 3 depicts the time course of the decays at room temperature for compound C and the oxyferryl species. The decay of compound C was measured by plotting the intensity decrease in the 607-nm band of the compound C minus reoxidized absorbance difference spectrum. The decay of the oxyferryl intermediate was monitored by the decrease in intensity of the 580-nm band of the ferryl minus reoxidized absorbance difference spectrum. Figure 4 shows the compound C minus reoxidized and oxyferryl minus reoxidized absorption difference spectra in the visible region of the spectrum. The

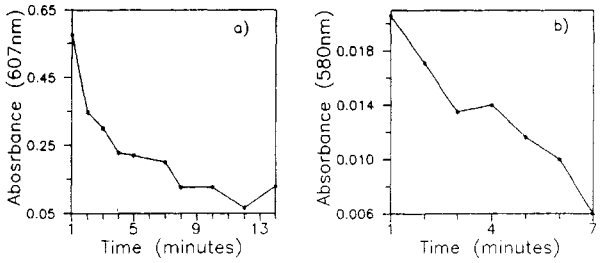

FIGURE 3: (a) Room temperature decay kinetics of compound C formed by photolysis of the CO mixed-valence enzyme in the presence of O_2. Enzyme concentration, ~75 μM (per a_3). (b) Room temperature decay kinetics of the oxyferryl intermediate formed by reoxidation of fully reduced enzyme by excess H_2O_2. Enzyme concentration, ~75 μM (per a_3); path length, 2 mm.

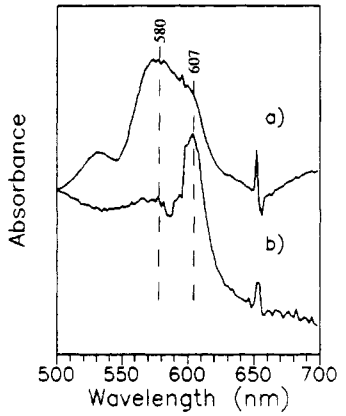

FIGURE 4: Absorbance difference spectra: (a) oxyferryl minus 420-nm reoxidized, ΔA at 580 nm is 0.018, and (b) compound C minus 420 nm reoxidized, ΔA at 607 nm is 0.07. Conditions are the same as in Figure 3. The feature at ~650 nm is an instrumental artifact.

compound C difference spectrum displays a band at 607 nm, while the oxyferryl difference spectrum has a band at 580 nm, consistent with previous data (Witt, 1988).

The absolute absorption spectra of the resting, compound C, oxyferryl, 420-nm reoxidized, and 415-nm reoxidized forms of cytochrome *c* oxidase are shown in Figure 5. The positions of the bands in the Soret and visible regions of the spectra are also consistent with previous studies by Witt (1988) and Chan et al. (1988).

Discussion

Compound C. Transient absorption studies by Hill and Greenwood indicate that, upon dioxygen binding to the fully reduced cytochrome *c* oxidase, the first event may be a concerted two-electron transfer from both heme a_3 and Cu_B to O_2, forming a peroxo-like bridged intermediate. This mechanism circumvents the thermodynamically unfavorable superoxide radical. It should be pointed out, however, that binding of the dioxygen to ferrous heme iron alters the energetics of the situation dramatically. In any case, it is well-known that a peroxo intermediate of the enzyme may also be formed from the CO-inhibited mixed-valence complex (Chance et al., 1975). Since this species contains two reducing equivalents at the O_2 binding site (cyt a_3, Cu_B), a two-electron reduced O_2 intermediate may be formed by photolyzing CO in the presence of O_2. The data presented in Figure 1b indicate that the peroxo intermediate formed in this manner is relatively long-lived at room temperature. The resonance Raman spectrum of compound C formed at room temperature is similar to that of the transient species (Carter et al., 1981) recorded at low temperatures. Because ν_4 is sensitive to both oxidation state of Fe and to π^* density on the porphyrin ring,

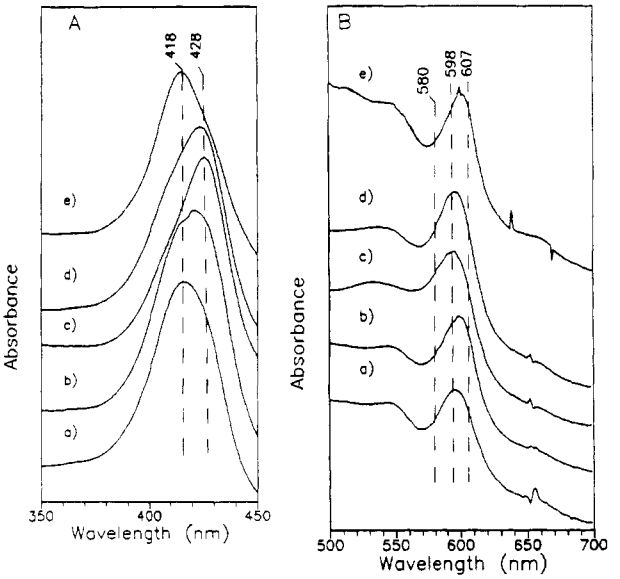

FIGURE 5: Absolute absorption spectra for (a) resting, (b) compound C, (c) oxyferryl, (d) 420-nm reoxidized, and (e) 415-nm reoxidized forms of cytochrome *c* oxidase. Enzyme concentration was 75 μM for (a)–(c) and 100 μM for (d) and (e); path length, 2 mm. Spectra were normalized for presentation purposes. Panel A: Soret band region. Panel B: visible band region.

the increase in frequency of this mode relative to that of the resting enzyme indicates a decrease in the electron density at the Fe in heme a_3. This effect may be accentuated by the electron-withdrawing effects of the bound peroxide.

The spectral region from 1500 to 1700 cm^{-1} in the resting form of cytochrome *c* oxidase is complex and contains overlapping spectral contributions from both heme *a* and heme a_3. Previous studies of heme *a* model compounds (Choi et al., 1983; Callahan & Babcock, 1981) and cytochrome *c* oxidase (Callahan & Babcock, 1981; Woodruff et al., 1981) have shown that the band centered at ~1585 cm^{-1} contains predominant contributions from the spin-state-sensitive band, ν_2, of both low-spin heme *a* (ν_2 ~1589 cm^{-1}) and high-spin heme a_3 (ν_2 ~1572 cm^{-1}). In addition, the band at 1650 cm^{-1} is attributed to an in-plane C=O vibration from the formyl group of cytochrome *a*. The formyl vibration from heme a_3 is weak in the oxidized forms of the enzyme and appears at 1676 cm^{-1}.

Upon formation of compound C, the intensity at 1572 cm^{-1} (ν_2, high-spin heme a_3) decreases with an apparent increase in relative intensity at 1588 cm^{-1} (ν_2, low spin). This is consistent with a change in spin state at the ligand-binding heme a_3 center. Similar spectral changes accompany CN^- binding to the resting form of the enzyme in which heme a_3 is converted to a low-spin complex (Ching et al., 1985).

Although the high-frequency spectrum of compound C clearly indicates changes associated with the heme a_3 site, the exact spin state of heme a_3 remains uncertain. Carter et al. (1981) suggested that compound C may be an intermediate-spin ($S = 3/2$) complex superexchange coupled to cupric Cu_B, on the basis of magnetic circular dichroism data. In such a complex, the Fe d_{z^2} orbital would be occupied, while the Fe $d_{x^2-y^2}$ would be empty. The porphyrin core size (Ct–N distance) in the intermediate-spin complex would be similar to that of the low-spin complex (i.e., contracted core) due to the unoccupied Fe $d_{x^2-y^2}$ orbital. Since ν_2 is sensitive to the core size of the porphyrin macrocycle, both low-spin ($S = 1/2$) Fe(+3) and intermediate-spin ($S = 3/2$) Fe(+3) complexes would have similar frequencies for ν_2. Thus, the data presented are consistent with either an intermediate or low-spin ferric

heme a_3 complex in compound C.

Oxyferryl Species. Previous optical and EPR experiments revealed that reoxidation of the fully reduced enzyme with excess H_2O_2 produced a low-spin Fe(IV) at the heme a_3 ligand binding site with a characteristic absorption band at 580 nm in the H_2O_2 reoxidized minus the 415-nm reoxidized difference spectrum (Witt & Chan, 1987). The room temperature resonance Raman spectrum of this H_2O_2 reoxidized enzyme confirms the presence of a low-spin heme a_3. Little or no intensity is observed in the ν_2 region for high-spin heme a_3 (1572 cm^{-1}). The room temperature Fe(IV) species also has an oxidation-state marker band (ν_4) significantly upshifted from that of the resting or 420-nm reoxidized forms of the enzyme (from 1372 to 1376 cm^{-1}). Low-temperature studies of this intermediate also show an increase in the frequency of ν_4 (from 1372 to 1377 cm^{-1}) (Witt, 1988). Similar shifts in ν_4 have been observed in other Fe(IV) species. When metmyoglobin is treated with H_2O_2, an Fe(IV) species is produced with ν_4 at ~1380 cm^{-1}. In contrast, HRP compound II has ν_4 ~1378 cm^{-1} and cytochrome *c* peroxidase compound II displays ν_4 at ~1375–1377 cm^{-1} (Campbell et al., 1980; Oertling & Babcock, 1988; Hashimoto et al., 1986). The shift in ν_4 from 1372 to 1376 cm^{-1} in the H_2O_2-reoxidized form of cytochrome *c* oxidase is consistent with significantly reduced electron density on the Fe of heme a_3, resulting from the oxidation of ferric Fe. This reduced electron density at the heme Fe diminishes the interaction between the d–Π orbitals of the Fe_{a_3} and the porphyrin Π* molecular orbitals. The somewhat lower frequency observed for ν_4 in the Fe(IV) form of cytochrome *c* oxidase relative to HRP compound II and Fe(IV) myoglobin may be attributed to possible distal interactions with the Fe^{IV}=O center, such as H bonding to distal residues or possibly the ligands of Cu_B (His or Cys). Such effects could decrease the electron density on the Fe and further weaken the d–Π/porphyrin Π* orbital interactions. Since Cu_B and Fe_{a_3} are known to be close (3–5 Å), distal perturbations of the Fe^{IV}=O by the ligands of Cu_B are not unreasonable (Wikstrom et al., 1981), although further experimentation is required to verify this point.

The 420- and 415-nm Reoxidized Forms of Oxidase. Previous studies have shown that when fully reduced cytochrome *c* oxidase is oxidized by O_2, a spectroscopically distinct product is formed that displays a slight red shift in the α-band region of the visible spectrum and a Soret band located at 420 nm (Kumar et al., 1984). The room temperature resonance Raman spectrum of the 420-nm reoxidized form of cytochrome *c* oxidase appears similar to that of both compound C and the oxyferryl form in the high-frequency region, indicating that heme a_3 is either low or intermediate spin in this species. Carter et al. (1981) have characterized the "pulsed" enzyme at low temperature in the presence of glycerol by resonance Raman spectroscopy. Their data indicate that the "pulsed" enzyme is a ferric, intermediate-spin cytochrome a_3 species similar to compound C (ν_4 ~1374 cm^{-1}, ν_2 1591 cm^{-1} with a shoulder at 1572 cm^{-1}). These authors attribute the intermediate-spin state of heme a_3 to a weakly coordinated ligand at the sixth axial ligation site. Recent studies by Han et al. (1989) have shown that an Fe_{a_3}–OH complex forms subsequent to reoxidation of the reduced enzyme. In addition, OH^--bound hemoglobin is known to form a low-spin, Fe^{3+}–OH complex (Iizuka & Kotani, 1969). The position of ν_4 in the 420-nm reoxidized form of the enzyme (1372 cm^{-1}), however, indicates that this species does contain an Fe^{3+} heme, which is similar to the resting and 415-nm reoxidized forms of the enzyme. However, the high-frequency resonance Raman spectrum of the 420-nm reoxidized enzyme obtained in this study is consistent with that of a low-spin, ferric heme a_3 complex. The increase in bandwidth of ν_4 in the 420-nm derivative may be attributed to a subpopulation of compound C formed by the reaction of the 420-nm reoxidized enzyme and H_2O_2, since peroxide is generated by the reaction of O_2 with sodium dithionite.

When the 420-nm form of the enzyme was incubated for several hours at 4 °C, a new species formed, with a blue-shifted Soret absorption band (~415 nm) relative to the resting form of the enzyme (418 nm). The resonance Raman spectrum of this species is nearly identical with that of the resting form of the enzyme. In particular, the increased intensity at ~1572 cm^{-1} suggests that this species contains a five- or six-coordinate, high-spin heme a_3 complex similar to that of the resting form of the enzyme. The oxidation-state marker band (ν_4) is in a position (1372 cm^{-1}) identical with that of 420-nm reoxidized and resting forms of the enzyme. Brudvig et al. (1981) have proposed a model on the basis of EPR (electron paramagnetic resonance) data in which three oxidized conformations of the enzyme form sequentially upon reoxidation. The first species is a transient ($t_{1/2}$ 400 s), which displays a $g = 5$ EPR resonance. This species delays into an EPR-silent, "oxygenated" form. Both of these conformations are proposed to contain an $Fe_{a_3}^{3+}$–OH complex, the latter being magnetically coupled to Cu_B. The oxygenated form then delays to the resting conformation with $t_{1/2}$ ~1 h. The resonance Raman data imply that the decay of the 420-nm reoxidized (oxygenated) form to the 415-nm reoxidized species may involve displacement of the OH^- group (possibly by Cl^-) (Z. Y. Li and S. I. Chan, manuscript in preparation), producing a six-coordinate high-spin Fe_{a_3} complex, which is structurally similar to the resting form of the enzyme.

Photodynamics of the Room Temperature Dioxygen Intermediates. The photolability of bound dioxygen in the catalytic intermediates of cytochrome *c* oxidase provides information concerning the heme–oxygen interactions in the enzyme. Previous studies by Babcock et al. (1985) and Varotsis et al. (1989) have shown that the species formed at early times (<10 μs) in the reaction of fully reduced cytochrome *c* oxidase with dioxygen is photolabile. It was suggested that this intermediate (designated compound A) contains a dioxygen reduction site with an O_2 bound heme (heme a_3) similar to that of oxyhemoglobin. This intermediate subsequently decays into a nonphotolabile species at longer times (>40 μs). The resonance Raman data of the room temperature dioxygen intermediates demonstrate that both compound C and the oxyferryl species are not photolabile under conditions where carbon monoxide is fully photolyzed from the fully reduced enzyme. This is evident by the position of ν_4 in these species. The frequency of this band is higher in both the compound C and oxyferryl forms (relative to the resting form of the enzyme), indicating that an electron-withdrawing ligand (presumably oxygen) is bound to the heme a_3 site.

In addition to photolability, the photoreduction characteristics of the room temperature dioxygen intermediates are also of interest. The resting form of cytochrome *c* oxidase can be reduced under intense laser illumination (Adar & Yonetani, 1978; Ogura et al., 1985). Copeland et al. (1985) recently reported that the "pulsed" enzyme possesses enhanced photoreducibility relative to the resting form of the enzyme. The Raman spectrum of the 420-nm form of the enzyme at room temperature displays a weak band at 1355 cm^{-1} (ν_4 for reduced heme *a*). This band may be due to a small amount of pho-

toreduced 420-nm form of the enzyme since multiple laser pulses were incident upon a given sample volume at the slow flow rates used in this experiment (2 mL/h). The oxyferryl species and compound C show no evidence of photoreduction under similar experimental conditions. This suggests that the 420-nm form of the enzyme may be more photoreactive than either compound C or the oxyferryl intermediate, although further experimentation is required to confirm these results.

Conclusion

The room temperature resonance Raman spectra of reoxidized oxidase, compound C, and the oxyferryl intermediate of cytochrome *c* oxidase presented in this study provide an excellent starting point for elucidating the mechanism of dioxygen reduction under physiological conditions. Our data support the assignment of a peroxide-bound cytochrome a_3 in compound C. The oxyferryl intermediate formed by reoxidation of the fully reduced enzyme with H_2O_2 resembles the Fe(IV) intermediate of cytochrome *c* peroxidase compound ES, with a low-spin heme a_3 and an oxidation-state marker band, ν_4, at higher frequency than in either resting/reoxidized enzyme or compound C. Finally, the Raman spectrum of the 420-nm form of the enzyme is consistent with a six-coordinate, low- or intermediate-spin heme a_3 site, which decays to a five- or six-coordinate species similar to the high-spin complex in the resting form of the enzyme.

Registry No. O_2, 7782-44-7; cytochrome *c* oxidase, 9001-16-5.

References

Babcock, G. T. (1988) in *Biological Applications of Raman Spectroscopy* (Spiro, T. G., Ed.) Vol. 3, Wiley and Sons, New York.

Babcock, G. T., & Salmeen, I. (1979) *Biochemistry 18*, 2493–2499.

Babcock, G. T., Jean, J. M., Johnston, L. N., & Woodruff, W. H. (1985) *J. Inorg. Biochem. 23*, 243–251.

Bickar, D., Bonaventura, J., & Bonaventura, C. (1982) *Biochemistry 21*, 2661–2666.

Blair, D. F., Witt, S. N., & Chan, S. I. (1985) *J. Am. Chem. Soc. 107*, 7389–7399.

Brudvig, G. W., Stevens, T. H., Morse, R. H., & Chan, S. I. (1981) *Biochemistry 20*, 3912–3921.

Callahan, P. M., & Babcock, G. T. (1981) *Biochemistry 20*, 952–956.

Campbell, J. R., Clark, R. J. H., Clore, G., & Lane, L. N. (1980) *Inorg. Chem. Acta 46*, 77–84.

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.

Chan, S. I., Witt, S. N., & Blair, D. F. (1988) *Chem. Scr. 28A*, 51–56.

Chance, B., Saronio, C., & Leigh, J. S. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1635–1640.

Ching, Y., Argade, P. V., & Rousseau, D. L. (1985) *Biochemistry 24*, 4936–4946.

Choi, S., Lee, J. J., Wei, Y. H., & Spiro, T. G. (1983) *J. Am. Chem. Soc. 105*, 3692–3707.

Dyer, R. B., Einarsdottir, O., Killough, P. M., Lopez-Garriga, J. J., & Woodruff, W. H. (1989) *J. Am. Chem. Soc. 111*, 7657–7660.

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

Findsen, E. W. (1986) Ph.D. Dissertation, University of New Mexico, Albuquerque, NM.

Findsen, E. F., Centeno, J. Babcock, G. T., & Ondrias, M. R. (1987) *J. Am. Chem. Soc. 109*, 5367–5372.

Han, S., Ching, Y., & Rousseau, D. L. (1989) *Biophys. J. 55*, 559a.

Han, S., Ching, Y., & Rousseau, D. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 2491–2495.

Hartzell, R., & Beinert, H. (1974) *Biochim. Biophys. Acta 368*, 318–338.

Hashimoto, S., Teraoka, J., Inubushi, T., Yonetani, T., & Kitagawa, T. (1986) *J. Biol. Chem. 261*, 11110–11118.

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

Hill, B. C., Greenwood, C., & Nicholls, P. (1986) *Biochim. Biophys. Acta 853*, 91–113.

Iizuka, T., & Kotani, M. (1969) *Biochim. Biophys. Acta 194*, 351.

Iizuka, T., & Yonetani, T. (1970) *Adv. Biophys. 1*, 157–182.

Kumar, C., Naqui, A., & Chance, B. (1984) *J. Biol. Chem. 259*, 2073–2076.

Oertling, W. A., & Babcock, G. T. (1988) *Biochemistry 27*, 3331–3338.

Palmer, G., Babcock, G. T., & Vickery, L. (1979) *Proc. Natl. Acad. Sci. U.S.A. 73*, 2206–2210.

Varotsis, C., Woodruff, W. H., & Babcock, G. T. (1989) *J. Am. Chem. Soc. 111*, 6439–6440.

Wikstrom, M. (1977) *Nature 266*, 217–273.

Wikstrom, M., Krab, K., & Saraste, M. (1981) *Cytochrome Oxidase: A Synthesis*, Academic Press, New York.

Witt, S. N. (1988) Ph.D. Dissertation, California Institute of Technology, Pasadena, CA.

Witt, S. N., & Chan, S. I. (1987) *J. Biol. Chem. 262*, 1446–1448.

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