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Time Evolution of the Intermediates Formed in the Reaction of Oxygen with Mixed-Valence Cytochrome *c* Oxidase

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Abstract: The intermediates formed in the reaction of oxygen with mixed-valence cytochrome *c* oxidase were followed as a function of time by monitoring the resonance Raman and optical absorption spectra with a flow–flash–probe technique. At early times (50 μs), the optical absorption spectra confirm the presence of the primary intermediate (I_m). This intermediate has an Fe–O_2 stretching mode in the resonance Raman spectrum at 568 cm^{-1} that was used as a marker for its population. By following the change in intensity of this mode, the first-order rate constant for the decay of I_m was measured to be 4.5 × 10^3 s^{-1}. The formation of the next intermediate (II_m) of the enzyme with a 607-nm peak in the optical absorption difference spectrum was monitored by its optical absorption spectrum and displays a first-order rate constant for its formation, which is the same as the rate constant for the decay I_m. The resonance Raman spectrum of I_m and II_m are nearly identical, indicating that the π-electron depletion of the porphyrin macrocycle in cytochrome a_3 is the same for these two intermediates. The absorption spectrum and the resonance Raman spectrum of an intermediate formed by mixing oxygen with CO-bound mixed-valence cytochrome *c* oxidase and allowing O_2 to spontaneously replace CO are the same as those of II_m formed in the photolysis experiments. II_m, formed in this way, decays biphasically to the resting form of the enzyme.

Introduction

Cytochrome *c* oxidase, the terminal enzyme in the electron-transport chain, catalyzes the four-electron reduction of dioxygen to water.[1] Concomitantly, it translocates protons across the inner mitochondrial membrane. The enzyme contains four redox centers: two heme groups and two copper atoms. Oxygen binds to a binuclear site formed by one of the heme groups (cytochrome a_3) and one of the copper atoms (Cu_B). The other two redox centers, cytochrome *a* and Cu_A, are involved in the electron-transfer function from cytochrome *c* to the binuclear oxygen binding site and have been postulated to play essential roles in proton translocation.[2] The molecular mechanism of oxygen reduction is not yet fully understood because it is difficult to obtain structural information on the state of the oxygen in the various intermediates that are formed during its reduction. In the past, most information has been obtained from optical absorption and EPR measurements. However, the detailed structure of the various intermediates has been difficult to determine by these techniques. More recently, resonance Raman scattering has been applied to this problem and has yielded some useful information concerning the early intermediates in the reduction process.[3-7] A full elucidation of the structure and the electron configuration of various intermediates is of utmost importance for understanding the mechanism by which this enzyme reduces oxygen to water so

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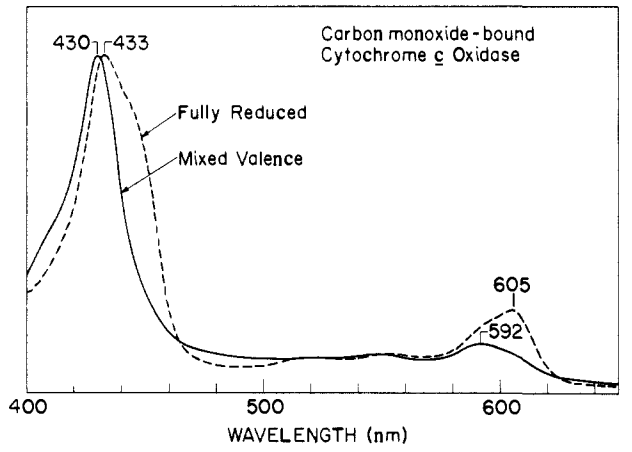

Figure 1. Optical absorption spectra of carbon monoxide bound cytochrome *c* oxidase in the fully reduced [$a^{2+}Cu_A^{+}a_3^{2+}$–CO Cu_B^{+}] and the mixed-valence [$a^{3+}Cu_A^{2+}a_3^{2+}$–CO Cu_B^{+}] forms. The intensity of the mixed-valence form was lowered to be on the same scale as that of the fully reduced form.

rapidly as well as for understanding the mechanism of proton translocation.

In order to isolate the various electron-transfer events and the effects of the redox states of specific sites on the others, it is useful to study forms of the enzyme in which some of the redox centers are oxidized, and thereby incapable of transferring electrons to the oxygen, and compare them to forms of the enzyme in which all of the redox centers are reduced. In particular, "mixed-valence" cytochrome oxidase is a useful form of the enzyme that has been studied extensively.[8-13] In this form of the enzyme the low-potential sites, cytochrome *a* and Cu_A, remain in the oxidized state whereas cytochrome a_3 and Cu_B in the binuclear binding site are reduced. In addition, in the mixed-valence form of the enzyme the decay of the early oxygenated intermediate is much slower than that in the fully reduced enzyme. It therefore serves as a useful model for the study of the early events in the dioxygen reduction process.

Recently, we obtained the resonance Raman spectrum of the primary intermediate (I_m) in the reaction of oxygen with the mixed-valence enzyme.[6] We were able to confirm that I_m was formed under our conditions by obtaining an optical absorption spectrum. The Raman spectrum in the high-frequency region, as well as the frequency of the Fe–O_2 stretching mode at 568 cm^{-1} indicate that I_m has properties and a structure very similar to those of oxyhemoglobin and oxymyoglobin. Similarly, the Fe–O_2 stretching mode in the fully reduced enzyme was observed at the same frequency (568 cm^{-1}), indicating that there is no difference in the structure at the binuclear site for I_m in the mixed-valence and the fully reduced forms of the enzyme.[7] By following the intensity of the Fe–O_2 stretching mode, the first-order rate constant for the decay of I_m in the fully reduced enzyme was determined[7] to be 3.5×10^4 s^{-1}.

In this report we present the time evolution of the intermediates generated by the reaction of oxygen with the mixed-valence form of the enzyme. By following the intensity of the Fe–O_2 stretching mode, the rate constant for the decay of I_m can be determined.

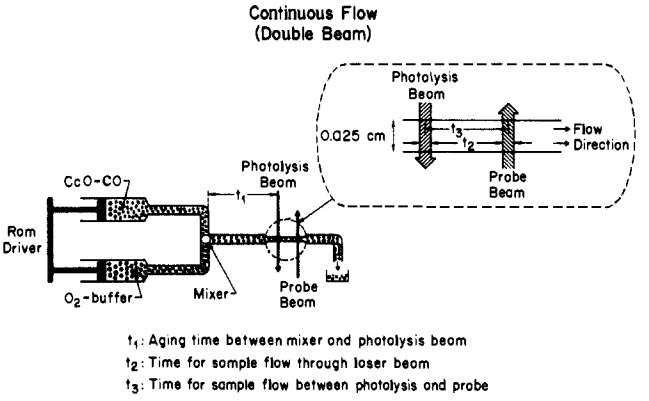

Figure 2. Continuous-flow apparatus for obtaining time-resolved resonance Raman and optical absorption spectra. Samples of CO-bound cytochrome *c* oxidase are mixed in a Wiskind four-grid mixer (Update Instruments) and flow into the observation cell where the CO is photodissociated to initiate the reaction between oxygen and the enzyme. The cell is 2 cm long and has a internal cross section of 0.25 mm by 0.25 mm. The time resolution is determined by the flow rate, the cell cross-sectional area, and the spacing between the sample and probe beams. For resonance Raman measurements the probe is a laser beam, and for optical absorption it is a tungsten lamp. In the experiments reported here, the aging time between the mixer and the photolysis beam, t_1, was 0.15 s and the time for sample flow through the photolysis of probe beams, t_2, was 25 μs. The probe minus photolysis delay time could be varied from 0 to several milliseconds.

By following the change in the intensity of the optical absorption spectrum, the rate constant for the formation of the next intermediate (II_m) can also be obtained. We find that these two rate constants are the same as would be expected if there is a direct conversion from one to the other. In addition, by following both the resonance Raman spectrum and the optical absorption spectrum as a function of time after the formation of II_m, the decay of this intermediate may be monitored. Our data demonstrate that II_m decays to an oxidized form of the enzyme, which further decays to the resting enzyme.

Methods and Materials

Cytochrome *c* oxidase was isolated from bovine heart muscle by the method of Yoshikawa et al.[14] It was stored under liquid nitrogen until ready to use. The enzyme was solubilized in phosphate buffer (100 mM) at pH 7.4 with 0.2% dodecyl β-D-maltoside. Samples of anaerobic resting cytochrome *c* oxidase were exposed to carbon monoxide and incubated for several hours. The absorption spectrum resulting from this incubation is shown in Figure 1. The loss of intensity at about 444 and 605 nm confirms the presence of cytochrome *a* in the ferric state, and the absorption bands at 430 and 592 nm confirm the presence of CO-bound cytochrome a_3.[10] The CO incubation procedure that we used has been shown by others[10,15] to result in the two-electron-reduced enzyme (i.e., $a^{3+}Cu_A^{2+}a_3^{2+}$–CO Cu_B^{+}).

To study the reaction of oxygen with mixed-valence cytochrome *c* oxidase, we use a continuous-flow technique. This is an adaptation of the flow-flash-probe method originally pioneered by Gibson and Greenwood.[16] In our previous study[6] of the mixed-valence enzyme, a single laser beam was used both to photolyze the CO-bound enzyme and to give rise to the scattering for the resonance Raman spectrum. In the present study we use two laser beams, the first to photolyze the sample and the second to probe the resulting species. A schematic diagram of this method is shown in Figure 2. A sample of the CO-bound enzyme (200 μM before mixing) is placed in one syringe of the mixing apparatus. Oxygen-saturated buffer is placed in the other syringe. The solutions pass through the mixer and then flow into the sampling area. The first beam (413.1 nm) photodissociates the CO from the enzyme, allowing O_2 to react with it. A second beam (413.1 nm), which may be continuously translated with respect to the first, probes the resulting spectrum. There are three time regimes that are important in this apparatus. The first

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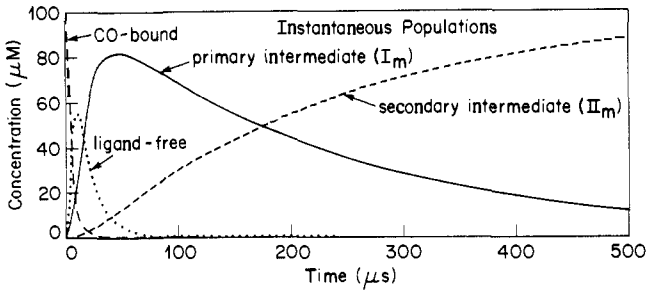

Reaction Scheme for Generating Populations

$$E_m\text{-}CO \xrightarrow[h\nu]{k_p = 2 \times 10^5\ s^{-1}} E_m + O_2 \underset{k_{-1} = 2 \times 10^3\ s^{-1}}{\overset{k_1 = 1 \times 10^8\ M^{-1}\ s^{-1}}{\rightleftharpoons}} I_m \xrightarrow{k_2 = 4.5 \times 10^3\ s^{-1}} II_m$$

Figure 3. Populations of the early intermediates in the reaction of oxygen with mixed-valence cytochrome *c* oxidase. In the reaction scheme, k_p, the rate of photolysis of the CO-bound mixed-valence enzyme (E_m–CO) was determined from measurements of the photolysis in the absence of oxygen; the association, k_1, and dissociation, k_{-1} rate constants in the reaction of the ligand-free mixed-valence enzyme, E_m, with oxygen were adopted from those reported by other workers;[37,38] and the rate constant, k_2, for the decay of I_m to II_m was determined by the experiments reported here.

time, t_1, is the time for the sample to pass from the mixer to the photolysis beam. This time must be short enough to avoid significant spontaneous replacement of CO by O_2. With the flow rates used in the experiments reported here, this time was 0.15 s. The characteristic time for spontaneous CO replacement by O_2 is approximately 5 s under our conditions. This measured time in our apparatus is similar to that reported by others.[17] Therefore, the flow is sufficiently fast so that there should be very little spontaneous replacement of CO by O_2. The second time, t_2, is the time it takes for the sample to pass through the photolysis beam or the probe beam. With our geometry and flow rate used in the present investigation, these time windows are 25 μs. The third time of interest, t_3, is the time for the sample to flow from the photolysis beam to the probe beam. We are able to vary this time continuously up to a few milliseconds by translating the photolysis beam away from the probe beam. We used this technique rather than the single-beam technique that was used in our prior work,[6,7] since to study long-lived intermediates with a single beam, the sample flow rate has to become very slow so that some spontaneous replacement of CO by O_2 occurs. Furthermore, with a single beam at a low flow rate, the sample residence time in the laser is long, and thus, photoeffects of the sample by the incident laser light may occur.

Direct replacement of CO by O_2 in the mixed-valence enzyme was done by first forming the CO-bound form as described above. The enzyme was then exposed to an atmosphere of O_2 and shaken vigorously for several seconds. The sample was split into two for simultaneous measurements of the resonance Raman spectra and the optical absorption spectra. The entire process of mixing the enzyme with oxygen and loading the observation cells took ~30 s.

Resonance Raman spectra were obtained by projecting the scattered light from the probe region onto the entrance slit of a single monochromator for dispersion and subsequent detection by a linear photodiode array (PAR Reticon). With our optics, a window of about 500 cm^{-1} could be obtained. Optical absorption spectra were measured with the same apparatus by replacing the probe beam by a white light source that was directed through the sample directly onto the entrance slit of the monochromator. In this way, absorption spectra in the presence of the lasers were recorded under exactly the same conditions as the Raman measurements. Conventional absorption spectra were obtained on an SLM Aminco DW-2000 spectrometer.

Results

In order to understand the results of experiments on molecules that are evolving in time, it is necessary to have a framework so that appropriate time scales can be examined. Such a framework is given by the population curves shown in Figure 3. The rate constants used to calculate these curves will be discussed in the next section of this paper. It should be noted that I_m rises to its maximum population at about 50 μs and it decays slowly over a several hundred microsecond time period.[18] Concomitantly, II_m steadily rises throughout a several hundred microsecond time course. We have carried out measurements throughout this time range in order to obtain the spectra of each of these intermediates and determine their kinetic properties.

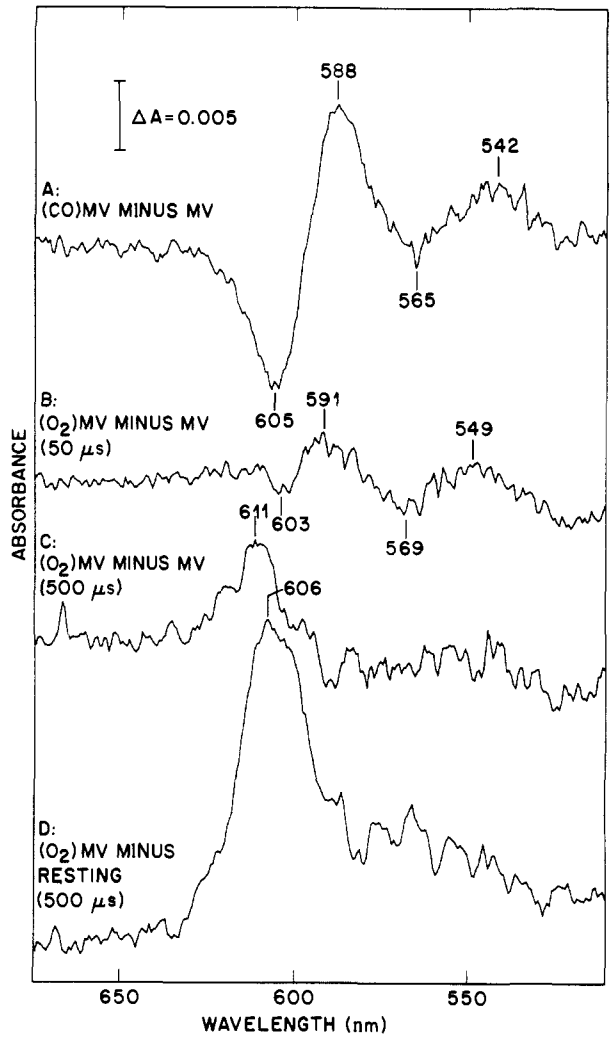

Figure 4. Visible absorption difference spectra for various states of cytochrome *c* oxidase obtained in the continuous-flow apparatus: A, CO-bound mixed-valence enzyme minus the mixed-valence enzyme generated by photodissociating the CO-bound form; B, reaction product of photodissociated mixed-valence enzyme with oxygen minus the ligand-free mixed-valence enzyme at a 50-μs delay between the probe and photolysis beams; C, as in B but with a 500-μs delay; D, difference spectrum generated by subtracting the spectrum of the resting enzyme from the reaction product at 500 μs.

Decay of I_m. To confirm the presence of I_m and II_m, we obtained optical absorption difference spectra throughout the time range of interest (Figure 4). In spectrum A, the difference spectrum between the mixed-valence CO-bound species and the photolyzed species is presented. This difference spectrum with maxima and minima at 565, 588, and 605 nm agrees with those that have been reported by others in room-temperature measurements.[10,11] In spectrum B, the difference spectrum of I_m is obtained by subtracting the photolyzed spectrum from the oxygenated species formed after photolysis resulting in features at 569, 591, and 603 nm, in agreement with those reported by others for I_m.[11,12,19] This

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(18) In our nomenclature, the first intermediate in the reaction of the mixed-valence enzyme with oxygen is I_m and is equivalent to intermediate I_m in the nomenclature of Clore et al.[9] and to compound A in the nomenclature of Chance et al.[8] The next intermediate that we detect with a maximum in the visible difference spectrum at 607 nm has been labeled as intermediate III_m by Clore et al.[9] and compound C by Chance et al.[8]

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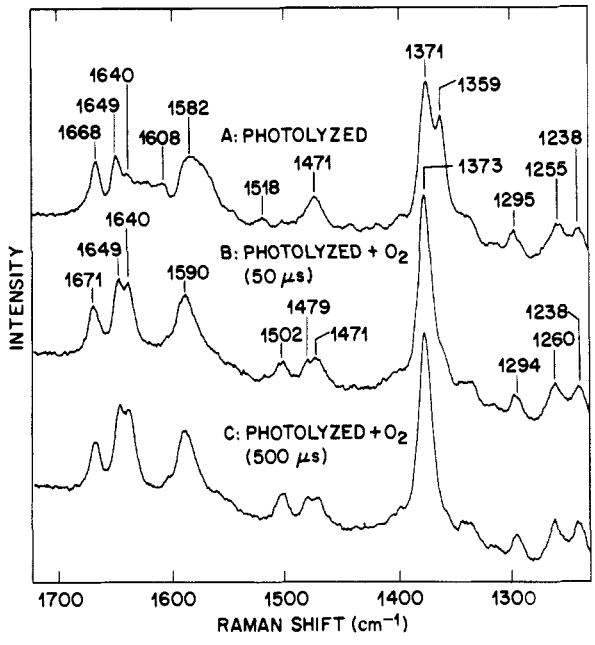

Figure 5. Resonance Raman spectra (413.1-nm excitation) of various forms of cytochrome *c* oxidase: A, ligand-free mixed-valence cytochrome *c* oxidase generated by photodissociating the carbon monoxide bound enzyme; B, reaction of photodissociated enzyme with oxygen at a separation between the probe and photolysis beams resulting in a time delay of 50 μs; C, same as B but with a 500-μs delay. The integration time for each spectrum was 6 s.

spectrum was obtained with a 50-μs delay between the probe beam and the photolysis beam. In spectrum C, the difference spectrum of the oxygenated intermediate minus the fully photolyzed spectrum is presented at a 500-μs delay. Significant differences are evident in the comparison between this spectrum and that obtained at a 50-μs delay. Most evident is the loss in the peak at 591 nm and the growth of a peak at 611 nm. Spectrum D is the same as spectrum C, except that it is referenced to a spectrum of the resting enzyme showing a strong absorption at 606 nm. We label this as II_m.[18] It has the same spectral properties as intermediate III_m of Clore et al.[9] and compound C of Chance et al.[8] detected in cryogenic measurements. From these data, it is clear that on going from 50 to 500 μs the difference spectrum characteristic of I_m is converted to one characteristic of II_m.

Resonance Raman spectra of the intermediates corresponding to the optical absorption spectra are presented in Figure 5. Spectrum A is characteristic of the mixed-valence photolyzed species with two peaks in the region of the spectrum that is sensitive to the π-electron density in the porphyrin macrocycle. We assign the 1371-cm^{-1} line as originating from cytochrome *a* in its ferric state and the line at 1359 cm^{-1} as originating from cytochrome a_3 in its ligand-free ferrous state. Similarly, in the higher frequency region, the line at 1668 cm^{-1} results from the formyl group of cytochrome a_3 in its ferrous state while the line at 1649 cm^{-1} results from the formyl group of cytochrome *a* in its ferric state. The oxygenated intermediate examined at 50 μs after CO photolysis is shown in spectrum B of Figure 5. Marked differences are seen in the lines at 1260, 1479, 1502, 1590, 1640, and 1671 cm^{-1}. The intermediate at 500 μs after photolysis is shown in spectrum C. From visual inspection of spectra B and C it is clear that the differences between them are minor. However, confirmation that spectrum B in Figure 5 is indeed that of I_m is obtained by examining the region of the spectrum in which the $Fe-O_2$ stretching mode is located (Figure 6). In these data there is a line at 568 cm^{-1} which we previously assigned as the $Fe-O_2$ stretching mode in this intermediate.[6] Also shown is the decay of the intensity of this line as a function of time. Thus, the species that we are examining at early time (50 μs) is clearly very different from that at long time (500 μs), even though the high-frequency region of the spectrum displayed in Figure 5 is virtually identical.

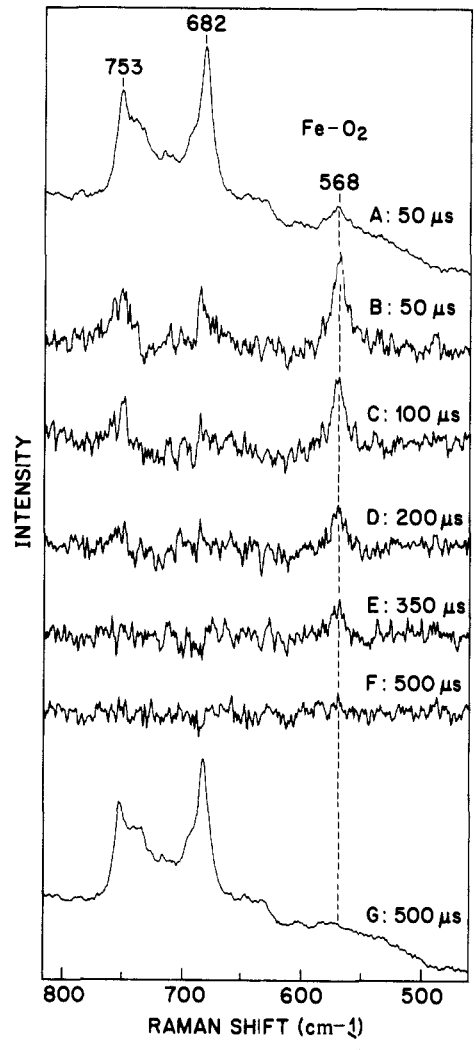

Figure 6. Resonance Raman spectra showing the decay of the $Fe-O_2$ stretching mode (568 cm^{-1}) in mixed-valence cytochrome *c* oxidase as a function of the delay time between the probe and photolysis beams. A and G are the absolute spectra, and B–F are the difference spectra generated by subtracting the spectrum at 750 μs from the spectrum obtained at the indicated time delay. Each spectrum is a result of a 20-s accumulation.

Decay of II_m. II_m may be generated by direct mixing of oxygen with CO-bound mixed-valence cytochrome oxidase.[20] This complex has been observed by others to have a relatively long lifetime.[21] Therefore, it can be studied by using a conventional scanning absorption spectrometer and can be compared with that observed in the flow–flash–probe technique. The visible region optical absorption spectra and resonance Raman spectra in the high-frequency region of II_m generated in this way are shown in Figures 7 and 8, respectively. The optical difference spectra in Figure 7 show the decay of II_m as a function of time starting from spectrum B′, which is obtained at about 80 s after the start of the formation of the complex, progressing through to spectrum E′, which is obtained at 1900 s after formation of the complex. In Figure 8, the corresponding Raman spectra (spectra B–E) were obtained from a parallel sample. Since at 80 s some of the intermediate has decayed and the spectrum of the final form appears to be very similar to that of the resting enzyme as seen in the comparison between spectra E and F of Figure 8, in spectrum A of Figure 8 we have subtracted a small amount of the spectrum of the resting enzyme from the 80-s spectrum. The resulting spectrum (A) agrees well with spectrum C reported in Figure 5, which is that from II_m generated in the continuous-flow

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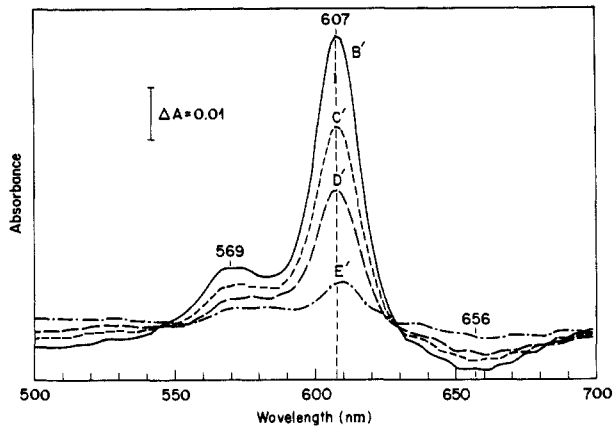

Figure 7. Optical absorption difference spectra showing reaction product minus the resting form of cytochrome *c* oxidase. The reaction was initiated by mixing oxygen with the carbon monoxide bound enzyme. Spectra B′–E′ were obtained at time delays of 80, 170, 300, and 1900 s, respectively, and correspond to the parallel Raman measurements (spectra B–E) shown in Figure 8. The concentration of the enzyme was 100 μM, and the path length of the cell was 0.1 cm.

apparatus by the flow-flash-probe method. The progression of changes in the optical absorption spectrum (Figure 7) indicates that the decay of II_m occurs on a time scale of many tens of seconds. A similar decay is observed in Figure 8 in the resonance Raman measurements. The final Raman spectrum in the decay (spectrum E) is similar to that of the resting form of the enzyme (spectrum F).

Discussion

Properties of the Intermediates. An intriguing finding of these results is the observation (Figure 5) that the Raman spectrum of the oxygenated cytochrome a_3 heme of I_m is nearly identical with that of II_m. The decay in the intensity of the Fe–O_2 stretching mode (Figure 6) and the changes in the optical absorption difference spectrum (Figure 4) confirm that over this time period conversion from I_m to II_m has occurred although only small changes are observed in the Raman spectrum.

The structure of I_m is now well established as being very similar to that of oxyhemoglobin and oxymyoglobin,[6-8] and II_m is believed to be a peroxide-bound form of the heme.[8,11-13] At cryogenic temperatures, the latter has been labeled[8] compound C and has a Raman spectrum[22] similar to that reported here. The data present a dilemma of how two apparently very different ligands can lead to nearly identical Raman spectra of the heme. To resolve this dilemma, we first consider possible structures of the Fe–O_2 complex in I_m. Since it has been established that I_m has the same properties as oxyhemoglobin and oxymyoglobin,[6-8] we examine available data on these oxygen-storage and -transport proteins to address the structural question for I_m. In oxyhemoglobin and oxymyoglobin a long-standing controversy has been whether these oxy complexes are most appropriately described as a ferrous-dioxygen complex or as a ferric-superoxide complex.[8,23-25]

As a first step in understanding the electronic structure involved in the oxygen binding we consider the reported molecular orbital calculations. These indicate that for essentially all forms of the heme group the iron atom has near neutrality.[25] This is achieved by the interplay between the σ-donation from the ligands (including the porphyrin macrocycle and the axial ligands) and the associated π-back-donation to these ligands. Consequently, the degree of electron donation to the bound dioxygen is determined

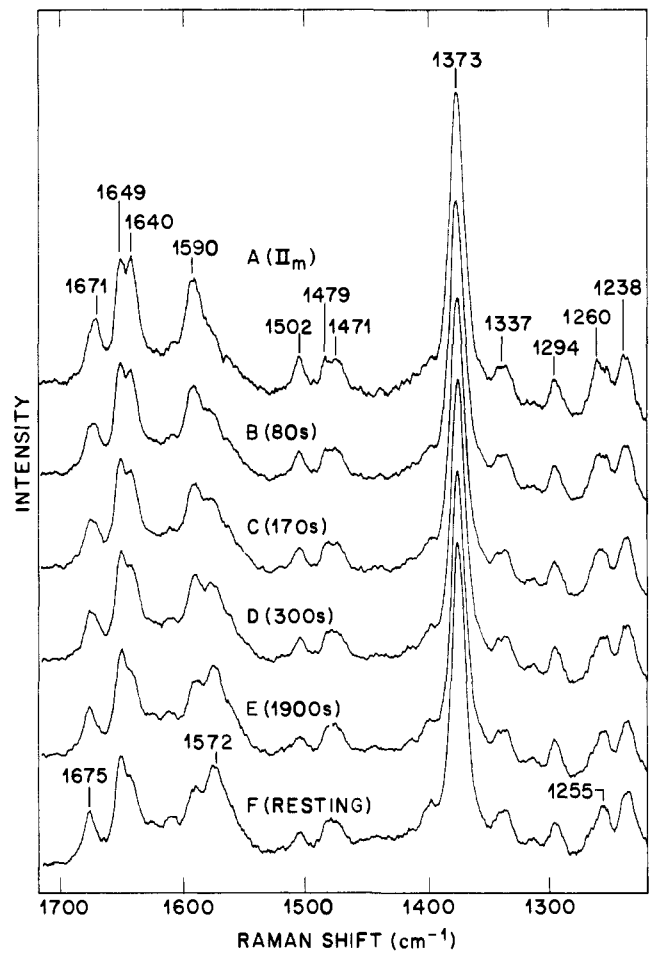

Figure 8. Resonance Raman spectra of the reaction product of cytochrome *c* oxidase with oxygen in a parallel sample to that used for the optical absorption spectra in Figure 7. The indicated time is that subsequent to the moment of the initial exposure of the CO-bound sample to oxygen.

not by examination of the iron atom but by examination of the porphyrin macrocycle and the bound oxygen, since in any case the iron would be expected to retain its neutrality.

All of the available measurements on the oxy complexes are consistent with the concept that significant electron density is transferred to the oxygen in stable oxy complexes. Specifically, the optical absorption spectra of low-spin ferric hemoglobin and myoglobin are very similar to those of oxyhemoglobin and oxymyoglobin.[24] Moreover, the resonance Raman spectra indicate that the same degree of electron density has been withdrawn from the heme in the oxy complexes of these proteins as in their formally ferric forms.[26] Concomitantly, the infrared absorption spectrum indicates that the bound dioxygen has gained electron density. In infrared spectra the O–O stretching frequency is detected at $\sim$1100 cm^{-1}, very close to the superoxide frequency in KO_2 at 1145 cm^{-1}.[27] Finally, the electronic charge density in oxyhemoglobin, as inferred from Mössbauer spectroscopy, is consistent with a low-spin heme.[28] With this technique, however, there is no distinction between ferric and ferrous forms.

Since in the oxygen-transport and -storage proteins many spectroscopic indicators are consistent with a significant degree of electron transfer from the heme group to the bound dioxygen, we infer that in I_m in cytochrome *c* oxidase, a similar degree of electron transfer has occurred and assign the complex as a superoxide. With the assumption that the Fe–O_2 complex is a

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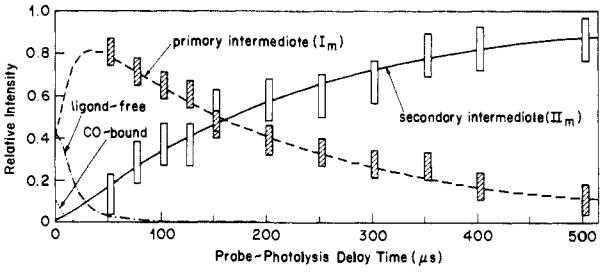

Figure 9. Comparison of the optical absorption and the resonance Raman data to a computer simulation of the intermediate populations under our experimental conditions. The shaded bars are the intensity of the $Fe-O_2$ stretching mode at 568 cm^{-1} in the resonance Raman spectrum as a function of delay time after photolysis. The open bars are the intensities in the optical absorption difference spectrum of the peak in the 607-nm region attributed to II_m. The first-order rate constant for the decay of I_m (or the formation of II_m) is determined to be 4.5×10^3 s^{-1} from a log plot of the Raman (or optical) intensity as a function time. The other constants used for the computer simulation are described in Figure 3.

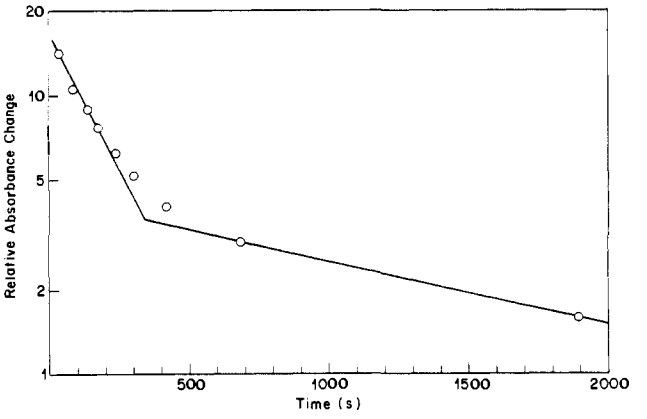

Figure 10. Decay of absorption of 607 nm from II_m as a function of time. The data may be described by a fast and a slow first-order process with rate constants of 5×10^{-3} and 5×10^{-4} s^{-1}, respectively. These data are interpreted as a decay of II_m to the pulsed enzyme and its subsequent decay to the resting state.

superoxide, the similarity between I_m and II_m becomes more easily understood. For both the superoxide complex of I_m and the peroxide complex of II_m, the ligand confers a low-spin configuration on the iron atom and extracts electron density from the heme macrocycle. To account for the observations that the resonance Raman spectra of the porphyrin macrocycle are the same for these two intermediates in the enzyme, it is only necessary to assume that both confer a low-spin configuration on the iron atom and that the electron-withdrawing properties of a superoxide and a peroxide are similar. With the model it should be noted that the decay of I_m does not take place by a concerted two-electron transfer but by what may be formally considered a one-electron transfer to a superoxide complex.

Decay of I_m. In our prior study of mixed-valence cytochrome *c* oxidase, we assigned the $Fe-O_2$ stretching mode in I_m formed by the reaction of oxygen with the enzyme.[6] In the present investigation, we follow the change in intensity of the $Fe-O_2$ stretching mode from which we may determine the kinetic constant for the decay of I_m. Data such as that in Figure 6 were used to determine the change in intensity of I_m as a function of time. The intensities were obtained by subtracting the photodissociated form of the mixed-valence enzyme from the oxygenated intermediate. The resulting data are plotted in Figure 9. In this figure, the dashed curve is the calculated intensity of I_m as a function of time with a rate constant for its decay of 4.5×10^3 s^{-1}. By monitoring the intensity of the band in the 607-nm region of the optical absorption difference spectrum, the formation of II_m may also be followed. These data are plotted as the open bars in Figure 9. The agreement of the data with the solid curve generated with the same rate constant demonstrates that the decay of I_m leads directly to the formation of II_m without formation of an intervening intermediate. The value we obtained, 4.5×10^3 s^{-1}, for the rate constant for the decay of I_m is very consistent with previous reports for this constant.[11-13]

The observation that the relaxation of I_m in the mixed-valence enzyme occurs with a rate constant of 4.5×10^3 s^{-1} shows that the enzyme behaves very differently from the fully reduced enzyme where the decay of I_m has a rate constant of 3.5×10^4 s^{-1}, nearly 1 order of magnitude larger. The functional implication of this difference in rate constant is not known nor is the structural origin for this difference. It has been suggested in the past that the change in oxidation states of the cytochrome *a* and Cu_A sites brings about a conformational transition of the protein, which facilitates an electron transfer from Cu_B to the bound $Fe-O_2$ complex.[13-29] However, the identical frequency for the $Fe-O_2$ stretching mode in the fully reduced[7] and mixed-valence forms[6] of the enzyme argues against a conformational change. Moreover, we have found that direct electron transfer from cytochrome *a* in the fully reduced enzyme can account for this difference without the need to invoke a conformational transition. The implications of direct electron transfer from cytochrome *a* to the binuclear site are discussed in a separate publication.[30]

Decay of II_m. The resonance Raman data in Figure 8 indicate that after 1900 s the spectrum of II_m has converted to that of a high-spin oxidized form of the enzyme that is very similar to that which we obtain for the resting enzyme. However, it is not clear what differences, if any, exist in the resonance Raman spectrum of resting and pulsed oxidase, two high-spin forms of the oxidized enzyme. Data reported by Copeland et al.[31] showed no difference between these forms, whereas differences between a "fast" and "slow" reacting form of the oxidized enzyme were reported by Schoonover et al.[32] The visible optical absorption spectra demonstrate that II_m does not directly decay to the resting state, since at 1900 s the difference spectrum of II_m minus the resting form has a maximum that has moved from 607 to ~610 nm and the isosbestic point at ~628 nm has moved to shorter wavelength. Therefore, it appears that II_m decays to an oxidized form of the enzyme, which then undergoes a subsequent decay.

To study the kinetics of the decay of II_m, in Figure 10 we plot the log of its change in absorbance versus time. The early fast decay has a decay time of ~150 s ($k \sim 5 \times 10^{-3}$ s^{-1}) whereas the slower decay has a decay time of ca. 20 min ($k \sim 5 \times 10^{-4}$ s^{-1}). These decay constants are only considered rough guides as a first-order approach to understanding the possible mechanisms of the decay of II_m.

Other workers have studied the properties of peroxide binding to cytochrome *c* oxidase. The K_d for the reversible peroxide complex has been reported[33,34] to be in the range of 2–10 μM, and the on-rate has been observed[34] to be 700 M^{-1} s^{-1}. At high peroxide concentration, the peroxide complex decays to the ferryl form,[34] but at low peroxide concentration from the reported values of K_d and k_{on} the k_{off} should be in the range of $(1-7) \times 10^{-3}$ s^{-1} (100–700 s). This is consistent with the value of the rate constant for the fast phase we observed in the decay of II_m, i.e., ~150 s. Thus, the decay of II_m may be interpreted as a unimolecular dissociation of the peroxide complex leaving a fully oxidized form of the enzyme.

We interpret the slow decay ($t_{1/2} \sim 20$ min), which we detect subsequent to the decay of II_m, as a conformational change of high-spin oxidized cytochrome a_3. Residual intensity in and a

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slight shift of the visible band in spectrum E′ of Figure 7 suggest the existence of the pulsed state.[35,36] The slow decay rate constant that we observe is consistent with the pulsed to resting transition reported by others[35] so we tentatively make that assignment. The overall decay of II_m under our conditions may, therefore, be written as

$$II_m \underset{k_{on} = 7 \times 10^2\ M^{-1}\ s^{-1}}{\overset{k_{off} = 5 \times 10^{-3}\ s^{-1}}{\rightleftharpoons}} H_2O_2 + \text{pulsed} \xrightarrow{k = 5 \times 10^{-4}\ s^{-1}} \text{resting}$$

Additional experiments are needed to confirm this decay mechanism.

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

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Single-Crystal Electron Spin-Echo Envelope Modulation Study of Cu(II)-Doped Zinc Bis(1,2-dimethylimidazole) Dichloride

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Abstract: Single crystals of copper-doped zinc bis(1,2-dimethylimidazole) dichloride were studied with electron spin-echo envelope modulation (ESEEM) spectroscopy. Modulation frequencies apparent through Fourier transformation of the ESEEM decay curves were attributed to interactions between the Cu(II) unpaired electron and remote ^{14}N nuclei of the two coordinated dimethylimidazole ligands. Hyperfine and quadrupole coupling tensors were fit to the observed magnetic field angular dependencies of these frequencies according to a least-squares criterion. The two remote nitrogen atoms, although formally magnetically distinct, give essentially equivalent coupling tensor principal values. This agrees with the noncrystallographic, 2-fold rotation relationship between the two dimethylimidazole rings around the metal ion in the host crystal structure. The isotropic part of the ^{14}N hyperfine tensor was found to be smaller than reported in other model imidazole compounds previously studied by ESEEM spectroscopy in frozen solutions. This trend is consistent with the change in geometry of ligands around the copper from presumed approximate square planar in frozen solution to near tetrahedral in the present system. The maximum hyperfine principal direction is not along the Cu(II)-N(remote) vector but subtends an angle of ≈40° from this direction. A simple multipoint electron spin-nucleus dipolar hyperfine calculation failed to reconcile this difference. This result and others suggest that a nonnegligible contribution to the hyperfine coupling originates from spin density located on the imidazole ring. The quadrupole tensor principal directions, on the other hand, correlate with the local geometry about the remote nitrogen. In addition, the quadrupole principal values were found to be similar to those reported previously for solid *N*-benzylimidazole.

I. Introduction

X-ray crystallographic analysis of numerous blue copper proteins[1] show metal ion geometries that deviate greatly from the normally found distorted octahedral ligand arrangement around copper in model compounds. These copper sites known as type I[2] have geometries best described as elongated C_{3v} whose equatorial coordination consists of two histidinyl imidazole nitrogens and a cysteinyl sulfur in an approximate trigonal plane; the Cu(II) is displaced toward a distant apical methionyl sulfur to complete the arrangement.[1] In view of this biological prevalence for low-symmetry copper sites it was of our interest to study the magnetic parameters of remotely coupled nitrogens in copper models having geometries that differ from the most commonly found near-square-planar coordination.[3] We have therefore initiated a systematic single-crystal electron spin-echo envelope modulation (ESEEM) analysis of remote nitrogen couplings to copper in known geometrical sites in the hope that accurate hyperfine and quadrupole tensors may aid similar studies of copper models and metalloproteins.

The present report deals with copper doped into single crystals of Zn(II)(1,2-dimethylimidazole)$_2$Cl$_2$. X-ray diffraction analysis has shown the zinc complex to exhibit a distorted tetrahedral geometry.[4,5] Although previous ESEEM studies have been performed on imidazole models bound to copper in frozen solutions,[6-8] the analysis of an imidazole compound in a tetrahedral

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