throughout the whole structure. In this study, the large changes in the spectral properties of the polypeptide backbone that are observed by FT-IR spectroscopy can be directly assigned to the formation of well-defined hydrogen bonds in α-helices and β-sheets in split α_1-AT. The magnitude of these changes for two forms of a water-soluble protein measured under physiological conditions is unusual. Our data suggest that split α_1-AT is stabilized relative to the native form by a more complete hydrogen bonding of its secondary structure (particularly α-helical). The lability of the native serpin structure is consequent on a far more extensive degree of stress than has previously been supposed.

ACKNOWLEDGMENTS

We thank the Wellcome Trust for support and the Science and Engineering Research Council for beamtime at the Institut Laue Langevin, Grenoble. We are grateful to Dr. J. Feeney and Dr. C. Bauer for use of the 400-MHz ^{1}H NMR spectrometer at the National Institute of Medical Research, Mill Hill, London, and Dr. A. de Geyer for support at ILL.

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Primary Intermediate in the Reaction of Mixed-Valence Cytochrome *c* Oxidase with Oxygen

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Received November 15, 1989; Revised Manuscript Received December 18, 1989

ABSTRACT: The reaction of dioxygen with mixed-valence cytochrome *c* oxidase was followed in a rapid-mixing continuous-flow apparatus. The optical absorption difference spectrum and a kinetic analysis confirm the presence of the primary oxygen intermediate in the 0–100-μs time window. The resonance Raman spectrum of the iron–dioxygen stretching mode (568 cm^{-1}) supplies evidence that the degree of electron transfer from the iron atom to the dioxygen is similar to that in oxy complexes of other heme proteins. Thus, the Fe–O_2 bond does not display any unique structural features that could account for the rapid reduction of dioxygen to water. Furthermore, the frequency of the iron–dioxygen stretching mode is the same as that of the primary intermediate in the fully reduced enzyme, indicating that the oxidation state of cytochrome *a* plays no role in controlling the initial properties of the oxygen binding site.

Cytochrome *c* oxidase (CcO), the terminal enzyme in the electron transport chain, transfers four electrons to dioxygen to reduce it to water. This process involves the generation of several intermediates in a very complex series of changes. Until now, it has been difficult to identify and follow the progress of these intermediates at physiological temperatures owing to their short lifetimes coupled with difficulties in detecting them spectroscopically. Reliance was made primarily on the optical absorption difference spectrum. Recently, preliminary resonance Raman spectra have been obtained on some of the early intermediates (Babcock et al., 1984, 1985;

‡Partially supported by Grant GM-39359 from the National Institute for General Medical Sciences.

0006-2960/90/0429-1380$02.50/0

Ogura et al., 1985, 1989; Varotsis et al., 1989). This technique gives greater structural detail than does optical absorption spectroscopy, and it thereby has the potential of being able to reveal the identity of the metastable intermediates and determine their kinetic properties.

The conditions for the generation of the primary intermediate in the reaction of CcO with oxygen and the structure of this intermediate are not agreed upon. For the fully reduced enzyme, Hill et al. (1986) reported that the lifetime of the primary intermediate is too short (first-order decay constant = 10^6 s^{-1}) to allow for its detection. However, Orii (1984, 1988) reported that in his optical absorption measurements the primary intermediate has a first-order decay constant of $\sim 10^3$ s^{-1} and may, therefore, be detected readily. On the other hand, there is modest agreement on the kinetic properties of the primary oxygen compound when generated from the mixed-valence species (Hill & Greenwood, 1983, 1984; Hill et al., 1986; Orii, 1984, 1988). Since there is a lack of agreement on the lifetime of the primary intermediate formed by the reaction of oxygen with fully reduced CcO, we have studied the reaction of oxygen with the mixed-valence enzyme. The kinetic analysis and the optical absorption difference spectrum confirm the formation of the primary intermediate under our conditions, and thereby allow characterization of the intermediate by its resonance Raman spectra. Analyses of these spectra clarify many of the properties of the primary oxygen intermediate.

Experimental Procedures

Cytochrome *c* oxidase was isolated by the methods of Yonetani (1960) and Yoshikawa et al. (1977). It was stored under liquid nitrogen until ready for use. The enzyme was solubilized in phosphate buffer (100 mM) at pH 7.4 with 1% dodecyl β-D-maltoside and deoxygenated in an anaerobic chamber. Samples of anaerobic resting CcO were exposed to carbon monoxide (CO) and incubated for several hours. In some cases a small amount of ferricyanide was added to the sample after the incubation. No differences in the properties of the final product were detected between these two preparations of the mixed-valence species. Optical absorption spectra of the samples confirmed the presence of the mixed-valence carbon monoxide bound CcO [a^{3+},a_3^{2+}(CO)].

To study the reaction of dioxygen with mixed-valence CcO, we use an adaptation of the flow–flash–probe method originally pioneered by Gibson and Greenwood (1963). The CO-bound mixed-valence enzyme (CcO_m–CO) at a concentration of 200 μM was placed in one syringe of the rapid-mixing apparatus. In the other syringe a solution of buffer saturated with oxygen at atmospheric pressure (~1.4 mM) was placed. The two solutions were mixed in a Wiskind four-grid mixer (Update Instruments) and passed into a 2 cm long, 0.25 × 0.25 mm cross-section experimental cell. A continuous laser was sharply focused with a cylindrical lens on the flowing sample such that a 0.025 mm thin "slab" of the sample was exposed to the laser. Partial photolysis of the CcO_m–CO occurs in this region so that the reaction with oxygen is initiated. For the fastest flow rates used in our measurements, these conditions result in a transit time of 10 μs for a molecule passing through the laser-irradiated region. The flow rate could be varied, giving much longer transit times when desired. Resonance Raman spectra were obtained by projecting the scattered light from this region on the entrance slit of a single monochrometer for dispersion and subsequent detection by a linear photodiode array (PAR Reticon). This technique is related to that reported previously by Ogura et al. (1985, 1989), but owing to their sample geometry, the fastest time window available was

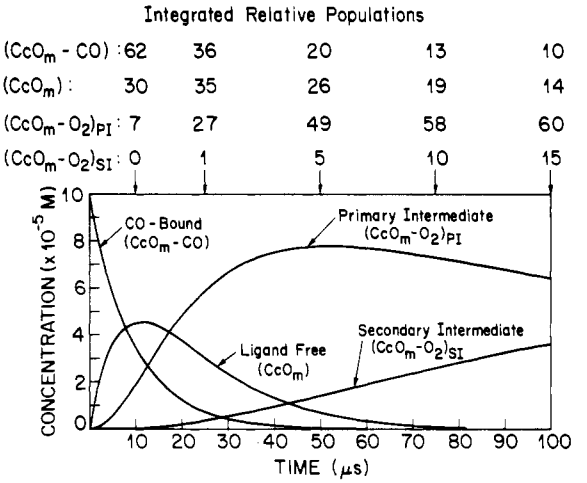

FIGURE 1: Enzyme populations of reaction intermediates in continuous-flow apparatus. The curves are the calculated populations as a function of time of the various forms of the enzyme under our conditions. The initial carbon monoxide bound cytochrome *c* oxidase concentration was 100 μM, and the dioxygen concentration was 0.7 mM. The numbers at the top are the relative populations integrated over the time windows indicated by the arrows.

150 μs. Optical absorption spectra were obtained by passing a white light through the laser-irradiated region followed by dispersion and detection with the same apparatus.

Results and Discussion

Calculated populations of a given intermediate expected under our conditions, on the basis of the reported kinetic constants (Hill & Greenwood, 1983, 1984; Hill et al., 1986), are shown in Figure 1. To calculate these curves, we have simulated the photodissociation as a rapid first-order kinetic process. The value of the "kinetic" constant k_{photo} used for this simulation was selected to approximate the photodissociation process in the absence of oxygen. The following expression was used to calculate the population of each component of the reaction:

$$CcO_m\text{–}CO \xrightarrow{k_{photo}} CcO_m + O_2 \xrightarrow{k_1} (CcO_m\text{–}O_2)_{PI} \xrightarrow{k_2} (CcO_m\text{–}O_2)_{SI} \quad (1)$$

where k_{photo} is 1×10^5 s^{-1} and the values of the kinetic constants, k_1 and k_2, are 1×10^8 M^{-1} s^{-1} and 6×10^3 s^{-1}, respectively, as reported by Hill and Greenwood (1963). PI and SI refer to the primary and secondary intermediates, respectively.

The experimental measurements give the integrated population of all forms of the enzyme present in the time interval determined by the geometry and flow rate. Therefore, in order to be able to compare the calculations in Figure 1 with experiments, the percentage of each species is written above the instantaneous population curves for a series of time intervals. It is evident that at the 50-μs time interval there is a large population of the primary oxygen compound but not too much accumulation of the secondary intermediate. In most of the measurements reported here we have used this time interval. The time dependence of the phenomena will be discussed in a separate publication.

To assure that the primary oxygen intermediate was generated, the optical difference spectrum was obtained (Figure 2) by measuring the absorption spectrum of the photodissociated sample in the presence of oxygen and in the absence of oxygen. Since photodissociation is incomplete under our conditions, photolysis in the absence of oxygen gives a mixture of the CO-bound sample [(CcO_m–CO)] and ligand-free ma-

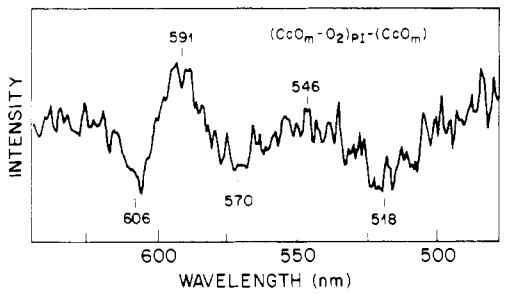

FIGURE 2: Difference optical absorption spectrum of the primary oxygen intermediate in the mixed-valence enzyme minus the ligand-free form. The flow duration of the enzyme through the sample region was 50 μs, and the total exposure time was 3.3 s.

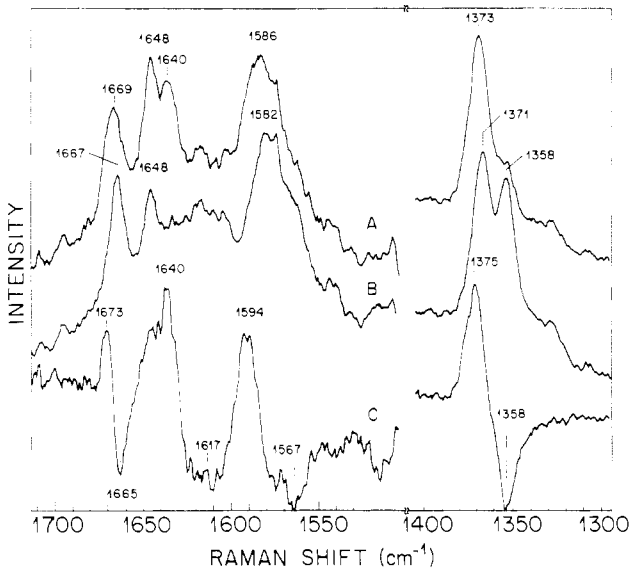

FIGURE 3: Resonance Raman spectra of cytochrome *c* oxidase reaction products. The partially photodissociated sample of spectrum B when reacted with oxygen yields spectrum A. The unreacted material cancels out of the difference spectrum (C), yielding a spectrum of the primary intermediate minus the ligand-free form of the enzyme. The flow duration time of the enzyme through the laser-irradiated region was 50 μs, and the total exposure time on the detector was 4 s. The laser (413.1 nm, 100 mW) was focused on the flowing sample with a cylindrical lens.

terial, CcO_m. If p is the fraction of the enzyme that is photolyzed, then the population of CcO_m–CO is $1 - p$ and that of CcO_m is p. In the presence of oxygen, if n is the fraction of CcO_m that reacts with oxygen to form an intermediate $[(CcO_m–O_2)_I]$, then the population of CcO_m–CO is still $1 - p$, that of CcO_m is $p - n$, and that of $(CcO_m–O_2)_I$ is n. The aerobic minus anaerobic difference spectrum then becomes a difference spectrum between the oxygen intermediate and the ligand-free sample, i.e., $n[(CcO_m–O_2)_I - CcO_m]$. The magnitude in the difference spectrum is a measure of the amount of sample that is converted into intermediate. The remaining unreacted and unphotolyzed material cancel from the difference spectrum. Thus, the absorption difference spectrum reported in Figure 2 may be compared directly to published reports. The data agree well with those reported for the primary oxygen intermediate in both low-temperature (Chance et al., 1975a,b) and room-temperature studies (Hill & Greenwood, 1983; Hill et al., 1986; Orii, 1988). Therefore, we may identify $(CcO_m–O_2)_I$, the dominant intermediate present in our 50-μs time window, as the primary intermediate, $(CcO_m–O_2)_{PI}$.

The resonance Raman spectra were obtained on the same samples and under the same conditions as the optical absorption spectra. In Figure 3 the high-frequency region of the

Table I: Frequencies (cm^{-1}) of Cytochrome a_3–O_2 (Primary Oxygen Intermediate) of Mixed-Valence Cytochrome *c* Oxidase Compared to Frequencies of Cytochrome a_3 in Other Forms of the Enzyme[a]

mode	$(CcO_m–O_2)_{PI}$	CcO–CO	$(CcO–NO)_{mv}$	$CcO_{reduced}$	$CcO_{resting}$
ν_4	1373	1371	1371	1358	1371
ν_2	1590	1589	1587	1569	1572
ν_{10}	1640		1636		
$(C=O)_{formyl}$	1672	1668	1669	1666	1675

[a] The values for the NO adduct are for the mixed-valence form of the enzyme.

Raman spectra is presented. The top two spectra compare the CO photodissociated samples in the presence (A) and absence (B) of oxygen. Spectrum B of Figure 3 confirms the presence of the mixed-valence compound. The frequency of the formyl stretching mode at 1667 cm^{-1} is that of reduced cytochrome a_3 (Salmeen et al., 1978). The line at 1648 cm^{-1} is assigned as the formyl stretching mode of ferric cytochrome *a* (Salmeen et al., 1978). We assign the residual lines in the 1610–1625-cm^{-1} region as originating from cytochrome a_3, not cytochrome *a*, since lines from cytochrome a_3 have been detected in this region in the past (Ching et al., 1985). Finally, the spectrum of the ν_4 region (Argade et al., 1986) has a doublet (1371 and 1358 cm^{-1}), an expected characteristic of the photodissociated mixed-valence enzyme.

In the comparison of spectra A and B, changes are evident due to the formation of the primary oxygen intermediate. Most prominent is a shift and broading of the carbonyl stretching mode (1669 cm^{-1}) of the formyl group of cytochrome a_3, the growth of a strong new line at 1640 cm^{-1}, and a shift in the spin marker line at 1586 cm^{-1}. Changes were also detected in the electron density marker line in the 1350–1380-cm^{-1} region in which the doublet at 1371 and 1358 cm^{-1} in the photodissociated enzyme is replaced by a strong peak at 1373 cm^{-1} with a weak shoulder at 1358 cm^{-1} in the presence of oxygen. By the magnitude of the changes detected, it is clear that a significant amount of the oxygen intermediate has been formed. From the same procedures used with the optical absorption spectra, a Raman difference spectrum may be generated. This spectrum is that of the primary intermediate minus that of the ligand-free enzyme. Major maxima are detected at 1673, 1640, 1594, and 1375 cm^{-1}, and minima are detected at 1665, 1617, 1567, and 1358 cm^{-1}. In the only prior report (Babcock et al., 1985) of the oxygen reaction with the mixed-valence enzyme, pulsed lasers were used, but the power had to be kept low to avoid photodissociation of the intermediate. Therefore, the signal to noise ratio was too low to make clear identification of the modes.

Spectrum A of Figure 3 is a mixture of the primary intermediate, unphotolyzed CO-bound material, and unreacted ligand-free material. It is desirable to have a spectrum of the pure intermediate in order to compare the properties of it to those of other forms of the enzyme. Since the difference spectrum is that of the intermediate $(CcO_m–O_2)_{PI}$ minus the ligand-free form of the enzyme, (CcO_m), an absolute spectrum of the intermediate may be generated by adding to the difference spectrum (spectrum C of Figure 3) a spectrum of the ligand-free mixed-valence enzyme (CcO_m). We obtained this spectrum by slowly flowing the CO-bound sample in the apparatus in the absence of oxygen. Under these conditions full photodissociation takes place so a spectrum of the ligand-free mixed-valence enzyme is generated. By adding this to the difference spectrum, spectrum B in Figure 4 is obtained. This spectrum is that of $[a^{3+},a_3^{2+}–O_2]$, i.e., the mixed-valence primary oxygen intermediate. We compare this to the spectra of the resting and the fully reduced enzyme. The frequencies

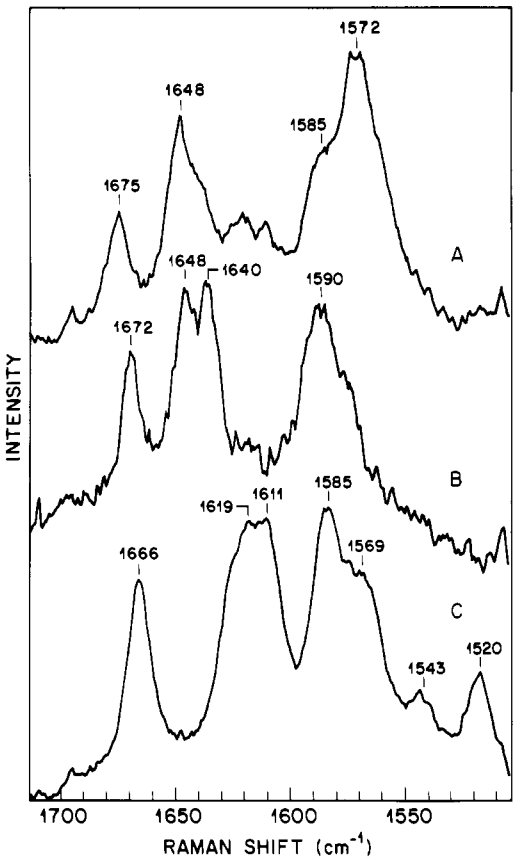

FIGURE 4: Comparison of the spectrum of the mixed-valence primary oxygen intermediate (B) with resting (A) and fully reduced (C) cytochrome *c* oxidase. The primary intermediate spectrum was generated by adding to the difference spectrum in Figure 3 the spectrum of the ligand-free mixed-valence enzyme.

of the cytochrome a_3–oxygen complex from this spectrum are compared to those of other forms of cytochrome a_3 in Table I.

The spectrum of the primary intermediate (spectrum B of Figure 4) demonstrates that this intermediate is low spin since there is an intense line at 1590 cm^{-1} and no intensity in the high-spin region (1560–1570 cm^{-1}) (Van Steelandt-Frentrup et al., 1981) (see spectra A and C). Furthermore, the absence of intensity in the 1605–1625-cm^{-1} region demonstrates that there is no contribution from any reduced cytochrome *a* in the spectrum (see spectrum C). The intensity of ν_{10} from the intermediate is quite high as indicated by the strength of the line at 1640 cm^{-1}. A high intensity for ν_{10} was also detected in NO-bound cytochrome *c* oxidase (Rousseau et al., 1988). The strong line at 1648 cm^{-1} originates from the carbonyl stretching mode of the formyl group on ferric cytochrome *a*.

Comparison of the spectrum of the primary intermediate to that of CO-bound and NO-bound forms of the enzyme is quite interesting. As may be seen from Table I, the frequencies of the lines agree well. Indeed, the spectrum reported for mixed-valence NO-bound CcO is very similar to that of the primary intermediate. This serves to confirm the similarity between these structures. The frequencies of the carbonyl stretching mode of the formyl group and of the electron density marker line (ν_4) in the primary oxygen intermediate are slightly higher than the corresponding frequencies in the NO-bound enzyme. This may be an indication of more electron transfer from the iron to the oxygen in the primary intermediate or may reflect conformational differences. Conformational differences affecting the frequency of the formyl mode have been reported in other forms of the enzyme (Sassaroli et al., 1988).

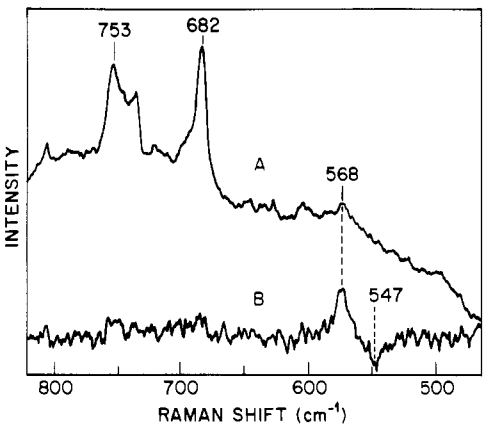

FIGURE 5: Spectrum of the cytochrome *c* oxidase plus oxygen reaction product in the $Fe–O_2$ stretching mode region (A). The assignment of this mode at 568 cm^{-1} is confirmed by the 21-cm^{-1} isotopic shift shown in the reaction product difference spectrum ($^{16}O_2 - {}^{18}O_2$) (B). The data are the sume of a 50- and a 100-μs duration experiment. The total integration time is 20 s.

To further characterize the primary oxygen intermediate, we have measured the low-frequency region of the resonance Raman spectrum to examine the iron–oxygen stretching mode. To locate and assign this mode, CcO_m–CO samples were mixed with either oxygen-16 or oxygen-18 prior to initiating the reaction by photolysis. The spectrum of $(CcO_m–{}^{16}O_2)_{PI}$ and the $^{16}O_2 - {}^{18}O_2$ difference spectrum are shown in Figure 5. From these spectra the $Fe–O_2$ stretching mode for oxygen-16 is located at 568 cm^{-1}. The frequency of this mode and its oxygen isotopic shift (21 cm^{-1}) are, within experimental error, the same as those reported for oxymyoglobin and oxyhemoglobin (Brunner, 1974; Nagai et al., 1980; Van Wart & Zimmer, 1985). Therefore, the degree of electron transfer from the iron to the dioxygen in the primary intermediate is similar to that in oxyhemoglobin and oxymyoglobin, the oxygen transport and storage proteins, in agreement with similar conclusions drawn from studies of low-temperature intermediates several years ago by Chance et al. (1975a,b). This demonstrates that the rapid reduction of oxygen to water in CcO is not a consequence of the $Fe–O_2$ structure of cytochrome a_3. Instead, it is a consequence of the amino acid structure surrounding the bound O_2 and the copper atom in the binuclear site. Thus, the anomously high value of the Fe–CO stretching mode reported previously (Argade et al., 1984) must result from distal interactions of the CO in the binding pocket. The finding that the $Fe–O_2$ stretching mode is the same as that in hemoglobin and myoglobin suggests that there is no bridging from Cu_B to the oxygen in this intermediate. If the dioxygen were bridged to the Cu_B atom, the $Fe–O_2–Cu_B$ unit would be expected to have a very different stretching frequency than that of hemoglobin or myoglobin as has been reported in other bridged complexes [Paeng et al. (1988) and references cited therein].

An interesting result from this work is the comparison of the $Fe–O_2$ stretching mode of the primary intermediate in the mixed-valence enzyme (568 cm^{-1}) with the corresponding frequency in the fully reduced enzyme. We have recently assigned this mode in the fully reduced enzyme at 568 cm^{-1}, the same frequency as that which we detect in the mixed-valence enzyme. The properties of the $Fe–O_2$ stretching mode for the primary intermediate and its relationship to the oxygen isotope sensitive mode in the fully reduced enzyme reported by Varotsis et al. (1989) are discussed in a separate publication (Han et al., 1990). The similarities in the $Fe–O_2$ stretching mode frequency for the primary intermediate in the fully

reduced and the mixed-valence preparations indicate that the iron–oxygen bonding is the same in these two forms of the enzyme. Thus, the oxidation state of the cytochrome *a* plays no role in controlling the structure of the iron–oxygen moiety at the binuclear site. A similar conclusion concerning the effect of the redox state of cytochrome *a* on the cytochrome a_3–CO moiety was reached recently by Einarsdottir et al. (1988). Additional studies of both of these forms of CcO by the technique described here will allow for a determination of the differences in the structures and the associated time evolution of all of the intermediates.

By obtaining the resonance Raman spectrum of the primary intermediate and the corresponding difference spectrum, we have established a new way of characterizing this intermediate. The difference spectrum we obtain for the primary intermediate of the mixed-valence enzyme minus the ligand-free preparation only involves cytochrome a_3. Unless there is some heretofore undetected heme–heme interaction in which oxygen binding changes the cytochrome *a* spectrum, there should be no contributions from cytochrome *a* in the difference spectrum. Thus, the difference spectrum of the primary intermediate consists only of oxygen-bound cytochrome a_3 minus ligand-free cytochrome a_3. The features in this spectrum may be used to assess the formation of the primary intermediate in other forms of the enzyme and evaluate the structure of the subsequent intermediates. Finally, when applied to the study of the reaction of oxygen with the fully reduced enzyme, the spectra reported here should allow for a clear determination of the kinetic properties and electron-transfer rates in that system.

Acknowledgments

We thank Dr. G. Blondin of Water Resources Center for mitochondria.

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