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Cytochrome *c* Oxidase: Biphasic Kinetics Result from Incomplete Reduction of Cytochrome *a* by Cytochrome *c* Bound to the High-Affinity Site†

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ABSTRACT: The electron-transfer kinetics of cytochrome *c* oxidase were probed by measuring the reduction levels of bound cytochrome *c*, cytochrome *a*, and cytochrome a_3 during steady-state turnover. Our experimental approach was to measure these reduction levels as a function of (1) the rate of electron input into tightly bound cytochrome *c* by varying the concentration of TMPD (*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine) and/or cytochrome *c* and (2) the rate of electron efflux out of cytochrome *a* (true k_{cat}) by changing the detergent surrounding cytochrome *c* oxidase. In most detergent environments, the rate of electron input into cytochrome *c* is not faster than the rate of electron efflux from cytochrome *a*. The relatively slow rate of electron input results in incomplete reduction of both cytochrome *a* and cytochrome *c* bound at the high-affinity site unless k_{cat} is very slow. When the high-affinity site is saturated with cytochrome *c*, the steady-state reduction level of cytochrome *a* defines $V_{max,1}$, which is the maximum velocity of the high-affinity phase. The remaining fractional oxidation level of cytochrome *a* determines $V_{max,2}$, the maximum velocity of the low-affinity phase. Therefore, it is the sum $V_{max,1} + V_{max,2}$ which defines the maximum rate of electron transfer between cytochrome *a* and the bimetallic center, i.e., k_{cat}. We also were able to evaluate the true k_{cat} of cytochrome *c* oxidase in each detergent environment directly from the steady-state reduction levels without any of the complications introduced by the analysis of the polarographic kinetic data. By comparison of the steady-state reduction levels of the redox centers with the polarographically measured kinetics, we conclude that the second kinetic phase is present only when cytochrome *a* cannot be fully reduced by the primary high affinity site pathway. Therefore, the biphasic cytochrome *c* kinetics of bovine heart cytochrome *c* oxidase should not be thought of as a necessary and essential part of the enzymatic mechanism. Rather, the biphasic kinetics are a direct consequence of limited electron input into cytochrome *c* from the artificial electron donor TMPD.

Cytochrome *c* oxidase (ferrocytochrome *c*:O_2 oxidoreductase, EC 1.9.3.1) is a multisubunit, inner mitochondrial membrane protein complex that catalyzes electron transfer from reduced cytochrome *c* to oxygen. Energy derived from this process is utilized to generate the electrochemical gradient that drives ATP synthesis. The entire process can be summarized as

$$4\text{Cyt } c^{2+} + O_2 + (4 + n)H^+(\text{mito matrix}) \rightarrow 4\text{Cyt } c^{3+} + 2H_2O + nH^+(\text{inter membrane space})$$

Details of the individual electron transfers within cytochrome *c* oxidase are fairly well understood; however, the enzyme kinetics are complex, and the mechanism by which electron transfer is coupled to the proton pump is not fully understood (Hosler et al., 1993).

Electron transfers within cytochrome *c* oxidase involve five functional metal centers, two hemes and three coppers. Electrons are first transferred from reduced cytochrome *c* bound at the high-affinity site to Cu_A (Hill, 1991, 1993, 1994) which is in fast equilibrium with cytochrome *a*. This electron transfer is rapid and probably involves a Cu_X bridge (Alleyne et al., 1992; Lappalainen & Saraste, 1994). From cytochrome *a* the electrons are transferred directly to the bimetallic cytochrome a_3−Cu_B center where the oxygen reduction chemistry occurs (Hill, 1991, 1993, 1994; Verkhovsky et al., 1992; Pan et al., 1991). This final electron transfer from cytochrome *a* to the bimetallic center is thought to be the rate-limiting step in the overall transfer of electrons from cytochrome *c* to oxygen (Cooper, 1990; Sarti et al., 1988; Mahapatro & Robinson, 1990; Hill, 1988; Hill & Greenwood, 1984a,b).

Cytochrome *c* oxidase steady-state kinetics have been studied extensively (Malmström, 1990a,b; Cooper; 1990; Myers & Palmer, 1988; Wikström et al., 1981), and it has always been obvious that the kinetics exhibit biphasic or multiphasic rather than classical hyperbolic behavior (Smith & Conrad, 1956; Minnaert, 1961; Ferguson-Miller et al., 1976; Errede et al., 1976; Errede & Kamen, 1978). Three models have been proposed to explain this biphasic behavior: (1) two catalytically active cytochrome *c* binding sites per cytochrome *c* oxidase monomer with different binding affinities, i.e., one with $K_{d,1} = K_{m,1} = 10-50$ nM[2], the other with $K_{d,2} = K_{m,2} \cong 1$ μM (Nicholls, 1965; Ferguson-Miller et al., 1976); (2) a single large binding site that accommodates two molecules of cytochrome *c* (the second is not an electron donor, but its binding regulates the binding of the first) (Wilms et al., 1981; Antalis & Palmer, 1982;

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Osheroff et al., 1983; Nalecz et al., 1983; Speck et al., 1984; Bolli et al., 1985a,b); and (3) a single binding site with two different affinities for cytochrome c (the binding affinity depends upon which one of two cytochrome c oxidase conformations is present) (Brzezinski & Malmström, 1986, 1987; Garber & Margoliash, 1990). None of these three models, however, gives a completely satisfactory explanation of the biphasic steady-state kinetic behavior of cytochrome c oxidase; e.g., $K_{d,2}$ is an order of magnitude greater than $K_{m,2}$, which is inconsistent with the two binding site model (Garber & Margoliash, 1990).

One possible explanation for the biphasic kinetics is the inability of electron transfer through a single, tightly bound cytochrome c to fully reduce cytochrome a. Cytochrome c, cytochrome a, and Cu_A are all known to be partially oxidized during steady-state turnover when the artificial electron donor N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD)[1] is used as the electron donor (Thörnström et al., 1988; Morgan & Wikström, 1991; Alleyne et al., 1992; Nicholls, 1993). Incomplete reduction of these redox centers is probably due to the rate-limiting reduction of tightly bound cytochrome c by TMPD (Osheroff et al., 1983). In fact, both the magnitude of the high-affinity phase, $V_{max,1}$, and the percent reduction of the redox centers are dependent upon the concentration of TMPD (Ferguson-Miller et al., 1978; Morgan & Wikström, 1991).

The rate limiting reduction of tightly bound cytochrome c by TMPD certainly complicates the polarographic kinetics and makes interpretation of the rate constants in terms of intrinsic catalytic constants nearly impossible. In the present study we have taken a different experimental approach for evaluating the true intrinsic catalytic constants of cytochrome c oxidase. The steady-state reduction levels of both tightly bound cytochrome c and cytochrome a were each measured as a function of the rate of electron entry into tightly bound cytochrome c and electron exit from cytochrome a. Because the steady-state reduction levels of these redox centers are directly dependent upon the rate of electron exit from cytochrome a, we were able to evaluate the true k_{cat} of cytochrome c oxidase in a variety of detergent environments without any of the complications associated with the normal kinetic assay. From these studies we conclude that incomplete reduction of cytochrome a, due to limited electron entry into tightly bound cytochrome c, is clearly responsible for the biphasic cytochrome c kinetics of cytochrome c oxidase. The enzymatic maximum velocity measures the true intrinsic electron-transfer rate within cytochrome c oxidase only when reduction of tightly bound cytochrome a is much faster than its oxidation by the binuclear center.

MATERIALS AND METHODS

Materials. Horse heart cytochrome c (type III), cholic acid, sodium deoxycholate, and N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) were obtained from Sigma Chemical Co. Sodium deoxycholate and sodium cholate, prepared from cholic acid, were each recrystallized from ethanol before they were used. Cytochrome c was used for the enzyme activity determinations without further purification, and its concentration was determined using $\epsilon_{410} = 106.1$ mM^{-1} cm^{-1}. Tris base (enzyme grade) and ammonium sulfate (ultrapure) were both obtained from GIBCO BRL. Dodecyl maltoside was purchased from Anatrace, Inc.; Triton X-100 (specially purified) was from Boehringer-Mannheim; Tween-80, Tween-20, and n-decanoyl sucrose were from Calbiochem; and $C_{12}E_8$ was from Fluka. Sephadex G-100 was obtained from Pharmacia. Other chemicals were analytical grade.

Cytochrome c Oxidase. Keilin-Hartree particles were prepared from frozen beef heart by the method of Yonetani (1960). Cytochrome c oxidase was isolated from the heart particles by the method of Fowler *et al.* (1962) with the modifications described by Mahapatro and Robinson (1990). Usually a second ammonium sulfate precipitation from cholate was required for the preparation of adequately pure enzyme. After the final ammonium sulfate precipitation, the pellet containing cytochrome c oxidase was dissolved in 0.1 M NaH_2PO_4, pH 7.4, buffer containing 1% sodium cholate and 1 mM EDTA. The purified cytochrome c oxidase was stored at −70 °C at approximately 30 mg/mL. The isolated complex contained 9.5−10.0 nmol of heme A per milligram of Biuret protein on the basis of $\Delta\epsilon_{(606-630)red} = 16.5$ mM^{-1} cm^{-1} as a measure of total heme A (Griffiths & Wharton, 1961). The purified oxidase had a molecular enzyme activity of 340−350 μmol of cytochrome c oxidized (μmol of cytochrome c oxidase)$^{-1}$ s^{-1} when assayed spectrophotometrically at 25 °C in 0.025 M, pH 7.0, phosphate buffer containing 1 mg/mL dodecyl maltoside. Activities were calculated from the first-order decrease in reduced cytochrome c as described by Vanneste et al. (1974) and Robinson et al. (1985). The sodium cholate in the stock enzyme was exchanged for the appropriate detergent, i.e., dodecyl maltoside, decanoyl sucrose, Triton X-100, $C_{12}E_8$, Tween-80, or Tween-20, by (1) the addition of 1 mg of the second detergent per milligram of protein and (2) removal of the sodium cholate by extensive dialysis at 4 °C vs 25 mM Tris−acetate, pH 7.9, buffer containing 0.1 mg/mL of the second detergent (0.5 mg/mL when the detergent was decanoyl sucrose).

Enzymatic Assays and Kinetics. The electron-transfer rate of cytochrome c oxidase was measured by both the spectrophotometric and the polarographic methods, both at 25 °C. The spectrophotometric assay was done in 0.025 M phosphate buffer with saturating cytochrome c as previously described (Robinson et al., 1985). The polarographic assay was done as follows: Cytochrome c oxidase at a final concentration of 0.01−0.03 μM was mixed with 5 mM sodium ascorbate and 1.0 mM TMPD in 25 mM Tris−acetate, pH 7.9, buffer containing 1 mg/mL of either dodecyl maltoside, $C_{12}E_8$, or Triton X-100 or 2 mg/mL of decanoyl sucrose (higher concentrations were used because the CMC of n-decanoyl sucrose is 1.25 mg/mL). Oxygen consumption was measured using a YSI oxygen electrode with a temperature-controlled cell and a Model 53 oxygen electrode monitor, all purchased from Yellow Spring Instrument Co., Inc. The reaction was started by adding 0.05−80 μM cytochrome c. The oxygen concentration was measured as a function of time, and the analog voltage, which is

[1] Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; dodecyl maltoside, n-dodecyl β-D-maltoside; decanoyl sucrose, α-D-glucopyranoside β-D-fructofuranosyl monodecanoate; EDTA, ethylenediaminetetraacetic acid; TMPD, N,N,N′,N′-tetramethyl-1,4-phenylenediamine; Tris−acetate buffer, tris(hydroxymethyl)aminomethane base titrated to the appropriate pH with acetic acid.

[2] The identity of $K_{d,1}$ and $K_{m,1}$ is not surprising since $K_{m,1}$ is not a true Michaelis constant, but rather the dissociation constant of cytochrome c from cytochrome c oxidase as measured by a kinetic assay.

proportional to the oxygen concentration, was collected at 13 Hertz using a DASH-16 I/O analog/digital card (Metra-Byte Inc.) and a PC6300 AT&T computer. The digitized data, which were collected for 1 min before and 1 min after addition of cytochrome *c*, were analyzed using Quattro Pro for Windows (Borland International, Inc.). The rate of oxygen consumption was calculated from the difference in the rate of oxygen depletion after and before the addition of cytochrome *c* (each rate was calculated by linear regression analysis of the slope from at least 700 data points). The kinetic constants $K_{m,1}$, $K_{m,2}$, $V_{max,1}$, and $V_{max,2}$ were evaluated from a nonlinear least squares biphasic hyperbolic fit to the velocity vs substrate data using MINSQ 4.02 PC software (MicroMath Inc.) and a 386DX microcomputer equipped with a math coprocessor using the equation

$$v_i = \frac{V_{max,1}[\text{Cyt c}]}{K_{m,1} + [\text{Cyt c}]} + \frac{V_{max,2}[\text{Cyt c}]}{K_{m,2} + [\text{Cyt c}]}$$

Steady-State Redox Level Measurements. The steady-state redox states of cytochrome *c*, cytochrome *a*, and cytochrome a_3 were measured at pH 7.9 with 1 μM cytochrome *c* and 1 μM cytochrome *c* oxidase in 25 mM Tris−acetate buffer containing 5 mM sodium ascorbate and 1 mg/mL of either dodecyl maltoside, Triton X-100, $C_{12}E_8$, or Tween-80. The temperature of the solution was controlled at 25.0 ± 0.05 °C with a water-jacketed cuvette holder and a circulating water bath. The reaction was started by the addition of 0.1−2.0 mM TMPD. Absorbance values were simultaneously monitored at 444, 550, 578, 604, 620, and 630 nm with a Hewlett Packard 8452 diode array spectrophotometer interfaced with a Packard Bell PB VX88 computer. Data were collected every second when the TMPD concentration was less than 0.75 mM and every 0.25−0.5 s when the concentration was greater than 0.75 mM until at least 20 s after anaerobiosis. The fractional reduction of each cytochrome was calculated using $\Delta\Delta\epsilon_{[(550-578)_{red} - (550-578)_{ox}]} = 20.7$ mM^{-1} cm^{-1} for cytochrome *c*, $\Delta\Delta\epsilon_{[(604-620)_{red} - (604-620)_{ox}]} = 20$ mM^{-1} cm^{-1} for cytochrome *a*, and $\Delta\epsilon_{444(red-oxid)} = 143$ mM^{-1} cm^{-1} for cytochrome a_3.

These delta extinction coefficients were evaluated from the absorbance values of oxidized and dithionite-reduced cytochrome *c* and cytochrome *c* oxidase at the appropriate wavelengths, and the values agree with accepted literature values (Antonini et al., 1991; Thörnström et al., 1988). The fractional reduction of each cytochrome was calculated as a function of time from the digitized absorbance data using Quattro Pro for Windows. The fractional reduction of cytochrome *c* was calculated within the spreadsheet using the formula

$$\%\ \text{reduction Cyt } c = A_{[(550-578)_{reaction} - (550-578)_{ox}]} / \Delta\Delta\epsilon_{[(550-578)_{red} - (550-578)_{ox}]}$$

The fractional reduction levels of cytochrome *a* and cytochrome a_3 were evaluated using a system of two linear simultaneous equations. Reduced cytochrome *a* was assumed to contribute 33% of the reduced minus oxidized absorbance at 444 nm (Gibson & Greenwood, 1967; Antalis & Palmer, 1982; Mahapatro & Robinson, 1990) and 80% of the reduced minus oxidized absorbance at either 604−620 or 604−630 nm (Thörnström et al., 1988), values that were confirmed in the present study. The A_{444} data were corrected for the contribution of reduced cytochrome *c* prior to evaluating the percent reduction of each cytochrome. The absorbance difference at 604−620 nm was used to evaluate the fractional reduction of cytochrome *a* and a_3 to minimize the possible interference of $TMPD^+$ as suggested by Morgan and Wikström (1991). However, the same result was obtained whether the fractional reduction of cytochrome *a* was calculated from the delta absorbance of 604−620 or 604−630 nm. Using this approach, cytochrome a_3 was found to be fully oxidized during the aerobic reaction and cytochromes *a*, a_3 and *c* were each fully reduced after anaerobiosis. These results confirm the validity of our calculations. In the experiments where the ratio of cytochrome *c* to cytochrome *c* oxidase was greater than 1, it was assumed that (1) cytochrome a_3 was fully oxidized during the aerobic steady state; (2) 80% of the $\Delta A_{(604-630)_{rxn-ox}}$, after correction for the contribution of reduced cytochrome *c*, was due to reduced cytochrome *a*; and (3) there was no interference from $TMPD^+$.

Determination of k_{cat} from Redox Measurements. The rate of electron transfer from cytochrome *a* to the bimolecular Cu_B−cytochrome a_3 center (k_{cat}) was evaluated from the steady-state reduction values using the equation

$$\frac{[\text{Cyt a}]_{red}}{[\text{Cyt c}]_{ox}} = \frac{k_1}{k_{cat}}[\text{TMPD}] + C \qquad (1)$$

where k_1 = the second-order rate constant for the TMPD reduction of cytochrome *c* bound at the high-affinity site, and *C* is a small constant that results from the very slow rate of reduction of tightly bound cytochrome *c* by ascorbate. This equation was obtained by assuming that the rate of electron flux into cytochrome *c* was equal to the rate of electron flux out of cytochrome a during steady-state conditions and solving for $[\text{Cyt } a]_{red}/[\text{Cyt } c]_{ox}$, i.e.,

$$\text{flux}_{in} = k_1[\text{TMPD}][\text{Cyt c}]_{ox} + k_{Asc}[\text{AscH}][\text{Cyt c}]_{ox}$$

$$\text{flux}_{out} = k_{cat}[\text{Cyt a}]_{red}$$

Evaluation of $[Cyt\ a]_{red}$ and $[Cyt\ a]_{ox}$ from k_{cat}, [TMPD], and K_{eq}. In a 1:1 complex of cytochrome *c* and cytochrome *c* oxidase, the fractional reduction and oxidation of cytochrome *a*, $[\text{Cyt } a]_{red}/[\text{Cyt } a]_{tot}$ and $[\text{Cyt } a]_{ox}/[\text{Cyt } a]_{tot}$, are determined by the values of k_{cat}, k_1, [TMPD], and K_{eq} (the equilibrium constant between cytochrome *c* and cytochrome *a*), which is

$$K_{eq} = \frac{[\text{Cyt } a]_{red}[\text{Cyt } c]_{ox}}{[\text{Cyt } a]_{ox}[\text{Cyt } c]_{red}} = \frac{([\text{Cyt } a]_{red}/[\text{Cyt } a]_{tot})([\text{Cyt } c]_{ox}/[\text{Cyt } c]_{tot})}{(1 - [\text{Cyt } a]_{red}/[\text{Cyt } a]_{tot})(1 - [\text{Cyt } c]_{ox}/[\text{Cyt } c]_{tot})} \qquad (2)$$

A quadratic equation can be written for $[\text{Cyt } a]_{red}/[\text{Cyt } a]_{tot}$ as a function of these parameters by substituting into eq 2 the expression for $[\text{Cyt } c]_{ox}$ in terms of $[\text{Cyt } a]_{red}$ that is obtained from eq 1 after assuming that the constant $C = 0$. The resulting quadratic equation is

$$a\left(\frac{[\text{Cyt } a]_{red}}{[\text{Cyt } a]_{tot}}\right)^2 + b\left(\frac{[\text{Cyt } a]_{red}}{[\text{Cyt } a]_{tot}}\right) + c = 0$$

Table 1: Enzyme Activity of Cytochrome *c* Oxidase in Different Detergents

detergent	enzyme activity		% reduced[c]	
	spectrophotometric[a] (s^{-1})	polarographic[b] (s^{-1})	cytochrome *c*	cytochrome *a*
dodecyl maltoside	341	107	30	49
$C_{12}E_8$	204	58	50	64
Tween-80	151	53	48	61
decanoyl sucrose		34	81	84
Triton X-100	8	4	94	98

[a] Spectrophotometric assay in 25 mM phosphate buffer, pH 7.0, with 30 μM reduced cytochrome *c* and 1 mg/mL of the appropriate detergent. Refer to Materials and Methods for details. [b] Polarographic assay in 25 mM Tris−acetate buffer, pH 7.9, with 5 mM ascorbate, 0.7 mM TMPD, 30 μM cytochrome *c*, and 1 mg/mL of the appropriate detergent (2 mg/mL of decanoyl sucrose). Refer to Materials and Methods for details. [c] The percent reduction was measured under aerobic steady-state conditions, with a 1:1 cytochrome *c*:oxidase complex (1 μM each) in 25 mM Tris−acetate buffer, pH 7.9 with 5 mM ascorbate, 0.7 mM TMPD, and 1 mg/mL of each detergent (2 mg/mL of decanoyl sucrose). Refer to Materials and Methods for details.

where

$$a = \left(\frac{k_{cat}}{k_1[\text{TMPD}]}\right)(K_{eq} - 1)$$

$$b = -K_{eq}\left(1 + \frac{k_{cat}}{k_1[\text{TMPD}]}\right)$$

and

$$c = K_{eq}$$

Only one of the roots of the quadratic equation leads to a physically relevant value of $[\text{Cyt } a]_{red}/[\text{Cyt } a]_{tot}$ between 0 and 1. This value then determines $[\text{Cyt } a]_{ox}/[\text{Cyt } a]_{tot}$ which is equal to $1 - [\text{Cyt } a]_{red}/[\text{Cyt } a]_{tot}$. The corresponding terms for cytochrome *c* are obtained from K_{eq}.

RESULTS

The amphiphilic environment surrounding detergent-solubilized cytochrome *c* oxidase affects the maximum electron-transfer rate from cytochrome *c* to oxygen by as much as 2 orders of magnitude (Table 1). These activities, measured by either the spectrophotometric or the polarographic assay, are consistent with previous reports (Rosevear et al., 1980; Thompson & Ferguson-Miller, 1983; Robinson et al., 1985; Gregory & Ferguson-Miller, 1988; Michel & Boshard, 1989; Mahapatro & Robinson 1990). As expected, the highest enzyme activity is obtained when the enzyme is solubilized in dodecyl maltoside, while the lowest enzyme activity is obtained for enzyme solubilized in Triton X-100. Intermediate enzyme activities are obtained for oxidase solubilized in $C_{12}E_8$, Tween-80, or decanoyl sucrose.

The type of detergent used to solubilize cytochrome *c* oxidase also greatly alters the biphasic behavior of the cytochrome *c* kinetics (Figure 1). The maximum velocity of the high-affinity phase ($V_{max,1}$) becomes smaller as the solubilizing detergent is changed from dodecyl maltoside to other detergents, with the magnitude of $V_{max,1}$ being in the order dodecyl maltoside > $C_{12}E_8$ > decanoyl sucrose > Triton X-100. However, $K_{m,app}$ for the high-affinity phase is approximately the same in each detergent. The behavior of the low-affinity second phase is somewhat different. The maximum velocity for the low-affinity second phase, $V_{max,2}$, is more sensitive to changes in the detergent environment than is $V_{max,1}$ (Table 2). Once again the order is dodecyl maltoside > $C_{12}E_8$ > decanoyl sucrose > Triton X-100. $V_{max,2}$ is larger than $V_{max,1}$ when dodecyl maltoside is the

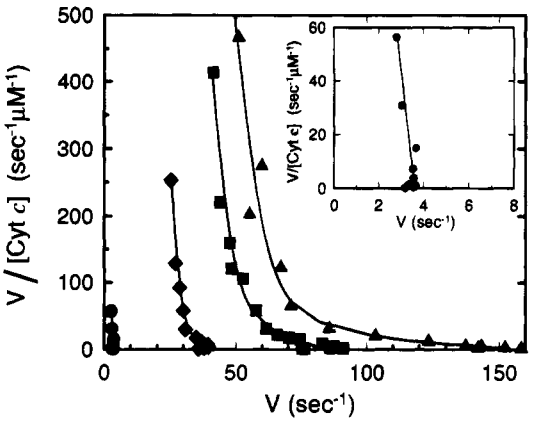


FIGURE 1: Eadie−Hofstee plot of cytochrome *c* oxidase steady-state kinetics at low ionic strength and 25 °C in four detergents. The rate measurements were done polarographically as described in Materials and Methods. In all cases, the buffer was 25 mM Tris−acetate, pH 7.9, 1.0 mM TMPD with 1 mg/mL dodecyl maltoside (▲), 1 mg/mL $C_{12}E_8$ (■), 2 mg/mL decanoyl sucrose (◆) and 1 mg/mL Triton X-100 (●). The continuous lines represent the best fits to a biphasic model except for the Triton X-100 data where there was no second phase.

solubilizing detergent, is about the same as $V_{max,1}$ with $C_{12}E_8$, and is nearly absent in decanoyl sucrose. In fact, the second phase is completely absent and the enzyme kinetics are monophasic when cytochrome *c* oxidase is solubilized with Triton X-100 (inset to Figure 1).

The type of detergent used to solubilize cytochrome *c* oxidase also alters the steady-state reduction levels of cytochrome *c* and cytochrome *a* as measured by multiwavelength visible spectroscopy of a 1:1 complex of cytochrome *c* and cytochrome *c* oxidase (Table 1). After initiation of electron transfer by addition of TMPD, the kinetic steady-state is maintained until the oxygen in the solution is exhausted (Figure 2). The reduction levels of each of the three cytochromes is fairly constant during the steady-state aerobic phase, and as expected, cytochrome a_3 remains fully oxidized. However, in dodecyl maltoside neither cytochrome *c* nor cytochrome *a* is fully reduced; i.e., each is approximately 50% oxidized using the normal polarographic assay conditions of 0.5−0.7 mM TMPD (Figure 2B). Complete reduction of all three cytochromes occurs when the oxygen is exhausted, which confirms the validity of the multiwavelength analysis procedure in assessing the reduction level of each cytochrome.

In the other detergents, the steady-state reduction levels of cytochrome *c* and cytochrome *a* are different. With Triton X-100, in which the activity of cytochrome *c* oxidase is very

Table 2: Comparison of Polarographic Values of $V_{max,1}$, $V_{max,2}$, and $V_{max,total}$ with Steady-State Reduction Values of $V_{max,1}$, $V_{max,2}$, and k_{cat}

	polarographic assay			steady-state assay			
detergent	$V_{max,1}$[a] (s^{-1})	$V_{max,2}$[a] (s^{-1})	$V_{max,total}$[b] (s^{-1})	k_1/k_{cat}[c]	k_{cat}[d] (s^{-1})	$V_{max,1}$[e] (s^{-1})	$V_{max,2}$[e] (s^{-1})
dodecyl maltoside	65	102	167	560	170	69	101
Tween 80				1000	95	54	41
$C_{12}E_8$	48	40	88	1040	91	53	38
decanoyl sucrose	28	11	39	5500	17	15 (31)[f]	2 (8)[f]
Triton X-100	4	0	4	14000	6.8	6.5 (3.9)[f]	0.3 (0.1)[f]

[a] Evaluated from the polarographic data in Figure 1 as described in Materials and Methods. [b] $V_{max,total} = V_{max,1} + V_{max,2}$. [c] Evaluated from the slopes of plots in Figure 5. [d] Evaluated from k_1/k_{cat} using $k_1 = 0.95 \times 10^5$ M^{-1} s^{-1} obtained from the inverse of the slope in the inset to Figure 5. [e] $V_{max,1} = k_{cat}([Cyt\ a]_{red}/[Cyt\ a]_{tot})$, and $V_{max,2} = k_{cat}([Cyt\ a]_{ox}/[Cyt\ a]_{tot})$, where $([Cyt\ a]_{red}/[Cyt\ a]_{tot})$ and $([Cyt\ a]_{ox}/[Cyt\ a]_{tot})$ were calculated from k_{cat}, K_{eq}, k_1, and [TMPD] as described in Materials and Methods. [f] Calculated as described in footnote *e*, but using $V_{max,total}$ derived from polarographic data instead of k_{cat} derived from steady-state reduction data.

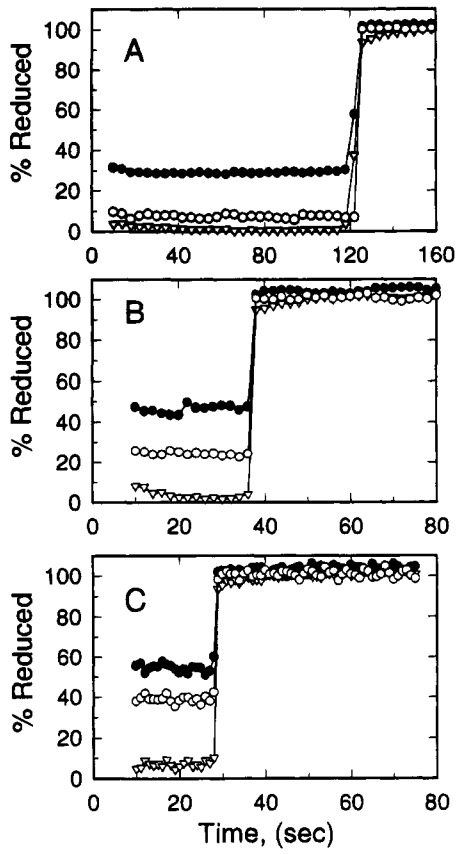


FIGURE 2: Time course of reduction levels of cytochrome *a* (●), cytochrome *c* (○), and cytochrome a_3 (▽) before and after anaerobiosis at different TMPD concentrations. Cytochrome *c* oxidase and cytochrome *c* were mixed in a cuvette at final concentrations of 1 μM each and 5 mM ascorbate in 25 mM Tris–acetate, pH 7.9 and 25 °C, with 1 mg/mL dodecyl maltoside and 0.1 (A), 0.5 (B), or 1.0 mM TMPD (C). The determination of percent reduction is described in Material and Methods. Data were acquired at intervals of 1 (panels A and B) and 0.25 s (panel C); every fourth data point is plotted. Fluctuations in steady-state reduction levels were always less than ±4%. The average steady-state reduction levels had standard deviations closer to ±2%, e.g., steady-state reduction of cytochrome *a* in panel C was 54.1 ± 1.9%.

low (4–8 s^{-1}) and the cytochrome *c* kinetics are completely monophasic, both cytochromes are nearly 90–100% reduced during aerobic turnover. With $C_{12}E_8$, Tween-80, or decanoyl sucrose the reduction levels of cytochrome *c* and cytochrome *a* are intermediate between those obtained in dodecyl maltoside and Triton X-100.

The percent reduction of cytochrome *c* and cytochrome *a* both decrease in detergents that support the highest steady-state turnover and have the greatest amount of the second low-affinity phase. Exactly the opposite is true in detergents that support the lowest turnover. The most probable explanation for this apparent inverse correlation is that the fixed rate of electron transfer into cytochrome *c* bound at the high-affinity site becomes rate limiting with high cytochrome *c* oxidase activity. If this is the case, one would predict that increasing the concentration of TMPD would increase the rate of reduction of bound cytochrome *c* and increase the steady-state reduction levels of both cytochrome *c* and cytochrome *a*. Furthermore, if incomplete reduction of cytochrome *a* via the high-affinity site is due to a limited rate of electron input into the tightly bound cytochrome *c*, then introduction of electrons into cytochrome *a* via any secondary pathway should increase the total rate of electron import into cytochrome *a* and increase its steady-state reduction level.

To test these predictions, the percent reduction of both cytochrome *c* and cytochrome *a* were measured during steady-state turnover as a function of the solubilizing detergent, the concentration of TMPD, and the concentration of cytochrome *c*. As expected, the time required to reach anaerobiosis in each condition was directly related to the enzymatic rate and was a function of all three variables (Figure 2). The steady-state reduction levels of cytochrome *c* and cytochrome *a* were also both strongly influenced by the nature of the bound detergent and the concentration of the reductant TMPD (Figure 3). In detergents that only supported very low enzymatic activity, e.g., Triton X-100, full steady-state reduction of both cytochrome *c* and cytochrome *a* (Figure 3, panels A and B) occurred even with very low concentrations of TMPD, i.e., 0.5–0.75 mM. However, in detergents that support high enzymatic turnover, e.g., dodecyl maltoside, steady-state reduction of both cytochromes was strongly dependent upon the TMPD concentration, and only 50% of the cytochrome *c* and 70% of the cytochrome *a* was reduced even with 2 mM TMPD. Once again, with detergents that support intermediate enzymatic activity, the dependence of steady-state reduction was intermediate between those observed with Triton X-100 and dodecyl maltoside.

With all concentrations of TMPD and for all detergents, the percent reduction of cytochrome *a* was always greater than the percent reduction of cytochrome *c*, a result that is consistent with their individual reduction potentials. This is best expressed as the ratio of reduced to oxidized cytochrome *a* divided by the ratio of reduced to oxidized cytochrome *c*, a ratio that is proportional to the difference between their midpoint potentials. This ratio is relatively constant under all conditions, particularly if the TMPD

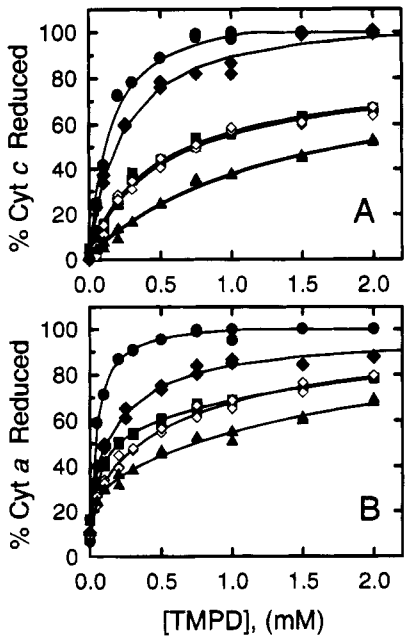


FIGURE 3: Steady-state reduction of cytochrome *c* (A) and cytochrome *a* (B) as a function of TMPD concentration and detergent used to solubilize cytochrome *c* oxidase. Cytochrome *c* oxidase and cytochrome *c* were mixed to a final concentration of 1 μM each, in 25 mM Tris−acetate, pH 7.9, containing 5 mM ascorbate and 1 mg/mL dodecyl maltoside (▲), Tween-80 (◇), $C_{12}E_8$ (■), and Triton X-100 (●) or 2 mg/mL decanoyl sucrose (◆). Measurements were done in duplicate using multiwavelength visible spectroscopy. Differences between duplicate samples were never greater than 4%. Refer to Materials and Methods for calculations and experimental details.

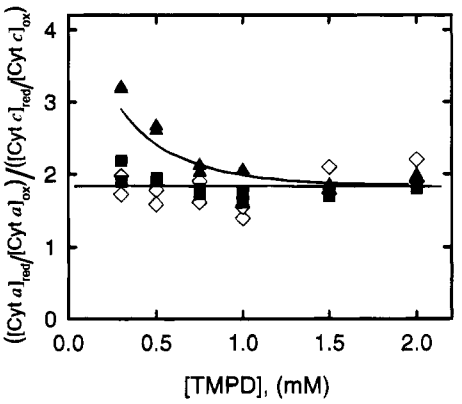


FIGURE 4: Ratio of $[\text{Cyt } a]_{red}/[\text{Cyt } a]_{ox}$ to $[\text{Cyt } c]_{red}/[\text{Cyt } c]_{ox}$ under aerobic steady-state conditions as a function of the TMPD concentration and the type of solubilizing detergent. These ratios were calculated from data in Figure 3: dodecyl maltoside (▲), Tween 80 (◇), and $C_{12}E_8$ (■).

concentration is greater than 0.5 mM (Figure 4), and suggests that the steady-state reaction is near redox equilibrium in each experiment with a $K_{eq} = 1.8$. Apparently, neither the type of detergent nor the concentration of TMPD significantly alters either the midpoint potential or the attainment of redox equilibrium.

The fractional reduction levels of cytochrome *c* and *a* can also be used to calculate a theoretical k_{cat} for cytochrome *c* oxidase when electron entry is restricted to cytochrome *c* at the high-affinity site. At steady state, the flux of electrons into tightly bound cytochrome *c* equals the flux of electrons out of cytochrome *a*. As described in Materials and Methods, this assumption leads to the prediction that the ratio

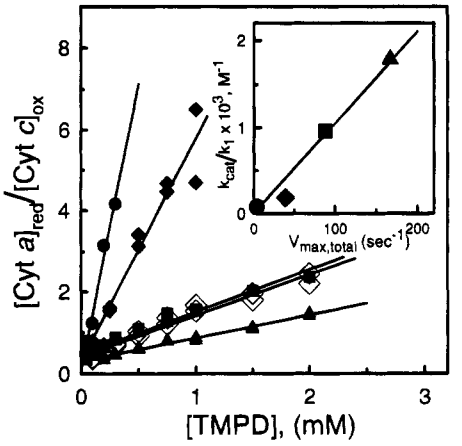


FIGURE 5: Determination of k_{cat}/k_1 from a plot of $[\text{Cyt a}]_{red}/[\text{Cyt c}]_{ox}$ as a function [TMPD]. Steady-state reduction and oxidation levels were measured using a 1:1 complex of cytochrome *c* and cytochrome *c* oxidase in dodecyl maltoside (▲), Tween-80 (◇), $C_{12}E_8$ (■), decanoyl sucrose (◆), and Triton X-100 (●). The reduction levels were obtained from the data in Figure 3. Inset: Plot of k_{cat}/k_1 obtained from the slopes of linear regression fits to the data in the main panel versus $V_{max,tot}$ evaluated from the polarographic data as described in Materials and Methods. Symbols used in the inset panel are the same as in Figure 1 and the main panel of Figure 5.

$[\text{Cyt } a]_{red}/[\text{Cyt } c]_{ox}$ is a linear function of the TMPD concentration with a slope of k_1/k_{cat}, where k_1 is the bimolecular rate constant for electron transfer from TMPD to oxidized cytochrome *c* and k_{cat} is the first-order rate constant for electron transfer out of cytochrome *a*. As predicted, in each detergent a plot of $[\text{Cyt } a]_{red}/[\text{Cyt } c]_{ox}$ versus the TMPD concentration is linear with the greatest slope in Triton X-100 and the smallest slope in dodecyl maltoside (Figure 5 and Table 2). Furthermore, a plot of the polarographically determined total maximum velocity in each detergent, i.e., $V_{max,tot} = V_{max,1} + V_{max,2}$ (Table 2), versus the reciprocal of the corresponding slopes of Figure 5 (k_{cat}/k_1) is linear (inset in Figure 5). This indicates that $V_{max,total}$ is directly proportional to k_{cat} and permits evaluation of the second-order rate constant for reduction of cytochrome *c* by TMPD from the inverse of the slope of the inset to Figure 5. The value of $k_1 = (9.5 \pm 0.5) \times 10^4$ M^{-1} s^{-1} obtained by this method is intermediate between the value obtained by Hill and Nicholls (1980) (1.3×10^5 M^{-1} s^{-1}) and that obtained by Osheroff et al. (1983) (2.2×10^4 M^{-1} s^{-1}). Direct comparison of $V_{max,tot}$ with the steady-state evaluation of k_{cat} (Table 2) is also quite good, as one would expect from the linear relationships in Figure 5.

The finding that k_{cat} is equivalent to $V_{max,tot}$ permitted us to predict $V_{max,1}$ and $V_{max,2}$ at any value of $V_{max,tot}$ using the known values for k_1 and K_{eq} between cytochrome *c* and cytochrome *a*. All we must do is calculate the fractional reduction and oxidation of cytochrome *a* (refer to Materials and Methods) and assume that

$$V_{max,1} = V_{max,tot}\left(\frac{[\text{Cyt } a]_{red}}{[\text{Cyt } a]_{tot}}\right)$$

and

$$V_{max,2} = V_{max,tot}\left(\frac{[\text{Cyt } a]_{ox}}{[\text{Cyt } a]_{tot}}\right)$$

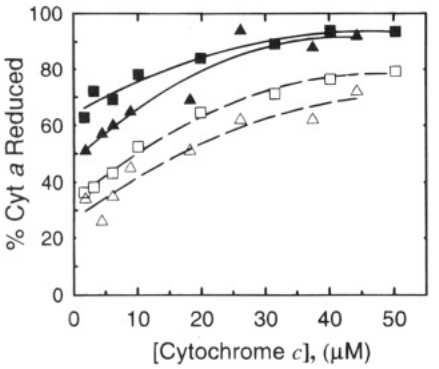


FIGURE 6: Steady-state reduction level of cytochrome *a* as a function of cytochrome *c* concentration in two different detergents. Cytochrome *c* oxidase, at a final concentration of 1 μM, was mixed with 1.5−50 μM cytochrome *c* in 25 mM, pH 7.9, Tris−acetate buffer at 25 °C containing 5 mM ascorbate and either 1 mg/mL dodecyl maltoside (△, ▲) or $C_{12}E_8$ (□, ■). The reaction was started by addition of either 0.1 mM TMPD (△, □) or 0.5 mM TMPD (▲, ■). Refer to Materials and Methods for calculations.

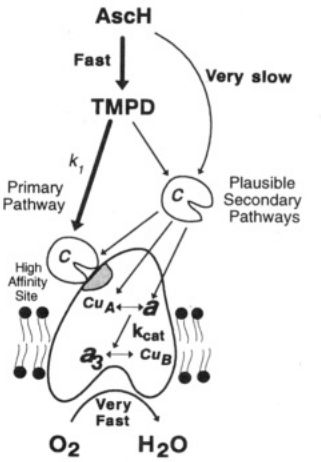
Using this approach, $V_{max,1}$ and $V_{max,2}$ were calculated from the steady-state reduction level data in each detergent (Table 2). The agreement with the polarographic data is excellent.

The prediction that the biphasic cytochrome *c* kinetic behavior of cytochrome *c* oxidase results from increased reduction of cytochrome *a* at high cytochrome *c* concentrations was directly tested and confirmed by measuring the effect of increased cytochrome *c* upon the steady-state reduction of cytochrome *a*. In this case the fractional reduction of cytochrome *c* could not be measured because of its high concentration, but the reduction level of cytochrome *a* was measured as a function of the cytochrome *c* concentration while keeping the TMPD constant at either 0.1 or 0.5 mM (Figure 6). In either dodecyl maltoside or $C_{12}E_8$, detergents in which the low-affinity phase is clearly present, increasing the cytochrome *c* concentration greatly increased the reduction level of cytochrome *a*. For example, in dodecyl maltoside with 0.1 mM TMPD, 80% of the cytochrome *a* could be reduced with a 50-fold molar excess of cytochrome *c*.

DISCUSSION

The present study quantifies the kinetic consequences of the rate-limiting reduction of cytochrome *c* by TMPD and the incomplete reduction of cytochrome *c* and cytochrome *a* during steady-state turnover in the absence of excess cytochrome *c*. We also were able to evaluate the intrinsic rate of electron transfer from cytochrome *a* to the bimetallic center (true k_{cat}) in a variety of detergent environments by measuring the steady-state reduction level of cytochrome *a* as a function of electron input into cytochrome *a*. By comparison of k_{cat} with the kinetically derived $V_{max,1}$ and $V_{max,2}$, we conclude that $k_{cat} = V_{max,1} + V_{max,2}$ in all of the detergent environments that were tested. These results confirm that biphasic kinetics and the requirement for additional electron transfer from excess cytochrome *c* (weakly bound or unbound) during the low-affinity phase are a direct consequence of the limited electron input into cytochrome *a* via tightly bound cytochrome *c*. The functional role of excess cytochrome *c* during the low-affinity kinetic phase is to provide additional reducing equivalents to cytochrome *a*.

Scheme 1: Main Electron-Transfer Reactions Involved in Polarographic Measurement of Cytochrome *c* Oxidase Enzymatic Turnover[a]



[a] The primary source of electrons is ascorbate (AscH). Electrons are transferred from AscH by way of the artificial electron donor TMPD either to cytochrome *c* that is bound to cytochrome *c* oxidase at the high-affinity site (primary pathway) or to cytochrome *c* that is free in solution. In the standard polarographic assay ascorbate is kept in excess (5 mM). TMPD (usually 0.70−1.00 mM) greatly increases the rate of electron transfer into either bound or free cytochrome *c*. Electron transfer from tightly bound cytochrome *c* into cytochrome *a* is via Cu_A and probably Cu_X (refer to text for details and references). The enzymatic turnover number, k_{cat}, is the first-order rate of electron transfer from cytochrome *a* to the binuclear cytochrome a_3−Cu_B center. The pathway of electron transfer from free cytochrome *c* to cytochrome *a* via the low-affinity site is not known. Direct electron transfer from excess cytochrome *c* to tightly bound cytochrome *c*, Cu_A, cytochrome *a*, and the binuclear center are all plausible secondary pathways, as is the proposal that excess cytochrome *c* increases the off rate of oxidized cytochrome *c* from the high-affinity site (refer to the discussion of alternate pathways in Discussion). However, the low-affinity binding of cytochrome *c* to cytochrome *c* oxidase does not involve cardiolipin as proposed by Vik et al. (1981) (J. Ortega-Lopez and N. C. Robinson, unpublished results).

Our approach was to (1) control the rate of electron transfer between cytochrome *a* and the binuclear center by changing the detergent environment surrounding the solubilized complex and (2) control the rate of electron entry into tightly bound cytochrome *c* by changing the concentration of TMPD. In this way we were able to regulate both the rate of electron entry into cytochrome *a* and the rate of electron exit from cytochrome *a* (k_{cat}) and to determine the effect of each upon the steady-state reduction levels of cytochrome *a* and the tightly bound cytochrome *c*. Under conditions of low enzymatic turnover, i.e., low TMPD, it is not the rate of electron transfer between the two cytochromes that is slow but rather the transfer of electrons from TMPD into the tightly bound cytochrome *c* (k_1) that limits the fractional reduction of both cytochromes (Primary Pathway in Scheme 1). Because electron entry into tightly bound cytochrome *c* is not rapid enough to keep up with electron transfer out of cytochrome *a* (k_{cat}), both cytochromes remain partially oxidized during normal steady-state turnover even with quite high concentrations of TMPD (refer to Table 1 and Figure 3). In fact, the true k_{cat} cannot be attained by electron entry from TMPD into the tightly bound cytochrome *c* without an additional source of electrons that further reduces cytochrome *a*. Only when cytochrome *a* is fully reduced does the enzymatic rate equal the true rate-limiting catalytic step, i.e., the electron-transfer step between cyto-

chrome a and the bimetallic center (k_{cat}). In the low-affinity kinetic phase, excess reduced cytochrome c is the source of these additional electrons.

The data in this study support these conclusions and Scheme 1 since they explain all of the following observations: (1) Biphasic cytochrome c kinetics occur only under high-turnover conditions, i.e., when internal electron transfer (k_{cat}) is as fast as or faster than the rate of electron transfer into tightly bound cytochrome c (k_1). (2) Monophasic cytochrome c kinetics occur when k_{cat} is much slower than k_1. (3) The fractional reduction of cytochrome a is high under low-turnover conditions, i.e., $k_{cat} \ll k_1$, but can be quite low under high-turnover conditions, i.e., $k_{cat} \geq k_1$. (4) The rate of oxygen reduction is equal to the k_{cat} of cytochrome c oxidase times the fractional reduction of cytochrome a. The basis of the model and the data that support it are discussed in the following sections.

Reduction of Cytochrome a Is Usually Rate Limiting. The data in Table 1 and Figure 3 indicate that both cytochrome a and cytochrome c are almost always partially oxidized during normal steady-state conditions with 0.5−1.0 mM TMPD. This is true not only when entry of electrons into cytochrome a is limited to the primary pathway, i.e., with a one to one complex of cytochrome c and cytochrome c oxidase (Figure 3), but also with higher concentrations of cytochrome c when electrons can enter by both pathways (Figure 5). These data are consistent with a number of studies on both detergent-solubilized and membrane-bound cytochrome c oxidase that have shown cytochromes c and a are partially oxidized during steady-state turnover (Kimelberg & Nicholls, 1969; Thörnström et al., 1988; Morgan & Wikström, 1991; Nicholls, 1993).

Limited electron input into cytochrome a via the primary pathway from tightly bound cytochrome c, Cu_A, and Cu_X at first seems improbable since the rate of electron transfer from cytochrome c bound at the high-affinity site to cytochrome a via Cu_A and Cu_X is known to be rapid. Pan et al. (1993) report first-order rate constants of $>10^5$ s^{-1} for cytochrome $c \rightarrow Cu_A$ and 2.3×10^4 s^{-1} for $Cu_A \rightarrow$ cytochrome a; Pan et al. (1991) report a first-order rate constant of 2600 s^{-1} for the oxidation of tightly bound cytochrome c. The present data support a rapid redox equilibrium between tightly bound cytochrome c and cytochrome a since the ratio of [cytochrome a_{red}/cytochrome a_{ox}] to [cytochrome c_{red}/cytochrome c_{ox}] remained constant in almost all experiments (refer to Figure 4). However, as proposed by Osheroff et al. (1983), it is not the rate of electron transfer from tightly bound cytochrome c to cytochrome a that is rate limiting; it is the rate of electron transfer into tightly bound cytochrome c that is insufficient to maintain both cytochrome c and cytochrome a in a fully reduced state. Reduction of tightly bound cytochrome c by TMPD is relatively slow with a bimolecular rate constant, k_1, of $(0.95-1.3) \times 10^5$ M^{-1} s^{-1} [this study and Hill and Nicholls (1980)], a rate that corresponds to a pseudo-first-order rate constant of only 100 s^{-1} with 0.7−1.0 mM TMPD. This rate is certainly not sufficient to maintain fully reduced cytochrome c and cytochrome a if k_{cat} is greater than 50 s^{-1}, which is almost certainly the case in nearly all detergent and phospholipid environments.

Increasing the concentration of TMPD increases the steady-state reduction level of both cytochrome c and cytochrome a (refer to Figures 2 and 3), a result that is in agreement with both Kimelberg and Nicholls (1969) and Morgan and Wikström (1991). These increased reduction levels are, of course, due to the rate-limiting pseudo-first-order reduction of tightly bound cytochrome c by TMPD (Osheroff et al., 1983; this study). However, at any given concentration of TMPD, the fractional reduction of cytochrome a is not constant and also depends upon the solubilizing detergent. We attribute this to the different values of k_{cat} in each detergent (Robinson & Mahapatro, 1990). In detergents in which k_{cat} is large, the steady-state reduction of cytochrome a is always lower than in detergents in which k_{cat} is small (refer to Table 1).

Primary Pathway for Cytochrome a Reduction. In normal Michaelis−Menten kinetics, the observed velocity is directly proportional to the concentration of the enzyme−substrate complex only if all of the steps are rapid prior to the rate-limiting step. Within cytochrome c oxidase, all of the enzymatic steps are rapid prior to the rate-limiting transfer from cytochrome a to the binuclear center, but it is the reduction of the tightly bound substrate, cytochrome c, that is usually rate limiting. The consequences of this are (1) the substrate binding site is partially filled with oxidized cytochrome c, (2) cytochrome a is always partially oxidized (it is in rapid equilibrium with cytochrome c), and (3) the observed maximal velocity for the high-affinity site ($V_{max,1}$) is not a measure of the true maximal rate of catalysis; it is rather the true k_{cat} times the fractional reduction in cytochrome a during steady-state turnover of the one to one complex (Table 2). This underestimation of k_{cat} from $V_{max,1}$ is a direct consequence of the incomplete steady-state reduction of cytochrome a even with saturation of the high-affinity binding site. However, we were able to evaluate the true k_{cat} directly from the steady-state reduction levels of cytochrome c and cytochrome a (refer to Figure 5 and Table 2). This approach avoids all of the complications associated with evaluation of $V_{max,1}$ and $V_{max,2}$ from polarographic kinetic data such as shown in Figure 1 and confirms that the rate-limiting step during cytochrome c oxidase catalysis is the transfer of electrons between cytochrome a and the bimetallic center.

Alternate Pathway for Cytochrome a Reduction. When cytochrome c and cytochrome a are partially oxidized during normal steady-state turnover, which is true unless k_{cat} is very small, further reduction of cytochrome a can occur only if the rate of electron entry into the tightly bound cytochrome c can be increased or if electrons can enter cytochrome c oxidase via a secondary pathway. A number of alternative mechanisms for increased electron entry into cytochrome c and/or cytochrome a have been proposed, and each is certainly consistent with the present data (refer to Plausible Secondary Pathways in Scheme 1). (1) Electrons could enter directly into cytochrome a, or any of the redox centers prior to cytochrome a, either directly from cytochrome c free in solution or from cytochrome c bound at the low-affinity site. (2) Electrons could enter by way of the tightly bound cytochrome c if direct electron transfer from soluble, reduced cytochrome c to bound, oxidized cytochrome c occurs. (3) Electrons could enter via the high-affinity site if additional cytochrome c increases the off rate of oxidized cytochrome c from this site [model of Wilms et al. (1981); Antalis & Palmer, 1982; Osheroff et al., 1983; Nalecz et al., 1983; Speck et al., 1984; Bolli et al., 1985a,b]. According to any of these models, the second kinetic phase occurs only if electrons entering cytochrome c oxidase by the secondary

pathway result in the further reduction of cytochrome *a*; i.e., the low-affinity kinetic phase can occur only in environments that support high enzymatic turnover. The data in Figure 5 and Table 2 support this conclusion since $V_{max,1}$ is directly related to the fractional reduction of cytochrome *a* during steady-state turnover of a 1:1 complex, while $V_{max,2}$ is directly related to the corresponding fractional oxidation of cytochrome *a*. We conclude that the second phase would never exist if electrons could enter tightly bound cytochrome *c* rapidly enough to keep both cytochromes fully reduced.

Our data also rule out any conformational model as a mechanism to explain the biphasic kinetics (Brzezinski & Malmström, 1986, 1987; Garber & Margoliash, 1990) since the cytochromes in one or more low-activity conformers would be fully reduced with low TMPD and/or low cytochrome *c*. Such a subpopulation of fully reduced cytochrome *c* oxidase was not seen in any of our experiments. The data also exclude any mechanism by which electrons are directly transferred from the extra cytochrome *c* to the binuclear center since this would preclude the observed additional reduction of cytochrome *a* (Figure 6).

In summary, we conclude that the biphasic kinetics of cytochrome *c* oxidase are not an intrinsic property of the enzyme; rather the biphasic kinetics arise from an insufficient rate of reduction of cytochrome *c* by the artificial electron donor TMPD. Such a conclusion is indicated by (1) the monophasic or near monophasic kinetics of cytochrome *c* oxidase when the rate of electron transfer into tightly bound cytochrome *c* is faster than the rate of internal electron transfer, (2) the biphasic kinetics of the enzyme when the rate of electron transfer into tightly bound cytochrome *c* is slower than the rate of internal electron transfer, (3) the incomplete steady-state reduction of both cytochrome *c* and cytochrome *a* during high turnover, and (4) the direct relationship between $V_{max,1}$ and $V_{max,2}$ with the fractional reduction and oxidation in cytochrome *a* during steady-state turnover of the 1:1 complex. Of course, *in vivo* the cytochrome *c* reductant is not TMPD but cytochrome c_1. Electron transfer from cytochrome c_1 may be much faster, in which case electron transfers from the low-affinity binding site would not be necessary to fully reduce cytochrome *a* during coupled electron transport.

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