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The Mechanism by Which Oxygen and Cytochrome *c* Increase the Rate of Electron Transfer from Cytochrome *a* to Cytochrome a_3 of Cytochrome *c* Oxidase*

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When cytochrome *c* oxidase is isolated from mitochondria, the purified enzyme requires both cytochrome *c* and O_2 to achieve its maximum rate of internal electron transfer from cytochrome *a* to cytochrome a_3. When reductants other than cytochrome *c* are used, the rate of internal electron transfer is very slow. In this paper we offer an explanation for the slow reduction of cytochrome a_3 when reductants other than cytochrome *c* are used and for the apparent allosteric effects of cytochrome *c* and O_2. Our model is based on the conventional understanding of cytochrome oxidase mechanism (*i.e.* electron transfer from cytochrome *a*/Cu_A to cytochrome a_3/Cu_B), but assumes a relatively rapid two-electron transfer between cytochrome *a*/Cu_A and cytochrome a_3/Cu_B and a thermodynamic equilibrium in the "resting" enzyme (the enzyme as isolated) which favors reduced cytochrome *a* and oxidized cytochrome a_3. Using the kinetic constants that are known for this reaction, we find that the activating effects of O_2 and cytochrome *c* on the rate of electron transfer from cytochrome *a* to cytochrome a_3 conform to the predictions of the model and so provide no evidence of any allosteric effects or control of cytochrome *c* oxidase by O_2 or cytochrome *c*.

The electron transfer from cytochrome *a* to cytochrome a_3 is usually considered to be an "internal" electron transfer, that is an electron transfer within the enzyme. It, therefore, would be expected to have first-order kinetics, independent of the type of reductant and the reductant concentration. Several reductants other than cytochrome *c* will reduce cytochrome *c* oxidase, and although these reductants have conventional hyperbolic kinetics for cytochrome *a* reduction and so in appropriate concentrations will reduce cytochrome *a* rapidly, the subsequent reduction of cytochrome a_3 by electron transfer from cytochrome *a* is remarkably slow, over 100 times slower than the rate of electron transfer from cytochrome *a* to cytochrome a_3 during turnover of the enzyme (see Table I; Refs. 1-7). As long as the reduction of cytochrome *a* is substantially faster than the internal electron transfer, the rate of internal electron transfer and, therefore, presumably the rate of cytochrome a_3 reduction should be independent of the original source of electrons. Contrary to this prediction, the rate of cytochrome a_3 reduction can be increased by about 20 times by using cytochrome *c* as the reductant (1, 3, 7, 8). Although several authors have noted the unusually slow rate of electron transfer to cytochrome a_3 when reductants other than cytochrome *c* are used (1, 3, 7, 8), no particularly satisfactory theory has been proposed to account for this phenomena.

The rate of internal electron transfer from cytochrome *a* to cytochrome a_3 should also be independent of reactions which occur after this step; yet the rate of electron transfer to cytochrome a_3 is significantly faster in the presence of O_2 ((8, 9) also see "Results").

In this paper we propose that the observed rate of cytochrome a_3 reduction is the product of a relatively rapid rate of electron transfer between cytochrome *a* and cytochrome a_3 and an oxidation-reduction equilibrium between cytochrome *a* and cytochrome a_3 which, in the "resting" enzyme (the enzyme as isolated), favors reduced cytochrome *a* and oxidized cytochrome a_3. Since the redox potential of cytochrome *a* is not altered significantly in going from the resting to "pulsed" enzyme (the enzyme after reduction and reoxidation), this implies that the redox potential of cytochrome a_3 in resting cytochrome oxidase is significantly below that of cytochrome a_3 in pulsed cytochrome oxidase. A kinetic model of the reduction of cytochrome *c* oxidase was developed and used to predict the rate of reduction of cytochrome *a* and cytochrome a_3. Based on the kinetic parameters which are known for these systems, we are able to use this model to demonstrate that the puzzling kinetics of cytochrome a_3 reduction can be easily described. Application of this model also predicted some unexpected results which we were able to confirm. These include (*a*) a dithionite concentration dependence for the rate of cytochrome a_3 reduction, (*b*) a CO concentration dependence for the rate of cytochrome a_3 reduction, and (*c*) the fact that pulsed cytochrome oxidase will show, at any dithionite concentration, reduction of cytochrome a_3 which is simultaneous with cytochrome *a* reduction.

MATERIALS AND METHODS

Reduction of cytochrome *a*, Cu_A, and cytochrome a_3 was monitored spectrophotometrically. Cu_A was monitored at 820 nm and cytochrome *a* at 600 nm. Both cytochrome *a* and cytochrome a_3 were monitored at 432 and 440 nm. At 440 nm cytochrome *a* and cytochrome a_3 both cause an increase in absorbance upon reduction and can be distinguished only by their relative rates. At 432 nm the reduction of cytochrome *a* and cytochrome a_3 causes absorbance changes which are smaller but opposite in sign and approximately

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TABLE I

The effect of various reductants capable of reducing cytochrome a on the rate of reduction of cytochrome a_3 in resting beef heart cytochrome oxidase

Compound	Rate of cytochrome a_3 reduction	Reference
	s^{-1}	
$Na_2S_2O_4$	0.1–0.4	1
$Ru(NH_3)_6$	0.02	2
Phenazine methosulfate	0.2	3
$Cr(H_2O)_6$	0.3	4
$Fe(CN)_6$	Slow	5
Cytochrome *c*	5	6
Methyl viologen	7.5	7
Benzyl viologen	7.5	7

equal in magnitude. This wavelength is also useful because it is isosbestic for cytochrome *c*.

Porphyrin cytochrome *c* was prepared according to the method described in Ref. 8, except that after iron removal the dry protein was dissolved in 50 mM Hepes,[1] pH 8.0, and later equilibrated with 10 mM Hepes, pH 7.4, by passing down a Sephadex G-25 column.

The metal to be incorporated was prepared by dissolving 100 mM divalent metal salt in 250 mM imidazole and adjusting the pH to 7.0. Dimethylformamide was added to a final concentration of 10% v/v. An equal volume of ~100 μM porphyrin cytochrome *c* was added, and the solution was warmed to 60 °C in a foil-covered test tube. Metal incorporation was monitored spectrally and was usually complete within an hour. After the reaction was complete the reaction mixture was passed down a Sephadex PD-10 column equilibrated with 10 mM Hepes, 1 mM EDTA, pH 7.0. The cytochrome *c* was collected and passed down a Bio-Rad P-10 column, 1.5 × 10 cm, equilibrated with 10 mM Hepes, pH 7.0, at a flow rate of 12 ml/h. Most samples passed as a single tight band, although occasionally a band of porphyrin cytochrome *c* preceded the metal-containing cytochrome *c*.

Cytochrome *c* was added to the cytochrome oxidase either before the reductant or after cytochrome *a* reduction. To observe cytochrome a_3 reduction, the solution must be anaerobic, and this is most easily achieved if the reductant used is dithionite, which quickly reacts with both dissolved O_2 and cytochrome *a*. Dithionite reduction was performed by adding 5–50 μl of 1 M dithionite to 2.5 ml of 2.3 μM cytochrome oxidase (aa_3) in a closed stirred cuvette. 1 M dithionite solutions were prepared daily in anaerobic 0.01 M NaOH. The enzyme solution contained 0.1 M Hepes, 1 mM diethyltriaminepentaacetic acid, pH 7.4, and 0.1% Tween 80.

When 10 mM ascorbate, 1 mM dimethylferrocene was used as the reductant, dissolved O_2 was removed by one of two procedures. In the first, O_2 was removed by several (~10) cycles of evacuation followed by saturation with O_2-free N_2 or argon, all in a septum-sealed cuvette. If CO was to be used in the reaction, it was used for the final saturation of the solution. In the second procedure, 0.5 M glucose was added to the buffer. After briefly stirring (~5 min) the solution under a stream of argon in a sealed cuvette, 0.2 mg/ml glucose oxidase containing catalase was added. After 20 min the reductants and cytochrome *c*, also treated with glucose oxidase, were added with rapid mixing. The reactions were monitored using an Aminco-Chance DW-2A spectrophotometer.

The kinetic modeling was performed using an iterative program to numerically approximate the differential equations describing various reactions (10). By repeating the approximations after very short time intervals in which small changes in reactant concentrations have occurred, the differentials can be closely approximated. Time intervals of less than 20 μs were found to give identical results, and intervals of 1–10 μs were routinely used to model the reactions. Modeling was performed using a Hewlett-Packard model 9845B computer.

RESULTS

Experimental Determination of the Rates of Cytochrome a and Cytochrome a_3 Reduction—In Fig. 1*A* the reduction of cytochrome *a* and cytochrome a_3 by dithionite are shown. At the wavelength used in this figure (432 nm), reduction of cytochrome *a* causes a decrease in absorbance while cytochrome a_3 reduction causes an increase. It is clear from this figure that under these conditions cytochrome *a* is reduced rapidly ($k_{obs} = 0.4\ s^{-1}$), causing a decrease in absorbance, while cytochrome a_3 is reduced very slowly. The rate of cytochrome a_3 reduction was dependent on the dithionite concentration with an approximate K_m of 2 mM (results not shown). As shown in Fig. 1*A*, the $k_{obs} = 0.003\ s^{-1}$ at 2 mM dithionite. The addition of cytochrome *c* increases the rate of cytochrome a_3 reduction (Fig. 1*B*) to about 0.015 s^{-1}. When we examined this phenomena we found that only cytochrome *c* containing a metal capable of a one-electron redox reaction causes an increase in the rate of cytochrome a_3 reduction (see Table II), so it seems clear that the effect of cytochrome *c* involves its electron-carrying capacity rather than an allosteric influence through binding.

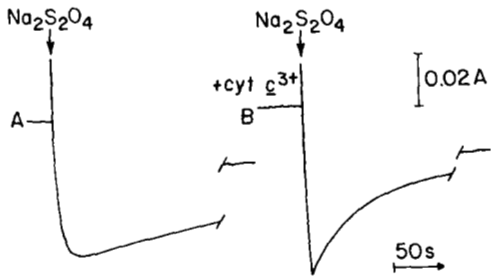

FIG. 1. **Acceleration of cytochrome a_3 reduction by cytochrome *c*.** *A*, cytochrome *c* oxidase solution was 2.3 μM aa_3 in 0.1 M Hepes, 1 mM diethyltriaminepentaacetic acid, 0.1% Tween 80 at pH 7.4, 25 °C. To 2.5 ml of the solution in a closed round cuvette was added 5 μl of 1 M $Na_2S_2O_4$ with rapid stirring. Absorbance was monitored at 432 nm, where reduction of cytochrome *a* causes a decrease in absorbance and reduction of cytochrome *c* causes an increase in absorbance. *B*, like *A* but with cytochrome c^{3+} added equimolar to cytochrome aa_3 prior to the $Na_2S_2O_4$.

TABLE II

Effect of metal replacements in cytochrome c on the rate of reduction of cytochrome a_3

Derivatives	Rate
	s^{-1}
Undergoing reversible redox cycles	
Cytochrome *c* (native)	1.0
Fe^{2+} cytochrome *c* (Fe^{2+} reinserted)	1.0
Mn^{2+} cytochrome *c*	1.0
Co^{2+} cytochrome *c*	1.0
Not undergoing reversible redox cycles	
None	0.06
Porphyrin cytochrome *c*	0.06
Cu^{2+} cytochrome *c*	0.06
Cu^{2+} cytochrome *c* (20-fold excess)	0.07
Ru^{2+} cytochrome *c*	0.06
Sn^{4+} cytochrome *c*	0.06
Zn^{2+} cytochrome *c*	0.06

Similar results were observed using anaerobic cytochrome *c* oxidase with other reductants. Cytochrome *c* oxidase was made anaerobic by using a glucose/glucose oxidase/catalase system (see "Materials and Methods"), and anaerobic ascorbate and dimethylferrocene were added as reductants. As in the previous case, we observed rapid reduction of cytochrome *a* with adequate mediator concentrations and slow cytochrome a_3 reduction (Fig. 2*A*). As in the other experiments, reduction of cytochrome a_3 was greatly accelerated in the presence of cytochrome *c* (Fig. 2*B*).

Using resting cytochrome *c* oxidase in an O_2-free CO atmosphere, dithionite reduction of cytochrome a_3 occurred with

[1] The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

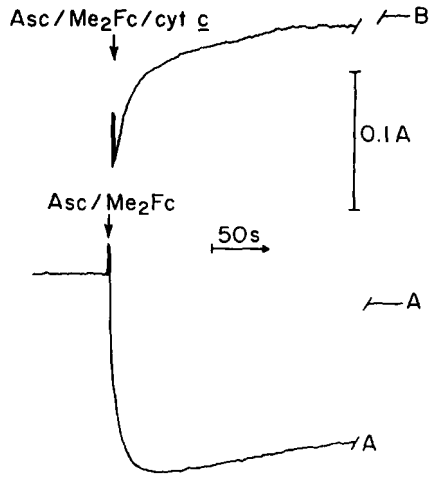

FIG. 2. **Cytochrome c induced acceleration of cytochrome a_3 reduction with reductants other than $Na_2S_2O_4$.** *A*, conditions were the same as in Fig. 1*A* except the buffer contained 0.5 M glucose and 0.2 mg/ml glucose oxidase with catalase added to the sealed cuvette (see "Materials and Methods"). 10 mM ascorbate (*Asc*) with 1 mM dimethylferrocene (*Me_2Fc*) was used as the reductant. *B*, same as *A* but cytochrome *c* equimolar to the oxidase concentration was added with the reductants.

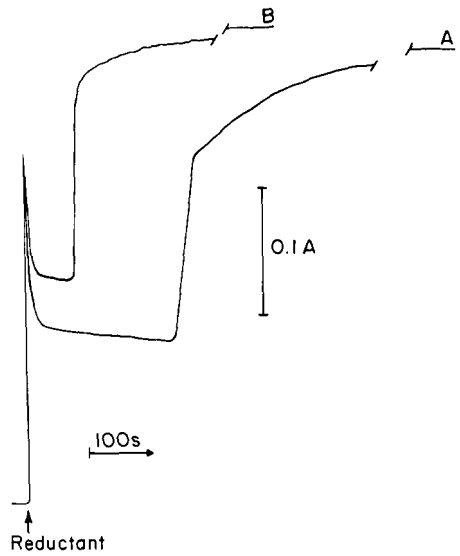

FIG. 3. **Acceleration of electron transfer to cytochrome a_3 by O_2 and by cytochrome c.** Conditions were as in Fig. 2 but without glucose oxidase/catalase and, therefore, with ~250 μM O_2 present at the beginning of the reaction. The reaction was monitored at 444 nm, where reduction of cytochrome *a* and cytochrome a_3 both cause an increase in absorbance. Upon addition of reductant the enzyme maintained a steady state level of reduction until O_2 depletion. *A*, reductant was 10 mM ascorbate, 0.5 mM dimethylferrocene; *B*, reductant was 10 mM ascorbate, 0.5 mM dimethylferrocene, 0.5 μM cytochrome *c*.

a slightly faster rate than that seen with dithionite alone. In the presence of cytochrome *c* the rate of cytochrome a_3 reduction greatly increased (not shown).

In the experiments shown in Fig. 3, cytochrome *c* oxidase was allowed to consume the O_2 in solution, using either dimethylferrocene or cytochrome *c* as electron donor. Fig. 3*A* shows that in the absence of cytochrome *c*, anaerobic reduction of cytochrome a_3 (after O_2 depletion) is much slower than the rate of turnover in the presence of O_2. The average turnover rate of electrons was estimated to be 2 s^{-1}, while the rate of the final reduction of cytochrome a_3, after O_2 depletion, was 0.008 s^{-1}, only 1/250 of the rate in the presence of O_2.

In the presence of O_2 and equimolar cytochrome *c* (Fig. 3*B*) the turnover rate was estimated to be 16 s^{-1}. (The rate was well below V_{max} for cytochrome *c* oxidase due to the low concentration of cytochrome *c*.) The rate of the final reduction of cytochrome a_3 after O_2 depletion is difficult to estimate but is considerably enhanced over the rate without cytochrome *c* and obviously less than the rate of electron transfer to cytochrome a_3 in the presence of O_2.

Kinetic Modeling—A simple kinetic model, similar to that described by Malmström and Andréasson (11), was used in the approximation of the reactions of cytochrome *c* oxidase. In our model electron transfer to cytochrome *c* oxidase was assumed to be essentially irreversible, because the experiments were done in the presence of a large excess of reductant (R).

Like Malmström and Andréasson (11), we used *E*(0000) to represent the fully oxidized enzyme, with the zeros representing the oxidized cytochrome *a*, Cu_A, Cu_B, and cytochrome a_3, in that order. Reduction of any of the redox centers is shown by changing the appropriate digit to a numeral 1. *E*(1000) represents, therefore, cytochrome oxidase with cytochrome *a* reduced and all other redox sites oxidized. The reaction sequence we used is as follows.

$$\underset{E(0000)}{A} + R \xrightarrow{k_1} \underset{E(1000)}{B}$$

$$\underset{E(1000)}{B} \underset{k_3}{\overset{k_2}{\rightleftharpoons}} \underset{E(0100)}{C}$$

$$\underset{E(0100)}{C} + R \xrightarrow{k_4} \underset{E(1100)}{D}$$

$$\underset{E(1100)}{D} \underset{k_6}{\overset{k_5}{\rightleftharpoons}} \underset{E(0011)}{E}$$

$$\underset{E(0011)}{E} + R \xrightarrow{k_7} \underset{E(1011)}{F}$$

$$\underset{E(1011)}{F} \underset{k_9}{\overset{k_8}{\rightleftharpoons}} \underset{E(0111)}{G}$$

$$\underset{E(0111)}{G} + R \xrightarrow{k_{10}} \underset{E(1111)}{H}$$

Using this model and assuming $k_1 = k_4 = k_7 = k_{10}$, the following differentials can be defined.

$$\frac{d[B]}{dt} = k_1[A][R] + k_3[C] - k_2[B]$$

$$\frac{d[C]}{dt} = k_2[B] - (k_3[C] + k_1[C][R])$$

$$\frac{d[D]}{dt} = k_1[C][R] + k_6[E] - k_5[D]$$

$$\frac{d[E]}{dt} = k_5[D] - (k_6[E] + k_1[E][R])$$

$$\frac{d[F]}{dt} = k_1[E][R] + k_9[G] - k_8[F]$$

$$\frac{d[G]}{dt} = k_8[F] - (k_9[G] + k_1[G][R])$$

$$\frac{d[H]}{dt} = k_1[G][R]$$

Although the analytical solution to this set of differential equations is not available, the time course of the reaction can be determined by using sequential iterative approximations. Approximation of the rate of electron transfer from cyto-

chrome *a* to cytochrome a_3 in the presence of O_2 or CO can also be determined by assuming that these can bind to forms E, F, G, H, in which cytochrome a_3 is reduced, and adding the appropriate differential equations.

The values for k_{1-10} were derived from a number of sources. The estimate for the rate constant for the reduction of cytochrome *a* reduction ($k_{1,4,7,10}$) was 4×10^5 M^{-1} s^{-1} for $SO_2^{\cdot}$ (1) and 1×10^7 M^{-1} s^{-1} for cytochrome *c* (12) (see Table III, Refs. 12–15). The rate of electron transfer between cytochrome *a* to Cu_A (k_2, k_3, k_8, k_9) is reported to be 700 s^{-1} (14).

The value for the rate of electron transfer from cytochrome a/Cu_A to cytochrome a_3/Cu_B (k_5) was estimated by using the approximate rate of cytochrome *c* oxidation during enzyme turnover under these conditions, $k_5 \approx 50$ s^{-1}, and then adjusted to give the best fit for the data.

The rate constant for electron transfer from cytochrome a_3/Cu_B to cytochrome a/Cu_A ($k_6 = 7000$ s^{-1}) was also from the literature (14). Similarly, values for the rate constants of O_2 and CO binding and their sources are shown in Table III, as are estimates of the rate of CO dissociation. The rate for dissociation of O_2 was assumed to be zero.

In Fig. 4*A* the actual time course of cytochrome oxidase reduction by dithionite is compared with the modeled reduction time course. The modeled time course closely fits the actual reaction. The observed rate constants for cytochrome *a* and a_3 reduction derived from the known kinetic constants were 0.4 s^{-1} and 0.006 s^{-1}, respectively, equal to the rate constants for cytochrome *a* and a_3 reduction observed experimentally.

Fig. 4*B* shows the actual time course of cytochrome oxidase reduction by cytochrome *c* and the modeled time course. The kinetic constants used in the modeled time course were the same as those used in Fig. 4*A*, except the rate constants for cytochrome *a* reduction ($k_{1,4,7,10}$) were those for cytochrome *c* in Fig. 4*B* (1×10^7 M^{-1} s^{-1}) and those for $SO_2^{\cdot}$ (4×10^5 M^{-1} s^{-1}) in Fig. 4*A*.

Fig. 5*A* shows the theoretical time course predicted by our kinetic model for dithionite reduction of cytochrome oxidase in the presence of 2 mM CO. In Fig. 5*B* the theoretical time course for the formation of $a^{2+}a_3^{2+}\cdot CO$ is shown for different concentrations of CO. For comparison, the theoretical rate of formation of $a^{2+}a_3^{2+}\cdot O_2$ is shown, neglecting the subsequent rapid reduction of bound O_2. Both CO and O_2 caused an increase in the rate of cytochrome oxidase reduction. The increase in rate was distinctly concentration dependent for CO, but for above 5 μM O_2 the increase in rate, though greater than that for CO, was O_2 concentration independent.

DISCUSSION

Reduction of cytochrome a_3 occurs by electron transfer from cytochrome *a*, and since in the conventional models of cytochrome oxidase mechanisms neither cytochrome *c* nor O_2 are involved in this step, the ability of O_2 and cytochrome *c* (Fig. 1) to catalyze this step is puzzling. As shown in Table II, only cytochrome *c* containing metal atoms capable of reversible one-electron redox reactions increased the rate of cytochrome a_3 reduction. Therefore, it appears that cytochrome *c* acts in a catalytic rather than allosteric fashion with its activation of the internal electron transfer due to its ability to reduce cytochrome *a*. Reductants other than dithionite also show slow reduction of cytochrome a_3, so the phenomena is not due to some unique property of dithionite (Fig. 2). Conversely, CO cannot replace O_2 to give the maximum rate of electron transfer to cytochrome a_3. Several alternative models of cytochrome oxidase mechanisms have been examined (1), but we find them to require hypotheses for which there is rather little evidence (for example, direct electron transfer from cytochrome *c* to cytochrome a_3) and, therefore, somewhat unsatisfactory as explanations of this phenomena. Our kinetic model is based on a rapidly attained two-electron equilibration between cytochrome *a* and cytochrome a_3 but with the thermodynamic equilibrium favoring reduced cytochrome *a* and oxidized cytochrome a_3. This model suggests that in partially reduced cytochrome oxidase, most (99.5%) of the time the electrons will reside at cytochrome a/Cu_A. Full reduction of the enzyme can occur, however, only during the intervals the electrons reside on the cytochrome a_3/Cu_B binuclear center. Consequently the rate of reduction of cytochrome a_3 will depend on the rate of electron entry into cytochrome *a*, which in turn is dependent on the second-order rate constants and the concentration of reductant, *i.e.* dithionite or cytochrome *c*. In other words, the rate at which the enzyme goes from half-reduced to fully reduced depends on the rate of formation of the a^{3+}/a_3^{2+} species, and the relative rates at which electrons are transferred to cytochrome a^{2+} from cytochrome a_3^{2+} or an external reductant. The kinetic model also predicts an increase in the rate of observable electron transfer to cytochrome a_3 caused by O_2 and, to a lesser extent, by CO, because these ligands can react with cytochrome a_3/Cu_B center during the time it is reduced.

The ability to "trap" the reduced cytochrome a_3 depends on the rate at which the ligand binds. CO, due to its relatively slow binding, had a smaller effect than O_2. O_2, which binds very rapidly, caused a large increase in rate, which was O_2 concentration independent above 5 μM O_2. These theoretical predictions agree with our experimental results shown in Fig. 3.

A further prediction of our kinetic model, that the rate of enzyme reduction by dithionite will have a distinct CO concentration dependence, remains to be tested.

Various authors have suggested that cytochrome *c* or O_2 may fill an additional allosteric role in the regular function of

TABLE III

Rate constant reactions of cytochrome c oxidase

Reaction	Reactants	Rate constants	Reference
$k_{1,4,7,10}$	$a^{3+} + SO_2^{\cdot} \rightarrow a^{2+}$	4×10^5 M^{-1} s^{-1}	1
	$[SO_2^{\cdot}]^2 = 0.85 \times 10^{-9} \times [S_2O_4]$		13
$k_{1,4,7,10}$	a^{3+} + cytochrome $c^{2+} \rightarrow a^{2+}$ + cytochrome c^{3+}	1×10^7 M^{-1} s^{-1}	12
$k_{2,3,8,9}$	a^{2+} $Cu_A^{2+} \rightleftharpoons a^{3+}$ Cu_A^{1+}	700 s^{-1}	14
$k_{O_2\text{-}ON}$	$a_3^{2+} + O_2 \rightarrow a_3^{2+} - O_2$	1×10^8 M^{-1} s^{-1}	12
$k_{CO\text{-}ON}$	$a_3^{2+} + CO \rightarrow a_3^{2+} - CO$	7×10^4 M^{-1} s^{-1}	14
$k_{CO\text{-}OFF}$	$a_3^{2+} - CO \rightarrow a_3^{2+} + CO$	0.07 s^{-1}	15
k_5	$a^{2+}a_3^{3+} \rightarrow a^{3+}a_3^{2+}$	35 s^{-1}	See text
k_6	$a^{3+}a_3^{2+} \rightarrow a^{2+}a_3^{3+}$	7000 s^{-1}	14

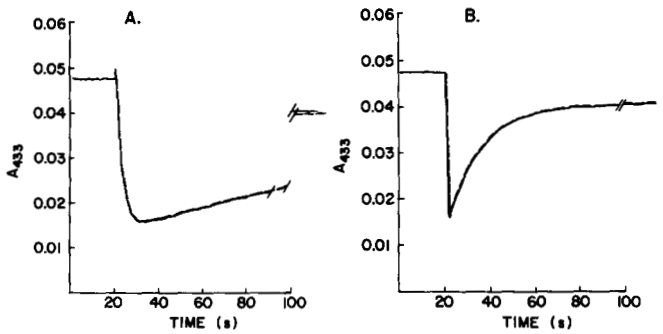

FIG. 4. **Kinetic model of reduction of cytochrome *a* and cytochrome a_3.** *A*, dithionite reduction (——) with superimposed kinetic model (- - -) oxidase solution was 2 μM aa_3. Dithionite was 20 μl of 1 M stock solution. Other conditions were as described in the legend to Fig. 1. *B*, cytochrome *c* reduction (——) with superimposed kinetic model (- - -). Conditions as in *A* but with cytochrome c^{3+} added equimolar to cytochrome aa_3 prior to the $Na_2S_2O_4$. Kinetic constants used in the modeled time course are given in the text.

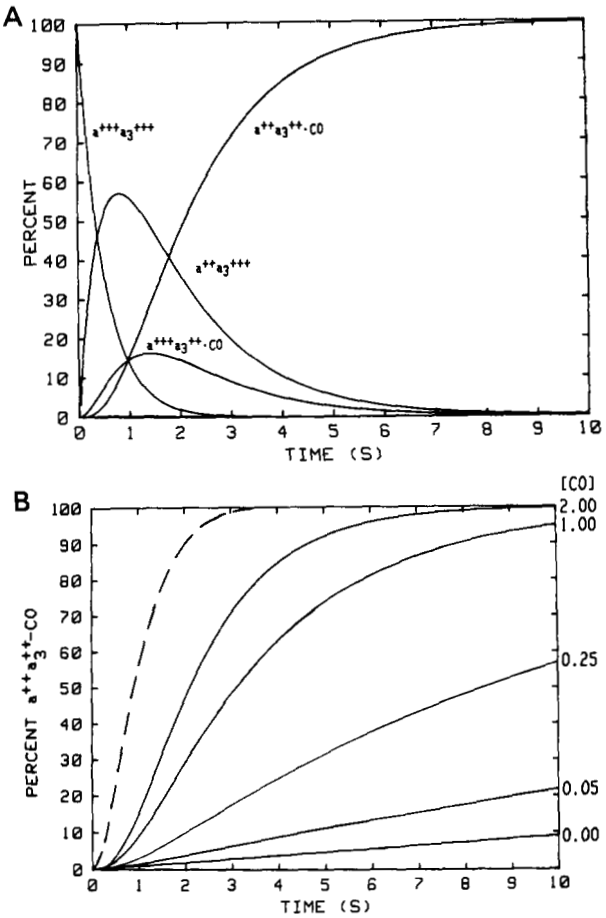

FIG. 5. **Kinetic model of dithionite reduction of cytochrome oxidase.** *A*, theoretical time course of cytochrome oxidase reduction by dithionite in the presence of 2 mM CO. The percentage of each cytochrome redox and liganding state is shown. A fifth state, a^{3+} a_3^{2+}, is obscured by the *x* axis. *B*, theoretical time course of cytochrome oxidase reduction in the presence of different CO concentrations. The CO concentration (mM) is shown on the *left side* of the figure. The *dashed line* is the time course of reduction in the presence of ≥5 μM O_2, assuming O_2 bound, but did not oxidize, reduced cytochrome a_3.

cytochrome oxidase (1, 5, 8, 9, 16–19). It appears that the kinetic anomalies which seemed to require these additional functions for cytochrome *c* and O_2 may have a simpler explanation. The increased rate of cytochrome a_3 reduction in the presence of cytochrome *c* and the apparent increase in the rate of electron transfer to cytochrome a_3 in the presence of O_2 are not evidence of regulation or an allosteric effect by either of these substrates.

In addition to explaining the peculiarities of cytochrome *c* oxidase reduction by artificial reductants, our proposal that resting cytochrome *c* oxidase has a rapid electron transfer between cytochrome *a* and cytochrome a_3 but a redox equilibrium favoring reduced cytochrome *a* and oxidized cytochrome a_3, may clarify the conversion of resting to pulsed cytochrome *c* oxidase. This change may reflect, at least in part, the difference in liganding of cytochrome a_3 of the two states. Chance and co-workers (20) have proposed that resting cytochrome oxidase has a bridging ligand between cytochrome a_3 and Cu_B. This may act to restrict cytochrome a_3 to a low potential state.

A low potential for cytochrome a_3 of resting cytochrome oxidase explains why resting cytochrome oxidase often has Cu_A partially reduced (21), but upon reducing and reoxidizing (*i.e.* converting to the pulsed form) Cu_A is fully oxidized (21). With the low potential for cytochrome a_3 in resting oxidase, any trace reductants could be expected to reduce cytochrome *a* or Cu_A without electron transfer to cytochrome a_3 and oxygen reduction. The negative cooperativity between cytochrome *a* and Cu_A (22) predicts that Cu_A would be the first site reduced.

Upon conversion from the resting to pulsed form, the redox potential of cytochrome a_3 increases, shifting the a/a_3 toward reduction of cytochrome a_3 and allowing a more rapid electron transfer from external reductants to cytochrome *a*. If the kinetic as well as thermodynamic barrier is lower for pulsed cytochrome *c* oxidase, then cytochrome a_3 reduction could be rapid as well as thermodynamically favorable. This is borne out by experimental observation. Reduction of pulsed cytochrome oxidase by dithionite, unlike the reduction of resting cytochrome oxidase, shows no difference between the rate of cytochrome *a* reduction and the rate of cytochrome a_3 reduction. The interactions between the redox potentials of the cytochromes of pulsed cytochrome oxidase would also aid in rapid reduction of cytochrome a_3 (17, 23).

Wilson *et al.* (6) have proposed a model for the transition of cytochrome *c* oxidase from the resting to the pulsed form of the enzyme, in which both the resting and pulsed enzymes are capable of enzymatic turnover, but the resting form is slower; the resting form gradually converts to the pulsed form, with the rate depending on the fraction of time spent in the reduced state during turnover. This model appears to be compatible with the redox potential-dependent description of resting and pulsed oxidase which we propose. It is also compatible with the results of Chance and co-workers (20) if brief periods of reduction of the resting oxidase do not cause exchange of the ligands of cytochrome a_3. Longer periods of reduction or repeated periods of reduction experienced during turnover allow the transition from the resting to pulsed state by a slow replacement of a ligand of cytochrome a_3.

In their initial description of resting and pulsed cytochrome *c* oxidase, Antonini *et al.* (24) suggested that the different forms of the enzyme may exist to provide a mechanism for regulation. Our results support this hypothesis, and we suggest that the slow internal electron transfer observed with resting cytochrome oxidase is a result of the thermodynamic barrier provided by the low redox potential of cytochrome a_3. We believe the purpose of resting and pulsed forms of cytochrome oxidase is to allow the size of the pool of active cytochrome oxidase to vary with demand; under conditions of high electron flow, the cytochrome oxidase molecules are converted to

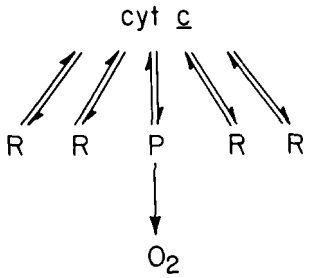

FIG. 6. **"Funneling" of electrons to pulsed cytochrome oxidase by a mixed pool of resting and pulsed enzymes.**

the active pulsed form. When electron flow is slow, some of the oxidase molecules shift to the resting form in which the redox potential of cytochrome a_3 is considerably below that of cytochrome *a*. This acts to keep cytochrome a_3 oxidized and to inhibit electron flow through these molecules, instead funneling the electrons to the enzymes still in the pulsed form (see Fig. 6).

In vitro evidence for electron funneling was actually shown several years ago. In 1970 Antonini and co-workers (25) showed that when the concentration of cytochrome c^{2+} was low, the cytochrome *a* pool became more reduced and the rate of internal electron transfer to cytochrome a_3 decreased. The authors suggested that for full reduction of the cytochrome oxidase, electron transfer between different oxidase molecules was required. This is exactly what would be predicted from electron funneling by a mixed pool of resting and pulsed cytochrome oxidase molecules.

The channeling of electrons to only some of the oxidase molecules has two protective effects; it increases the rate of electron flow through the active pulsed molecules and it decreases the frequency of reduction of the cytochrome a_3 of the resting cytochrome oxidase molecule. Both of these effects act to ensure complete reduction of molecular oxygen to water, decreasing the chance of generating the deleterious species formed by partial oxygen reduction.

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