Functional Intermediates in Reaction of Cytochrome Oxidase with Oxygen
(cytochrome oxidase—oxygen intermediates/flash photolysis/peroxide compounds)
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ABSTRACT The development of a low temperature kinetic method for the flash photolysis of the compounds of membrane-bound cytochrome a, with carbon monoxide in the presence of oxygen affords evidence for three categories of functional intermediate compounds of cytochrome a, and oxygen. The three classes are identified as follows: Compounds of Type A are considered to be "oxy" compounds of the ferrous heme. They have the composition a\(^{1+}\cdot O\). Compounds of Type B are considered to be peroxide compounds (Cu\(^{1+}\cdot a\cdot O\)\(_2\) or Cu\(^{1+}\cdot a\cdot O\)\(_2\)) or the equivalent heme Fe-Cu peroxide bridge structures. Compounds of Type C are formed from the ferriyanide pretreated oxidase and may involve higher oxidation states of the heme iron such as quadrivalent iron, and peroxide. Kinetic and equilibrium studies show these compounds to be functional in oxygen reduction in the sequence A → B → cytochromes a, c, c\(_1\), etc.

The observation of intermediates in enzyme–substrate interactions has led to a verification of the Michaelis–Menten theory upon which enzyme mechanisms are based (1–5). The most likely enzyme–substrate compounds (6) to be discovered first are those that are abortive, nonfunctional, or "dead-end." History tells us that Kurt Stern's (7) red enzyme–substrate compound in the catalase–ethyl hydrogen peroxide reaction was discovered only because it was inactive; the active compound was too labile (2) for the techniques at hand. Peroxidase was also found to form an inactive stable enzyme–substrate compound IV (8) which has recently been restudied by Yamasaki (9).

A similar development seems to have occurred in the case of cytochrome oxidase, where Okunuki (10), Lemberg (11), Wainio (12), and others have studied what they term an "oxygenated" form of the oxidase. The possibility that the "oxygenated" form could be preceded by more active forms, just as in the case of the catalases and peroxidases, has led us to the current study.

Gibson and Greenwood have studied the detergent-solubilized oxidase and have discovered a time separation of components a, a\(_2\), and Cu (13). However, heterogeneity is present in such preparations and thus the membrane-bound oxidase of intact mitochondria has been used in these studies.

Some success with this approach was obtained in experiments with Erecinska (14–16) where a 10% occupancy of membrane-bound oxidase with oxygen was obtained at ~25°C. Somewhat greater occupancy (up to 25%) was achieved in later experiments (17) but the method was limited by the lowest temperature that could be reached in the liquid phase, about ~40°C, with the maximum concentration of nonaqueous solvent permissible without denaturing the oxidase. In short, liquid phase studies of the cytochrome oxidase–oxygen compound gave clues to the existence of a partially occupied intermediate.

The study of enzyme–substrate kinetics in non-aqueous solvents was pioneered by Strother and Ackerman (18) who used ethylene glycol solutions of catalase to study its \(H_2O_2\) compound at temperatures down to ~20°C in times as short as 0.2 msec. Methanol was used as an "anti-freeze" in the study of cytochrome kinetics in a suspension of yeast cells down to temperatures of approximately ~4°C (19). These experiments were justified in detail by the work of Tyler and Estabrook (20) who explored a variety of nonaqueous solvents and showed that their effect upon electron transport was due to the replacement of water by the nonaqueous solvent. Dousou has most recently studied a variety of hemoproteins and microsomal electron transport systems (21).

Flash activation of electron transfer reactions in the solid state in frozen biological systems has been used extensively in the study of photosynthetic systems (22–24), where the study of cytochrome c down to ~290°C has revealed electron tunnelling in biological systems (25). Similarly, the flash photolysis and recombination of carbon monoxide compounds of hemoproteins in both the liquid crystalline and frozen states at room and low temperatures suggests that flash photolysis activation of the solid-state system in the presence of oxygen might be an approach appropriate to a wide variety of temperatures.

There remained the problem of how to oxygenate the material prior to freezing and without oxidizing ferrocytochrome \(a_\). This was solved by the serendipic observation that ligand exchange between oxygen and carbon monoxide bound to cytochrome oxidase does not proceed at a significant rate in the dark at ~15°C to ~35°C (26). Thus, addition of relatively low concentrations of nonaqueous solvents, sufficient to lower the freezing point to within this temperature range, allowed oxygenation of the mitochondrial suspension with saturating concentrations of oxygen without significant reaction of the CO-inhibited oxidase system. Oxygen was added by vigorous stirring or by the decomposition of \(H_2O_2\) by catalase.

Of greatest advantage in these studies was the fact that the frozen mixture of oxygen and the carbon monoxide compound of cytochrome oxidase can be chilled prior to photolysis to the point where the half-time of the reaction following photoly-
sis is seconds (−95°) or minutes (−120°). With such long half-times, a further step has been provided in which the process of combination with oxygen or the transformation of this compound into subsequent intermediates could be stopped or trapped at any desirable point by cooling the sample tube to temperatures of liquid nitrogen, where time is available for examination of the sample by electron paramagnetic resonance (EPR), laser Raman and Mössbauer spectroscopy, magnetic susceptibility, and other physical techniques.

The method is termed "triple trapping" (27) and has afforded optical and EPR spectroscopic evidence of a fully occupied cytochrome oxidase–oxygen compound, and of two further compounds as well (28, 29). These compounds are functional in oxygen reduction and afford a basis for understanding energy conservation at the molecular level in oxygen reduction by membrane-bound cytochrome oxidase.

MATERIALS, METHODS, AND RESULTS

Fig. 1 illustrates the methods and the results in four panels which present the first minute in the formation of the compounds of membrane-bound cytochrome oxidase with oxygen. Four temperatures are selected to illustrate the intermediates involved in this reaction, together with the kinetics of the formation and interconversion.

The sample is prepared by diluting pigeon heart mitochondria prepared according to the method of Chance and Hagihara (30) to a final concentration of 30 mg of protein per ml in a medium containing 20% ethylene glycol by volume, the remainder being mannitol–sucrose–phosphate buffer, pH 7.4 at 23°. The mitochondria are supplemented with 10 mM succinate, allowed to become anaerobic, and saturated with carbon monoxide (1.2 mM). The suspension is transferred to a 3.5 mm diameter quartz tube, chilled to just above its freezing point (−20°), and stirred vigorously for ten seconds at two stirs per second to give about 200 μM oxygen, as described elsewhere (27). Less than 2% oxidation of the cytochromes a and c occurs during the stirring interval (27).

At the end of the 10-sec oxygenation, the sample is rapidly chilled by vigorous agitation in a dry ice–ethanol mixture at −80°. The frozen sample is transferred to the Dewar flask of the spectrophotometer through which is flowing thermostatically regulated nitrogen at the temperatures indicated in Fig. 1. The Dewar flask (1 cm diameter) is surrounded by several hundred 1 mm light guides. A portion of these afford transmission measurements at the wavelength pairs indicated in the figure which are appropriate to measurements of the heme component of cytochrome oxidase (Traces a, b, and c) and its copper moiety (Trace g). These pairs of wavelengths are obtained by interlacing of light pulses from two motor-driven discs containing interference filters (2–4 nm spectral interval). The multiple traces recorded on a camera oscillograph are reproduced in Fig. 1, time proceeding from left to right. The remainder of the light guides afford optical coupling for efficient flash photolysis.

In Panel E at −92° in the absence of oxygen, flash photolysis by means of a 1 μsec, 100 mJ flash from a liquid dye laser at 585 nm shows an absorbancy decrease at 591 nm, (Trace a) and an increase at 604 nm (Trace b). The wavelengths for Trace c have been chosen so as to give a minimum response to the photolysis and recombination of cytochrome oxidase with CO. Trace g, which represents the infrared absorbance of the copper component of cytochrome oxidase, also shows no deflection. At −92°, the half-time for recombination of cytochrome oxidase with CO is about 30 sec (31); at −114°, the recombination proceeds at a negligible rate.

Compound A †. In Panel A at −114°, the sample has been oxygenated as described above, and flash photolysis causes a rapid reaction (t1/2 = 12 sec) in Trace a and more complex and slower reactions in Traces b and c; Trace g shows no reaction. A second flash 1.5 min later causes no significant photolysis, indicating that the compound formed in the presence of oxygen is much less light-sensitive than the CO compound. This is in accordance with the low quantum yield of oxy compounds of hemoproteins as compared with their carboxy compounds (32). Thus, oxygen reacts with cytochrome oxidase at −114° to form a compound identified as Compound A with a composition indicated in Table 1 as "oxycytochrome oxidase."

Panel B of Fig. 1 shows that the same absorbancy changes occur at −105° but on a 2-fold more rapid time scale at 591 nm (Trace a). The changes registered in Traces b and c are also more rapid, but Trace g shows no reaction. Thus, the formation of Compound A may involve two stages.

An additional feature of the method is that a sample activated as shown in Fig. 1 can readily be transferred from the Dewar flask at −114° to chilled isopentane or liquid nitrogen at −196°, thus trapping the reaction in progress. Such a sample, trapped as in Fig. 1 at −100°, has a difference spectrum with respect to the reduced form (Cu12+Fe3+) indicated as Spectrum no. 1 of Fig. 2. This spectrum shows a

† The designation "Compounds A, B, C" etc., has been adopted here in order to avoid confusion with the nomenclature of the peroxidase and catalase intermediates I, II, III (6, 8).
distinctive peak at 590 nm and a trough at 611 nm, characteristic of the presence of a ligand on the ferrous iron of cytochrome oxidase.

Table 1 summarizes the properties of this product of the oxygen reaction (26-29). Since the initial state of cytochrome a₃ is ferrous, and the optical and EPR data show no significant oxidation of the heme or the copper components of the oxidase, a possible structure of Compound A is Cu⁺⁺a₃⁺·O₂⁻, that entitled "A₁" in Table 1. This would correspond to a non-photolyzable compound with an absorption spectrum similar to that of the CO compound.

Of greatest importance in distinguishing Compound A from the parent CO compound are the kinetic and equilibrium constants: a second-order velocity constant of 685 M⁻¹ sec⁻¹ at -94°, combined with an equilibrium constant of 300 μM oxygen required for half-maximal saturation at -100°, and finally, an apparent energy of activation of 9.9 kcal (41.4 kJ). These parameters, together with its inherent instability (see below), identify a new compound formed by ligand replacement of oxygen for carbon monoxide in membrane-bound cytochrome oxidase.

The remarkable feature of these observations is the large apparent dissociation constant of oxygen from cytochrome oxidase at -100°: 300 μM as compared with 0.05 μM at room temperature (33). In view of the characteristically larger temperature coefficients of first-order as compared with second-order reactions (especially those near the diffusion limitation), it appears unlikely that cytochrome oxidase depends upon a tightly-bound oxygen molecule for its high oxygen affinity under physiological conditions.

The lack of involvement of copper in Compound A formation is verified by Spectrum no. 2 of Fig. 2, which shows the product of the reaction of cytochrome oxidase with oxygen under conditions where copper and all other components of mid-potentials of less than 300 mV have been oxidized by prior treatment with ferricyanide (26, 28, 29). The peak remains at 591 nm and the trough at 612 nm, indicating a composition similar to that of the compound of Spectrum no. 1. Thus, Compound A (entitled "A₁" in Table 1) can form with the heme component of cytochrome a₃ as the only spectrosopically detectable component capable of reacting with oxygen. For this reason, the two structures are postulated in

Table 1. Intermediates in the reaction of membrane-bound cytochrome oxidase and oxygen at low temperatures

<table>
<thead>
<tr>
<th></th>
<th>Compound A₁</th>
<th>Compound A₂</th>
<th>Compound B₁</th>
<th>Compound C₁</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possible name</td>
<td>&quot;Oxycytochrome oxidase&quot;</td>
<td>&quot;Peroxy cytochrome oxidase&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Possible composition</td>
<td>Cu⁺⁺a₃⁺·O₂⁻</td>
<td>Cu⁺⁺a₃⁺·O₂⁻</td>
<td>Cu⁺⁺a₃⁺·O₂⁻</td>
<td>Cu⁺⁺a₃⁺·O₂⁻</td>
</tr>
<tr>
<td>Absorption band</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum (nm)</td>
<td>591</td>
<td>591</td>
<td>606</td>
<td></td>
</tr>
<tr>
<td>Trough (nm)</td>
<td>611</td>
<td>612</td>
<td>609</td>
<td></td>
</tr>
<tr>
<td>% Absorbancy change relative to Ox-Red change</td>
<td>18</td>
<td>18</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>(605-650 nm)</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>40</td>
<td>~15-20</td>
</tr>
<tr>
<td>(830-960 nm)</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% EPR change relative to Ox-Red change</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(g = 3.05)</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>35-40</td>
<td>~20</td>
</tr>
<tr>
<td>(g = 2.08)</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>35-40</td>
<td>?</td>
</tr>
<tr>
<td>Second order velocity constant</td>
<td>685</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>at -94° (M⁻¹ sec⁻¹)</td>
<td>—</td>
<td>—</td>
<td>0.45</td>
<td>(1.1)</td>
</tr>
<tr>
<td>First order velocity constant</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>at -78° (sec⁻¹)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Equilibrium constant (M)</td>
<td>300</td>
<td>—</td>
<td>300</td>
<td>~350</td>
</tr>
<tr>
<td>Temperature</td>
<td>-100</td>
<td>—</td>
<td>-96</td>
<td>-81</td>
</tr>
<tr>
<td>Apparent energy of activation</td>
<td>9.9</td>
<td>—</td>
<td>12.5</td>
<td>11.5</td>
</tr>
<tr>
<td>(kcal)</td>
<td>(41.42)*</td>
<td>—</td>
<td>(52.3)</td>
<td>(48.12)</td>
</tr>
</tbody>
</table>

* Values in parentheses indicate apparent energy of activation in kJ.
Table 1, depending on whether the copper is in the oxidized or the reduced state. In Panels C and D of Fig. 1 at −96° and −84°, distinctive kinetics are seen in Traces g indicating the oxidation of the copper moiety of cytochrome a₃. Traces a of these two panels now clearly represent the conversion of Compound A to a second intermediate, from two standpoints. First, the steady state concentration of Compound A no longer rises to a maximum; instead, Compound A appears as a transient intermediate in its conversion to a new compound which is identified by a decrease in absorbancy (Compound B) rather than an increase in absorbancy (Compound A) at 591 nm. This absorbance decrease proceeds slightly beyond that of photolyzed cytochrome a₃++, as evidenced by comparing the level just after photolysis in Panel E with the plateau level at the end of Panels C and D. Traces c of both panels show a summation of the kinetics of formation of Compound A as a fast phase and Compound B as a slower phase.

Traces b are of interest because they show at this point the slower kinetics of a component of Compound B whose spectrum is included in that of no. 3 of Fig. 2, trapped at −70° when the reactions of Panels C and D will have proceeded to equilibrium. Again, the spectrum is recorded with reference to the ferrous cytochrome a₃. In accordance with the kinetic traces, there is a small net change of absorption at 591 nm; the absorbance band characteristic of Compound A is no longer present. In addition, the trough is displaced from 611 nm to 609 nm. This spectrum is identified with a second intermediate, Compound B (25−27). By the disappearance of absorption at 609 nm and the increase of absorption at 830 nm, one may conclude that a portion of both the copper and the iron of cytochrome oxidase has been oxidized, corresponding to a transfer of two electrons.

Table 1 lists a possible composition of Cu²⁺a₃⁺⁺ ·O₂⁻ for Compound B ("B₂") with the position of the absorbance trough and the appropriate fraction of the oxidized-minus-reduced changes that are observed to be altered according to both EPR and optical spectroscopy (34). The kinetic properties of Compound B resemble those of Compound A up to the point where the rate of conversion from A to B becomes rate-limiting, for example, at 0.45 sec⁻¹ at −78°. Since Compound B is the product of the conversion from Compound A, the equilibrium constants are not distinguishable, being 300 μM at −96°. The energy of activation for the first-order step in the formation of Compound B is 12.5 kcal (52.3 kJ) as compared with 9.9 kcal for Compound A.

Compound C. Whereas the absorption spectrum of Compound A appears to be unaffected by whether the copper of cytochrome oxidase has been oxidized by a prior supplement of ferricyanide (1 mM) as shown in Spectrum no. 2 of Fig. 2, the nature of the subsequent reaction product (Spectrum no. 4) is affected. The 591 nm band disappears as in the case of Compound B (Spectrum no. 3) but Spectrum no. 4 shows the absorbance in the region of 605 nm to be in the opposite direction to that observed with Compound B and of larger magnitude. A small but significant change of absorbance at 830 nm is also observed in the formation of Compound C (compare Table 1). Under these conditions, most, if not all, of the other components of cytochrome oxidase (cytochrome a and its associated copper, as well as the copper associated with cytochrome a₃) are initially oxidized and it is only the positive potential of a₃⁺⁺−·CO (35) that maintains it in the reduced state during ferricyanide treatment. On this basis, the heme of cytochrome a₃ is the chief electron donor, and a possible structure for Compound C, shown in Table 1, is Cu²⁺a₃⁺⁺ ·O₂⁻. The kinetics of formation of Compound C at a given temperature are about 2.5-fold faster than those of Compound A. The oxygen affinity of Compound C is close to that of Compound A (350 μM at −81°) and the energy of activation is again larger than that of Compound A.

**DISCUSSION**

The diagram of Fig. 3 focuses upon cytochrome oxidase, which we here term "Cu a₃" signifying the valencies in the usual manner; in the components preceding Step no. 1, the reduced state is indicated as Cu⁺⁺a₃⁺⁺ and the mixed valency state obtained by ferricyanide treatment as Cu³⁺a₃⁺⁺. Both species provide suitable starting points for studying the reaction with oxygen.

The other components of cytochrome oxidase, Cu and cytochrome a, are included in the cycle of electron transfer reactions at −60° and above. Thereafter follow the electron transfer reactions which result in the oxidation of cytochromes c and c₁ and the complete reduction of oxygen to water. It is obvious that a multitude of initial and intermediate states are feasible in a system containing six or more electron donors (four hemes, two copper atoms) and three valency states of iron and two of copper (and of iron sulfur proteins as well) (36−38, and manuscript in preparation). Thus, the diagram of Fig. 3 represents a minimum of intermediates and electron transfer steps and is not intended to be comprehensive.

Beginning the cycle at −125° and with the photolysis of the CO compound yielding completely reduced or mixed valency state cytochrome oxidase, the reaction with oxygen ensues to give the first intermediates, A₁ and A₅, respectively. These two compounds may be termed "oxycytochrome oxidase" and are proposed to be the functional intermediates.
in the reaction of cytochrome oxidase with oxygen. These compounds are distinct from the carbon monoxide compound, or from a functionally inactive compound, on the basis of the observation that there are (a) small but significant differences in the positions and intensities of the absorption bands of Compound A and the CO compound; (b) very large differences in kinetic and equilibrium constants; (c) very large differences in photosensitivities. The compounds are identified as functional by their inherent instability and by their ready conversion into the variety of other compounds shown in this cycle, such compounds serving as electron acceptors for the function of the respiratory chain.

As may be said of any "primary" intermediate, prior intermediates are possible. However, the linear relationship between the oxygen concentration and the rate of formation of Compound A (explored from 4 sec⁻¹ at −94° to 2000 sec⁻¹ at −16°) (14–17) speaks strongly in favor of Compounds A₁ and A₂ as the first step in the cytochrome oxidase–oxygen reaction.

The composition of Compounds A₁ and A₂ as a₃²⁺−O₂ is compellingly related to that of a₃²⁺−CO by virtue of the general similarity of the positions of the absorption bands and the amplitudes of the absorption changes in the O₂ and CO compounds. However, the variety of structures postulated for oxyhemoglobin (39) are equally applicable to oxyctyochrome oxidase. There is no evidence of oxidation of the heme or copper moiety of cytochrome a₁ in the formation of compounds of Type A.

The apparent inconsistency between the low temperature and room temperature properties of the cytochrome oxidase–oxygen reaction underlines its mechanism of action. At −100°, oxygen appears to be highly dissociated from the oxidase, whereas at 23° the apparent affinity is approximately 0.05 µM (33), a ratio of nearly 10⁴. It appears that the high affinity of cytochrome oxidase for oxygen at room temperature is due to the kinetic rather than a thermodynamic property. The rapid electron transfer at higher temperatures, reducing oxygen to the variety of compounds illustrated here, creates a trap which affords the key to the physiological function of cytochrome oxidase. Thus, practically irreversible utilization of all oxygen molecules available to combine with cytochrome oxidase occurs at body temperature. It is a matter of interest that both cytochrome oxidase and oxyhemoglobin (40) have a low affinity for oxygen in their primary reactions and a high affinity for oxygen in the final stages of the oxygen reaction. It appears that they accomplish this in different ways: hemoglobin by a change in the structure of the tetramer, and cytochrome oxidase by an electron transfer reaction which reduces the oxygen molecule and may alter the structure of the cytochrome oxidase molecule as well.

The electron donation to oxygen is indicated in Fig. 3 to occur at Step no. 2, from either the reduced state to form Compound B₁ or from the mixed valency state to form Compound C₁. These are hypothetical intermediates involving a one-step electron transfer and the formation of the superoxide anion from oxygen. Experimentally, however, electron donation from both the heme iron and the copper atom is observed and thus Step no. 3 follows Step no. 2 immediately, leading to intermediate B₂ in which the oxygen is reduced to the peroxide level and the copper and the heme are oxidized to formal valency states of Cu²⁺ and Fe³⁺. The detailed evidence in support of a two-electron transfer in Compound B₂ is the disappearance of a portion of the 605 nm band, and the appearance of a portion of the 530 nm band, suggesting partial oxidation of the iron and copper components, respectively. Furthermore, increased EPR signals at g = 3.05 and g = 2.03 support the optical data. The possibility that superoxide anion (O₂⁻) is present at an appreciable concentration in Compound B₂ does not appear to be supported by the EPR spectra of the iron at g = 3.05 and copper at g = 2.03, which do not exhibit distortion due to a highly paramagnetic ion such as O₂⁻. This is also consistent with reduction of oxygen to the peroxide level in Compound B₂.

A similar argument is used to indicate that Compound C₂ involves oxygen reduction to the peroxide level. This would require oxidation of the heme iron to the quadrivalent state in Compound C₃, since no other known electron donor is available in the mixed valency state of the oxidase, nor is there any evidence of a g = 2 free radical signal, as in the case of Complex ES, the peroxide compound of yeast cytochrome c peroxidase and peroxide (41). This may account for the distinct spectrum of Compound C₂. This configuration differs from that of the "ferry" ion, Fe⁴⁺−O₂⁻, which has been postulated as an intermediate in the reactions of myoglobin and peroxidase (6). However, a variety of mixed valency states (manuscript in preparation) is possible in Compound C. Donation of one electron to Compound C₂ converts it to B₃ and thereafter it follows the remainder of the catalytic sequence.

Compound B₂ is stable to −60° and Compound C₂ to much higher temperatures. Below −60°, there is no spectroscopic evidence for the oxidation of other components of the system, for example, from cytochrome a or its associated copper, or cytochromes c or c₁.

At temperatures above −60°, electron transfer reactions begin to occur; presumably, some rotational to translational motion is necessary for electron transfer between the a and a₃ units of the cytochrome oxidase molecule. Electron transfer from cytochromes c and c₁ begins to occur at the same temperature. Sequential steps are indicated in no. 4 and no. 5, resulting first in oxidation of the heme of cytochrome a and then of cytochromes c and c₁, returning the heme and copper of cytochrome a to the reduced state.

At this point, a wide variety of electron transfer steps is feasible, and oxygen is eventually reduced to the level of water via the heme and copper of cytochrome a₃, following Step no. 6. The possibility of a peroxide bridge structure between heme iron and copper for Compounds B₂ and C₃ is seriously to be considered (compare ref. 39): for Compound B₂, Cu²⁺−O−O−Fe⁴⁺, and for Compound C₃, Cu²⁺−O−O−Fe⁴⁺.

Protonation of partially or completely reduced oxygen may occur at any point in the sequence, and it is only for the sake of brevity and lack of direct evidence that this process is indicated to occur in a single step when oxygen has been reduced to the level of water. Preliminary experiments described elsewhere (17) suggest that hydrogen ions may be taken up as early as Step no. 3. However, since protonation of a bridge peroxide would not occur, it is possible that protonation occurs very late in oxygen reduction and thus is not energy-linked. In any case, the cycle is complete with the formation of water and the release of cytochromes a and a₃ and their associated copper atoms in the reduced form. The cycle can then be restarted along the A₁ → B₁ → B₂ pathway.

In the early stages of this work, Drs. N. Sato, B. Hagihara and J. Leonard used the split-beam spectrophotometer in this
laboratory to record the spectrum of what is now termed “Compound A”, and our thanks are due to them for their stimulation of the results described in this paper. The authors also wish to acknowledge the excellent technical assistance of Yuriko Mukai, Louis Tannen, and Alberto Salcedo. This research was supported by NIGMS 12202.
