The Mechanism by which Oxygen and Cytochrome c Increase the Rate of Electron Transfer from Cytochrome a to Cytochrome a3 of Cytochrome c Oxidase

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The Mechanism by Which Oxygen and Cytochrome c Increase the Rate of Electron Transfer from Cytochrome a to Cytochrome a₃ of Cytochrome c Oxidase*

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¶ Deceased.

The electron transfer from cytochrome a to cytochrome a₃ 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 a, but Cytochrome oxidase is significantly below that of cytochrome a and cytochrome a₃. When reductants other than cytochrome c are used, conventional hyperbolic kinetics for cytochrome a reduction and so in appropriate concentrations will reduce cytochrome a rapidly, the subsequent reduction of cytochrome a₃ by electron transfer from cytochrome a is remarkably slow, over 100 times slower than the rate of electron transfer from cytochrome a to cytochrome a₃ 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 a₃ reduction should be independent of the original source of electrons. Contrary to this prediction, the rate of cytochrome a₃ 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 a₃ when reductants other than cytochrome c are used (1, 3, 7, 8), no particularly satisfactory theory has been proposed to account for this phenomenon.

The rate of internal electron transfer from cytochrome a to cytochrome a₃ should also be independent of reactions which occur after this step; yet the rate of electron transfer to cytochrome a₃ is significantly faster in the presence of O₂ (18, 9) also see "Results".

In this paper we propose that the observed rate of cytochrome a₃ reduction is the product of a relatively rapid rate of electron transfer between cytochrome a and cytochrome a₃ and an oxidation-reduction equilibrium between cytochrome a and cytochrome a₃ which, in the "resting" enzyme (the enzyme as isolated), favors reduced cytochrome a and oxidized cytochrome a₃. 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 a₃ in resting cytochrome oxidase is significantly below that of cytochrome a₃ 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 a₃. 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 a₃ 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 a₃ reduction, (b) a CO concentration dependence for the rate of cytochrome a₃ reduction, and (c) the fact that pulsed cytochrome oxidase will show, at any dithionite concentration, reduction of cytochrome a₃ which is simultaneous with cytochrome a reduction.

**MATERIALS AND METHODS**

Reduction of cytochrome a, C₄a, and cytochrome a₃ was monitored spectrophotometrically. C₄a was monitored at 809 nm and cytochrome a at 600 nm. Both cytochrome a and cytochrome a₃ were monitored at 432 and 440 nm. At 440 nm cytochrome a and cytochrome a₃ 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 a₃ causes absorbance changes which are smaller but opposite in sign and approximately
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, 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, pH 7.0. The cytochrome c was collected and passed down a Bio-Rad P-10 column, 1.5 x 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 c reduction. To observe cytochrome a reduction, the solution must be anaerobic, and this is most easily achieved if the reductant used is dithionite, which quickly reacts with both dissolved O2 and cytochrome a. Dithionite reduction was performed by adding 5-50 μl of 1 mM dithionite to 2.5 ml of 2.3 mM cytochrome oxidase (aoa) in a closed stirred cuvette. 1 mM dithionite solutions were prepared 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 O2 was removed by one of two procedures. In the first, O2 was removed by several (>10) cycles of evacuation followed by saturation with O2-free N2 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 Amino-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 a3 Reduction—In Fig. 1A the reduction of cytochrome a and cytochrome a3 by dithionite are shown. At the wavelength used in this figure (432 nm), reduction of cytochrome a causes a decrease in absorbance while cytochrome a3 reduction causes an increase. It is clear from this figure that under these conditions cytochrome a is reduced rapidly (kobs = 0.4 s⁻¹), causing a decrease in absorbance, while cytochrome a3 is reduced very slowly. The rate of cytochrome a reduction was dependent on the dithionite concentration with an approximate Kd of 2 mM (results not shown). As shown in Fig. 1A, the kobs = 0.003 s⁻¹ at 2 mM dithionite. The addition of cytochrome c increases the rate of cytochrome a reduction (Fig. 1B) to about 0.015 s⁻¹. When we examined this phenomenon 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 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.

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 a3 reduction (Fig. 2A). As in the other experiments, reduction of cytochrome a3 was greatly accelerated in the presence of cytochrome c (Fig. 2B).

Using resting cytochrome c oxidase in an O2-free CO atmosphere, dithionite reduction of cytochrome a3 occurred with

### TABLE I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Rate of cytochrome a3 reduction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na2S2O4</td>
<td>0.1-0.4</td>
<td>1</td>
</tr>
<tr>
<td>Ru(NH3)₃⁺</td>
<td>0.02</td>
<td>2</td>
</tr>
<tr>
<td>Phenazine methosulfate</td>
<td>0.2</td>
<td>3</td>
</tr>
<tr>
<td>Cr(H2O)₆⁺</td>
<td>0.3</td>
<td>4</td>
</tr>
<tr>
<td>Fe(CN)₆³⁻</td>
<td>Slow</td>
<td>5</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Methyl viologen</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Benzyl viologen</td>
<td>7</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE II

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>Rate s⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undergoing reversible redox cycles</td>
<td></td>
</tr>
<tr>
<td>Cytochrome c (native)</td>
<td>1.0</td>
</tr>
<tr>
<td>Fe²⁺ cytochrome c (Fe³⁺ reinserted)</td>
<td>1.0</td>
</tr>
<tr>
<td>Mn²⁺ cytochrome c</td>
<td>1.0</td>
</tr>
<tr>
<td>Co³⁺ cytochrome c</td>
<td>1.0</td>
</tr>
<tr>
<td>Not undergoing reversible redox cycles</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.06</td>
</tr>
<tr>
<td>Porphyrin cytochrome c</td>
<td>0.06</td>
</tr>
<tr>
<td>Cu²⁺ cytochrome c</td>
<td>0.06</td>
</tr>
<tr>
<td>Cu³⁺ cytochrome c (20-fold excess)</td>
<td>0.07</td>
</tr>
<tr>
<td>Ru²⁺ cytochrome c</td>
<td>0.06</td>
</tr>
<tr>
<td>Sn²⁺ cytochrome c</td>
<td>0.06</td>
</tr>
<tr>
<td>Zn²⁺ cytochrome c</td>
<td>0.06</td>
</tr>
</tbody>
</table>

The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Slow Electron Transfer from Cytochrome a to Cytochrome a₃

FIG. 2. Cytochrome c induced acceleration of cytochrome a₃ reduction with reductants other than Na₂S₂O₄. A, conditions were the same as in Fig. 1A except the buffer contained 0.5 mM 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 (Me₂Fc) 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 a₃ by O₂ and by cytochrome c. Conditions were as in Fig. 2 but without glucose oxidase/catalase and, therefore, with ~250 μM O₂ present at the beginning of the reaction. The reaction was monitored at 444 nm, where reduction of cytochrome a and cytochrome a₃ both cause an increase in absorbance. Upon addition of reductant the enzyme maintained a steady state level of reduction until O₂ depletion. A, reductant was 10 mM ascorbate, 0.5 mM dimethylferrocene; B, reductant was 10 mM ascorbate, 0.5 mM dimethylferrocene, 0.5 mM cytochrome c.

a slightly faster rate than that seen with dithionite alone. In the presence of cytochrome c the rate of cytochrome a₃ reduction greatly increased (not shown).

In the experiments shown in Fig. 3, cytochrome c oxidase was allowed to consume the O₂ in solution, using either dimethylferrocene or cytochrome c as electron donor. Fig. 3A shows that in the absence of cytochrome c, anaerobic reduction of cytochrome a₃ (after O₂ depletion) is much slower than the rate of turnover in the presence of O₂. The average turnover rate of electrons was estimated to be 2 s⁻¹, while the rate of the final reduction of cytochrome a₃ after O₂ depletion, was 0.008 s⁻¹, only 1/25 of the rate in the presence of O₂.

In the presence of O₂ and equimolar cytochrome c (Fig. 3B) the turnover rate was estimated to be 16 s⁻¹. (The rate was well below Vₘₐₓ for cytochrome c oxidase due to the low concentration of cytochrome c.) The rate of the final reduction of cytochrome a₃ after O₂ 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 a₃ in the presence of O₂.

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, Cuₐ, Cuₜ, and cytochrome a₃, 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.

\[
\begin{align*}
A & \rightarrow E(0000) + R \\
B & \rightarrow E(1000) \\
C & \rightarrow E(0100) \\
D & \rightarrow E(0110) \\
E & \rightarrow E(0111) \\
F & \rightarrow E(1111)
\end{align*}
\]

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

\[
\begin{align*}
\frac{d[B]}{dt} &= k_1[A][R] + \frac{k_3[C]}{k_4} - k_2[B] \\
\frac{d[C]}{dt} &= k_2[B] - (k_3[C] + k_4)[R] \\
\frac{d[D]}{dt} &= k_1[C][R] + k_4[E] - k_3[D] \\
\frac{d[E]}{dt} &= k_2[D] - (k_3[E] + k_4)[R] \\
\frac{d[F]}{dt} &= k_4[E][R] + k_7[G] - k_5[F] \\
\frac{d[G]}{dt} &= k_5[F] - (k_7[G] + k_9[G])[R] \\
\frac{d[H]}{dt} &= k_9[G][R]
\end{align*}
\]

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-
than that for CO, was rapid reduction of bound cytochrome concentrations of CO. For comparison, the theoretical rate of cytochrome in the presence of the same as those used in Fig. 4A. The rate of electron transfer to cytochrome a to CuA, ( $k_2$, $k_3$, $k_4$, $k_5$) is reported to be 700 s$^{-1}$ (14).

The value for the rate of electron transfer from cytochrome a/CuA to cytochrome a/CuA ($k_3$) was estimated by using the approximate rate of cytochrome c oxidation during enzyme turnover under these conditions, $k_3 \approx 50$ s$^{-1}$, and then adjusted to give the best fit for the data.

The rate constant for electron transfer from cytochrome a/CuA to cytochrome a/CuA ($k_3$) 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. 4A 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. 4B 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. 4A, except the rate constants for cytochrome a reduction ($k_{1,4,7,10}$) were those for cytochrome c in Fig. 4B (1 x 10$^7$ M$^{-1}$ s$^{-1}$) and those for SO$_2$ (4 x 10$^6$ M$^{-1}$ s$^{-1}$) in Fig. 4A.

Fig. 5A 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. 5B the theoretical time course for the formation of a$^{2+}$a$^{2+}$-CO is shown for different concentrations of CO. For comparison, the theoretical rate of formation of a$^{2+}$a$^{2+}$-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 mM 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 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/CuA. Full reduction of the enzyme can occur, however, only during the intervals the electrons reside on the cytochrome a$_3$/CuB 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$^{2+}$/a$^{2+}$ species, and the relative rates at which electrons are transferred to cytochrome a$^{2+}$ from cytochrome a$^{2+}$ 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$_3$/CuB 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 $\mu$M 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**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate constant reactions of cytochrome c oxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reactants</td>
<td>Rate constants</td>
</tr>
<tr>
<td>a$^{2+}$ + SO$_2$ → a$^{2+}$</td>
<td>$4 \times 10^6$ M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>[SO$_2$]$^2-$</td>
<td>0.85 x 10$^{-10}$ x [SO$_2$]</td>
</tr>
<tr>
<td>a$^{2+}$ + cytochrome c$^{3+}$ → a$^{2+}$ + cytochrome c$^{3+}$</td>
<td>$1 \times 10^6$ M$^{-1}$ s$^{-1}$</td>
</tr>
<tr>
<td>a$^{2+}$Cu$^+$ $\rightleftharpoons$ a$^{3+}$Cu$^+$</td>
<td>700 s$^{-1}$</td>
</tr>
<tr>
<td>k$_{OR}$</td>
<td>a$^{2+}$ + O$_2$ → a$^{2+}$ - O$_2$</td>
</tr>
<tr>
<td>k$_{CO}$</td>
<td>a$^{2+}$ + CO → a$^{3+}$ - CO</td>
</tr>
<tr>
<td>k$_{CO}$</td>
<td>a$^{2+}$ - CO → a$^{3+}$ + CO</td>
</tr>
<tr>
<td>k$_{O2}$</td>
<td>a$^{3+}$a$^{3+}$ → a$^{3+}$a$^{3+}$</td>
</tr>
<tr>
<td>k$_{O2}$</td>
<td>a$^{2+}$a$^{2+}$ → a$^{2+}$a$^{2+}$</td>
</tr>
</tbody>
</table>
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 O₂ may have a simpler explanation. The increased rate of cytochrome a₃ reduction in the presence of cytochrome c and the apparent increase in the rate of electron transfer to cytochrome a₃ in the presence of O₂ are not evidence of regulation or an allosteric effect by either of these substrates.

In addition to explaining the peculiarities of cytochrome c oxidation by artificial reductants, our proposal that resting cytochrome c oxidase has a rapid electron transfer between cytochrome a and cytochrome a₃ but a redox equilibrium favoring reduced cytochrome a and oxidized cytochrome a₃, 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 a₃ of the two states. Chance and co-workers (20) have proposed that resting cytochrome oxidase has a bridging ligand between cytochrome a₃ and Cuo. This may act to restrict cytochrome a₃ to a low potential state.

A low potential for cytochrome a₃ of resting cytochrome oxidase explains why resting cytochrome oxidase often has Cuo partially reduced (21), but upon reducing and reoxidizing (i.e. converting to the reduced form) Cuo is fully oxidized (21). With the low potential for cytochrome a₃ in resting oxidase, any trace reductants could be expected to reduce cytochrome a or Cuo without electron transfer to cytochrome a₃ and oxygen reduction. The negative cooperativity between cytochrome a and Cuo (22) predicts that Cuo would be the first site reduced.

Upon conversion from the resting to pulsed form, the redox potential of cytochrome a₃ increases, shifting the a₃/a₄ toward reduction of cytochrome a₃ 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 a₃ 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 a₃ reduction. The interactions between the redox potentials of the cytochromes of pulsed cytochrome oxidase would also aid in rapid reduction of cytochrome a₃ (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 a₃. 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 a₃.

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 a₃. 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. 4. Kinetic model of reduction of cytochrome a and cytochrome a₃.** A, dithionite reduction (---) with superimposed kinetic model (-----) oxidase solution was 2 μM a₃. 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⁺ added equimolar to cytochrome a₃ prior to the Na₂S₂O₄. 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, a₃⁺ a₃⁻, 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 25 μM O₂, assuming O₂ bound, but did not oxidize, reduced cytochrome a₃.
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.

Acknowledgments—We thank Drs. M. T. Wilson and A. S. Mildvan for encouragement and helpful suggestions in the preparation of this manuscript.

REFERENCES