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# Oxygen "pulsed" cytochrome *c* oxidase: Functional properties and catalytic relevance

**(electron transfer/oxidase intermediate/$O_2$ utilization)**

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ABSTRACT **The kinetics of the reaction of cytochrome *c* with solubilized mammalian cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) has been studied by a stopped-flow technique under two different experimental situations: (*i*) the completely oxidized enzyme (*resting* oxidase as obtained from the preparation) was mixed with reduced cytochrome *c*, and (*ii*) the completely reduced enzyme in the presence of reduced cytochrome *c* was exposed to a "pulse" of $O_2$ (*pulsed* oxidase). Both sets of experiments were performed with either "limiting" or "excess" $O_2$ (relative to oxidase), in the presence or absence of CO. Both the pre-steady-state events and the steady-state kinetics of cytochrome oxidase are found to be different in the two cases. This shows that the product of the reaction of fully reduced oxidase with $O_2$ (*pulsed* oxidase) is functionally different from the oxidase as prepared (*resting* oxidase). These differences are interpreted with the assumption of a different rate of intramolecular electron transfer in the *pulsed* and *resting* oxidases.**

**Implications of these experimental findings are discussed in the general framework of a tentative model for the catalytic cycle of the oxidase.**

Recent kinetic studies on mammalian cytochrome oxidase have been aimed at the isolation of individual steps and the identification of intermediates involved in catalysis (1–3). Pre-steady-state measurements have so far been performed mainly on the reaction of the fully or partially oxidized enzyme with reduced cytochrome *c*, under aerobic or anaerobic conditions, and on the reaction of the fully reduced enzyme with oxygen (4–8).

In brief, these investigations have characterized the electron transfer reaction between cytochrome *c* and the oxidase and have shown that (*i*) electrons enter the oxidase through cytochrome *a* and (*ii*) they are transferred rapidly to a second site that has been identified with the visible (absorbing at 830 nm) copper. These reactions appear to be relatively insensitive to changes in the state of cytochrome $a_3$—whether oxidized (5, 6), oxidized in the presence of cyanide (6), or reduced in the presence of CO (9). The reaction of the fully, or partially, reduced enzyme with $O_2$ has been investigated and found to be rapid indeed, the slowest step being of the order of 700 $sec^{-1}$. Recently, low-temperature kinetic studies (10, 11) have revealed a number of spectrally distinct and functionally significant intermediates in this reaction. Both entry to and release of electrons from oxidase are therefore very rapid and fully consistent with the known catalytic rates of the enzyme.

On the other hand, the rate of reduction of cytochrome $a_3$, starting from the fully oxidized enzyme and occurring through electron transfer from another site within the molecule, has been measured by a photochemical method and found to be slow, with half-time of approximately 1.2 sec (at 20° and pH 7.4) (5). The first-order rate constant for this process is slow, clearly inconsistent with reported values for the catalytic rate.

The kinetic studies reported here differ from previous ones in that they have been designed to follow: (*i*) the steady-state behavior of the species formed when the fully reduced enzyme is exposed to a pulse of oxygen (called hereafter *pulsed* oxidase), and (*ii*) the pre-steady-state events following the reaction of the *pulsed* enzyme with reduced cytochrome *c*. Parallel experiments have been conducted using the oxidized enzyme as isolated (called the *resting* enzyme).

On the basis of the experiments reported below, it is clear that the *pulsed* enzyme is functionally different from the *resting* enzyme, and we suggest that the latter species is not *directly* involved in the catalytic cycle but may become so once it has been completely reduced. Thus, it is proposed that the *resting* enzyme represents a pool that may be brought into the catalytic cycle in response to an increased electron flux through the system. The balance between the catalytically competent forms of the enzyme and the *resting* species, which is catalytically incompetent and into which the pulsed enzyme may decay, is sensitive to the flow of electrons through the chain and may therefore constitute an important regulatory mechanism in mitochondrial respiration.

## EXPERIMENTAL

Cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) was prepared from beef heart according to the method of Yonetani (12). All experiments were carried out at 20° in 0.1 M phosphate buffer, pH 7.4, containing 1% (mol/mol) Emasol 4130.

The concentrations of cytochrome oxidase and cyctochrome *c* were determined spectrophotometrically in the presence of dithionite by using the following values of the extinction coefficients: $\epsilon_{mM} = 21$ at 605 nm for reduced cytochrome oxidase (total heme); and $\epsilon_{mM} = 27.6$ at 550 nm for reduced cytochrome *c* (13).

Kinetic experiments were performed with a Gibson-Durrum stopped-flow apparatus equipped with a 2-cm observation chamber (dead time, 3–4 msec).

## RESULTS

By taking advantage of the fact that the reaction of reduced cytochrome oxidase with molecular oxygen is a very fast process

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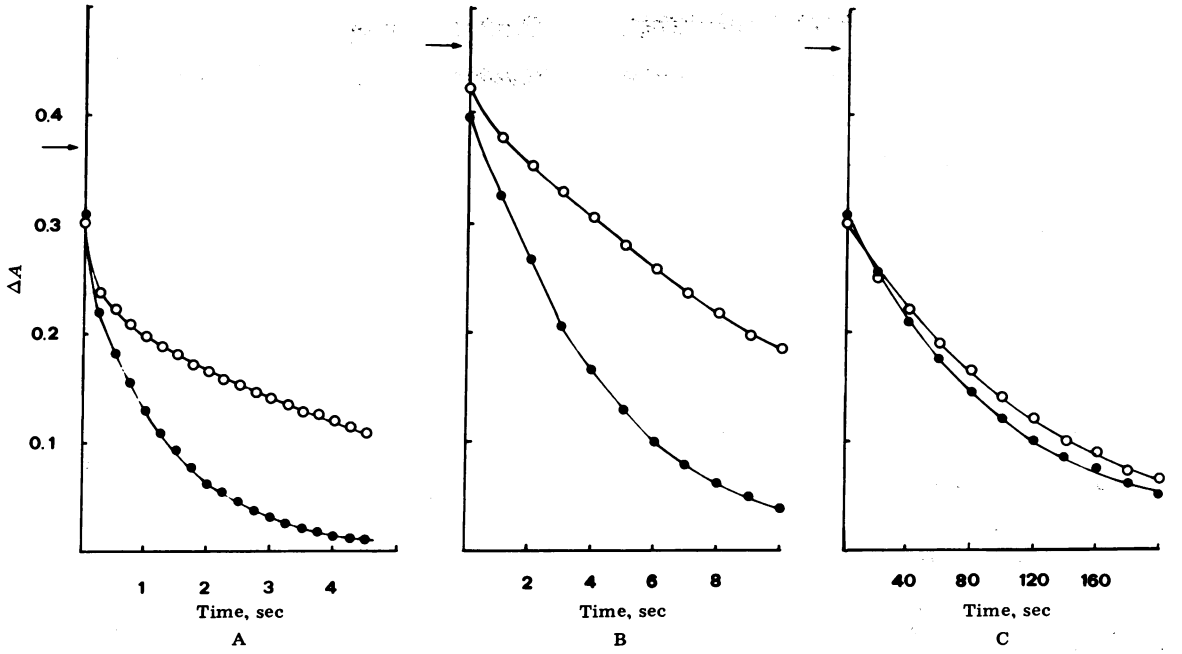


FIG. 1. Time course of oxidation of cytochrome *c* catalyzed by cytochrome oxidase. The reaction was followed at 550 nm in the stopped-flow apparatus (*A* and *B*) or in the Cary 14 spectrophotometer (*C*). The light path of observation was 2 cm in the former case and 1 cm in the latter. ●, *Pulsed* oxidase; ○, *resting* oxidase. The cytochrome *c* oxidase, cytochrome *c*, and $O_2$ concentrations after mixing were, respectively: 4, 10, and 135 μM in *A*; 0.95, 12.5, and 135 μM in *B*; and 0.12, 25, and 270 μM in *C*. Ascorbate was used as a reducing agent; its concentration always was 300 μM (after mixing). The buffer was 0.1 M potassium phosphate, pH 7.4, containing 1% (vol/vol) Emasol 4130. The temperature was 22°. The arrows indicate the expected total change in absorbance ($\Delta A$). Note difference in time scales.

(8–10), it is possible to investigate the kinetic properties of the product of this reaction *immediately* after formation of the reaction product (*pulsed* oxidase). In stopped-flow experiments, *pulsed* cytochrome oxidase was exposed to reduced cytochrome *c* in the absence or in the presence of excess $O_2$. The results of these experiments are compared, side-by-side, with results of similar experiments using cytochrome oxidase as prepared (*resting* oxidase).

**Steady-State Experiments.** Fig. 1 shows the time course of cytochrome *c* oxidation (followed at 550 nm) at different concentrations of cytochrome oxidase in the presence of excess oxygen. In one set of experiments (*pulsed* oxidase), reduced cytochrome *c* and reduced cytochrome oxidase were mixed with buffer equilibrated with air. Under these conditions, the pre-steady-state phase involved a series of events, including the very fast reaction of the fully reduced enzyme with $O_2$ (with the formation of *pulsed* oxidase) and subsequently the fast electron transfer from reduced cytochrome *c* to cytochrome *a* (and visible copper) in the *pulsed* oxidase. In a second set of experiments, reduced cytochrome *c* was mixed with oxidized oxidase (the *resting* enzyme) in the presence of air. Under these conditions, the pre-steady-state phase involved, as the first process, electron transfer to cytochrome *a* (and visible copper). In both sets of experiments, a steady-state phase was established after the fast pre-steady-state events.

It can be seen from the data in Fig. 1*A* that, at constant concentration of reduced cytochrome *c* and at the highest oxidase concentration, the initial velocity for the steady-state phase was very much faster when starting from the *pulsed* enzyme than when starting from the *resting* one, the difference being approximately 4- to 5-fold. On the other hand, at the lowest oxidase concentration (Fig. 1*C*), the steady-state process proceeded with approximately the same overall velocity with the *pulsed* and the *resting* oxidase.

Also, the time course of the steady-state phase was different in the two cases. Especially at some wavelengths (e.g., 563 nm), the mixing experiments with the *resting* enzyme seemed to show, after the fast pre-steady-state burst, a lag phase that was not apparent in parallel experiments with the *pulsed* enzyme.

Two features are clear from these experiments: (*i*) the dependence of the steady-state velocity, and its time course, on the past history of the oxidase, and (*ii*) the disappearance of the observed differences at very low oxidase/cytochrome *c* ratios. As indicated below, both features are fully consistent with the idea that the *resting* enzyme is not an intermediate in catalysis but may be transformed into an active species after being fully reduced in the presence of $O_2$.

**Pre-Steady-State Experiments: Reaction at High Concentration of Cytochrome *c* and Low Concentration of $O_2$.** Fig. 2 shows the observed changes in absorbance at 563 and 605 nm in the mixing experiments. These wavelengths were chosen because the former is isosbestic for the fully oxidized versus the fully reduced cytochrome oxidase and at the latter the contribution of cytochrome *c* is negligibly small compared that of the oxidase.

The time course of the reaction of the *resting* oxidase with cytochrome *c* in the absence of $O_2$ was in agreement with that obtained previously under the same conditions (6). The fast electron transfer from reduced cytochrome *c* to cytochrome *a* was essentially complete in the first 20 msec and, in view of the high value of the second-order rate constant (4–7), a substantial part of this process is lost in the dead time of the apparatus. A slower process, corresponding to more cytochrome *c* oxidized (about 1 electron equivalent), was observed at 563 nm; over the same time range, only a small absorbance change was observed at 605 nm. The pattern was the same in the absence (not shown) and in the presence of a large excess of CO although in the latter case the final product of the reaction is the CO complex of the fully reduced oxidase (5).

When reduced cytochrome oxidase in the presence of reduced cytochrome *c* was mixed with stoichiometric amounts

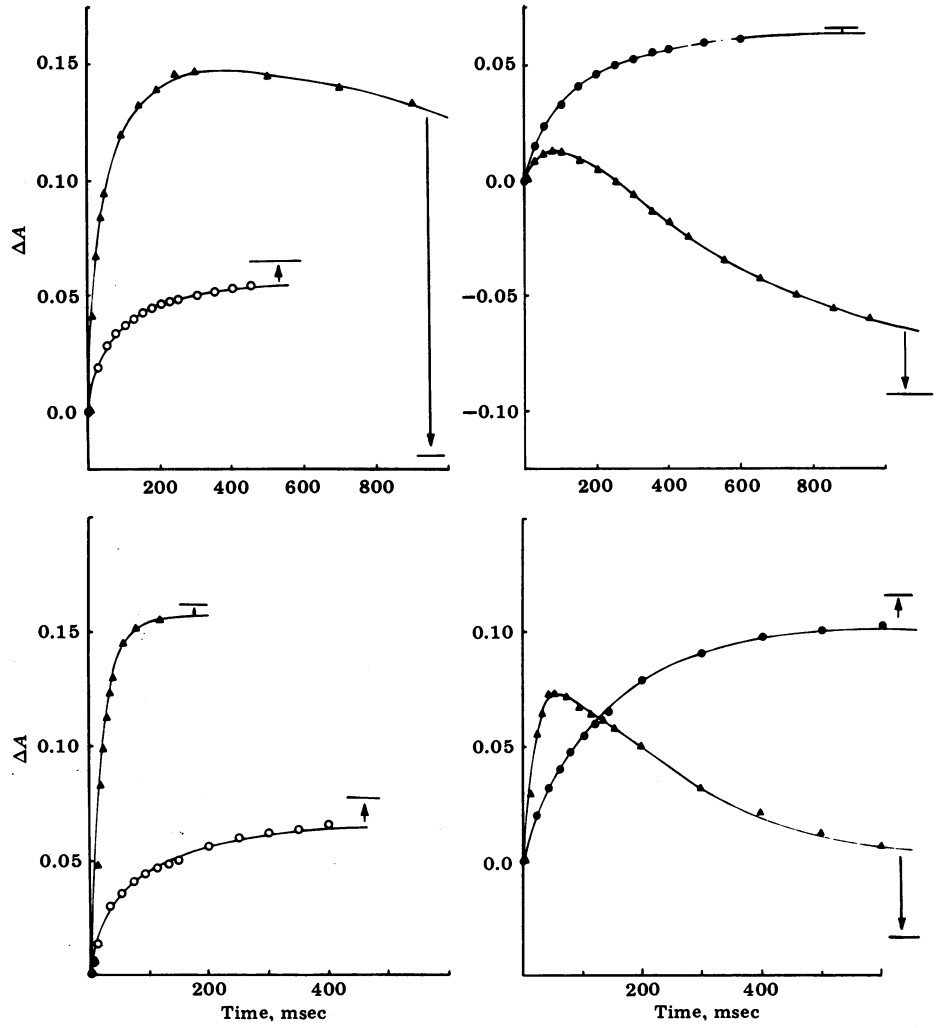


FIG. 2. Time course of the reaction between cytochrome *c* and cytochrome oxidase at low $O_2$ and high cytochrome *c* concentrations in the presence of CO. The reaction was followed at 563 (circles) and 605 nm (triangles) in the stopped-flow apparatus. Closed and open symbols refer to the *pulsed* and *resting* forms of the cytochrome oxidase, respectively. $\Delta A$ values are differences between the value measured at various times and the value measured at 0 time (i.e., 3–4 msec after mixing). The arrows indicate the $\Delta A$ observed about 1 min after mixing. Reagent concentrations ($\mu$M, before mixing) used in the mixing experiments were as follows: (*Upper Left*) oxidase 40, $O_2$ 275 plus cytochrome *c* 20, ascorbate 400, $O_2$ <4; (*Upper Right*) oxidase 38, cytochrome *c* 20, ascorbate 400, $O_2$ <4 plus $O_2$ 275; (*Lower Left*) oxidase 35.5, $O_2$ <4 plus cytochrome *c* 100, ascorbate 500, CO 900, $O_2$ <4; (*Lower Right*) oxidase 36, cytochrome *c* 100, ascorbate 500, $O_2$ <4 plus $O_2$ 15, CO 950. Other conditions as in Fig. 1.

of $O_2$ (1 mol of $O_2$ per oxidase unit), the initial phase of the reaction, after the rapid oxygen reaction (i.e., the fast electron transfer to cytochrome *a*), was similar to that described above. On the other hand, in the later stages of the reaction the *pulsed* oxidase behaved differently from the *resting* enzyme. This was especially evident in the presence of CO, when a process associated with a large absorbance decrease at 605 nm was evident, the half-time of the process being approximately 200 msec.

In order to eliminate the possibility that the unique properties of the *pulsed* oxidase result from a complex with ferrocytochrome *c* formed during the long period in which the two reside together in the stopped-flow syringe, we performed the following control study. Cytochrome oxidase was reduced with excess ascorbate containing a trace (1 $\mu$M) of cytochrome *c* and mixed, in the stopped-flow syringe, with a solution of ferrocytochrome *c* and oxygen. The enzyme formed as a result of the rapid oxidation showed all the kinetic properties that characterize the *pulsed* oxidase, thus excluding complications due to complex formation.

Fig. 3 reports the kinetic difference spectrum for the slower process (i.e., from 100 msec after mixing to about 1 min after mixing) in the region from 563 to 650 nm for the *pulsed* oxidase in the absence and presence of CO. Although the overall features of the two difference spectra were similar, the amplitude was much greater in the presence of CO. The difference between the two difference spectra, also shown in Fig. 3, corresponds to the difference spectrum between $a_3^{+2}$ CO and $a_3^{+2}$ [peak at 590 nm, trough at 605 nm, isosbestic at 598 nm, amplitude within 5% from calculated (9)]. This finding proves that the process occurring between 100 msec and infinity is indeed combination of CO with reduced cytochrome $a_3$.

Parallel controls showed, in accordance with the literature (4, 9), that under the conditions used (CO concentration $\simeq$ 500 mM) the combination of CO with reduced cytochrome $a_3$ is fast compared to the overshoot shown in Fig. 2 and therefore is not rate limiting. Thus, the rate-limiting process is elsewhere, and we presume that it is the internal electron transfer that limits the rate of the CO combination.

## DISCUSSION

The fast electron transfer between cytochrome *c* and cytochrome *a*, as well as the internal transfer from cytochrome *a*

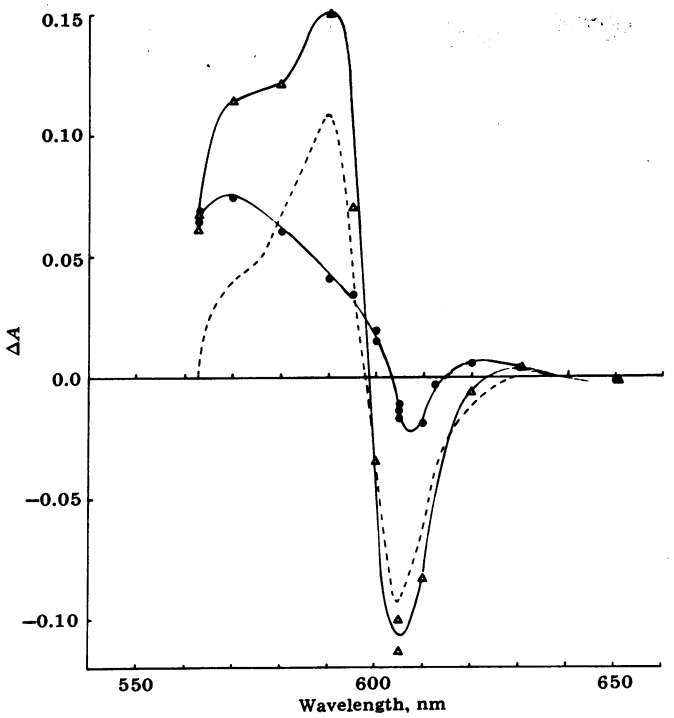


FIG. 3. Kinetic difference spectrum of the slow process (100 msec onward) observed in the reaction illustrated in Fig. 2. Experiments were run by mixing: (*i*) cytochrome oxidase (36 μM), cytochrome *c* (100 μM), and ascorbate (500 μM; free of $O_2$) with (*ii*) $O_2$ (15 μM), in the absence of CO (●) or in the presence of CO (950 μM) (Δ). Other experimental conditions as in Fig. 2. Δ*A* values were calculated as differences between *A* observed at 100 msec and at about 1 min after mixing. Dashed curve is the difference between (Δ - Δ -) and (- ● - ● ).

to visible copper, is similar for the fully oxidized enzyme (*resting* oxidase) and the mixed-valence CO complex of oxidase (6, 14). The data available to date indicate that these faster processes on the reductive pathway are similar, within a small factor (of perhaps 2), in the *pulsed* oxidase. Thus, it may be concluded that, apart from minor quantitative differences, the initial entry of electrons into the oxidase molecule and the subsequent transfer to the "830" absorbing copper are not grossly affected by the oxidation state of cytochrome $a_3$ (and presumably invisible copper) or by complex formation between reduced cytochrome $a_3$ and gaseous ligands. The possible differences in the initial steps between the various derivatives of oxidase (*resting, pulsed,* mixed-valence CO) may become important under conditions in which the turnover number is much higher, although this remains to be explored.

Subsequent phases of the reaction, however, are different for the *resting* and the *pulsed* oxidase. Thus, a primary conclusion to be drawn from the experiments reported in this paper is that the product of the reaction of fully reduced oxidase with $O_2$ has functional properties clearly different from those of the *resting* enzyme, the fully oxidized species obtained from the preparation. Therefore, although the spectral properties of the two species appear similar, as indicated by flow-flash experiments (8), their kinetic properties are different.

In the pre-steady-state experiments at low $O_2$ concentration, the formation of reduced cytochrome $a_3$ and its subsequent reaction with CO in the *pulsed* enzyme (evident from the spectral analysis shown in Fig. 3) occur with a half-time much shorter than that reported for the *resting* enzyme. Thus, although in parallel experiments starting with the *resting* enzyme

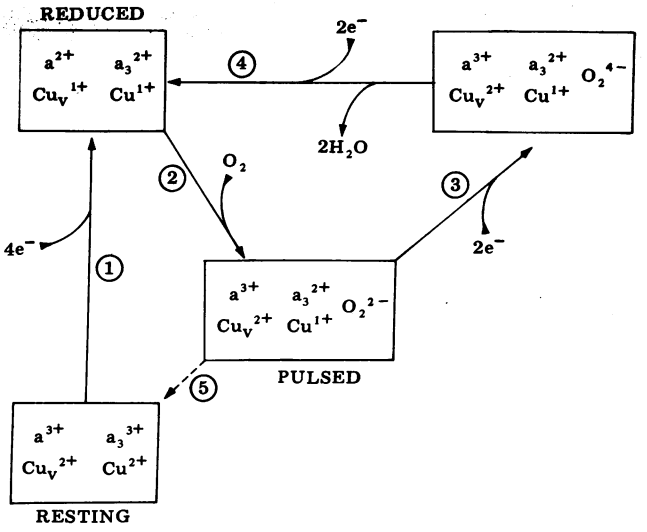


FIG. 4. Proposed scheme for system.

the reduction of cytochrome $a_3$ or its reaction with CO was not clearly discerned, previous studies indicate that ferric cytochrome $a_3$ is reduced to ferrous cytochrome $a_3$ in the presence of reduced cytochrome *c* with a half-time of about 1.2 sec (5). The experiments shown in Figs. 2 and 3 show instead that, in the presence of sufficient amounts of reduced cytochrome *c*, cytochrome $a_3$ becomes available for combining with CO about 100 msec after the formation of the *pulsed* oxidase.

The conclusion therefore seems to follow that the rate of internal electron transfer from cytochrome *a*, which produces a species capable of reacting with CO, is considerably faster in the *pulsed* oxidase than in the *resting* enzyme.

The shortening of the intramolecular electron transfer processes in the *pulsed* enzyme has, as expected, a corresponding effect on the steady-state properties of the enzyme. As shown by the data presented in Fig. 1, the initial velocity in the turnover experiments is considerably faster with *pulsed* oxidase, thus showing that this intermediate is catalytically competent. The difference between *pulsed* and *resting* enzymes in turnover experiments, which is a factor of 4 to 5 at high oxidase/cytochrome *c* ratios, disappears at low ratios. This is fully consistent with the idea that the *resting* oxidase is not an intermediate in the catalytic cycle, and only upon complete reduction and combination with $O_2$ does it become a catalytically active form.

A tentative representation of the system compatible with the results presented here, and with recent data in the literature (10, 11, 15), may be offered by the following overall scheme.

In this scheme (Fig. 4), a number of already identified intermediates—such as the $O_2$ adduct of reduced oxidase (10, 11)—are not indicated. The structure of the *pulsed* oxidase is difficult to assess. On the basis of low-temperature experiments on membrane-bound oxidase (10), and the fact that cytochrome *a* and visible copper ($Cu_v$) are oxidized in this intermediate, it may be represented as a peroxide-bound species. The suggestion that invisible copper (Cu) may itself be involved in the reaction of $O_2$ with reduced cytochrome $a_3$ comes from redox experiments with membrane-bound and solubilized oxidase in the presence of CO (15). However, it should be made clear that the distribution of the four electrons within this complex is not known, and alternative structures are equally justified. In particular, the earlier view of Greenwood and Gibson (8) would suggest that cytochrome $a_3$ is rapidly oxidized by molecular $O_2$.

In the absence of additional reducing equivalents the species generated by the reaction of reduced oxidase with $O_2$ will decay to the fully oxidized form (*resting* enzyme) through a slow process (or a set of processes, pathway 5). However, if in this intermediate the rate of donation of electron to the peroxide from reduced cytochrome $a_3$ is slow in comparison to that from reduced cytochrome *a* and "830" copper, a situation may arise in which cytochrome $a_3$ does not get significantly oxidized while electrons flow to the $O_2$ intermediate:

$$\begin{matrix} a^{3+} & a_3^{2+} & & & & & & & & & & \\ Cu_o^{2+} & Cu^{1+} & O_2^{2-} & \xrightarrow{2e} & a^{2+} & a_3^{2+} & O_2^{2-} & \rightarrow & a^{3+} & a_3^{2+} & & \\ & & & & Cu_o^{1+} & Cu^{1+} & & & Cu_o^{2+} & Cu^{1+} & O_2^{4-} & \end{matrix}$$

Thus, electrons entering the molecule rapidly through cytochrome *a* may be accepted directly by the $O_2$ intermediate, producing a species that, upon dissociation of $O_2^{4-}$, will be able to react with $O_2$ or CO.

According to the ideas proposed here, a species with oxidized cytochrome $a_3$ will not be present in significant amounts during the turnover, and the rate-limiting step in the catalytic mechanism will be electron transfer to the (peroxide) intermediate, leaving ferrous cytochrome $a_3$ [and "invisible" copper (15)] able to react with $O_2$.

The catalytic cycle (on the right in Fig. 4) will be maintained while a sufficiently high number of electrons flow through the system. When this is depressed, the *pulsed* oxidase will decay to the *resting* enzyme, which can be converted to an active species by a burst of electrons from the respiratory chain, giving rise to completely red oxidase. In this type of scheme, branching pathways are indicated to be key events in the regulation of respiration in mitochondria.


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