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Oxidation of ferrocytochrome *c* by mitochondrial cytochrome *c* oxidase

(kinetics/electron transfer mechanisms/dead-end complexes)

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ABSTRACT **Attempts to rationalize the kinetics of cytochrome *c* oxidation catalyzed by solubilized mitochondrial cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1) have been based on assumptions of productive complex formation (Michaelis–Menten approach). However, the range of substrate concentrations used has not, in general, been sufficient to establish a general rate equation. Data adequate to derive such a rate expression are presented, as well as a method for estimation of constants which appear in the rate law deduced and reported herein. It is shown that either of two types of mechanisms, one assuming productive complex formation, as opposed to the other postulating dead-end complex formation, accurately predict the rate equation as deduced from experiment.**

Clarification of the structural bases for mitochondrial function of cytochrome *c* remains a central problem in bio-energetics. Approaches to its solution by kinetic analyses of soluble cytochrome *c* interactions with the associated redox systems, particularly solubilized mitochondrial cytochrome *c* oxidase (ferrocytochrome *c*:oxygen oxidoreductase, EC 1.9.3.1), have generated a considerable literature (see ref. 1 for a recent review). A proposal to extend such studies to permit better understanding of the role of structural parameters in these interactions involves use of variant eukaryotic forms of cytochrome *c* as well as related proteins obtained from appropriate prokaryotes (2, 3). Implementing this approach has required a re-examination and extension of previous kinetic studies on the cytochrome *c*–cytochrome oxidase reaction.

A major impediment in all such studies to the present has been the finding that standard Michaelis–Menten analysis methods are not applicable for interpretation of kinetic data without many *ad hoc* assumptions. In this preliminary report, we present data over a greater range of substrate concentrations than reported previously and demonstrate that, in addition to the usual analysis based on the Michaelis–Menten postulate of productive complex formation, an alternative mechanism involving the opposed notion of nonproductive ("dead-end") complex formation can account as well for the observed rate equations.

MATERIALS AND METHODS

Oxidase was prepared from beef heart mitochondria according to the method of Fowler *et al.* (4). Protein was determined by both the modified biuret (5) and modified Lowry (6) procedures. Total heme *a* was determined using an extinction coefficient (Δ605–630 nm) of 16.5 mM^{-1} cm^{-1} for the sodium dithionite reduced enzyme (7). Concentrations of oxidase are expressed in terms of total heme *a*. The preparation was stored under liquid nitrogen in small aliquots (25 mg/ml in 10 mM Tris·HCl, 0.66 M sucrose, and 1 mM histidine, pH 8). Immediately before use, aliquots were diluted with ice-cold water to a final concentration of 0.1–0.4 mg/ml.

Horse heart cytochrome *c* (type VI) was purchased from Sigma Chemical Co. A stock solution of ferricytochrome *c* was dialyzed against three changes of 20 mM Tris base and 10 μM EDTA to remove bound ions (8). Immediately after dialysis the preparation was chromatographed on a Sephadex G-75 column equilibrated with 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6, and 10 μM EDTA. This procedure removed a small fraction of aggregated cytochrome which showed significant ($\geq$ 25%) CO binding. Prior to use, an appropriate amount of cytochrome *c* was reduced by the addition of excess sodium ascorbate. Excess reductant was removed by gel filtration chromatography on Sephadex G-25 equilibrated in the buffer to be used thereafter. Cytochrome *c* treated in this manner showed no change in oxidation state during the time normally required to complete an experiment. Total cytochrome *c* was determined spectrophotometrically, using ϵ_{550} nm = 27.6 mM^{-1} cm^{-1} for solution reduced with sodium dithionite (9).

The kinetics of oxidation of ferrocytochrome *c* by oxidase were studied spectrophotometrically by monitoring the decrease in absorbance of α, β, or γ bands. Under the conditions used the reaction being studied was restricted to electron transfer from cytochrome *c* to cytochrome oxidase. The reaction of oxidase with oxygen was very rapid relative to other electron transfer processes. Therefore, the acceptor, oxidase, was always available and oxygen was not limiting in the reaction. All experiments were performed in the presence of 0.1 M Mes, pH 6, and 10 μM EDTA at 25° with concentrations of cytochrome *c* and oxidase as specified. An Aminco-Chance model DW-2 dual wavelength recording spectrophotometer, operated in the split beam mode, was used throughout. A computer program enabled calculation of pseudo-first order rate constants. Estimates of kinetic constants with standard deviations were obtained using the linear regression method.

Mes and sodium ascorbate were purchased from CalBiochem Co. Sodium dithionite was obtained from Fisher Scientific Co.

RESULTS

Our results confirm the many previous observations of Smith and Conrad (10) as well as others (11, 12) that the reaction of ferrocytochrome *c* with the oxidase is pseudo-first order at all cytochrome *c* concentrations studied. First order kinetics from 0.7 μM to 160 μM cytochrome *c* is demonstrated by the linearity of plots of ln $[C_t - C_\infty]$ against time (Fig. 1A and B), C_t being the concentration of ferrocytochrome *c* at

Abbreviation: Mes, 2-(*N*-morpholino)ethanesulfonic acid.

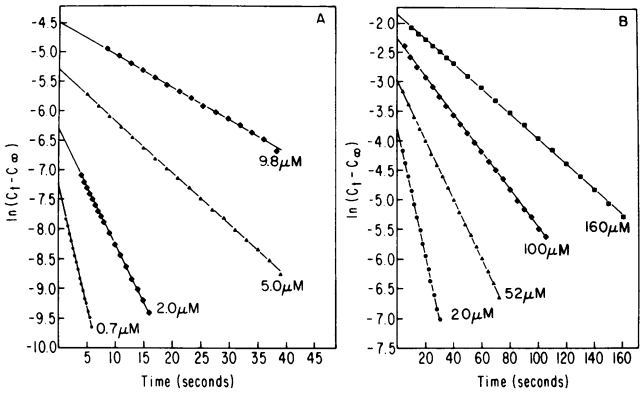

FIG. 1. Time course of oxidase reactions at varying total concentrations of cytochrome *c* (see *text*). (A) Concentrations of cytochrome *c* as noted (0.7–9.8 μM); 8.8 nM oxidase. (B) Concentrations of cytochrome *c* as noted (20–160 μM); 37 nM oxidase.

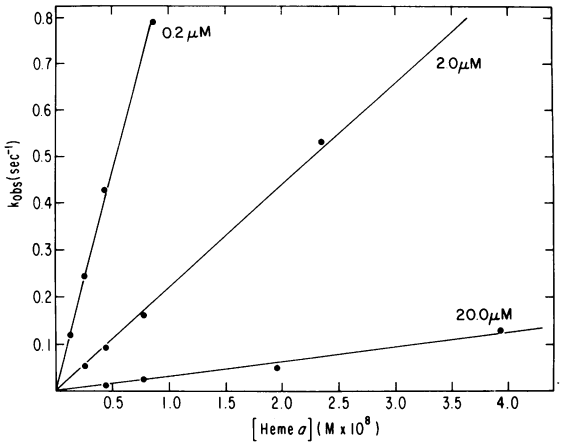

FIG. 2. Variations of k_{obs} (sec^{-1}) with oxidase concentration (see *text*). Three concentrations of total cytochrome *c* as noted (0.2 μM, 2 μM, and 20 μM).

time *t*. C_∞, the concentration of ferrocytochrome *c* at completion of reaction, was zero, as verified by the absence of further absorbance decrease upon addition of a small amount of ferricyanide when monitoring the α or β band.

The pseudo-first order nature of the steady state kinetics requires the general form for the rate equation to be:

$$\text{velocity} = k_{obs}[\text{ferrocytochrome } c] \quad [1]$$

The observed first order rate constant is directly proportional to total oxidase in the system. Plots of k_{obs} against total oxidase at different cytochrome *c* concentrations are linear with zero intercepts (Fig. 2), as represented by the the relation:

$$k_{obs} = k'[\text{oxidase}] \quad [2]$$

with slopes (k') dependent on the total cytochrome *c* concentration. The dependence of k' on cytochrome *c* concentration is demonstrated directly in Fig. 3. Furthermore, the data in Table 1 confirm the observation of Smith and Conrad (10) that k' is a function solely of *total* cytochrome *c* concentration (ferrocytochrome *c* plus ferricytochrome *c*). The pseudo-first order rate constant is independent of the initial ratio of ferrocytochrome *c* to ferricytochrome *c*.

Plots of $(k')^{-1}$ against cytochrome *c* concentration are nonlinear rather than linear. Eq. 3 closely fits the experimentally determined values of k'.

$$k' = \frac{N + QM[\text{C}]}{1 + Q[\text{C}] + QR[\text{C}]^2} \quad [3]$$

N, *M*, *Q*, and *R* are constants, and [C] is total cytochrome *c* concentration. The four constants in this equation can be approximated by satisfying two limiting conditions, as in cases I and II of Table 2. Case I applies at very low cytochrome *c* concentrations and case II at very high concentrations. At these extremes, plots of $(k')^{-1}$ against cytochrome *c* concentration become linear so that associated constants can be calculated (Table 2). Approximate values are deduced utilizing cytochrome *c* concentration ranges which by inspection appear linear for each case. These values are then refined by successively limiting the boundary values of cytochrome *c* concentrations included in the estimates until no further significant change occurs.

Refined estimates were deemed acceptable, if, for case I, $N \geq 10\ QM\ [C_i]$ and $1 + Q\ [C_i] \geq 10\ QR\ [C_i]^2$, and for case II, $QM\ [C_j] \geq 10\ N$ and $Q\ [C_j] + QR\ [C_j]^2 \geq 10$. ($C_i$ was the highest concentration of cytochrome *c* included in the estimate of *N* and *Q*, and C_j the lowest concentration of cytochrome *c* included in the estimates for *M* and *R*.) The values obtained by this method were: $N = 3.24 \times 10^8 \pm 0.27 \times 10^8\ sec^{-1}\ M^{-1}$, $M = 3.91 \times 10^6 \pm 0.28 \times 10^6\ sec^{-1}\ M^{-1}$, $Q = 9.21 \times 10^6 \pm 1.45 \times 10^6\ M^{-1}$, and $R = 3.58 \times 10^4 \pm 0.34 \times 10^4\ M^{-1}$. Substitution of these values into the reciprocal form of Eq. 3 allowed calculation of the solid curve in Fig. 4.

The constant *M* as the reciprocal of a *y*-intercept could not be determined unambiguously at $x \simeq 0$ because small variations in slope in this region of the curve strongly affected the value obtained. Therefore, this constant was further refined, using Eq. 3 with substitution of values for experimentally determined k' at concentrations of cytochrome *c* at which limiting case II applied, together with the above values of *N*, *Q*, and *R*. The value obtained for *M* in this

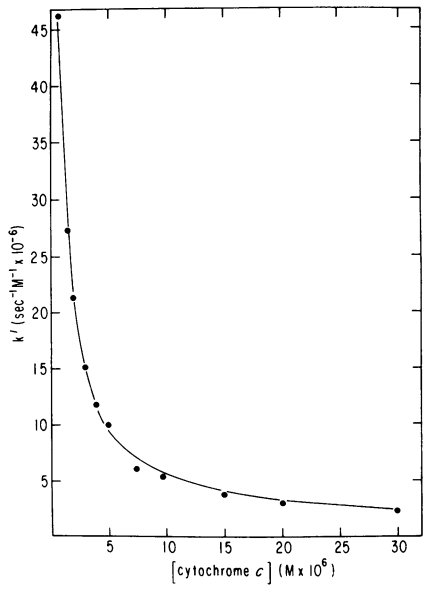

FIG. 3. Variation of k' ($sec^{-1}\ M^{-1} \times 10^{-6}$) with total concentration of cytochrome *c* (see *text*). Assay conditions: see legend of Fig 4.

Table 1. Pseudo-first order rate constants (k') for cytochrome *c* oxidations catalyzed by mitochondrial oxidase*

Initial concentration				
Ferro *c* (M × 10^{+6})	Ferri *c* (M × 10^{+6})	Ferro *c* / Ferri *c*	Total *c* (M × 10^{+6})	k' (sec^{-1} M^{-1} × 10^{-6})
1.99	0.04	50	2.04	21.3
2.96	0.06	50	3.02	15.1
3.91	0.08	50	3.99	11.8
4.96	0.10	50	5.06	10.0
4.04	1.01	4	5.05	10.4
3.23	1.86	1.7	5.09	10.1
2.41	2.66	0.9	5.07	10.0

* Assay conditions: 8.8 nM oxidase (heme *a*). (Other conditions as noted in *text*.)

manner was $3.62 \times 10^6 \pm 0.05 \times 10^6$ sec^{-1} M^{-1}. Substitution of this value for M along with those given for N, Q, and R into the reciprocal of Eq. 3 allowed calculation of the dashed curve in Fig. 4, so that a satisfactory fit to the experimental data resulted.

Substitution of the right-hand side of Eq. 3 for k' in Eq. 2 and further substitution for k_{obs} in Eq. 1 resulted in the complete rate equation:

$$\text{velocity} = \frac{N + QM[C]}{1 + Q[C] + QR[C]^2} \times [\text{ferrocytochrome } c][\text{oxidase}] \quad [4]$$

DISCUSSION

The results obtained confirm the central important finding of Smith and Conrad (10) that the oxidase does not show saturation by ferrocytochrome *c*. K_m values for cytochrome *c* reported in the literature vary from 1.5 μM to 20 μM (1). If this parameter is interpreted in the classical sense (half-maximal velocity), deviations (about 9%) from first order kinetics should be detectable at a cytochrome *c* concentration as low as 10% K_m. However, as demonstrated in Fig. 1, at concentrations as high as 160 μM (eight times the highest reported K_m value) the reaction remains strictly pseudo-first order.

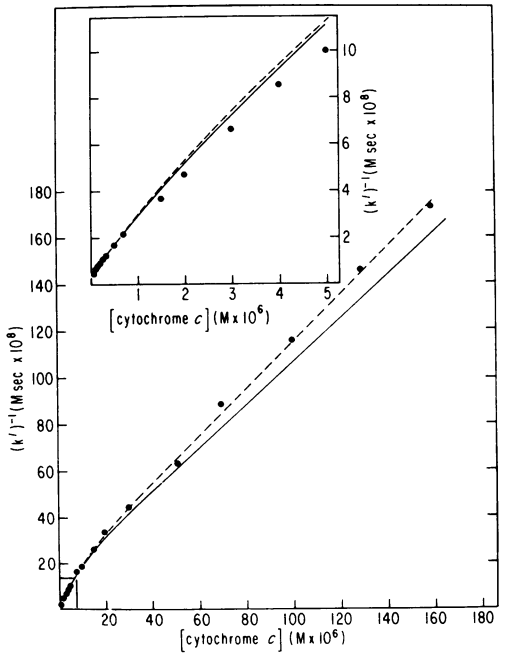

FIG. 4. Reciprocal plot of k' against total concentration of cytochrome *c* (see *text*). 0.061–0.7 μM cytochrome *c* with 1.3 nM oxidase, decrease in cytochrome *c* γ band; 0.5–10 μM cytochrome *c* with 4.2 nM oxidase, decrease in α band; 2–5 μM cytochrome *c* with 8.8 nM oxidase, decrease in α band; 7.5–160 μM cytochrome *c* with 37 nM oxidase, decrease in β band. *Inset:* expanded plot of boxed area.

The observed first order rate constant decreases with increasing cytochrome *c* concentration. Furthermore, the apparent inhibition by cytochrome *c* is independent of the oxidation state of the cytochrome *c*. Smith and Conrad have interpreted the inhibition of the reaction by total cytochrome *c* in terms of the formation of inactive complexes of cytochrome *c* with the oxidase. The possibility of inactive complex formation, together with the observed nonsaturation kinetics, has led these workers to suggest that K_m and V_{max} may not be meaningful parameters in analysis of the oxidase reaction (10).

Our results for the reaction of cytochrome *c* with purified solubilized oxidase are also consistent with the results of Nicholls (11) and Ferguson-Miller *et al.* (13), using Keilin-Hartree type submitochondrial particles. In both reports, nonlinear relationships are found. Nicholls has demonstrated that a variety of K_m and V_{max} values can be obtained depending upon the region of the gradually curving double reciprocal plot from which the estimation of constants is made (11). It is evident that accurate kinetic constants for this system can be obtained only by rigorous adherence to limiting conditions as defined in Table 2.

Table 2. Determination of characteristic constants for rate law of Eq. 4

Limiting case I	Limiting case II
If	If
$N \gg MQ[C]$	$MQ[C] \gg N$
$1 + Q[C] \gg QR[C]^2$	$Q[C] + QR[C]^2 \gg 1$
Then	Then
$k' = \frac{N}{1 + Q[C]}$	$k' = \frac{M}{1 + R[C]}$
$(k')^{-1} = \frac{1}{N} + \frac{Q}{N}[C]$	$(k')^{-1} = \frac{1}{M} + \frac{R}{M}[C]$
Plot $(k')^{-1}$ vs. [C]	Plot $(k')^{-1}$ vs. [C]
$(\text{Intercept})^{-1} = N$	$(\text{Intercept})^{-1} = M$
Slope/intercept = Q	Slope/intercept = R

Table 3. Comparison of alternative oxidase mechanisms

	A.	B.
Mechanism	$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k_3} V \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P$ $U + S \underset{k_8}{\overset{k_7}{\rightleftharpoons}} W \xrightarrow{k_9} X \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} U + P$ $V + S \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} Y \xrightarrow{k_{15}} Z \underset{k_{18}}{\overset{k_{17}}{\rightleftharpoons}} V + P$	$U \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E + S \xrightarrow{k_3} E + P \underset{k_6}{\overset{k_5}{\rightleftharpoons}} V$ $W \underset{k_8}{\overset{k_7}{\rightleftharpoons}} U + S \xrightarrow{k_9} U + P \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} X$ $Y \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} V + S \xrightarrow{k_{15}} V + P \underset{k_{18}}{\overset{k_{17}}{\rightleftharpoons}} Z$
Method	Steady state approximation $dE/dt = dU/dt = dV/dt = dW/dt = dX/dt = dY/dt = dZ/dt = 0$	Rapid equilibrium approximation
Assumptions	(1) Reoxidation of oxidase is fast (2) Reverse reaction is negligible (3) $k_1 = k_6; k_2 = k_5;$ $k_7 = k_{12} = k_{13} = k_{18};$ $k_8 = k_{11} = k_{14} = k_{17};$ $k_9 = k_{15}$	(1) Reoxidation of oxidase is fast (2) Reverse reaction is negligible (3) $k_1/k_2 = k_5/k_6$ $k_7/k_8 = k_{11}/k_{12} = k_{13}/k_{14} = k_{17}/k_{18}$ $k_9 = k_{15}$
Rate law velocity =	$\dfrac{\dfrac{k_1k_3}{(k_2+k_3)} + \dfrac{k_1k_7k_9}{k_2(k_8+k_9)}[S+P]}{1 + \dfrac{k_1}{k_2}[S+P] + \dfrac{k_1k_7}{k_2k_8}[S+P]^2}[S][E_0]$	$\dfrac{k_3 + \dfrac{k_1k_9}{k_2}[S+P]}{1 + \dfrac{k_1}{k_2}[S+P] + \dfrac{k_1k_7}{k_2k_8}[S+P]^2}[S][E_0]$
Kinetic constants*	$N = \dfrac{k_1k_3}{(k_2+k_3)}$ $M = \dfrac{k_7k_9}{(k_8+k_9)}$ $Q = k_1/k_2$ $R = k_7/k_8$	$N = k_3$ $M = k_9$ $Q = k_1/k_2$ $R = k_7/k_8$

Symbols: S = ferrocytochrome *c*; P = ferricytochrome *c*; E = free oxidase; U = ferrocytochrome *c*-oxidase complex; V = ferricytochrome *c*-oxidase complex; W = 2 ferrocytochrome *c*-oxidase complex; X=Y = ferrocytochrome *c* + ferricytochrome *c*-oxidase complex; Z = 2 ferricytochrome *c*-oxidase complex; $E_0 = E + U + V + W + X + Y + Z$ = total oxidase.
* Derived under limiting conditions of Table 2.

The formal mechanisms and rate laws deduced and previously proposed (12, 14–17) have considered only one binding site for cytochrome *c* on the oxidase, and require that linear double reciprocal plots should result for all cytochrome concentrations. Any of the five mechanisms proposed by Minneart (12) as well as that proposed by Yonetani and Ray (16) could be modified to include two oxidase binding sites. As an example, we have extended Minneart's "Mechanism IV" to include formation of an intermediate in which two cytochromes *c* are bound per oxidase molecule. The mechanism, assumptions, and derived rate law are summarized in Table 3, column A. The assumptions involving the various "on" and "off" rate constants, which require the binding of cytochrome *c* to the oxidase to be independent of the oxidation state of cytochrome *c*, are supported by the data given in Table 1. Pathways proceeding through intermediate formation of ferricytochrome *c*-oxidase (V) or ferrocytochrome *c*-oxidase (U) cannot be distinguished by kinetic analysis. Hence k_9 and k_{15} are essentially equivalent, as well as are the "on" and "off" rate constants for the formation of complexes involving two molecules of cytochrome *c* per molecule of oxidase. While electron transfer occurs in a stable complex(es), reversibility of association implies that not all complexes formed will be productive.

An alternative and equally plausible possibility is that in the *in vitro* systems studied, oxidase binds to one or more regions on the cytochrome molecule in a manner that makes interaction with the electron transfer portion of the cytochrome *c* unlikely (dead-end complex formation). Electron transfer then would require a transition-state type of complex. The parameters that define "binding" in this scheme would not be relevant to the electron transfer process as it occurs in intact mitochondria. Mechanism B in Table 3 extends the mechanism proposed by Nicholls (15) to include such dead-end complex formation with both ferri- and ferro-cytochrome *c* and to include two binding sites on the oxidase. Again, it is assumed that the binding of cytochrome *c* to oxidase is independent of the oxidation state of cytochrome *c*, and reaction pathways for cytochrome *c* with cytochrome *c*-oxidase complexes, *U* and *V*, are equivalent.

The two mechanisms presented in Table 3 are kinetically indistinguishable. It can be seen that the rate laws of either mechanism are of the general form required to predict the observed inhibition of k_{obs} by total cytochrome *c*, as well as the nonlinear plots that result from treatment of data for rate dependence on cytochrome *c* concentration. Both mechanisms are consistent with the observed nonsaturation kinetics. In mechanism A of Table 3, nonsaturation kinetics has been rationalized as a "combined effect of progressively decreasing substrate saturation and progressively increasing product inhibition" (16) with the provision that the equilibrium binding constants for complex formation with product and substrate are the same. The absence of saturation, however, is more clearly seen as a consequence of mechanism B in Table 3, in which the distribution of oxidase among its various forms (*E*, *U*, *V*, *W*, *X*, *Y*, and *Z*) can be inferred as established by a rapid equilibrium that is independent of the oxidation state of the cytochrome *c*, so that for any fixed concentration of cytochrome *c* there will be a constant amount of oxidase available for reaction. Hence, pseudo-first order kinetics will always result.

The two types of mechanism presented can be distinguished only on the basis of nonkinetic evidence. Some attempts designed to demonstrate catalytic competency of the

various isolated complexes of cytochrome *c* with oxidase have been reported (15, 18–20), but results are equivocal. Observed oxidations of ferrocytochrome *c* in isolated complexes could be the result of dissociation of a nonproductive complex and reformation of an active collision complex during the time course of the measurements.

These findings affect proposed comparative kinetic studies in that comparisons can be made only by properly estimating kinetic constants from an adequately extended range of cytochrome concentrations. Only constants that are estimated under the limiting conditions of Table 2 are meaningful. Because both mechanisms are plausible, kinetic constants for the oxidase reaction are not uniquely interpretable (Table 3). Although Q and R are defined as equilibrium binding constants, their physical meanings differ according to the mechanism proposed. In mechanism A electron transfer occurs only when cytochrome *c* is in a "stable" complex with the oxidase. Therefore, binding, which controls the rate of complex formation, would be important in relation to cytochrome *c* function in mitochondria. In mechanism B, the "binding" constants are parameters that limit the amount of free oxidase available for reaction. N and M in mechanism A are defined as the product of electron transfer rates (k_3 and k_9) and the steady state concentrations of U and W, respectively, while in mechanism B these same constants are defined only as electron transfer rates. Mechanism B appears more advantageous for comparative kinetic studies because the kinetic constants involved are more readily experimentally related to component processes.

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