Cytochrome c oxidase, the terminal enzyme in the electron transfer chain, catalyzes the reduction of oxygen to water in a multiple step process by utilizing four electrons from cytochrome c. To study the reaction mechanism, the resonance Raman spectra of the intermediate states were measured during single turnover of the enzyme after catalytic initiation by photolysis of CO from the fully reduced CO-bound enzyme. By measuring the change in intensity of lines associated with heme \( a \), the electron transfer steps were determined and found to be biphasic with apparent rate constants of \( -40 \times 10^3 \) s\(^{-1} \) and \( -1 \times 10^3 \) s\(^{-1} \). The time dependence for the oxidation of heme \( a \) and for the measured formation and decay of the oxy, the ferryl ("F"), and the hydroxy intermediates could be simulated by a simple reaction scheme. In this scheme, the presence of the "peroxy" ("P") intermediate does not build up a sufficient population to be detected because its decay rate is too fast in buffered \( \text{H}_2\text{O} \) at neutral pH. A comparison of the change in intensity of lines associated with heme \( a \) and the oxygen isotope-sensitive line at 355 cm\(^{-1} \), coincident with the presence of the F intermediate.

Cytochrome c oxidase catalyzes the four electron reduction of oxygen to water by cytochrome c (1). The enzyme contains four redox centers. One redox equivalent is in a binuclear copper center that accepts electrons from the enzyme’s redox partner, cytochrome c. Another redox center is located in a heme group, heme \( a \), through which the electrons pass on their pathway to the binuclear catalytic center consisting of a heme group (heme \( a_3 \)) and a copper atom (Cu\(_{III}\)) 4.6–5.1 Å away (2–7). In eukaryotic species, cytochrome c oxidase is located in the inner mitochondrial membrane. The energy associated with the oxygen reduction chemistry is harnessed by the enzyme for the translocation of protons across the inner mitochondrial membrane to generate the proton gradient leading to the formation of ATP.

Many studies have been carried out to determine the mechanism for \( O_2 \) reduction by cytochrome c oxidase. Typically, the reaction is initiated by the flow-flash-probe method, first developed by Greenwood and Gibson (8, 9). In this method, the reaction is triggered by photolysis of the CO inhibited enzyme in the presence of oxygen and the intermediates are examined spectroscopically by either continuous flow or stopped flow techniques. Optical absorption spectroscopy has been used to follow the kinetics of the reaction (10–15), and resonance Raman scattering has been used to identify the reaction intermediates (16–30). Other studies of the structures of the intermediates have been carried out by adding hydrogen peroxide to the oxidized enzyme, thereby generating some species that are identified as being the same as the intermediates that are formed during turnover (31–36).

Based on these experiments, structures for many intermediates have been proposed and the rates for the associated elementary steps have been determined. Some of the structures were inferred based on observed kinetic changes, and others were proposed based on spectroscopic properties. These studies led to a consensus on the structures of some of the intermediates and puzzling disagreements over others. The most controversial issues concern the identities of the so-called “peroxy” ("P") and “ferryl” ("F") intermediates. The P intermediate has an absorption maximum at \( 607 \) nm in the difference spectrum with respect to the oxidized enzyme and the F intermediate has a maximum at \( 580 \) nm in the absorption difference spectrum (37). Some investigators have identified properties attributed to a P intermediate during turnover (15), but, in contrast, others have reported that such an intermediate cannot be detected under turnover conditions (14). An oxygen intermediate at this two-electron reduced level has been assigned by different investigators as having both peroxo (Fe\(^{3+}\)-O-O\(^{-}\)- and Fe\(^{3+}\)-O-OH\(^{-}\)) and ferryl (Fe\(^{4+}\)-O\(^{2-}\)) structures (10, 11, 15, 16, 24, 27, 31–33, 38–40). Similar differences exist in the interpretation of the intermediate at the three electron reduced level, the F species. It has been proposed to be both a hydrogen-bonded and non-hydrogen-bonded ferryl intermediate, Fe\(^{4+}\)-O\(^{2-}\), (14, 16, 19, 23, 25, 27); it has been proposed to have a bridged peroxo (Fe\(^{3+}\)-O-O-Cu\(_B\)\(^{2+}\)) structure (36); it has been proposed that the intermediate at this redox level of oxidation is in an equilibrium in which heme \( a \) can be either oxidized or reduced (15, 36, 38).

Resonance Raman scattering has been shown to be very useful for the identification of intermediates during the catalytic turnover of cytochrome c oxidase. The iron-oxygen stretching modes for many of the intermediates can be identified, and their assignments can be confirmed by oxygen isotopic substitution (16–27). Therefore, structures containing one versus two oxygen atoms can be distinguished, and the presence of protons as well as the effect of hydrogen bonding can be assessed (16). Resonance Raman scattering is thus considered as the most
Catalytic Reaction Intermediates in Cytochrome c Oxidase

reliable technique to identify the intermediates. However, resonance Raman experiments were performed under different conditions in various laboratories, which in some cases has caused it to be difficult to make direct comparisons between the results because each has its own strengths and weaknesses. For example, the continuous flow method used by Kitagawa's group (16, 17, 23, 24, 33, 41) and by the present authors (18–22) gives a better signal-to-noise ratio than that of the pulsed laser technique used by Babcock's group (25–27, 29). However, with pulsed lasers, more accurate time delays can be measured. With the re-circulating system used by Kitagawa and co-workers, excellent signal-to-noise is obtained by use of long integration times, but it is difficult to generate a high concentration of molecular O₂, so the time course of the intermediates becomes obscured. In our work, it was felt that the continuous flow method without re-circulation is a good compromise that gives sufficient signal-to-noise and time resolution to address the central issues. The flow dynamics limitations of our continuous flow method are discussed under “Materials and Methods.” As a result of the different approaches and because the spectra of the P and F states are weak and difficult to obtain, there is no consensus on the P intermediate, although there is partial agreement on the other intermediates during the single turnover reaction. In addition, the kinetics of the formation and decay of the various intermediates detected in the resonance Raman spectra require further study and the relationship between the electron transfer events and the formation of each intermediate are needed to clarify the molecular mechanism of the O₂ reduction.

Identification of the P and F intermediates and determination of their structures are particularly important, because it was reported that the redox coupling that enables proton translocation only occurs at two of the steps in the reduction of oxygen to water: the P to F step and the F to the hydroxy step (40). However, only one half of the charge is translocated during this oxidative phase in which the enzyme becomes fully oxidized. The remaining charge is only translocated when the oxidized enzyme is immediately re-reduced (the reductive phase) (42). Proper understanding of the structures and kinetics of these intermediates is thereby extremely important in order to formulate the mechanism by which the redox events are coupled to proton translocation and how the energy is stored at the end of the oxidative phase to be released during the reductive phase (42, 43). In this article, we report the kinetics of the resonance Raman lines that are associated with the various intermediates during turnover to establish the mechanistic pathway for oxygen reduction and we report the associated changes in the oxidation state of heme a to monitor the electron transfer events. The kinetic behavior of the Raman mode at 355 cm⁻¹ was also measured to determine its time dependence, as there is a lack of consensus on the kinetics of its formation and decay.

MATERIALS AND METHODS

Cytochrome c oxidase was isolated from bovine heart muscle by the methods of Yonetani (44) and of Yoshikawa et al. (45) and stored under liquid nitrogen until ready for use. For the Raman measurements the enzyme was solubilized in phosphate buffer (100 mm) at pH 7.4 with 1% dodecyl β-D-maltoside. It was reduced with 20 mm ascorbate and catalytic amounts of cytochrome c, and then exposed to carbon monoxide.

To initiate the catalytic reaction, the CO-bound cytochrome c oxidase sample was placed in one syringe of the continuous flow apparatus described previously (22) and a buffer solution saturated with either natural abundance or isotopically substituted (¹⁸O₂) oxygen (1.4 mm) was placed in the other syringe. The two solutions were mixed in a Wiskind four grid mixer and passed into the flow cell with a 0.25 × 0.25-mm cross section. One laser beam at the entrance of the cell photodissociated the CO from the enzyme, and a second laser beam was used to probe the resonance Raman spectrum. The separation between the two beams could be changed so as to give a delay time ranging from ~15 μs (beam overlap) to ~3 ms. The actual time evolution of the intermediates is determined not only by the separation between the photolysis and the probe beam but also by the flow dynamics of the solution in the cell. Laminar flow gives a velocity distribution perpendicullar to the flow direction, and therefore a distribution in the time constants for the intermediates. On the other hand, “plug” flow gives a homogeneous velocity in the flow direction as if a plug were moving down the channel. A series of partial photolysis measurements across the cell were made to determine the variation in flow velocity, and it was found that the flow was intermediate between the plug and the laminar cases. By collecting data from only the central portion of the laser pathlength through the sample (~80% of the width of the cell), the velocity variation was within ±25% of the average velocity. Therefore, the measured time constants have an uncertainty of this magnitude.

For most of the experiments, the output from a krypton ion laser at 413.1 nm was used to both photodissociate the CO from the enzyme and probe the spectrum of the reaction products. By blocking the first beam, it was confirmed that no spontaneous replacement of the CO by oxygen was occurring under our conditions. By replacing the oxygen in the buffer by nitrogen, full photodissociation of the CO from the enzyme by the photolysis beam was observed. For the series of experiments to measure the line at 355 cm⁻¹, the output from a frequency doubled Ti-sapphire laser (pumped by an argon ion laser) with an output at 427 nm was used for both beams. The scattered light was dispersed by a 1.25-m polychromator and detected by either a linear photodiode array or a CCD camera.

RESULTS

The high frequency region (1000–1800 cm⁻¹) of the resonance Raman spectrum of heme proteins contains vibrational modes that are sensitive to the coordination, spin state and oxidation state of the heme moiety. For cytochrome c oxidase, the contributions to the spectrum from hemes a and a₃ overlap. However, several modes have been identified that have been demonstrated to be characteristic of each of the hemes and can thereby be used to monitor the properties of each without interference from the other (18, 46–50). In particular, we have shown that the lines in the spectrum at 1518, 1611, 1623, and 1647 cm⁻¹ can be used as markers of the oxidation state of heme a. The lines at 1518, 1611, and 1623 cm⁻¹ originate from the reduced heme, whereas the line at 1647 cm⁻¹ originates from the oxidized heme. The changes in the resonance Raman spectra of cytochrome c oxidase in the high frequency region during the reaction of the enzyme with oxygen are shown in Fig. 1 for several different time points. A rapid decrease in the intensity of the lines at 1518, 1611, and 1623 cm⁻¹ and a corresponding increase in the intensity of the line at 1647 cm⁻¹ occurs during the first ~100 μs. Subsequently, the changes are more gradual, such that the formation of the fully oxidized spectrum of heme a nears completion only at ~3 ms. Changes in the spin state, judged by the intensity of the low spin marker line at ~1584 cm⁻¹ and the high spin marker line at ~1570 cm⁻¹, occur on the 0.1–10-ms time scale. These data demonstrate a significant growth of the high spin population in this time range. Since heme a is always low spin, the change is attributed to heme a₃. The time dependence of the oxidation of heme a, plotted in Fig. 2, obtained by monitoring the change in intensity of the line at 1518 cm⁻¹, clearly demonstrates that heme a becomes oxidized by at least two separate processes. A rapid process occurs in which the heme is partially oxidized with an apparent rate constant of ~40,000 s⁻¹, and it is followed by a slower process with an apparent rate constant of ~1,000 s⁻¹.

In the low frequency region (100–1000 cm⁻¹) of the resonance Raman spectra, in addition to the modes associated with the heme macrocycles, oxygen isotope-sensitive modes associated with the reduction intermediates are also present during the reaction of cytochrome c oxidase with oxygen. However, the iron-oxygen modes are very weak, so except for the primary Fe-O₂ intermediate they cannot be readily identified in the
spectrum. By using oxygen isotope difference spectra (\(^{16}\text{O}_2 - ^{18}\text{O}_2\)), the modes involving the oxygen can be located as shown in Fig. 3 in which the laser excitation wavelength was 413.1 nm. Two oxygen isotope-sensitive lines are present in these data. The stronger line at 568 cm\(^{-1}\) for \(^{16}\text{O}_2\) that shifts to 547 cm\(^{-1}\) for \(^{18}\text{O}_2\) is assigned as the Fe-O\(_2\) stretching mode, as shown previously (20, 21, 26, 41, 51, 52). The other line in the difference spectrum is centered at \(-786\) cm\(^{-1}\) in \(^{16}\text{O}_2\) and shifts to \(-750\) cm\(^{-1}\) upon \(^{18}\text{O}_2\) isotopic substitution. Ogura et al. (24) reported multiple lines in the 780–805 cm\(^{-1}\) region. Although the line is broad, we detect it at 786 cm\(^{-1}\) in all of our data in buffered H\(_2\)O. Thus, the line is assigned as the ferryl (iron-oxo) stretching mode, in accord with prior assignments (16, 19, 23, 25, 27). The important observation in our new data is the strong contribution from the ferryl mode at an early reaction time (65 \(\mu\)s). At longer times the intensity of this mode diminishes and a line at 450 cm\(^{-1}\) appears, which we previously assigned (19) as the Fe-OH stretching mode (data not shown).

By measuring the amplitudes in the difference spectra, we have been able to determine the time dependence of three of the catalytic intermediates: the primary oxy (Fe-O\(_2\)) species, the ferryl (Fe-O) species, and the hydroxy (Fe-OH) species. These data are plotted in Fig. 4 versus the simulated time dependence based on the reaction scheme discussed below.

The weak mode at 355 cm\(^{-1}\) was discovered originally by Ogura et al. (53). In our hands, the line at \(-350\) cm\(^{-1}\) could not be detected with 413.1 nm excitation, but we have found that the relative intensity of all of the modes depends critically on the laser excitation wavelength. Consequently, only with laser excitation at 427 nm have we been able to detect the line at 355 cm\(^{-1}\) and follow its time dependence from 100 to 1000 \(\mu\)s (Fig. 5). Within the uncertainty of our data, the intensity of the mode has the same time dependence as the ferryl mode at \(-786\) cm\(^{-1}\). It is also noteworthy that the mode is very sharp in comparison to all of the other modes that have oxygen isotope dependence. The hydroxy intermediate with a Fe-OH stretching mode at \(-450\) cm\(^{-1}\) is not apparent in these data as it is very weak with this excitation frequency.

**DISCUSSION**

**Assignment of the Intermediates**—We adopt here the same assignment for the major intermediates as made in the past by several investigators (16, 19, 27). The line at 568 cm\(^{-1}\) has been clearly identified as the Fe-O\(_2\) stretching mode. It shifts to 547 cm\(^{-1}\) when the \(^{16}\text{O}_2\) is replaced by \(^{18}\text{O}_2\), in full agreement with the predicted isotope shift for a diatomic oscillator between an iron atom and an oxygen molecule. In addition, it is found at the same frequency in hemoglobins and myoglobins. Thus, this is an oxy intermediate, the primary intermediate in the reaction, and is also referred to as compound "A."

In most heme proteins, it has been argued that when molecular oxygen binds to the iron atom, it adopts a ferric-superoxide configuration. These arguments are based on the similarity of the heme optical and Raman spectra to those of ferric forms of the heme proteins and the similarity of the O-O stretching mode to that of inorganic superoxide (22). The high frequency resonance Raman spectra in Fig. 1 demonstrate that both of the heme groups have been largely converted to ferric or ferric-like structures within \(-40\) \(\mu\)s. The increase in the frequency of the electron density marker line from \(-1356\) cm\(^{-1}\) in the reduced heme to \(-1371\) cm\(^{-1}\) upon binding oxygen indicates a decrease in the electron density in the antibonding heme orbitals to the same degree as in the formation of the formal ferric iron oxidation state. This result, along with the identity of the
Fe-O\textsubscript{2} stretching mode in oxidase with that in hemoglobins and myoglobins, shows that in cytochrome \textit{c} oxidase, just as in the other heme proteins, the Fe-O2 moiety adopts the ferric-superoxide configuration (22). Thus, at this initial stage of the oxygen reaction, one electron has already been donated to the O\textsubscript{2} molecule.

The next intermediate we detect, which originally was assigned as a ferryl species, has a frequency of 786 cm\textsuperscript{-1} (19). This assignment has been confirmed by others (23, 25) and remains valid. The properties of the intermediates that appear in the 780–805 cm\textsuperscript{-1} frequency range are complicated, however (16, 24). Upon carrying out the reaction in deuterated buffers, the mode appears at higher frequency (801 cm\textsuperscript{-1}, data not shown). This apparent shift of 15 cm\textsuperscript{-1} to higher frequency was originally attributed by Han \textit{et al.} (19) to the loss of hydrogen bonding in deuterated buffers. However, Ogura \textit{et al.} reported that modes with frequencies at ~785 and 804 cm\textsuperscript{-1} were both present in protonated buffer (16), with the 804 cm\textsuperscript{-1} mode appearing prior to that at 785 cm\textsuperscript{-1} (24). Thus, rather than postulating that the 785 cm\textsuperscript{-1} mode was shifting in deuterated buffer, Ogura \textit{et al.} (16, 24) concluded that these two lines originated from distinct normal modes representing different intermediates and the intermediates had different temporal behavior in deuterated buffer as compared with protonated buffer. Correlation of these frequencies with the optical transitions was made by Proshlyakov \textit{et al.} (31), who, by adding hydrogen peroxide to the oxidized enzyme, demonstrated that the 607-nm species assigned as a peroxo intermediate (P) had only one oxygen atom and was therefore an oxo-ferryl species as well. They assigned the line in the resonance Raman spectrum at ~800–805 cm\textsuperscript{-1} to this P intermediate and the line in the spectrum at ~786 cm\textsuperscript{-1} to the F intermediate with the 580-nm band in the absorption difference spectrum. They proposed that there were additional reducing equivalents in the protein that could donate an electron to the heme oxygen complex resulting in the three-electron reduced P intermediate.

Similar conclusions were previously drawn by Weng and Baker (54), who observed that the Soret transition was identical in the P and the F species, whereas a large spectral change would be anticipated if the iron oxidation state changed from Fe\textsuperscript{3}\textsuperscript{+} for a peroxo intermediate to Fe\textsuperscript{4}\textsuperscript{+} for a ferryl intermediate. They concluded that the P and F species were analogous to compounds I and II in cytochrome \textit{c} peroxidase, both compounds having a ferryl structure but with the electron in compound I being supplied from an amino acid residue generating a radical species. It was suggested that detection of the radical by EPR could be difficult due to spin coupling. More recently from optical, MCD, and EPR experiments, Fabian and Palmer (36) also proposed that the P species has an oxo-ferryl structure with the extra reducing equivalent residing on Cu\textsubscript{II} giving rise
to a trivalent oxidation state. Finally, in cytochrome \( b_{2+} \), Wang et al. (55) proposed a radical amino acid species to account for the reaction of the enzyme with oxygen.

Recently, Proshlyakov et al. (30) followed the reaction of oxygen with the mixed valence enzyme. They observed the formation of a species with an 804 cm\(^{-1} \) line concomitant with the disappearance of the oxy intermediate. Thus, for the mixed valence enzyme, the lifetime of any species with a Fe-O(OH) structure is too fast to be observable under single turnover conditions and the P intermediate is formed. They postulated that the extra redox equivalent resides on a tyrosine radical, Tyr-244, which is linked to His-240 through a posttranslational modification (3, 6) and which stabilizes the bi-nuclear center (56). A similar conclusion was drawn by Sucheta et al. (38) from optical studies of the reaction of the fully reduced enzyme. They concluded that a P species, with a heme \( a_2 \) oxo-ferriyl structure, is formed during turnover through the formation of a tyrosine radical. This species is proposed to be in a rapid equilibrium with an F intermediate. Recent EPR experiments provide support for the presence of a tyrosine radical since a radical signal was detected that was changed drastically when deuterated tyrosine was incorporated into the protein (57).

Based on the above results, it now appears very likely that both the P and the F species have oxo-ferriyl structures. They have two different Fe-O stretching frequencies and two different visible optical transitions. The data also show that the chemical conditions of the reactions determine which of the intermediates can accumulate. One possible explanation to account for the difference in the Fe-O stretching frequencies of these two intermediates is the presence of two conformational states for the oxo-ferriyl species as observed with other ligands. These conformational states have been most intensively studied in the CO-bound form of the protein. It was found in the resonance Raman spectra that there are two different Fe-CO stretching frequencies, one at \(-520 \text{ cm}^{-1} \) and one at \(-495 \text{ cm}^{-1} \) (58). Similarly, two different conformations were detected in the FTIR spectra of the C-O stretching modes, both when the CO was coordinated to the iron atom and when it was coordinated to the Cu\(_B\) after photolysis (59, 60). These states have been attributed to a different distance between the iron atom of heme \( a_2 \) and Cu\(_B\) and have been shown to be sensitive to the chemical environment such as the pH. Interestingly, the relative proportions of the 801 and 786 cm\(^{-1} \) species, formed by the addition of hydrogen peroxide to the oxidized enzyme, are also \( \text{pH} \)-dependent (32) as are the relative proportions of the 607 and 580 nm species (36). Two structures have also been detected for the hydroxy intermediate (see below), which can be attributed to two different conformational forms of the protein (19, 61). The crystal structure supports the concept of two distinct conformations of the catalytic site. In the reduced state of the enzyme, the Fe-Cu\(_B\) distance is 5.2 Å, whereas, in the oxidized enzyme, it is only 4.9 Å (61). It is not known when this change occurs during the catalytic cycle. We propose that structure with the 801 cm\(^{-1} \) mode has a longer Fe-Cu\(_B\) distance, just as in the reduced enzyme, and the form with the 786 cm\(^{-1} \) ferryl mode has the shorter Fe-Cu\(_B\) distance, as in the oxidized protein. The change in the Fe-Cu\(_B\) distance can reflect significant differences in the heme environment, thereby resulting in the differences in the visible absorption spectra. In addition, a shortening (or shortening) of the Fe-Cu\(_B\) distance could easily cause the change in the Fe-O stretching frequency since it is well established that iron ligand frequencies in heme proteins are strongly modulated by the properties of the residues in the distal environment. The origin of the change in distance could be due to a radical formation on Tyr-244 near the heme in the P species as has been proposed (30). The radical changes the electrostatic environment near the heme, which could have significant structural consequences, and it also could directly effect the Cu\(_B\) via the cross-linking to His-240, a Cu\(_B\) ligand.

The last intermediate we are able to detect appears at 450 cm\(^{-1} \) in the spectrum and is assigned as the hydroxy intermediate. As compared with other heme proteins in which a hydroxy intermediate has been assigned, the frequency of the line in the oxidase is very low. For example, the Fe-OH stretching mode is found at 550 and 553 cm\(^{-1} \) in low spin metmyoglobin and methemoglobin, respectively, and at 491 and 492 cm\(^{-1} \) for the high spin forms of these proteins (62). On the other hand, the mode is detected at 503 cm\(^{-1} \) in low spin horseradish peroxidase (HRP), 50 cm\(^{-1} \) lower than for the low spin forms of the two globins. This low frequency has been attributed to very strong hydrogen bonding to the hydroxide in HRP (62).

From the very low frequency for the Fe-OH mode in the hydroxy intermediate, we postulate that it is high spin. However, its environment is different from that of most globins in which the mode appears in the 490 cm\(^{-1} \) region. Based on the observation that the Fe-OH stretching mode appears 40 cm\(^{-1} \) lower, at 450 cm\(^{-1} \), than in the high spin globins, we propose that there is a strong hydrogen bond between the proton on the hydroxide moiety and some other group, either the nearby copper atom or Tyr-244, which is located close to the ligand binding site. The smaller than predicted shift upon deuteration (6–8 cm\(^{-1} \) as compared with the predicted value of 10 cm\(^{-1} \) for a Fe-OH diatomic oscillator) is also indicative of strong hydrogen bonding. This is consistent with recent data reported by Yeh et al. on a unique hemoglobin discovered in *Mycobacterium tuberculosis*. In this hemoglobin, a tyrosine residue is in the distal pocket of the heme and, through strong hydrogen bonding, it gives rise to a high spin hydroxy species with a Fe-OH stretching mode at 454 cm\(^{-1} \) that disappears upon mutagenesis of the residue. Additional confirmation for the assignment of the hydroxy species in heme \( a_2 \) as having a high spin configuration comes from the coincidence of the time dependence of the low to high spin state change in cytochrome c oxidase with the formation of the hydroxy intermediate (see below).

Another hydroxy species with an Fe-OH stretching mode at 477 cm\(^{-1} \) in cytochrome c oxidase was reported previously in the presence of ferricyanide, oxygen, and sufficient laser power to photoreduce the enzyme (61). Under these conditions, the enzyme was forced to turnover repeatedly, until the molecular oxygen was depleted, terminating in an oxidized species that was hydroxide-bound. However, the Fe-OH mode is different from that detected in the single-turnover measurements. Thus, the enzyme can have two conformations for the hydroxy species as in the ferryl adduct and the CO adduct as discussed above.

In the CO derivatives, the two conformations have been attributed to changes in the position of the Fe-Cu\(_B\) distance. A similar assignment can be made with the hydroxide intermediate. In the form with a 450 cm\(^{-1} \) frequency, we postulate that the Fe-Cu\(_B\) distance is 4.9 Å, as found in the crystal structure of the ferric protein. This highly constrains the hydroxide, resulting in a strong hydrogen bond giving rise to the low Fe-OH stretching frequency. In the form of the hydroxide with the 477 cm\(^{-1} \) frequency, the Fe-Cu\(_B\) distance is relaxed so the hydrogen bond is weaker, resulting in a frequency closer to those in hemoglobin and myoglobin, which have a more open pocket.

The 355 cm\(^{-1} \) Mode—The observation and assignment of the oxygen isotope-sensitive mode at 355 cm\(^{-1} \) remains an enigma.

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\(^2\) Yeh, S.-R., Couture, M., Oullet, Y., Guertin, M., and Rousseau, D. L. (2000) *J. Biol. Chem.*, in press.
The reduced enzyme (R) is coordinated by CO. The CO is photodissocitated from the enzyme in the presence of oxygen. The oxygen first binds to CuA, and subsequently to the iron atom of heme $a_1$ forming a ferric superoxide complex (compound A). The reaction branches into the right-hand leg in which an electron from the heme $a$ -CuA sites is transferred to the heme $a_1$ -CuA binuclear center and the left-hand leg in which the initial electron transfer to the Fe-O2 complex comes from CuB. In this leg, a second electron is donated to the binuclear center from a redox site in the protein forming a radical designated as $R^*$. It is likely that this redox equivalent is stored in Tyr-244. This species is assigned as the $P$ intermediate. Both legs converge to the common $F$ intermediate. The final electron transfer leads to the formation of the $H$ intermediate, thereby completing the oxidative phase of the enzyme. The $H$ intermediate may convert to the oxidized state with undetermined ligands, or it may be re-reduced (reductive phase). The rate constants for the processes are listed in Table I.

This mode was originally detected by Kitagawa and co-workers and assigned as an iron-oxygen stretching mode of a peroxy species (53). They reported that it had a time dependence similar to that of the mode at 788 cm$^{-1}$ and assigned it as an Fe-O stretching mode of a hydroperoxo species. Very recently, they reported the line at a time delay of 400 $\mu$s in the reaction of the mixed valence enzyme with oxygen (30).

To address the differences in the results from those two laboratories, the new measurements of the 355 cm$^{-1}$ line reported here were carried out. The line, not detectable with 413.1 nm excitation in our laboratory but readily detected in the spectrum with 427 nm excitation, is present in our data from 130 $\mu$s to 980 $\mu$s. Since our experiments were carried out at ambient temperature rather than at 3°C, as reported by Ogura et al. (16), the lifetimes would be expected to be shorter and are thereby consistent with their results. Moreover, the

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**TABLE I**

**Rate constants reported for various intermediates in the reaction of cytochrome c oxidase with oxygen**

The redox centers in parentheses indicate the source of the electrons for each of the reactions. The (X) notation is that of Sucheta et al. (15). All of the numbered rate constants are those that were used in the calculations presented in this study. The "R" represents a redox equivalent residing on a protein residue.

| Reaction                              | Rate | This work     | Sucheta et al. (Ref. 38) | Sucheta et al. (Ref. 15) | Blackmore et al. (Ref. 65) | Hill (Ref. 69) | Babeck et al. (Refs. 27, 30, 63) |
|---------------------------------------|------|---------------|--------------------------|--------------------------|--------------------------|----------------|---------------------------------|
| CO off-rate (photolysis)              | $k_p$ | $1 \times 10^6$ s$^{-1}$ |                          |                          |                          |                |                                 |
| CO-CuB off-rate                       |      |               |                          |                          |                          |                |                                 |
| CO-CuB on-rate (reverse)              |      |               |                          |                          |                          |                |                                 |
| $O_2$-CuB on-rate                     | $k_1$ | $3.5 \times 10^4$ M$^{-1}$ s$^{-1}$ |                          |                          |                          |                |                                 |
| $O_2$-CuB off-rate                    |      |               |                          |                          |                          |                |                                 |
| Peroxy formation (a)                  | $k_3$ | $5 \times 10^4$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Peroxy formation (CuB)                | $k_4$ | $5 \times 10^4$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Peroxy back reaction                  |      |               |                          |                          |                          |                |                                 |
| Peroxy protonation                    |      |               |                          |                          |                          |                |                                 |
| Compound "P" formation (R)            | $k_5$ | $5 \times 10^4$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Compound "P" formation (CuB)          | $k_6$ | $5 \times 10^4$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Compound "F" formation (a)            |      |               |                          |                          |                          |                |                                 |
| Compound "F" back reaction (CuB)      | $k_7$ | $5 \times 10^5$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Ferryl to ferryl(X)                   |      |               |                          |                          |                          |                |                                 |
| Ferryl(X) back reaction               |      |               |                          |                          |                          |                |                                 |
| Hydroxy formation (ferryl)            | $k_8$ | $8 \times 10^2$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Hydroxy formation (ferryl(X))         |      |               |                          |                          |                          |                |                                 |
| Pulsed formation                      | $k_9$ | $6 \times 10^3$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Oxidation of CuB by CuA               | $k_{10}$ | $7 \times 10^3$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Oxidation of CuB by $a$               |      |               |                          |                          |                          |                |                                 |

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The redox centers in parentheses indicate the source of the electrons for each of the reactions. The (X) notation is that of Sucheta et al. (15). All of the numbered rate constants are those that were used in the calculations presented in this study. The "R" represents a redox equivalent residing on a protein residue.

| Reaction                              | Rate | This work     | Sucheta et al. (Ref. 38) | Sucheta et al. (Ref. 15) | Blackmore et al. (Ref. 65) | Hill (Ref. 69) | Babeck et al. (Refs. 27, 30, 63) |
|---------------------------------------|------|---------------|--------------------------|--------------------------|--------------------------|----------------|---------------------------------|
| CO off-rate (photolysis)              | $k_p$ | $1 \times 10^6$ s$^{-1}$ |                          |                          |                          |                |                                 |
| CO-CuB off-rate                       |      |               |                          |                          |                          |                |                                 |
| CO-CuB on-rate (reverse)              |      |               |                          |                          |                          |                |                                 |
| $O_2$-CuB on-rate                     | $k_1$ | $3.5 \times 10^4$ M$^{-1}$ s$^{-1}$ |                          |                          |                          |                |                                 |
| $O_2$-CuB off-rate                    |      |               |                          |                          |                          |                |                                 |
| Peroxy formation (a)                  | $k_3$ | $5 \times 10^4$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Peroxy formation (CuB)                | $k_4$ | $5 \times 10^4$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Peroxy back reaction                  |      |               |                          |                          |                          |                |                                 |
| Peroxy protonation                    |      |               |                          |                          |                          |                |                                 |
| Compound "P" formation (R)            | $k_5$ | $5 \times 10^4$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Compound "P" formation (CuB)          | $k_6$ | $5 \times 10^4$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Compound "F" formation (a)            |      |               |                          |                          |                          |                |                                 |
| Compound "F" back reaction (CuB)      | $k_7$ | $5 \times 10^5$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Ferryl to ferryl(X)                   |      |               |                          |                          |                          |                |                                 |
| Ferryl(X) back reaction               |      |               |                          |                          |                          |                |                                 |
| Hydroxy formation (ferryl)            | $k_8$ | $8 \times 10^2$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Hydroxy formation (ferryl(X))         |      |               |                          |                          |                          |                |                                 |
| Pulsed formation                      | $k_9$ | $6 \times 10^3$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Oxidation of CuB by CuA               | $k_{10}$ | $7 \times 10^3$ s$^{-1}$ |                          |                          |                          |                |                                 |
| Oxidation of CuB by $a$               |      |               |                          |                          |                          |                |                                 |
The decay of the primary (Fe-O$_2$) intermediate (A) to a peroxo species requires two electrons; one is supplied from the iron atom of heme $a_3$, which is formally converted to its ferric oxidation state as discussed above, and the other may originate from either Cu$_B$ or heme $a$. Thus, two separate pathways are indicated for the decay of the primary intermediate. In Fig. 6, the pathway via $k_4$ involves the oxygen getting the electron from Cu$_B$, and the $k_3$ pathway involves the electron transfer from heme $a$. The rate constants used in these parallel steps are those measured previously by comparing the decay of the primary intermediate in the fully reduced enzyme to that in the mixed valence enzyme (18). It has been established (66) that heme $a$ and Cu$_A$ are in equilibrium with rate constants for this redox coupled pair of $k_{10}$ and $k_{-10}$ for the transfer of an electron from Cu$_A$ to heme $a$ of $7 	imes 10^3$ s$^{-1}$ and $1 	imes 10^4$ s$^{-1}$, respectively. The maintenance of charge neutrality (67) is a necessity in the redox process. Thus, we have placed a proton on the peroxo intermediate in the pathway involving electron transfer from heme $a$ but not from Cu$_B$. In later steps, the charge neutrality is maintained by the presence of a hydroxide group on the Cu$_B$ atom.

Although, in the scheme depicted in Fig. 6, we show peroxo intermediates, there is no evidence for the build-up of such intermediates in our measurements. However, in different types of experiments reported by other groups, there is strong evidence for the presence of a 607-nm species, compound P, with an oxo-ferryl structure (31). We postulate that this arises when the first electron is supplied from Cu$_B$ (the $k_2$ pathway in Fig. 6) rather than the $k_3$ pathway involving the transfer of an electron from heme $a$. Thus, in the absence of electron transfer from heme $a$ to the $a_2$-Cu$_B$ site, the need of another electron for the decay of the peroxo species into the oxo-ferryl species presumably originates from an amino acid residue thereby forming a radical species (30, 31, 38, 68). This is consistent with the observation that in the mixed valence enzyme, the 607 nm species is generated and it has a $-800$ cm$^{-1}$ frequency of the Fe-O stretching mode (30).

The decay of the peroxo intermediates and intermediate P are the only steps for which we do not have well defined rate constants. We have been unable to detect these intermediates in our data, so we assume that their lifetimes are very short and assign rate constants ($k_{10}$, $k_{11}$, and $k_{-10}$) of $5 	imes 10^3$ s$^{-1}$ for the parallel steps leading to the ferryl intermediate, F. From the data presented here on the decay of the ferryl intermediate ($k_6$) to a hydroxide intermediate, “H,” we assign a rate constant of $8 \times 10^5$ s$^{-1}$. This value is very close to that reported by Hill (69) and by Varotsis et al. (27), as may be seen in Table I. Finally, we assign a rate constant of $6 \times 10^2$ s$^{-1}$ to the decay ($k_{9}$) of the hydroxy intermediate, although this needs to be confirmed by obtaining data at longer times. A similar value for this rate was reported by Varotsis et al. (27).

Under the conditions in which electron transfer from heme $a$ can take place, the enzyme rapidly forms the 580-nm species, F, via an electron from the heme $a$-Cu$_A$ center and one from Cu$_B$. Under our conditions, this is the dominant pathway. The same behavior appears to be present in the experiments reported by Babcock and co-workers (25, 27, 63). The absence of a “607-nm” intermediate in a single turnover experiment is also fully consistent with the earlier observations of Orii, who, by using optical absorption difference spectra, could not detect a line at 607 nm during the reaction and thereby concluded that the peroxo intermediate had a lifetime that was too short to detect under his conditions (14). We believe that the same is
true in our case. If the enzyme passed through an intermediate that had an oxo-ferryl structure, we would expect to see changes in the position of the mode in the 800 cm\(^{-1}\) region. Proshlyakov et al. have shown that the mode associated with the 607-nm band in the absorption spectrum appears at 804 cm\(^{-1}\), whereas that associated with the optical transition at 580 nm appears at 785 cm\(^{-1}\) (31). We detect no change in the position of the line in the 780–810 cm\(^{-1}\) region, as would be expected from the transition from a 607-nm intermediate to the 580-nm intermediate. However, in deuterated buffers, the pathway that leads to the 607-nm species with an \(\sim800\) cm\(^{-1}\) Fe-O stretching mode, intermediate P, is formed. It is clear from these experiments that the kinetics of the decay of the P intermediate are dependent on the experimental conditions. It is detectable in deuterated buffers and when electrons are apparently unavailable from heme a. In addition, it is detectable in the protocol used by Kitagawa and co-workers but not in that used by the present authors or that used by Babcock and co-workers.

Recently, Wikstrom and co-workers reported that in membranous preparations proton translocation occurred in two separate phases (42). During the oxidative phase, in which the enzyme becomes oxidized by the transfer of four electrons to the di-oxygen, approximately one half of the charge is translocated. The remaining charge is translocated only when the enzyme is re-reduced in the so-called reductive phase. Thus, at the end of the oxidative phase, the protein is in a metastable state in which energy released by the reduction of the oxygen is stored for the subsequent translocation of charge. In the scheme in Fig. 6, this metastable state would likely be the H intermediate. However, it is unclear whether or not under solution conditions a metastable state could exist. Thus, the H intermediate may not have the same structure as the “O-” energized state reported by Verhovsky et al. (42) in which the enzyme was located in vesicular membranes.

With the reaction scheme in Fig. 6 and the listed rate constants, we are able to simulate the time dependence of the populations of the various intermediates as shown in Fig. 7. Whether or not the P intermediate can be observed depends on the electron transfer rate from P to F, \(k_5\), as well as the relative rates of the \(k_7\), \(k_8\), \(k_9\) leg of the oxidative phase versus the \(k_3\), \(k_6\) leg. If the rate of formation of F via the \(k_9\), \(k_6\) leg is much faster than its rate of formation via the \(k_7\), \(k_8\), \(k_9\) leg, there will be no build-up in the population of P so it will not be detected. Similarly even if the dominant pathway is via the \(k_4\), \(k_5\), \(k_7\) leg, there will be no build-up of P if its decay rate, \(k_7\), is very fast compared with its formation rate. We assume that \(k_9\), \(k_6\), and \(k_7\) are all fast in Fig. 7 so that none of the intermediates on the pathway from A to F build up and the intermediate F is the dominant species. On the other hand, when no electrons are available from the cytochrome \(a\) -CuA site as in the mixed valence enzyme, then both \(k_9\) and \(k_7\) can be treated as zero and the population of the P intermediate can develop, making the intermediate detectable (30). In addition to the populations of the oxygen intermediates, the electron transfer events can be modeled by calculating the oxidation state of heme \(a\) as illustrated in both Figs. 2 and 7.

The simulations of the population of the intermediates and the oxidation states show several interesting features. First, there is significant overlap among the intermediates such that none can be isolated in a pure form. Second, two peroxo intermediates are postulated from the simulations, but at low concentrations. For the rate constants we used in the simulations, the peroxo and the P intermediates never attained populations greater than a few percent of the sample. Third, the ferryl species is dominant, reaching a population of over 80% of the sample at about 200 \(\mu s\). Its small decay rate allows for this population build-up. Fourth, heme \(a\) displays a rapid oxidation followed by a small but clear dip in its oxidation state and then a very gradual continued oxidation. Such changes have been also reported in the contribution to the optical absorption spectrum from heme \(a\) (69). This dip in the oxidation state of heme \(a\) results from the equilibrium between heme \(a\) and CuA.

**Time Dependence of the Intermediates**—To test the model in Fig. 6, we have measured the time dependence of three of the catalytic intermediates: the primary oxy species, the ferryl species, and the hydroxy species. These data were obtained by measuring the amplitudes in the \(^{16}\)O\(_2\) minus \(^{18}\)O\(_2\) difference spectra for each of these intermediates and are plotted in Fig. 4 versus \(t\) the calculated time dependence obtained from the scheme shown in Fig. 6 with the rate constants described above and listed in Table I. The time dependence supports the reaction scheme, but several points merit discussion. First, the decay data for the primary oxy intermediate (A) indicate that its actual decay rate could be slightly smaller than the one used in the simulations. However, “best” fit rate constants were used to accommodate the decay of the oxy intermediate, the formation of the ferryl intermediate, and the oxidation of heme \(a\), to be discussed below. Since the rate of formation of the primary intermediate is experimentally limited by our apparatus, the time dependence of data earlier than \(\sim15\) \(\mu s\) could not be determined. The second point regarding these data involves the absence of any peroxo intermediate. We do not detect a genuine peroxo intermediate or a P intermediate in our data. Within our model and the rate constants listed in Table I, the peroxo or P intermediates are present only at very low populations (a few percent) and thus would not be seen in our data.

Finally, the decay of the hydroxy intermediate is worthy of comment. We see in several data sets that the hydroxy intermediate decays on the few millisecond time scale. All of our experiments were carried out in \(H_2\)\(^{16}\)O, including those in which we introduced \(^{18}\)O\(_2\). If the hydroxide is able to exchange rapidly with the water, as has been proposed by others, then in our difference spectra we would detect a decrease in the intensity of the hydroxy intermediate although its true magnitude may continue to increase. Thus, its decay rate could be overestimated. However, we do know that at long times (seconds) there is no evidence for a hydroxy species since no oxygen isotope difference could be detected.\(^3\) The heme \(a_2\) in this form is likely to be either five-coordinated or six-coordinated with a water molecule as its sixth ligand. We have postulated that the hydroxy intermediate is a high spin species. To evaluate this, we measured the spin distribution of heme \(a_2\) from the data in Fig. 1 by measuring the relative intensities of the high spin marker line at about 1570 cm\(^{-1}\) and the low spin marker line at about 1584 cm\(^{-1}\) after subtracting out the contribution from low-spin heme \(a\). The high spin contribution from heme \(a_2\) is plotted in Fig. 5 versus \(t\) the calculated populations of the ferryl, the hydroxy and the oxidized intermediates. The data demonstrate that the high spin population grows with the formation of the hydroxy intermediate and continues to grow as it decays. The black dashed curve in the figure is the population of the sum of the hydroxy intermediate and the fully oxidized state. The agreement between the high spin formation and the sum of the hydroxy and oxidized intermediates is self-evident. We conclude that either both of these intermediates are high spin or that we are only detecting the high spin hydroxy intermediate due to the exchange of the \(^{16}\)OH\(^{-}\) with the \(^{18}\)OH\(^{-}\) as discussed above.

**Oxidation of Heme a**—From the scheme presented in Fig. 6,

\(^3\) T. K. Das, R. B. Gennis, and D. L. Rousseau, unpublished results.
the oxidation of heme $a$ can be modeled as shown in Fig. 7. By monitoring the high frequency region of the resonance Raman spectrum, changes in the oxidation state of heme $a$ may be determined experimentally. Resonance Raman scattering is an extremely reliable technique to monitor the oxidation state because each oxidation state has isolated lines in the spectrum (18). In Fig. 2 we compare the experimental data with the model. The agreement between the data and the calculation validates the model. The rapid oxidation corresponds to the decay of the primary oxy intermediate via electron transfer from heme $a$ (18); the plateau, which actually contains a slight dip in the intensity, results from the equilibrium between heme $a$ and CuA, and the gradual increase beyond a few hundred microseconds corresponds to the decay of the ferryl intermediate to the H intermediate associated with the electron transfer to the heme $a$-CuB center from the heme $a$-CuA sites. These results are fully consistent with the time-dependent changes in the optical spectra at 445 and 605 nm that are most sensitive to the oxidation state of heme $a$ and that show qualitatively similar changes in the intensity (69).

The model and the data reported here demonstrate that at times longer than $\sim 100$ $\mu$s, approximately 70% of heme $a$ is oxidized. This is in sharp contrast to the conclusions drawn by Sucheta et al. (15, 38). They report that, at about 500 $\mu$s when the ferryl intermediate reaches its maximum population in both our data and theirs, heme $a$ is only approximately 30% oxidized. Thus, their interpretation that there are two ferryl intermediates, the second of which has heme $a$ in its reduced state is inconsistent with our data. The optical changes that Sucheta et al. detect and attribute to a state in which heme $a$ is reduced could originate from a conformational change of the enzyme that affects its optical properties. Additional experiments are needed to clarify this issue.

Conclusions—The determination of the structures of the intermediates in the reduction of oxygen by cytochrome $c$ oxidase and their kinetics remains an important problem that is central to elucidating the mechanism of proton translocation in this enzyme. This is especially important now in light of the recent discovery that proton translocation occurs in two distinct phases, one during the oxidative phase and one during the reductive phase of the enzymatic cycle (42). It suggests that the chemical energy released by oxygen reduction is stored in the protein at the end of the oxidative phase prior to the re-reduction and is subsequently utilized for the proton translocation.

This energy may be stored locally in bonds between the metals in the bi-nuclear center and exogenous ligands, such as the oxygen reduction products (43). Comparisons of the structures of intermediates and their kinetics for intermediates formed under solution conditions with those formed in membranous preparations could help to identify the location of the stored energy.

The kinetics of the formation and decay of the oxy, ferryl, and hydroxy intermediates and the oxidation of heme $a$ can be accounted for by the simple model presented here. The presence of the $(607$ nm) intermediate, P, was not detected in our measurements so we have inferred that it is rapidly converted to the F intermediate under our conditions as well as those of others. The development of a measurable population of P clearly depends on the availability of electrons from the heme $a$-Cu$_B$ site as well as on the chemical conditions. Although possible structural differences between the P and F states that result in large differences in their optical properties remains undetermined, the presence of differing stable conformational states in other liganded forms of heme $a_2$ suggests that the P and the F intermediates may represent conformational differences in the binuclear center resulting from changes in the distance between the iron of heme $a$ and Cu$_B$. The change in distance may be modulated by the formation of a radical on Tyr-244 which through a posttranslational modification is bonded to His-240, one of the ligands to Cu$_B$ (3, 6). Experiments are in progress to test this possibility.

Acknowledgments—We thank Drs. Syun-Ru Yeh and Tapan Das of Albert Einstein College of Medicine for helpful discussions.

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