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Appearance of the $\nu(Fe^{IV}=O)$ Vibration from a Ferryl–Oxo Intermediate in the Cytochrome Oxidase/Dioxygen Reaction†

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ABSTRACT: Time-resolved resonance Raman spectra have been recorded during the reaction of fully reduced ($a^{2+}a_3^{2+}$) cytochrome oxidase with dioxygen at room temperature. In the spectrum recorded at 800 μs subsequent to carbon monoxide photolysis, a mode is observed at 790 cm^{-1} that shifts to 755 cm^{-1} when the experiment is repeated with $^{18}O_2$. The frequency of this vibration and the magnitude of the $^{18}O_2$ isotopic frequency shift lead us to assign the 790-cm^{-1} mode to the Fe^{IV}=O stretching vibration of a ferryl–oxo cytochrome a_3 intermediate that occurs in the reaction of fully reduced cytochrome oxidase with dioxygen. The appearance and vibrational frequency of this mode were not affected when D_2O was used as a solvent. This result suggests that the ferryl–oxo intermediate is not hydrogen bonded. We have also recorded Raman spectra in the high-frequency (1000–1700 cm^{-1}) region during the oxidase/O_2 reaction that show that the oxidation of cytochrome a^{2+} is biphasic. The faster phase is complete within 100 μs and is followed by a plateau region in which no further oxidation of cytochrome *a* occurs. The plateau persists to ~500 μs and is followed by the second phase of oxidation. These results on the kinetics of the redox activity of cytochrome *a* are consistent with the branched pathway discussed by Hill et al. [Hill, B., Greenwood, C., & Nichols, P. (1986) *Biochim. Biophys. Acta 853*, 91–113] for the oxidation of reduced cytochrome oxidase by O_2 at room temperature.

In the mitochondrial electron-transport chain of eucaryotic organisms, cytochrome oxidase functions as the oxygen-activating enzyme. The overall reaction catalyzed by the enzyme is the rapid reduction of dioxygen to water. The free energy released in the electron-transfer reactions that occur during O_2 reduction is conserved as an electrochemical proton gradient across the inner mitochondrial membrane and is used ultimately for adenosine triphosphate synthesis (Wikstrom et al., 1981). Mitochondrial cytochrome *c* oxidase contains two hemes, cytochrome *a* and a_3, and two copper atoms, designated Cu_A and Cu_B. The low-potential sites, cytochrome *a* and Cu_A, function together in the sense that they oxidize cytochrome *c* and subsequently transfer the reducing equivalents to the high-potential binuclear site, which contains cytochrome a_3 and Cu_B, where oxygen binding and reduction to H_2O take place.

Although the cytochrome oxidase/dioxygen reaction has been extensively studied by various spectroscopic techniques at room and low temperatures (Gibson & Greenwood, 1963; Hill & Greenwood, 1983, 1984; Hill et al., 1986; Orii, 1984, 1988; Oliveberg et al., 1990; Chance et al., 1975a,b; Clore et al., 1980; Blair et al., 1985; Chan et al., 1988), the reaction mechanism is not yet fully understood. For example, the precise chemical identity of various intermediates and the pathway(s) by which electrons are transferred to the binuclear site are important mechanistic questions for which further information is required. The reaction is rapid under physiological conditions ($t_{1/2}$ = 1 ms) (Gibson & Greenwood, 1963;, Greenwood & Gibson, 1967). Nonetheless, the room-temperature flow/flash technique developed by Gibson and Greenwood (1963) has provided a way to resolve the reaction kinetically. Hill and Greenwood (1983, 1984), Orii (1984, 1988), and Oliveberg et al. (1990) used this technique to show that partially reduced intermediates were generated at room temperature. Chance et al. (1975a,b), Clore et al. (1980), Blair et al. (1985), and Chan et al. (1988) studied intermediates involved in dioxygen reduction at low temperatures and characterized them by using optical and EPR spectroscopies.

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Wikstrom (1981, 1989) was able to reverse flow through the enzyme to trap what was postulated to be a peroxy intermediate that precedes ferryl–oxo formation in the reaction scheme he proposed.

Wikstrom (1989), Blair et al. (1985), and Chan et al. (1988) have postulated heterolytic cleavage of the O=O bond to form a cytochrome a_3 ferryl–oxo (Fe^{IV}=O) intermediate as electrons from the cytochrome a/Cu_A sites enter the oxygen-bound binuclear site. The formation and decay rates of intermediate species in this process are not well determined at room temperature, although recently Orii (1988) proposed that a ferryl–oxo species occurs at ~100 μs in the oxidase/dioxygen reaction, consistent with the rapid oxidation of a fraction of the a/Cu_A centers that has been inferred from optical measurements (Hill & Greenwood, 1984; Hill et al., 1986). Furthermore, the protonation steps involved, as well as the specific structure of the cytochrome a_3/O_2 adducts, are not yet well defined, although a variety of structures have been postulated (Hill & Greenwood, 1983, 1984; Hill et al., 1986; Chance et al., 1975a,b; Clore et al., 1980; Blair et al., 1983, 1985; Chan et al., 1988; Oliveberg et al., 1990).

Raman spectroscopy is a structure-specific vibrational technique and, relative to optical or EPR spectroscopies, has the potential to provide more detailed information on the intermediates structures that occur during the oxidation of cytochrome oxidase by O_2. Babcock et al. (1984, 1985), using pulsed excitation, and later Ogura et al. (1985, 1989), with continuous-wave lasers, showed that a time-resolved Raman approach was feasible. Although the measurements were confined to the high-frequency region, a photolabile cytochrome a_3^{2+}–O_2 species was postulated as the initial intermediate in O_2 reduction in this work (Babcock et al., 1984). Recently, Varotsis et al. (1989a,b; 1990a), Han et al. (1990a,b), and Ogura et al. (1990) have confirmed the occurrence of this oxy species by monitoring the $\nu(Fe_{a_3}^{2+}–O_2)$ vibration directly in the reaction of both the mixed-valence and fully-reduced enzyme with O_2.

In the experiments reported here, we have continued the two-color, pulsed irradiation Raman approach to intermediates that occur at later times in the fully reduced cytochrome oxidase/dioxygen reaction. The pulsed technique is particularly useful in this application, as the time resolution is determined by the programmable time delay between the short (10 ns) pump and probe laser flashes. This contrasts with a c.w. laser approach in which the time resolution is determined by the residence time of the reacting sample in the beam. The latter approach becomes ambiguous kinetically as this residence time increases. Our results indicate that cytochrome *a* is oxidized in a biphasic manner. Partial oxidation occurs at early times after mixing ($t < 100$ μs), consistent with optical data reported by several workers (Hill & Greenwood, 1984; Hill et al., 1986; Orii, 1988; Brunori & Gibson, 1983; Oliveberg et al., 1990) and with branched schemes for the overall reaction (Hill & Greenwood, 1984; Hill et al., 1986; Clore et al., 1980; Blair et al., 1983, 1985; Chan et al., 1988). Despite this agreement with the absorption experiments, there is no clear indication of a conventional ferryl–oxo species at this three-electron level of reduction. Instead, we detect ν-(Fe^{IV}=O) at later times in the reaction ($t \approx 800$ μs), as the more slowly reacting fraction of cytochrome *a* is oxidized. The frequency of this ferryl–oxo stretching motion, 790 cm^{-1}, is insensitive to H_2O/D_2O exchange, suggesting that it is not hydrogen bonded. Although the late appearance of the ferryl vibration may reflect heterogeneity in the reaction time course, within the context of the branched kinetic schemes that have been developed from the earlier spectroscopic work, the appearance of this ferryl accompanies the arrival of the fourth electron in the oxygen-bound binuclear site.

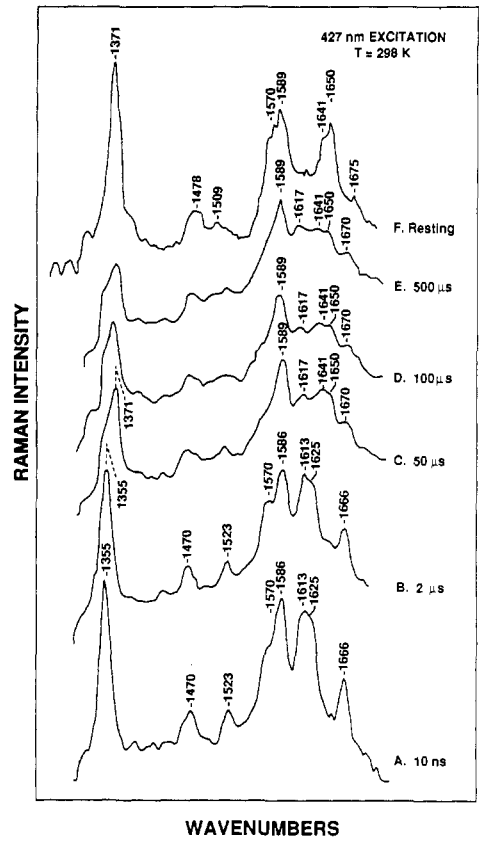

FIGURE 1: Time-resolved resonance Raman spectra of fully reduced cytochrome oxidase at the indicated times. The energy of the 532-nm photolysis pump/pulse was 1.3 mJ, sufficient to photolyze the enzyme–CO complex and initiate the O_2-reduction reaction. The energy of the 427-nm probe beam was 0.8 mJ for spectra A and F and 0.3 mJ for spectra B–E. The repetition rate for both the pump and probe pulses (10-ns duration) was 10 Hz. The accumulation time was 15 min for spectra A and F and 50 min for spectra B–E. The enzyme concentration was 50 μM after mixing, pH 7.4.

Experimental Procedures

Cytochrome oxidase was prepared from beef hearts from a modified Hartzell and Beinert (1974) preparation and was frozen under liquid N_2 until ready for use. The enzyme was solubilized in 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] at pH 7.4 with 0.5% dodecyl β-D-maltoside. The absorption spectrum of resting cytochrome oxidase shows a maximum at 421 nm, which is characteristic of rapidly reacting enzyme. The fully reduced, carbon monoxide bound enzyme was prepared by anaerobic reduction with 4 mM sodium ascorbate and 1 μM cytochrome *c* under CO and shows a Soret maximum at 430 nm, as expected (Vanneste, 1966). The pD of solutions prepared in D_2O buffer was measured by using a pH meter and assuming pD = pH(observed) + 0.4. The experimental techniques used for the measurements of time-resolved Raman spectra have already been reported (Varotsis et al., 1989a,b; 1990a,b). The probe wavelength (441 nm) was provided by pumping coumarin 440 with the third harmonic output (355 nm) of a Quanta Ray DCR 2A pulsed laser.

Results

Figure 1 shows high-frequency resonance Raman spectra of fully reduced ($a^{2+}a_3^{2+}$) cytochrome oxidase at various delay times subsequent to carbon monoxide photolysis in the presence

of O_2. With 427-nm excitation, cytochrome a^{2+} and cytochrome a_3^{2+} contribute roughly equally to the resonance Raman spectrum of the fully reduced enzyme through a 0–1 enhancement mechanism because of their coincident absorption maxima near 443 nm (Babcock et al., 1981; Woodruff et al., 1981; Argade et al., 1986; Babcock, 1988). Excitation at 427 nm also enhances vibrations of cytochrome a^{3+} and those of oxygenated cytochrome a_3, which have absorption maxima in the 427-nm range. In the 10-ns photoproduct spectrum (Figure 1A), the oxidation state marker is at 1355 cm^{-1}, establishing that both cytochromes are in the ferrous state. The core expansion region shows two vibrations at 1570 and 1586 cm^{-1}. The 1622-cm^{-1} mode arises from the C=C stretching vibration of cytochromes a^{2+} and a_3^{2+}. The 1613 and 1666-cm^{-1} modes have been assigned (Babcock et al., 1981; Babcock, 1988) as the C=O stretching vibration of the formyl group (–CHO) of a^{2+} and a_3^{2+}, respectively. Spectrum B shows that no significant changes are detected at 2 μs in the reaction, consistent with our earlier result (Varotsis et al., 1989a,b, 1990a).

As the reaction proceeds, oxidation of cytochrome a_3 and a occurs. At 50 μs, the oxidation-state marker has shifted to 1371 cm^{-1}, and the shoulder at 1358 cm^{-1} is substantially decreased, indicating that an oxygen adduct of cytochrome a_3 is formed in this reaction (Babcock et al., 1984; Varotsis et al., 1989a,b; Han et al., 1990b; Ogura et al., 1990). The cytochrome a_3 core-size band, ν_2, which appears at 1570 cm^{-1} in the 10-ns and 2-μs spectra, shifts to higher frequency in the 50-μs spectrum and overlaps with the ν_2 vibration of cytochrome a^{2+}, which is located at 1589 cm^{-1}. Evidence for six-coordination may be seen by the appearance of the ν_{10} of cytochrome a_3 at 1641 cm^{-1}. The decrease in intensity of the 1613-cm^{-1} mode and the concomitant increase in scattering at 1650 cm^{-1} indicate that partial oxidation of cytochrome a has occurred at 50 μs. A similar conclusion concerning partial oxidation of cytochrome a was reached by Hill and Greenwood (1984), Hill et al. (1986), and Orii (1988) in their optical absorption measurements. The formyl vibration of cytochrome a_3, which is located at 1666 cm^{-1} in the photoproduct spectrum, has lost intensity and shifted to 1670 cm^{-1} as oxycytochrome a_3, and subsequent oxidation products are formed. Following this burst of redox activity in the first 100 μs, the Raman spectra show little change in the 100–500-μs time range. In particular, oxidation of cytochrome a remains partial, as indicated by the 1358-cm^{-1} shoulder in the ν_4 region in the 500-μs spectrum. The second phase of a oxidation occurs in the 500-μs to 5-ms time range to produce the oxidized enzyme (Babcock et al., 1984).

The kinetic behavior of the enzyme, as detected by Raman spectroscopy, is similar to behavior that has been observed in optical experiments. Hill and Greenwood (1984) and Hill et al. (1986) observed biphasic oxidation of cytochrome a with half-times of 35 and 860 μs. The amplitudes of the two phases were 0.4 and 0.6, respectively. These authors also noted biphasic Cu_A oxidation with similar rate constants but with relative amplitudes of 0.6 and 0.4, respectively. Phenomenologically similar data on heme redox activity were reported by both Orii (1988) and Brunori and Gibson (1983), i.e., a rapid phase of cytochrome oxidation, followed by a plateau period extending to about 500 μs, and subsequent completion of heme oxidation. To explore further the oxidation of cytochrome a, we have obtained Raman spectra with 441-nm excitation (Figure 2), which should enhance vibrational modes of cytochrome a^{2+} extensively in the reaction time course, as oxygen-bound cytochrome a_3 intermediates are expected to

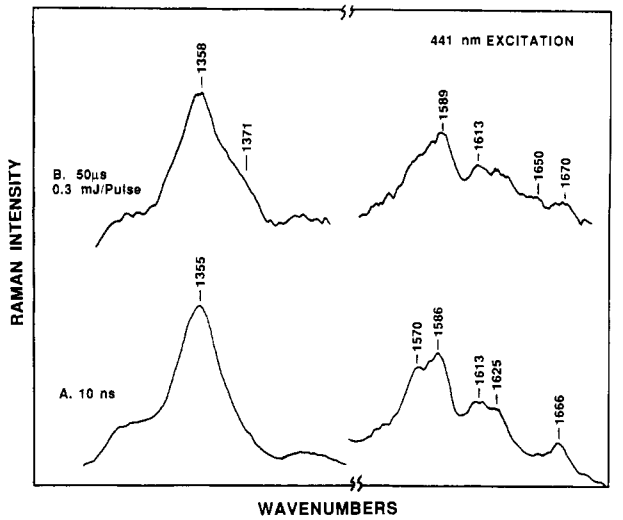

FIGURE 2: Time-resolved resonance Raman spectra of fully reduced cytochrome oxidase at the indicated times. The energy of the 532-nm photolysis pump/pulse was 1.3 mJ. The energy of the 441-nm probe beam was 0.8 mJ for spectrum A and 0.3 for spectrum B. The repetition rate for both the pump and probe pulses was 10 Hz. The accumulation time was 15 min for spectrum A and 65 min for spectrum B. The enzyme concentration was 50 μM after mixing, pH 7.4.

have substantially blue-shifted absorption spectra. The 50-μs spectrum indicates that oxidation of cytochrome a, as judged by the decreased scattered intensity of modes characteristic of cytochrome a^{2+} and the increased scattered intensity of the cytochrome a^{2+} formyl mode at 1650 cm^{-1}, has occurred and further supports the data obtained with 427-nm excitation.

The high-frequency data indicate partial oxidation of cytochrome a at 100 μs. Coupled with the complementary partial oxidation of Cu_A in the same time range that has been observed optically, these results indicate the formation of a three-electron-reduced $a_3/Cu_B/O$ site in a large fraction of the reacting enzyme. A number of chemically interesting species have been suggested for the three-electron-reduced intermediate including ferryl–oxo and ferrous–hydroperoxide structures (Wikstrom, 1981; Wikstrom et al., 1981; Blair et al., 1983, 1985; Chan et al., 1988), and indeed, Orii (1988) interpreted his optical data to indicate cytochrome a_3 ferryl–oxo formation at 100 μs in the reaction. In extensive investigations of the intermediate frequency region at 100, 300, and 500 μs in the reaction, however, we have seen no clear and reproducible indication of a conventional ferryl species, which is expected in the 740–850-cm^{-1} range (see Table I). Our failure, thus far, to observe such a mode unambiguously is certainly not an indication that a ferryl species does not occur at the three-electron level of reduction, and we are continuing our studies of this time regime.

By extending the pump–probe delay to times longer than 500 μs, we have continued our study of the reaction time course by recording Raman spectra in the 550–850-cm^{-1} frequency range during the slower phase of cytochrome a oxidation. These spectra are shown in Figure 3. Spectrum 3A is that of the photodissociation product of the fully reduced carbonmonoxy enzyme (t_d = 10 ns). The ν_7 and ν_{16} modes located at 685 and 749 cm^{-1} have contributions from both cytochrome a_3^{2+} and cytochrome a^{2+} (Argade et al., 1986). Spectrum B (t_d = 500 μs) and spectrum C (t_d = 800 μs) are similar to the 10-ns spectrum with the exception that a new mode appears at 790 cm^{-1} in the 800-μs spectrum. Figure 3D shows that the 790-cm^{-1} mode in the $^{16}O_2$ spectrum disappears when the experiment is repeated with $^{18}O_2$. By subtracting the $^{18}O_2$

Table I: Vibrational Frequencies for Ferryl–Oxy Complexes[a]

species	ν-(Fe^{IV}=O)	ref
cytochrome oxidase (a_3^{4+}=O)	790	this work
Fe^{IV}=O (TPP)	852	Proniewicz et al. (1986); Bajdor et al. (1984)
(NMI) Fe^{IV}=O (PPDME)	820	Kean et al. (1987); Oertling et al. (1990)
(NMI) Fe^{IV}=O (OEP)	820	Kean et al. (1987); Oertling et al. (1990)
(NMI) Fe^{IV}=O (TPP)	820	Kean et al. (1987); Oertling et al. (1990)
(NMI) Fe^{IV}=O ($T_{piv}PP$)	807	Schappacher et al. (1986)
(THF) Fe^{IV}=O ($T_{piv}PP$)	829	Schappacher et al. (1986)
Mb Fe^{IV}=O (pH 8.5)	797	Sitter et al. (1985a)
HRP-II (pH 7)	775	Terner et al. (1985); Hashimoto et al. (1986)
HRP-II (pH 11)	787	Hashimoto et al. (1984); Sitter et al. (1985b)
MPO-II (pH 11)	782	Oertling et al. (1988)
LPO-II (pH 6–10)	745	Reczek et al. (1990)

[a] Abbreviations: TPP, *meso*-tetraphenylporphyrin; $T_{piv}PP$, tetrakis-(pivaloylphenyl)porphyrin; PPDME, protoporphyrin IX dimethyl ester; NMI, 1-methylimidazole; OEP, octaethylporphyrin; Mb, myoglobin; HRP-II, horseradish peroxidase compound II; MPO-II, myeloperoxidase compound II; LPO-II, lactoperoxidase compound II.

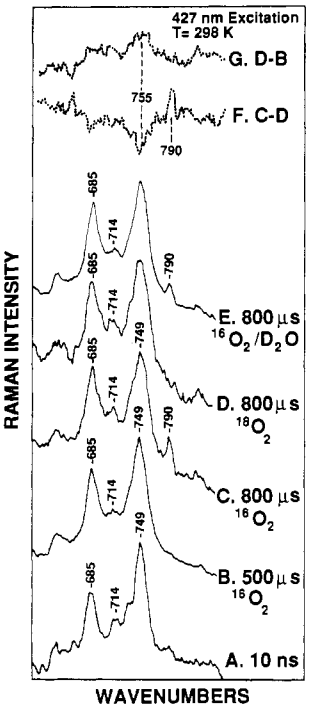

FIGURE 3: Time-resolved resonance Raman spectra of fully reduced cytochrome oxidase following initiation of the reaction with oxygen at room temperature. The energy of the 532-nm photolysis pump/pulse was 1.3 mJ. The energy of the 427-nm probe beam was 0.8 mJ for all spectra. The repetition rate for both the pump and probe pulses was 10 Hz. The accumulation time was 15 min for spectrum A and 20 min for spectra B–E. The measurements were repeated three times. A linear sloping background was subtracted from spectrum G, but no smoothing was done. The enzyme concentration was 50 μM after mixing, pH 7.4.

spectrum (Figure 3D) from that obtained with $^{16}O_2$ (Figure 3C), we obtain Figure 3F. This difference spectrum shows that the 790-cm^{-1} mode in the $^{16}O_2$ spectrum shifts down by 35 cm^{-1} to 755 cm^{-1} in the presence of $^{18}O_2$; in the absolute spectrum of the heavier oxygen isotope, the 755-cm^{-1} mode is obscured by the ν_{16} macrocycle mode at 749 cm^{-1}. Further evidence for the appearance of the 755-cm^{-1} mode in the $^{18}O_2$ experiment is shown in Figure 3G, which is the difference spectrum obtained by subtracting the 500-μs $^{16}O_2$ spectrum (Figure 3B) from the 800-μs $^{18}O_2$ spectrum (Figure 3D). These spectra demonstrate that an oxygen isotope sensitive mode occurs at 790 cm^{-1} at 800 μs in the reaction between O_2 and reduced cytochrome oxidase. The frequency of this mode and the 35-cm^{-1} isotope shift, which is in good agreement with that predicted by a two-body FeO harmonic oscillator approximation, indicate that this vibration arises from a ferryl–oxo (Fe^{IV}=O) cytochrome a_3 species (see below). Figure 3E shows the 800-μs spectrum of the cytochrome oxidase/$^{16}O_2$ solution prepared in D_2O. Comparison of spectrum C with spectrum E of Figure 3 shows that similar results for ν-(Fe^{IV}=O) are obtained in H_2O and D_2O buffers. This implies that there is no hydrogen bonding of the ferryl–oxo intermediate at pH 7.4.

Discussion

The oxidation of cytochrome oxidase has been studied extensively by both optical and EPR spectroscopies, and a variety of schemes has been proposed for the intramolecular transfer of electrons to the dioxygen-reducing site. The original formulation of Gibson and Greenwood (1963) involved sequential transfer to the oxygen-bound binuclear site, with Cu_A and then cytochrome *a* undergoing oxidation. Hill and Greenwood (1984) later attempted to reconcile this scheme with the extinction coefficients that have been established for *a* and a_3 and found that in order to do this it was necessary to introduce a branch in the room-temperature reaction pathway such that oxidation of both cytochrome *a* and Cu_A occurred biphasically with a fast phase of $\leq$100 μs and a slower phase in the 800-μs time range. Clore et al. (1980) and Chan and co-workers (Blair et al., 1985; Chan et al., 1988) were led to a similar branched pathway in order to interpret low-temperature optical and EPR data. In terms of the dioxygen-reducing site, these schemes suggest that the third electron in O_2 reduction is transferred in a branched fashion from either *a* or Cu_A and that the fourth electron is then transferred from the remaining, unoxidized center.

In room-temperature, time-resolved optical work, this branched pathway is manifested by a burst of cytochrome oxidation at $t < 100$ μs followed by a plateau, and final cytochrome oxidation in the slower phase. Thus, the three-electron intermediate would form in times less than 100 μs and be accompanied by partial cytochrome *a* oxidation. The fourth electron would be transferred in the 800-μs range. Although the details of the interpretations vary, observations phenomenologically similar to the time course of cytochrome oxidation have been made at room temperature by Orii (1988), by Malmstrom and co-workers (Oliveberg et al., 1990), and by Brunori and Gibson (1983).

The Raman data we report in Figures 1 and 2 are consistent with this branched mechanism. At early times in the reaction ($t < 100$ μs), we observe partial oxidation of cytochrome *a* with both 427- and 441-nm excitation, along with oxygenation and oxidation of cytochrome a_3. Following this rapid phase, which we associate with the formation of a three-electron-reduced dioxygen site, the system undergoes little detectable change to 500 μs. Entry of the fourth electron is associated with the slow phase that extends from 500 μs to about 5 ms.

Within the context of this scheme, which is consistent with the bulk of the optical, EPR, and resonance Raman data, the three-electron-reduced intermediate would persist from $\sim$100 to $\sim$500 μs. Wikstrom (1981, 1989), by reversing the dioxygen reaction in mitochondria, noted the appearance of a

580-nm-absorbing species which he proposed as the one-electron oxidation product of the a_3^{3+}/Cu_B^{2+} couple. In addition, he proposed ferryl–oxo structures for both the three- and four-electron-reduced intermediates. Chan and co-workers (Blair et al., 1985; Chan et al., 1988) detected a 580/537-nm species in the three-electron-reduced enzyme/O_2 reaction and suggested the formation of two three-electron-reduced intermediates. They proposed that the first species, which is EPR detectable, is a hydroperoxide-bridged Cu_B^{2+}/a_3^{2+} adduct and that the second intermediate is a Fe^{IV}=O/Cu_B^{2+} species formed by cleavage of the O–O bond in the hydroperoxide adduct with uptake of a proton. Clore et al. (1980) have also postulated the formation of a ferryl–oxo adduct. In addition, the 100-μs difference spectrum reported by Orii (1988) resembles the optical spectra of Chan et al. (1988), Clore et al. (1980), and Wikstrom (1981, 1989). He noted that his results indicated the early occurrence of a three-electron-reduced intermediate and proposed an oxo–ferryl cytochrome a_3 structure for this adduct. As already discussed, we see no clear evidence for a conventional ferryl–oxo species in our 550–850-cm^{-1} data at 100, 300, and 500 μs in the reaction. The three-electron level of reduction is likely to involve substantial nuclear rearrangement in the dioxygen site (Blair et al., 1985), however, and thus it is quite possible that the bound dioxygen species undergoes valence change, bond rearrangement, and protonation reactions before the arrival of the fourth electron. If the rates for these reactions are comparable, then at any given time the site will exist in a variety of different configurations. Such a situation would render Raman detection, already difficult for relatively homogeneous populations, problematic.

It is clear, however, from our data that a ferryl–oxo intermediate, detected by its iron–oxygen stretching vibration, does occur during dioxygen reduction. The data of Figure 3 indicate that this species is formed at 800 μs in the reaction sequence, which is substantially later than three-electron-reduced intermediates are expected in the majority of the reacting enzyme molecules. The iron–oxygen stretching mode is located at 790 cm^{-1} in the 800-μs spectrum and downshifts to 755 cm^{-1} when the experiment is repeated with $^{18}O_2$. The frequency of the iron–oxygen vibration and the magnitude of the isotope shift are both consistent with the assignment of this species as the ferryl–oxo cytochrome a_3 adduct. Moreover, the ferryl–oxo intermediate is absent in the 500-μs spectrum but present at ~800 μs, which is consistent with the half-times reported by Hill and Greenwood (1984), Hill et al. (1986), and Oliveberg et al. (1990) for the formation of a four-electron-reduced intermediate. Thus, although heterogeneity in the reaction may be such that the 790-cm^{-1} mode arises from a minority, three-electron-reduced species, a more reasonable assignment of the 790-cm^{-1} mode is that it arises from a $Cu_A^{2+}a^{3+}a_3^{4+}$=O/Cu_B^{1+} complex. Such a structure has been proposed for the four-electron-reduced complex by Wikstrom (1981). From a purely chemical point of view, such a set of valences in the binuclear site seems unusual and may have mechanistic significance. Unfortunately, we are unable to monitor the Cu_B redox state directly, so some ambiguity remains as to its valence.

Table I summarizes $\nu(Fe^{IV}$=O) frequencies for several heme proteins and model compounds. The 790-cm^{-1} frequency for the ferryl–oxo cytochrome a_3 adduct is similar to the 787-cm^{-1} frequency reported by Terner and co-workers (Sitter et al., 1985b) as well as by Hashimoto et al. (1984) and Oertling et al. (1988) for $\nu(Fe^{IV}$=O) of HRP compound II but slightly lower than that observed by Sitter et al. (1985a) for Mb=O [$\nu(Fe^{IV}$=O) = 797 cm^{-1}] and ~30 cm^{-1} lower than that observed by Kean et al. (1987) for the ferryl–oxo complexes of imidazole-ligated iron octaethylporphyrin (OEP), protoporphyrin IX dimethyl ester (PPDME), and tetraphenylporphyrin (TPP). Furthermore, the lack of an H_2O/D_2O exchange effect indicates that hydrogen bonding to the Fe^{IV}=O moiety is weak or absent and that the ferryl intermediate is relatively unperturbed by interactions with nearby amino acid residues or ligands bound to Cu_B. Decay of the ferryl–oxo intermediate may reflect formation of a μ-oxo $a_3^{3+}Cu_B^{2+}$ adduct or of a cytochrome a_3–hydroxide intermediate upon reduction and protonation. Such intermediates have been postulated by Wikstrom (1981), Blair et al. (1983), and Chan et al. (1988). More recently, Han et al. (1989), by photoreducing the resting enzyme under intense laser illumination, observed a mode at 477 cm^{-1} that they assigned to the Fe^{3+}–OH intermediate. Its role in the catalytic function of cytochrome *c* oxidase under physiological conditions, however, was not established.

Combining the results above with the earlier optical, EPR, and Raman results on the reduction of dioxygen by fully reduced cytochrome oxidase, the following points emerge. First, oxygen binding occurs rapidly to form oxycytochrome a_3 and is followed by one-electron intramolecular transfer from the cytochrome a/Cu_A centers to form a three-electron-reduced binuclear site within 100 μs. The nature of the bound oxygen intermediates following the oxy species remains to be determined. Second, the oxidation of *a* and Cu_A follows a branched pathway and is biphasic under most experimental conditions that have been used. The slower phase of oxidation occurs in the 500–1000-μs time range and produces a four-electron-reduced a_3/Cu_B/oxygen center. Third, a cytochrome a_3 ferryl intermediate can be detected in the reaction time course. Subsequent redox and ligand rearrangement produces the oxidized enzyme.

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