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Primary intermediate in the reaction of oxygen with fully reduced cytochrome *c* oxidase

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ABSTRACT **The primary intermediate in the reaction of oxygen with cytochrome *c* oxidase was generated by photodissociating carbon monoxide in a continuous flow rapid mixing apparatus. The presence of the primary intermediate was confirmed by a comparison of the iron–dioxygen stretching frequency with that obtained in the reaction of oxygen with the mixed-valence enzyme. For both of these preparations, the Fe–O_2 stretching mode is detected at 568 cm^{-1}, the same frequency as that found in oxyhemoglobin and oxymyoglobin. These data illustrate that the primary intermediate may be generated and detected at room temperature in the fully reduced enzyme and that the oxidation state of cytochrome *a* does not affect the structure of the iron–dioxygen complex. By following the changes in the intensity of the Fe–O_2 stretching mode in the resonance Raman spectrum as a function of time, the first-order rate constant for the decay of the primary intermediate was found to be 3.5×10^4 s^{-1} ($t_{1/2}$ = 20 μs).**

Cytochrome *c* oxidase is the terminal enzyme in the electron transport chain. It catalyzes the four-electron reduction of dioxygen to water in a complex series of steps (1). To uncover the mechanism of this process, the structure and properties of each of these intermediates, starting with the primary intermediate, must be determined. However, there is no agreement as to whether or not the primary intermediate in the reaction of oxygen with the fully reduced enzyme (CcO_{fr}) may even be detected at room temperature. Reported values for the first-order rate constant for the decay of the primary intermediate range from 3×10^6 s^{-1}, resulting in an experimentally undetectable level of the primary intermediate (2), to 1×10^3 s^{-1}, a value allowing for substantial accumulation of the primary intermediate (3, 4).

Because of the difficulties in studying the structure and properties of the reaction intermediates of cytochrome *c* oxidase at room temperature with only optical absorption spectroscopy, time-resolved resonance Raman spectroscopy has been applied to the problem (5–9). Resonance Raman spectra of an early intermediate that was photolabile and, thereby, argued to be the primary intermediate was first detected by Babcock *et al.* (5, 6), who used a flow-flash-probe technique with pulsed lasers. However, the signal-to-noise ratio in these data was low because of the low laser power that had to be used to avoid photodissociation of the oxygenated complex. Varotsis *et al.* (9) using the same technique were able to obtain the spectrum of an early intermediate in which an oxygen-isotope-sensitive line was detected at 589 cm^{-1} at a time delay of 10 μs after photodissociation. However, the identity of this intermediate was not confirmed by its optical absorption spectrum.

Another approach to the study of cytochrome *c* oxidase intermediates was taken by Kitagawa and coworkers (7, 8) who continuously recirculated the enzyme through an observation cell that was constantly illuminated by a laser beam. The time resolution was determined by the residence time of the sample in the laser beam. The time limit reported for this apparatus was 150 μs, resulting in a cumulative spectrum from 0 to 150 μs. This time resolution is long for the clarification of the properties of the primary intermediate. Furthermore, their spectrum was complicated by the presence of cytochrome *c*, used in their recirculating system to maintain the proper oxidation state of the cytochrome *c* oxidase (8).

We have developed (10) a continuous-flow apparatus that can be used for the study of short-lived intermediates of cytochrome *c* oxidase and that has time-resolution capabilities of less than 10 μs. We applied (10) it first to the study of the primary intermediate in the mixed-valence enzyme (cytochrome a^{3+}/a_3^{2+}). The presence of the primary intermediate was confirmed by its optical absorption spectrum and a kinetic analysis. Characteristic resonance Raman spectra of the intermediate were obtained. A most notable feature of the primary intermediate in the mixed valence enzyme is the presence of an Fe–O_2 stretching mode at 568 cm^{-1}, a frequency that is the same as that observed in oxyhemoglobin and oxymyoglobin. In this paper we use the same technique to generate and study the primary intermediate in the reaction of oxygen with the fully reduced enzyme. By following the time dependence of the intensity of the isolated Fe–O_2 stretching mode in the primary intermediate, the first-order rate constant for the decay of this intermediate was determined unambiguously.

EXPERIMENTAL METHODS

Cytochrome *c* oxidase was isolated by the method of Yoshikawa *et al.* (11). The enzyme was stored under liquid nitrogen. It was solubilized in potassium phosphate buffer (100 mM at pH 7.4) with 1% dodecyl β-D-maltoside and deoxygenated in an anaerobic chamber. Samples of the anaerobic enzyme (200 μM) were reduced with ascorbate (50 mM) and catalytic amounts of cytochrome *c* (3 μM) and then exposed to carbon monoxide to form the fully reduced carbon monoxide-bound species (CcO_{fr}—CO). Optical absorption spectra of the enzyme confirmed the integrity of the samples at each step.

To obtain the resonance Raman spectra, samples of CcO_{fr}—CO (200 μM) were placed in one syringe of a continuous flow rapid mixing apparatus and dioxygen-saturated (1.4 mM O_2) buffer (100 mM potassium phosphate, pH 7.4) was placed in the other syringe. The solutions were mixed in a Wiskind four-grid mixer of an Update Instruments (Madison, WI) rapid mixing apparatus and flowed into the small (cross section, 0.25 mm × 0.25 mm) Raman sampling cell where the incident laser photodissociated the carbon monoxide from the enzyme and probed the resonance Raman spectrum. The flow rate could be varied so that the sample residence time in the laser-irradiated region ranged from 10 μs to 500 μs, allowing time-evolution studies to be made.

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Abbreviation: CcO_{fr}, fully reduced cytochrome *c* oxidase.

The scattered light was detected with an array detection system consisting of a 1.25-m spectrograph (Spex Industries, Metuchen, NJ) and an intensified linear photodiode array detector (Princeton Applied Research, Inc., Princeton, NJ). The spectral window for the accumulation of each spectrum was about 500 cm^{-1}. The only correction made to the data was a division by a white light spectrum so as to correct for the pixel-to-pixel variation in the sensitivity of the detector. Since spectra obtained on an array detector are linear in wavelength, it is essential to calibrate the detector for each new experimental setup. To minimize any errors due to uncertainty in the frequency calibration, reference samples were run through our flow apparatus at the start of each experiment. The reference samples used (cytochrome *c* and carboxymyoglobin) have the similar optical properties as the cytochrome *c* oxidase and lines in the same region of the Raman spectrum. The spectral frequencies of the same reference materials were calibrated against indene on a scanning Raman instrument using the same laser excitation frequency. This procedure yielded an accuracy of ± 2 cm^{-1} on the array detector.

In our experimental protocol the incident laser intensity (100 mW at 413.1 nm) was adjusted to obtain significant, but not complete, photolysis of the CO-bound cytochrome *c* oxidase at the fastest flow rate. This laser power level assures that the oxygen-bound complex will not be photodissociated by the laser, a problem encountered when high-power-pulsed lasers are used. Therefore, at any instant our samples consist of a mixture of unphotolyzed material (CcO_{fr}—CO), photodissociated but unreacted material (CcO_{fr}), the primary intermediate of the reaction with oxygen $(CcO_{fr}—O_2)_{PI}$, and later intermediates $(CcO_{fr}—O_2)_{LI}$. The relative proportions that we detect depend on the experimental conditions, the time of observation, and the kinetic properties of the enzyme. In Fig. 1*A* we present the instantaneous populations of the various species present in our experiment, based on a combination of reported kinetic constants (2–4, 12, 13) and those determined in the present study (see *Discussion*). To carry out this calculation we used the following scheme:

$$[CcO_{fr}—CO] \xrightarrow[k_p]{h\nu} CcO_{fr} + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}}$$

$$[CcO_{fr}—O_2]_{PI} \xrightarrow{k_2} [CcO_{fr}—O_2]_{LI} \quad [1]$$

The later intermediates (LI) formed subsequent to the primary intermediate (PI) are grouped together since in this paper we consider only the primary intermediate and its decay pathway is first order. The amount of photolysis is modeled as a first-order kinetic process with a constant k_p of 2×10^5 s^{-1} selected to fit the amount of photolyzed material in additional experiments that we carried out in the absence of oxygen.

The continuous-flow technique that we use does not give an instantaneous measure of the population but instead it gives an integration of the total population present in the time window being examined. To compare our experimental results to the theoretical expectations, the curves in Fig. 1*B* give the integrated populations as a function of a time window. As may be seen from Fig. 1*B*, the primary intermediate has its highest fractional contribution (≈40%) in the time windows ranging from 0–40 μs to 0–60 μs. At longer time windows, significant contributions to the total population result from the later intermediates.

RESULTS AND DISCUSSION

The resonance Raman spectra of the reaction products of dioxygen with the fully reduced enzyme in the region of the iron–dioxygen stretching modes of heme proteins (9, 10, 14–16) are shown in Fig. 2. The time window used to obtain these data was 0–50 μs. To determine if there are any isotope-sensitive lines in the spectrum, the CcO_{fr}—CO was mixed with either $^{16}O_2$- or $^{18}O_2$-saturated buffers. An oxygen-isotope-sensitive line is detected at 568 cm^{-1} with $^{16}O_2$ and at 547 cm^{-1} with $^{18}O_2$. This isotope shift is most clearly seen in the difference spectrum shown in Fig. 2, spectrum C. No other lines in this spectral region display any frequency shifts upon isotopic substitution. Without making any changes in the experimental setup, the mixed-valence form of the enzyme was reacted with each of the two isotopes of oxygen. The resulting difference spectrum is shown in Fig. 2, spectrum D. The difference spectrum shows that the reaction of oxygen with the mixed-valence enzyme has an oxygen-isotope-sensitive Raman line at the same frequency as that of the reaction product in the fully reduced enzyme.

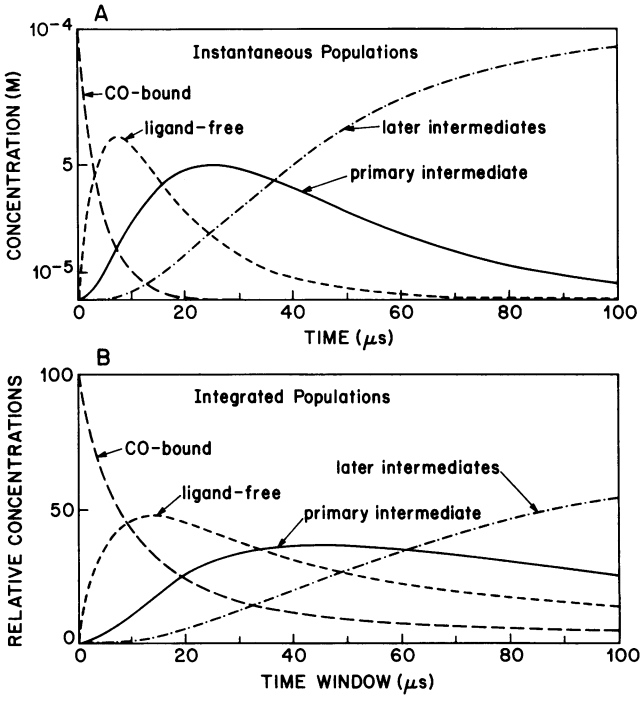

FIG. 1. Populations of cytochrome *c* oxidase species in the reaction of oxygen with the fully reduced enzyme after the photodissociation of carbon monoxide. (*A*) Instantaneous populations based on the kinetic scheme of Eq. 1. Since the decay of primary intermediate is first-order, all of the later intermediates are grouped together. (*B*) Relative populations of cytochrome *c* oxidase intermediate integrated over the time windows used in the experimental protocol reported here. The scale on the abscissa is the time window from zero to the indicated time.

The spectrum of an early intermediate in the reaction of oxygen with the mixed-valence enzyme was shown to be the primary intermediate in that form of the enzyme based on the optical absorption difference spectrum and a kinetic analysis (10). A characteristic feature of that spectrum is the presence of the $Fe–O_2$ stretching mode at 568 cm^{-1}. The identity of the $Fe–O_2$ stretching mode in the mixed-valence enzyme with that of oxyhemoglobin and oxymyoglobin was taken as further evidence that the early intermediate in the mixed-valence enzyme is indeed that of the primary intermediate with an iron–dioxygen bond analogous to that in the oxygen carrier and transport proteins. The $Fe–O_2$ stretching mode that we detect in the reaction of oxygen with the fully reduced enzyme has the same frequency as that of the $Fe–O_2$ stretching mode found in the reaction of oxygen with the mixed-valence enzyme. This isotope shift is in agreement with that predicted (≈20 cm^{-1}) for an $Fe–O_2$ stretching mode based on a harmonic oscillator approximation. We detect no other

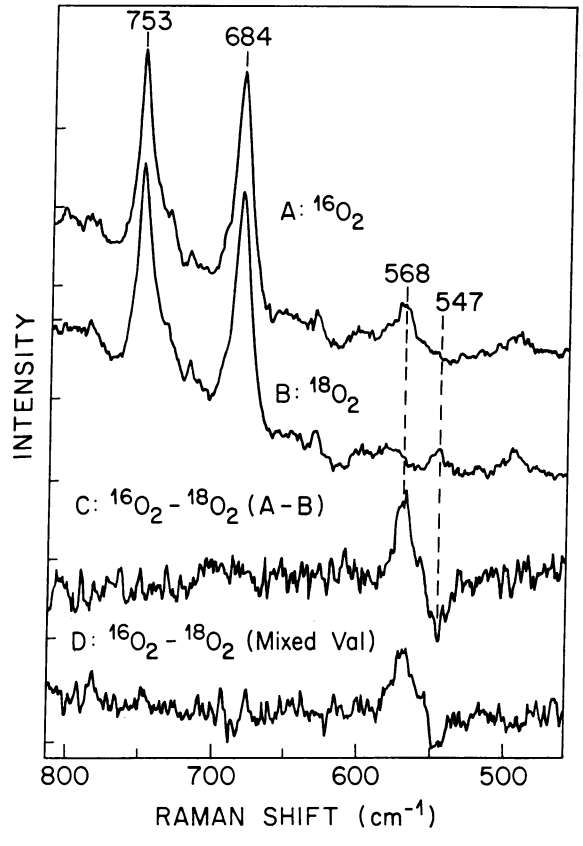

FIG. 2. Resonance Raman spectra and difference spectra of the primary intermediate is the reaction of cytochrome *c* oxidase with isotopes of oxygen observed in a time window of 0–50 μs. The spectral accumulation time was 30 s. Spectra: A, reaction of fully reduced cytochrome *c* oxidase with $^{16}O_2$; B, reaction of fully reduced cytochrome *c* oxidase with $^{18}O_2$; C, difference spectrum showing the isotopic shift of the Fe–O_2 stretching mode of the primary intermediate in the reaction of oxygen with the fully reduced enzyme (spectrum A minus spectrum B); D, difference spectrum showing the isotopic shift of the Fe–O_2 stretching mode of the primary intermediate in the reaction of oxygen with the mixed-valence (Mixed Val) enzyme.

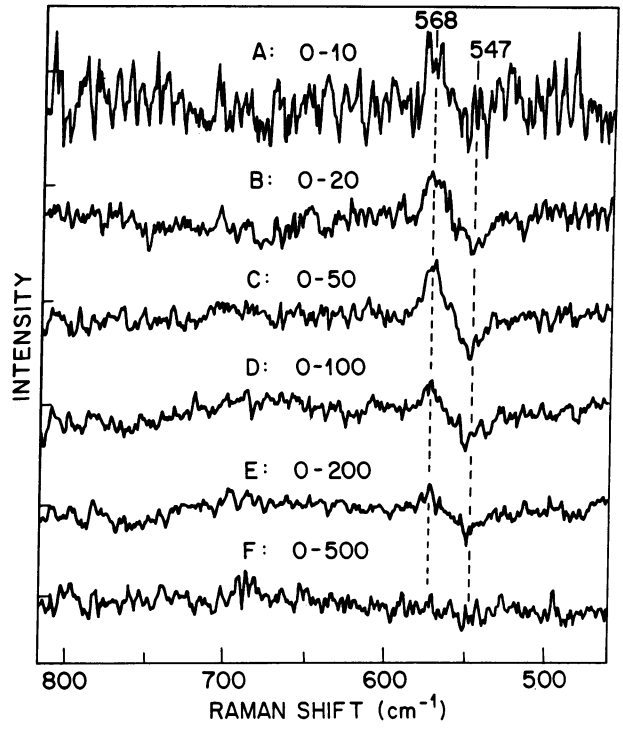

FIG. 3. Isotopic oxygen ($^{16}O_2$–$^{18}O_2$) resonance Raman difference spectra of the primary intermediate in the reaction of oxygen with fully reduced cytochrome *c* oxidase. Each difference spectrum is an integration over a different time window for laser exposure ranging from 0–10 μs (spectrum A) to 0–500 μs (spectrum F), as indicated. The accumulation time for the spectra ranged from 4 to 30 s. However, all of the intensities were normalized to the same scale.

oxygen-isotope-sensitive mode in any other time range that we examined (10–500 μs) in the 500–800 cm^{-1} region of the spectrum (Fig. 3). Consequently, we assign this line as the Fe–O_2 stretching mode of the primary intermediate in the reaction of the fully reduced enzyme with oxygen. It should be pointed out that in base-free model complexes an Fe–O stretching mode from the Fe–O–O–Fe moiety has been reported (17) at 574 cm^{-1}. However, the presence of a nitrogenous base in cytochrome *c* oxidase would be expected to cause the Fe–O mode to shift. Therefore, we cannot predict where the Fe–O stretching frequency would be expected in a bridged peroxide complex. Varotsis *et al.* (9) reported an oxygen-isotope-sensitive line at 589 cm^{-1} in an early intermediate of the reaction of cytochrome *c* oxidase with oxygen. We have searched for such a line in all of the time windows that we have examined (Fig. 3) and found no evidence for such a line in our data.*

The assignment of the Fe–O_2 stretching mode at 568 cm^{-1} in the primary intermediate of the reaction of oxygen with cytochrome *c* oxidase provides a clear description of its properties. As originally concluded by Chance *et al.* (18, 19) from the optical properties of intermediates stabilized at low temperature by the triple-trapping technique, the primary intermediate (compound A) has a structure similar to that of oxyhemoglobin and oxymyoglobin. There do not appear to be any unusual stereochemical influences in the binuclear site to modify the Fe–O_2 bonding; and the degree of electron transfer from the iron to the bound dioxygen also appears to be the same as that of the oxygen storage and transport proteins. In addition, no evidence for any direct interaction of the bound oxygen with the nearby copper atom is apparent. Thus, our data do not support the conclusions of Varotsis *et al.* (9) that, in binding at the binuclear site, the O=O bond is weakened in preparation for its subsequent reactions.

The properties of cytochrome *c* oxidase are known to depend on the oxidation state of the low-potential sites (cytochrome *a* and Cu_A). Reduction of these sites results in a protein transition from the closed to the open conformation (20–25). The binding of cyanide to the ferric heme at the binuclear site has been reported to be five orders of magnitude faster in the open than in the closed conformation (22). This remarkable change in kinetic properties has been thought to result from the opening of an access channel to the active site, a modification in the structure of the binuclear site, or both. If the structure of the binuclear site did change with the oxidation state of the low-potential centers, a difference between the Fe–O_2 stretching frequency would be expected in the comparison of the primary intermediate generated in the fully reduced enzyme with that generated in the mixed-valence enzyme. The absence of any such differences in the mode suggests that, for the reduced form of cytochrome a_3, the oxidation state of cytochrome *a* does not influence the position of any atom or group that interacts directly with the bound oxygen. We infer from this that the change in the cyanide on-rate between the open and the closed conformation is a consequence of a change in the

*After this manuscript was submitted for publication we learned from G. T. Babcock (private communication) that there was an error in the calibration of the spectrometer used by Varotsis *et al.* (9). After correcting for the error their data agree with ours.

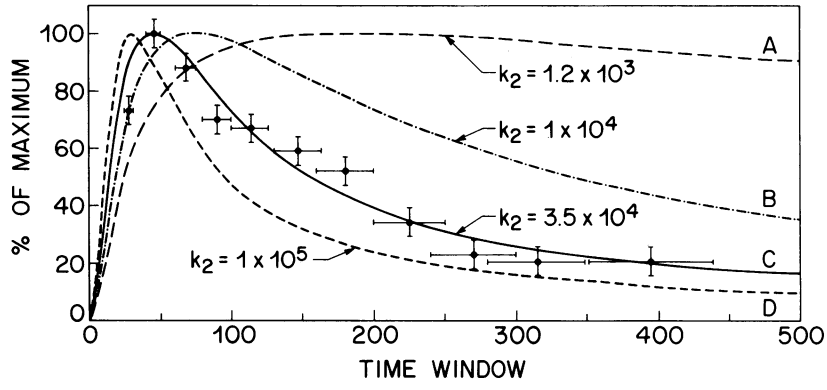

FIG. 4. Evaluation of first-order rate constant, k_2, in reciprocal seconds for the decay of the primary intermediate in the reaction of oxygen with fully reduced cytochrome *c* oxidase. The error bars on the data points in the vertical direction were determined by estimating the uncertainty in the Raman intensity due to the noise in the data. The error bars in the horizontal direction result from the uncertainty in the laser spot size. Since the latter uncertainty gives a constant percentage error in the integration time, it is larger at longer times. Each curve represents a different value for k_2 calculated as a function of time windows used in our experiments. The data and the calculated curves were normalized to their maximum values. The best agreement with the data gives a value for k_2 of 3.5×10^4 s^{-1} ($t_{1/2} \approx 20$ μs).

accessibility to the binuclear site or is a property of only the oxidized form of cytochrome a_3. A similar conclusion concerning the absence of changes in the cytochrome a_3–CO complex when the cytochrome *a* redox state is changed was reached by others (26).

In the past there has been a lack of agreement as to whether or not the primary intermediate in the reaction of oxygen with fully reduced cytochrome *c* oxidase could be observed at room temperature. Initially, Gibson and Greenwood (27), Greenwood and Gibson (28), and Hill and Greenwood (12, 13) determined that the first-order rate constant for the decay of the primary intermediate, k_2, was 3×10^4 s^{-1}. Later, Hill *et al.* (2) argued that the rate constant was so large that the intermediate could not be detected at room temperature and concluded that it was 3×10^6 s^{-1}. On the other hand, Orii (3) reported that he found a value for k_2 of 1.2×10^3 s^{-1} but more recently reported (29) a value of 3×10^4 s^{-1}. Since knowing the value of the first-order rate constant for the decay of the primary intermediate is necessary for studying the development of the subsequent intermediates and for understanding the relationship between the properties of the fully reduced enzyme and the mixed-valence enzyme, it is of utmost importance to obtain an accurate value of k_2.

The difficulty in determining the value of k_2 from the optical absorption spectra, as has been done in the past, is that the changes in the spectrum as a function of time, which are monitored to determine the constant, are complicated by the contributions from several species at the same wavelength. Thus, it is difficult to follow the change in intensity of the primary intermediate since other centers or other intermediates may have spectral variations at the same wavelengths. These difficulties may be overcome by using the resonance Raman spectrum to determine the kinetic properties of the primary intermediate. The Fe–O_2 stretching mode has a characteristic frequency that is uncomplicated by other spectral lines. In addition, the spectrum of the intermediate reacted with $^{16}O_2$ minus that reacted with $^{18}O_2$ corrects for instrumental artifacts and changes in the spectrum due to the formation of other intermediates leading to very flat difference spectra with major peaks and valleys at only the frequencies of the Fe–O_2 stretching modes of the primary intermediate. Thus, by following the development and decay of that mode, an accurate measure of the population of the primary intermediate may be obtained.

The changes in intensity of the Fe–O_2 stretching mode versus the length of the time window are shown in Fig. 3. The peak-to-valley intensity from such data, normalized to the maximum value, are plotted in Fig. 4. By using this data, the values of the kinetic constants may be determined. By using Eq. 1 and values of the other kinetic constants of 2×10^5 s^{-1}, 1×10^8 s^{-1}, and 2×10^3 s^{-1} for k_p, k_1, and k_{-1}, respectively, curves are plotted in Fig. 4 for various values of k_2. [At high O_2 concentration, the oxygen binding reaction can saturate (2, 12, 13). Calculations done with the kinetic constants reported under saturation conditions revealed only very small changes in the relative populations of the intermediate and were, therefore, neglected.] The calculated curves clearly show that the original constant reported by Orii (3) ($k_2 = 1.2 \times 10^3$ s^{-1}) and any constant larger than $\approx 1 \times 10^5$ s^{-1}, such as the value of 3×10^6 s^{-1} reported by Hill *et al.* (2), are not consistent with our data. The best fit to the data results in a value for k_2 of 3.5×10^4 s^{-1} ($t_{1/2} \approx 20$ μs). Within the uncertainties of these measurements, this value is in agreement with values reported by Greenwood and coworkers (12, 13, 27, 28) and recently reported by Orii (29) and by Oliveberg *et al.* (30). The curves in Fig. 1 were also calculated with this value of k_2. These curves illustrate the complexity of determining the properties of the various intermediates in that at any time there is considerable overlap in the population of several different species. Therefore, it becomes essential either to locate isolated spectral marker lines or to be able to do difference spectra that subtract out forms of the enzyme other than that which is being probed, if semiquantitative studies of the various intermediates are desired.

In conclusion, the data reported here demonstrate that the primary intermediate in the reaction of oxygen with the fully reduced enzyme may be formed and studied at room temperature. The kinetic constant for its decay, which we were able to measure, should be sufficiently accurate to allow for the determination of the conditions needed to generate the next intermediate and the study of its properties. Also, by carrying out a similar experiment on the mixed-valence enzyme, the decay of its primary intermediate may be determined, and the influence of the oxidation state of cytochrome *a* on the kinetics of the oxygen reduction may be assessed.

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