A Suggested Mechanism for the Catalytic Cycle of Cytochrome *bd* Terminal Oxidase Based on Kinetic Analysis

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ABSTRACT: The apparent oxygen affinity of cytochrome *bd* from *Escherichia coli* and *Azotobacter vinelandii* has been measured using oxymyoglobin as a sensitive monitor of oxygen concentration. In membrane preparations, the $K_{m(O_2)}$ and respiratory rate varied with the nature of the primary substrate used (malate, lactate, reduced nicotinamide adenine dinucleotide (NADH), or ubiquinol-1). At maximum respiratory rates, the $K_{m(O_2)}$ for cytochrome *bd* from *A. vinelandii* was 4.1 μM, approximately 2 times higher than the corresponding value for the *E. coli* enzyme. There were no significant differences between the $K_{m(O_2)}$ values for membrane-bound and purified cytochrome *bd* from *A. vinelandii* when ubiquinol-1 was used as primary substrate. The kinetic parameters $K_{m(O_2)}$ and V_{max} provide a value of 2.8×10^8 M^{-1} s^{-1} for the bimolecular rate constant for oxygen reaction with the enzyme, suggesting that this reaction is diffusion-controlled. Kinetic analysis indicates a mechanism involving a ternary complex. A scheme for the reaction mechanism of cytochrome *bd* is proposed.

Bacterial cytochrome *bd* terminal oxidase complexes are widely distributed, especially in Gram-negative heterotrophs. They function as quinol oxidases and contain three redox centers, hemes *b*-558, *b*-595, and *d*, but no copper [see Poole (1988) for a review]. Purifications of the cytochrome *bd* complex include the enzymes from *Escherichia coli* (Miller & Gennis, 1983; Kita *et al.*, 1984) and *Azotobacter vinelandii* (Jünemann & Wrigglesworth, 1994; Kolonay *et al.*, 1994). Most studies have been carried out on the *E. coli* oxidase, but the structurally and genetically similar *A. vinelandii* enzyme is of particular interest, because it is required for respiratory protection of the oxygen-labile nitrogenase during aerotolerant nitrogen fixation (Kelly *et al.*, 1990). The significance of the latter function has been emphasized by Jones and Redfearn (1967), who noted that the respiratory chain of *A. vinelandii* is one of the most active known.

In view of the function of the *bd* oxidases, knowledge of the K_m for oxygen is of considerable interest to characterize these enzymes. A wide range of $K_{m(O_2)}$ values has been reported, both between different species and for the same organism (Table 1). Some of this variation may reflect experimental problems of measurement, especially when using an oxygen electrode in the low micromolar range of oxygen concentrations, but the rest may indeed reflect molecular/structural differences relevant to the physiology of the organism. These data led to the belief that the apparent oxygen affinity of cytochrome *bd* from *A. vinelandii* is much lower than that for the *E. coli* enzyme (D'mello *et al.*, 1994). Interestingly, the expression of cytochrome *bd* in *E. coli* decreases in response to increased culture aeration, whereas the expression of the *A. vinelandii* enzyme increases when the oxygen supply is elevated (Poole, 1983). In *A. vinelandii*, the lack of a mutant with cytochrome *bd* as sole oxidase poses additional difficulties when attempting to interpret multiphasic oxygen consumption kinetics and to assign the kinetic phases to particular oxidases. It must also be borne in mind that, for two-substrate reactions, the apparent kinetic parameters for one substrate (O_2) are affected by the supply of the other substrate (in this case ubiquinol) which, in a sequence of reactions such as the respiratory chain, may in turn be controlled by other enzymes in the sequence and/or by diffusion processes.

We present here a comparative study of the Michaelis constants (K_m) for oxygen and ubiquinol-1 of cytochrome *bd* from *E. coli* and *A vinelandii,* using oxymyoglobin as a sensitive oxygen probe. Variations of the kinetic parameters $K_{m(O_2)}$ and V_{max} with ubiquinol-1 concentrations have been analyzed. The results allow us to propose a scheme for the catalytic cycle of the enzyme.

EXPERIMENTAL PROCEDURES

The *bd*-overproducing *A. vinelandii* strain MK8 was grown essentially as described by Kelly *et al.* (1990). Overnight cultures of the *bo* deficient *E. coli* strain GL101 (Lemieux *et al.*, 1992) were grown in Luria-Bertani medium (Maniatis *et al.*, 1982) containing 20 mg mL^{-1} kanamycin and 50 mg mL^{-1} tetracycline. Membrane vesicles were prepared by the method of Kelly *et al.* (1990). Cytochrome *bd* from *A. vinelandii* was purified from membrane vesicles as described previously (Jünemann & Wrigglesworth, 1995). The final preparation contained 10.3 nmol of total heme *b* per milligram of protein and was stored at concentrations of 10−15 mg/mL under liquid nitrogen. Horse heart myoglobin (Sigma Chemical Co. Ltd., Poole, U. K.) was oxygenated by passage of dithionite-reduced protein through a Sephadex G25 column equilibrated with 50 mM phosphate buffer (pH 7.2) containing 1 mM ethylenediaminetetraacetic acid (EDTA). The oxymyoglobin was then concentrated in a Centricon cell with YM10 membrane (Amicon Inc., Beverly, MA) and stored under liquid nitrogen. A visible spectrum was taken immediately before use to check the concentration of the oxygenated form ($\epsilon_{580\ nm}$ = 14.4 mM^{-1} cm^{-1}; Antonini, 1955). Myoglobin concentrations were also determined from pyridine haemochrome spectra (Berry & Trumpower, 1987).

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Table 1: Comparison of the K_m Values for Oxygen of the Cytochrome *bd* Terminal Oxidase Complexes from *E. coli* and *A. vinelandii*[a]

sample	substrate	$K_{m(O_2)}$ (μM)	reference
E. coli			
whole cells	succinate (20 mM)	0.024[b]	Rice & Hempfling, 1978
membranes Δ*cyo cyd*$^+$	lactate (10 mM)	0.26[b]	Kita *et al.*, 1984
purified *bd*	ubiquinol-1 (200 μM)	0.38[b]	Kita *et al.*, 1984
purified *bd*	ubiquinol-1	2[b]	Kolonay *et al.*, 1994
A. vinelandii			
membranes	NADH or malate	18[b]	Hoffman *et al.*, 1979
membranes/whole cells	malate (7.7 mM)	3−4.9, 0.25−0.27, 0.01−0.05[c]	D'mello *et al.*, 1994
whole cells	glucose (10 mM)	0.48, 20−50[c]	Bergersen & Turner, 1980
purified *bd*	ubiquinol-1	5[c]	Kolonay *et al.*, 1994

[a] For *A. vinelandii*, all K_m values found in the system are listed, with the ones assigned to cytochrome *bd* underlined. Where substrate concentrations are given by the authors, these are indicated. [b] O_2 electrode method. [c] Globin method.

The use of myoglobin as an oxygen probe essentially followed the method of Bergersen and Turner (1979). A cuvette containing approximately 20 μM oxymyoglobin and purified cytochrome *bd* (1−2 μg mL^{-1}), or membrane vesicles (approximately 50 μg of protein per milliliter), in 0.1 M phosphate buffer (pH 7.9), previously equilibrated with 1% oxygen in argon, was stoppered so that there was no gas phase above the solution. Purified enzyme was preincubated at a protein concentration of 2 mg mL^{-1} for 5 min on ice in 0.1 M phosphate buffer (pH 7.9) containing 1 mM L-α-phosphatidylcholine (from Soybean Type IV-S, Sigma Chemical Co. Ltd.). The reaction was started by addition of substrate (as specified under Results) and monitored at 575−560 nm in a Johnson Foundation four-filter air-turbine spectrophotometer (Biomedical Instrumentation Group, University of Pennsylvania). The time courses of deoxygenation of oxymyoglobin during respiration were analyzed as follows. From the fractional oxygenation at time t,

$$Y = (\Delta A_t - \Delta A_{t=\infty})/(\Delta A_{t=0} - \Delta A_{t=\infty})$$

The concentration of free oxygen can be calculated as

$$[O_2\ \text{free}] = K_d Y/(1 - Y)$$

The dissociation constant, K_d, for the reaction of myoglobin with oxygen was taken to be 1.2 μM (Antonini & Brunori, 1969). The total oxygen concentration in the mixture is

$$[O_2\ \text{total}] = [O_2\ \text{free}] + Y[\text{myoglobin}]$$

Finally, the change of total oxygen concentration with time gives the oxygen consumption rate

$$v = \Delta[O_2\ \text{total}]/\Delta t$$

Kinetic parameters were then determined from the values of v and [O_2 free] with the use of standard methods (e.g. Hanes plots).

RESULTS

Membrane Vesicles Respiring on Different Physiological Substrates. From the summary of the reported values for the apparent $K_{m(O_2)}$ for cytochrome *bd* from *E. coli* in Table 1, it is clear that a crucial difference in experimental conditions used by different authors is the nature of the respiratory substrate. We have therefore measured the apparent V_{max} and $K_{m(O_2)}$ on the same preparation of *E. coli* membranes, using different physiological substrates [malate, lactate, or reduced nicotinamide adenine dinucleotide (NADH)] at concentrations found to be saturating. Typical traces for two substrates, lactate and NADH, are shown in Figure 1A. The corresponding calculated v *vs* [O_2] plots are shown in Figure 1B. It can clearly be seen that the rate of deoxygenation of myoglobin is very different for the two substrates, and this is reflected in the values for V_{max}. The apparent K_m and V_{max} values for all the conditions used are summarized in Table 2 and show the expected close relationship between the apparent K_m and turnover for multisubstrate reactions (Segel, 1975). Increasing turnover leads to increased apparent $K_{m(O_2)}$. It can also be concluded from Table 2 that, even though the added respiratory substrate, lactate or NADH, is present at saturating levels for the primary

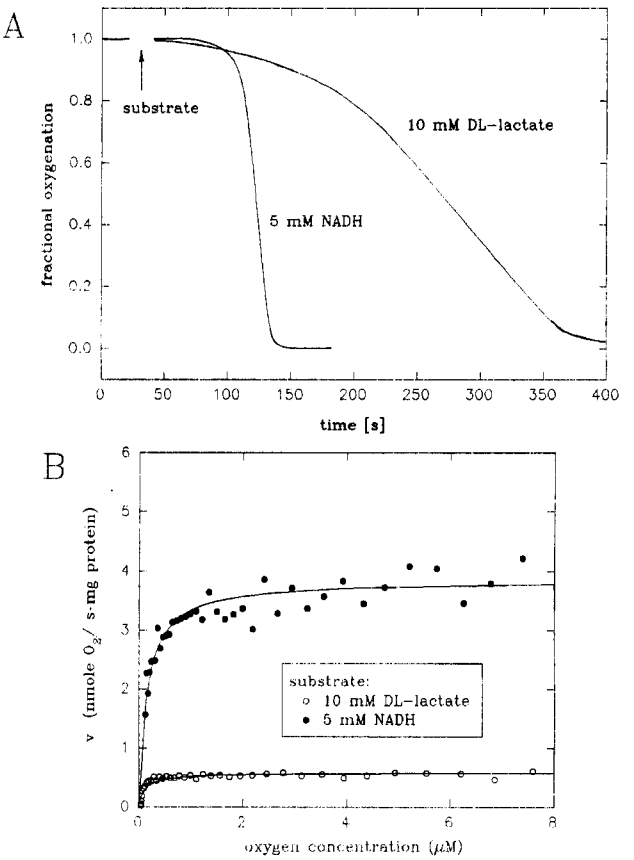


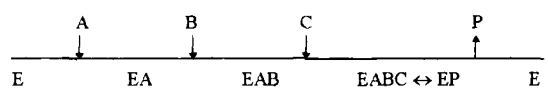
FIGURE 1: Respiratory kinetics of *E. coli* GL101 membranes (0.35 mg mL^{-1}, 57 nM *bd*) using either NADH (5 mM) or lactate (10 mM) as respiratory substrate in 0.1 M potassium phosphate buffer (pH 7.2) at 23 °C. (A) Time courses of deoxygenation of oxymyoglobin. (B) v *vs* [O_2] plots derived from deoxygenation rates as described in the text.

dehydrogenase, this does not necessarily mean that the level of reduced ubiquinone is also saturating for the terminal oxidase. The added substrates give different apparent V_{max} values for cytochrome *bd*. We conclude that the values quoted in the literature for the $K_{m(O_2)}$ of cytochrome *bd* vary because in many cases no account has been taken of the effect of added substrate on the steady state reduction levels of reduced ubiquinone. With this in mind, we have studied the kinetics of oxygen reduction as a function of oxygen and ubiquinone concentrations in both membrane and purified preparations of cytochrome *bd*.

Kinetic Measurements with Ubiquinol-1 as Substrate. In order to determine the limiting values of V_{max} and K_m for the multisubstrate reaction of oxygen reduction/quinol oxidation, the kinetic data were measured with respect to the substrate oxygen at different concentrations of ubiquinol-1. In any individual experiment, the concentration of ubiquinol-1 was kept at a constant level by the presence of 2 mM dithiothreitol (DTT) in the reaction mixture. Increasing the dithiothreitol concentration to 5 mM did not increase the rate of oxygen reduction, and no respiration was observed if dithiothreitol or ubiquinone-1 was omitted. Individual experiments gave primary data to plot v *vs* [O_2 free]. From sets of curves at different ubiquinol-1 (Q_1) concentrations, the corresponding v *vs* [Q_1] plots at different O_2 concentrations could then be derived.

Kinetic Analysis. In the schemes of enzyme mechanisms presented below, E, F, and G denote different forms of the enzyme, A, B, and C are substrates, and P represents any products, not necessarily one, and not necessarily identical ones. The definitions of symbols in steady state rate equations are as follows: v, rate; S, substrate (undefined); V_{max}, limiting rate; K_{iA}, K_{iB}, and K_{iC}, dissociation constants of the EA, EB, and EC complex, respectively; K_{mA}, K_{mB}, and K_{mC}, Michaelis constants for substrate A, B, and C, respectively; K_{iq1} and K_{iq2}, dissociation constants of the EQ complex for the first and second ubiquinol-1 molecule, respectively; $K_{i(O_2)}$, dissociation constant of the EO_2 complex; K_{mq1} and K_{mq2}, Michaelis constants for the first and second molecule of ubiquinol-1, respectively; $K_{mQ} = K_{mq1} + K_{mq2}$, "total" Michaelis constant for ubiquinol-1; and $K_{m(O_2)}$, Michaelis constant for oxygen. With a substrate stoichiometry of one molecule of oxygen and two molecules of ubiquinol, the reaction could, in principle, proceed by a purely sequential scheme, a purely ping-pong scheme, or a mixed ping-pong/sequential reaction involving a ternary complex. Analysis of these schemes gives the following equations.

Sequential Scheme. Assuming an ordered system, a purely sequential scheme has the general form:



(1)

The steady state rate equation for this reaction, in the absence of product (Segal, 1975), is

$$v/V_{max} = 1/(K_{iA}K_{iB}K_{mC}/[A][B][C] + K_{iB}K_{mC}/[B][C] + K_{iA}K_{mB}/[A][B] + K_{mA}/[A] + K_{mB}/[B] + K_{mC}/[C] + 1) \quad (1a)$$

or with Q substituted for A and B and oxygen for C,

Table 2: Apparent Values for V_{max} and $K_{m(O_2)}$ of Cytochrome *bd* from *E. coli* GL101 Membranes Using Different Respiratory Substrates[a]

substrate	V_{max} [nmol of O_2 s^{-1} (mg of protein)$^{-1}$]	$K_{m(O_2)}$ (μM)
10 mM malate	0.02	≤0.01
10 mM DL-lactate	0.56	0.07
5 mM NADH	3.92	0.13

[a] The kinetic parameters were derived using data from deoxygenation curves of oxymyoglobin. The conditions of measurement were as described in Figure 1.

$$v/V_{max} = 1/(K_{iq1}K_{iq2}K_{m(O_2)}/[O_2][Q]^2 + K_{iq2}K_{m(O_2)}/[O_2][Q] + K_{iq1}K_{mq2}/[Q]^2 + K_{m(O_2)}/[O_2] + K_{mQ}/[Q] + 1) \quad (1b)$$

Rearranging eq 1b in the form $[O_2]/v$ *vs* $[O_2]$ or $[Q]/v$ *vs* [Q] gives

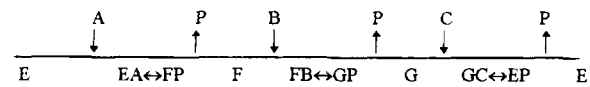
$$[O_2]/v = [O_2](1/V_{max})(K_{iq1}K_{mq2}/[Q]^2 + K_{mQ}/[Q] + 1) + (K_{m(O_2)}/V_{max})(K_{iq1}K_{iq2}/[Q]^2 + K_{iq2}/[Q] + 1) \quad (1c)$$

and

$$[Q]/v = [Q](1/V_{max})(K_{iq1}K_{iq2}K_{m(O_2)}/[O_2][Q]^2 + K_{iq1}K_{mq2}/[Q]^2 + K_{m(O_2)}/[O_2] + 1) + K_{mQ}/V_{max} + K_{iq2}K_{m(O_2)}/[O_2] \quad (1d)$$

It is clear from eqs 1c and 1d that, in a series of $[S]/v$ *vs* [S] plots for different fixed concentrations of the other substrate, the lines would not intersect on the y-axis, but in the third or fourth quadrant (equivalent to intersecting lines in a Lineweaver–Burk double reciprocal plot). This is also the case if oxygen is substituted for substrates A or B.

Ping-Pong Scheme. The second possible reaction mechanism is a pure ping-pong scheme



(2)

with the steady state rate equation

$$v/V_{max} = 1/(K_{mA}/[A] + K_{mB}/[B] + K_{mC}/[C] + 1) \quad (2a)$$

Substitution of A,B and C for ubiquinol-1 and oxygen, respectively, gives

$$v/V_{max} = 1/(K_{m(O_2)}/[O_2] + K_{mQ}/[Q] + 1) \quad (2b)$$

or rearranged

$$[O_2]/v = [O_2](1/V_{max})(K_{mQ}/[Q] + 1) + K_{m(O_2)}/V_{max} \quad (2c)$$

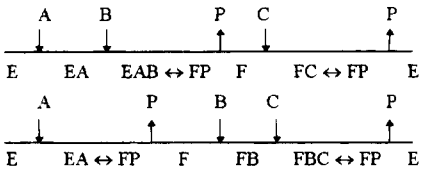
and

$$[Q]/v = [Q](1/V_{max})(K_{m(O_2)}/[O_2] + 1) + K_{mQ}/V_{max} \quad (2d)$$

These equations predict that the ratio K_m/V_{max} is constant for either substrate if the second substrate is varied. Thus, in a series of $[O_2]/v$ *vs* $[O_2]$ plots at different fixed ubiquinol-1

concentrations and in a series of $[Q_1]/v$ *vs* $[Q_1]$ plots at different fixed oxygen concentrations, the lines would converge to a common intersect on the *y*-axis (equivalent to parallel lines in the double reciprocal plot).

Ternary Complex Schemes. If the reaction mechanism involves a ternary complex, two such schemes are possible:



(3.1)

(3.2)

These mechanisms formally have identical steady state rate equations because scheme 3.1 can be converted into scheme 3.2 if the sequence starts with F instead of E. The steady state rate equation, in the absence of product (Segal, 1975), is

$$v/V_{max} = 1/(K_{iA}K_{mB}/[A][B] + K_{mA}/[A] + K_{mB}/[B] + K_{mC}/[C] + 1) \quad (3a)$$

Substitution of A,B and C for oxygen and ubiquinol-1 yields slightly different equations, depending on whether oxygen is added between two ubiquinone-1 release steps (C = O_2 and A,B = Q_1) or not (A or B = O_2). In the latter case, the steady state rate equation is of the form

$$v/V_{max} = 1/(K'/[Q][O_2] + K_{mQ}/[Q] + K_{m(O_2)}/[O_2] + 1) \quad (3b)$$

or rearranged

$$[O_2]/v = [O_2](1/V_{max})(K_{mQ}/[Q] + 1) + K_{m(O_2)}/V_{max} + K'/V_{max}[O_2] \quad (3c)$$

and

$$[Q]/v = [Q](1/V_{max})(K_{m(O_2)}/[O_2] + 1) + K_{mQ}/V_{max} + K'/V_{max}[O_2] \quad (3d)$$

where K' is either $K_{i(O_2)}K_{mq1}$ for A = O_2 or $K_{iq1}K_{m(O_2)}$ for B = O_2.

A series of such $[S]/v$ *vs* [S] plots will not converge on the *y*-axis, if either substrate is the varied one. If, however, C = O_2, then the following rate equation applies:

$$v/V_{max} = 1/(K_{iq1}K_{mq2}/[Q]^2 + K_{mQ}/[Q] + K_{m(O_2)}/[O_2] + 1) \quad (3e)$$

Rearranging the equations in the form $[S]/v$ *vs* [S] gives

$$[O_2]/v = [O_2](1/V_{max})(K_{iq1}K_{mq2}/[Q]^2 + K_{mQ}/[Q] + 1) + K_{m(O_2)}/V_{max} \quad (3f)$$

$$[Q]/v = [Q](1/V_{max})(K_{iq1}K_{mq2}/[Q]^2 + K_{m(O_2)}/[O_2] + 1) + K_{mQ}/V_{max} \quad (3g)$$

and reveals that in this case $[S]/v$ *vs* [S] plots will intersect on the ordinate irrespective of which substrate is the varied one, thus at this stage resembling plots for a ping-pong mechanism.

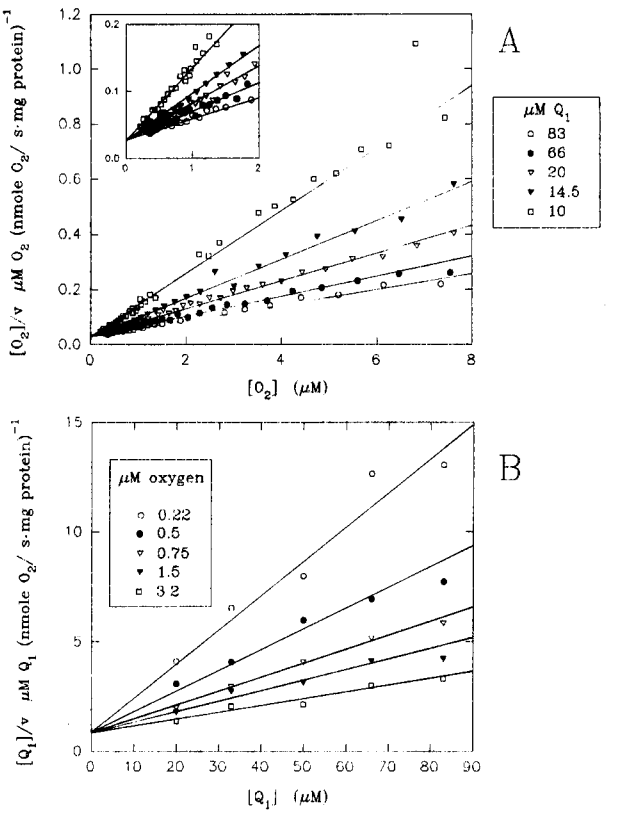


FIGURE 2: Kinetic plots for *E. coli* membranes (35 μg mL^{-1}, 5.7 nM *bd*) respiring on ubiquinol-1 plus 2 mM dithiothreitol in 0.1 M potassium phosphate buffer (pH 7.9). (A) $[O_2]/v$ *vs* $[O_2]$ (inset, enlarged intersect region). (B) $[Q_1]/v$ *vs* $[Q_1]$.

Figures 2 and 3 show $[O_2]/v$ *vs* $[O_2]$ plots at various ubiquinol-1 concentrations (A) and $[Q_1]/v$ *vs* $[Q_1]$ plots at various oxygen concentrations (B) for cytochrome *bd* from *E. coli* and *A. vinelandii*, respectively. The graphs show intersecting lines on the *y*-axis which is compatible with either a pure ping-pong (scheme 2) or a mixed mechanism involving a ternary complex (scheme 3 with C = O_2).

The *y*-axis intercepts of primary (K_m/V_{max}) and secondary ($1/V_{max}$) plots provide the basis for calculating values of $K_{m(O_2)}$ and K_{mQ}. These plots can also distinguish between the two mechanisms possible at this stage. Whereas the secondary plot would be linear in the case of a ping-pong mechanism (see eq 2c), deviations from linearity are expected in a mechanism involving a ternary complex (eq 3g).

Replots of the slopes against 1/[Q], from the primary plots for oxygen as the varied substrate, are indeed nonlinear for enzyme from *E. coli* (Figure 4A) and from *A. vinelandii* (Figure 4B), indicating therefore a mechanism involving a ternary complex (type 3 in the schemes above). The slopes from primary plots of $[Q]/v$ *vs* [Q] were not used for a replot because, for a mixed mechanism (3), they depend on the concentrations of both substrates (see eq 3g).

A further approximation of both Michaelis constants can also be obtained by nonlinear curve fits (Marquardt–Levenberg) of the dependence of the apparent K_m values for oxygen on ubiquinol-1 concentration (Figure 5) which obeys the equation

$$K^{app}_{m(O_2)} = K_{m(O_2)}/(K_{iq1}K_{mq2}/[Q]^2 + K_{mQ}/[Q] + 1) \quad (4)$$

The final kinetic parameters are summarized in Table 3. For *A. vinelandii*, there were no differences between the K_m

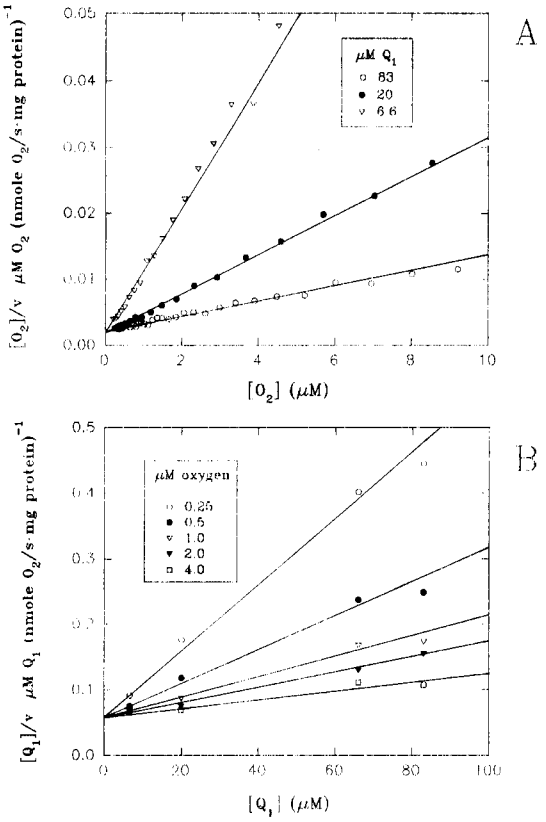


FIGURE 3: Kinetic plots for *A. vinelandii* MK8 membranes (23 μg mL^{-1}, 34 nM *bd*) respiring on ubiquinol-1 plus 2 mM dithiothreitol in 0.1 M potassium phosphate buffer (pH 7.9). (A) $[O_2]/v$ *vs* $[O_2]$. (B) $[Q_1]/v$ *vs* $[Q_1]$.

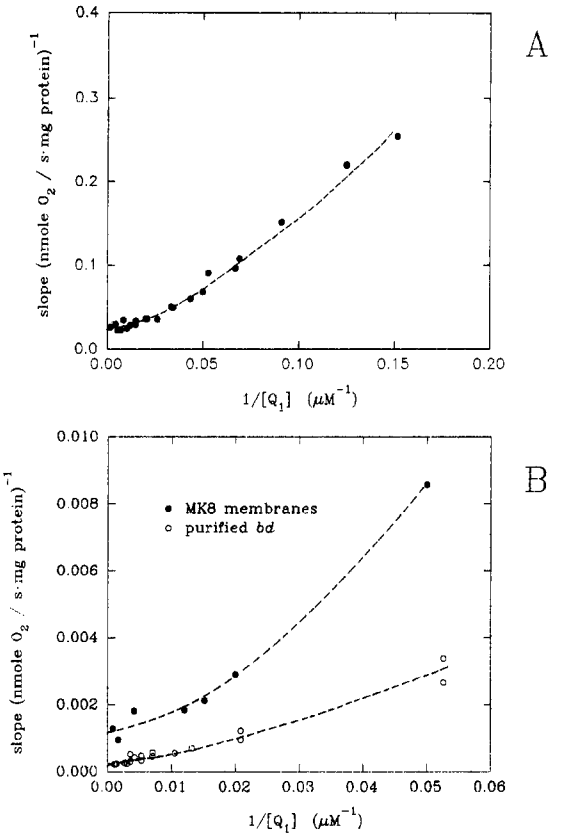


FIGURE 4: Secondary plots of the slopes ($1/V_{max(app)}$) from Figures 2 and 3 *vs* $1/[Q_1]$.

values for membrane-bound and purified cytochrome *bd*. It should be noted that, for ubiquinol-1, only the sum of the two individual Michaelis constants, $K_{mQ} = K_{mq1} + K_{mq2}$ can be obtained, not the individual values. Similarly, the term $K_{iq1}K_{mq2}$ cannot be resolved.

DISCUSSION

It is clear from the results shown in Table 2 that the apparent K_m values for oxygen, $K_{m(O_2)}$, of cytochrome *bd* in membrane preparations vary with the nature of the reducing substrates used. This variation can be mimicked in purified preparations by varying the concentration of ubiquinol. Lower ubiquinol levels result in lower values of the apparent $K_{m(O_2)}$. This is a well-known property of multisubstrate reactions, where the K_m value for one substrate depends on the concentration of the other. The maximum/limiting K_m value for one substrate is that measured under, or extrapolated to, conditions of saturating concentration of the other substrate. For quinol oxidases, the kinetic parameters for ubiquinol are usually measured under saturating oxygen concentrations. For oxygen, the reverse is not always true, especially when using substrates donating electrons into the chain at sites not directly linked to the oxidase. Under conditions of low electron transfer rates, the apparent $K_{m(O_2)}$ of an oxidase can fall to low values (D'mello *et al.*, 1995). However, it is useful to recall that K_m values are phenomenological parameters and so an apparent K_m can have physiological significance to the organism even though it will not have been measured under optimal conditions. In organisms with branched electron transfer chains and more than one type of oxidase, a comparison between the apparent K_m values under "operating" levels of electron flow can reveal useful information about function. The present results clarify the apparent discrepancies reported in the literature (see Table 1) and give values for $K_{m(O_2)}$ extrapolated to saturating concentrations of ubiquinol for cytochrome *bd* from *E. coli* and *A. vinelandii*. For the two organisms, these differ only by a factor of 2 (Table 3). It is interesting to compare these values with the corresponding value for cytochrome *c* oxidase where the $K_{m(O_2)}$ at infinite cytochrome *c* concentration is of the same order of magnitude, 0.95 μM (Petersen *et al.*, 1976). More variation between species is seen in the K_m for ubiquinol-1 which may reflect differences in structure at the quinone binding site(s) of enzyme from the two organisms, although it is known that some of the primary amino acid sequence of the putative Q-binding site is conserved (Moshiri *et al.*, 1991). The results also show that, under optimal conditions, the V_{max} for the enzyme is high compared with those of other oxidases. For example, the maximum turnover (see Table 3) is approximately 6 times higher than the corresponding value for mammaliam cytochrome *c* oxidase (Hill *et al.*, 1986). The turnover number for the enzyme from the two sources differs by a factor of approximately 2. This means that the value of the turnover number divided by the $K_{m(O_2)}$ is on the order of 2.8×10^8 M^{-1} s^{-1} for the enzyme from both *E. coli* and *A. vinelandii*. The parameter (turnover number)/$K_{m(O_2)}$ is indicative of the bimolecular rate constant for oxygen reaction with the enzyme. Since molecular collision rates in aqueous media are on the order of 10^8–10^9 s^{-1}, the reaction is approaching diffusion control and the enzyme is acting as a "perfect catalyst". The present value of 2.8×10^8 M^{-1} s^{-1} can be compared with a value of 1.9×10^9 M^{-1} s^{-1} calculated from the results of rapid reaction spectrophotometry of CO-ligated

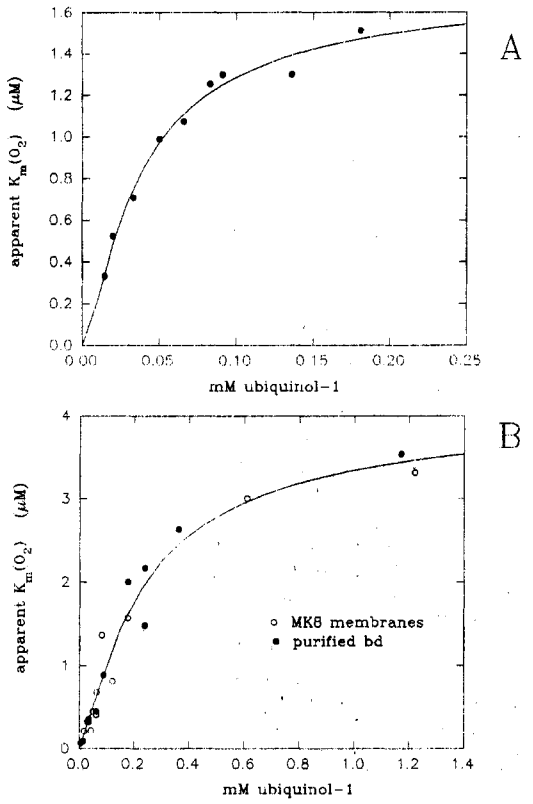


FIGURE 5: Dependence of the apparent $K_{m(O_2)}$ of cytochrome *bd* on ubiquinol-1 concentrations. Conditions of measurement as in Figures 2 and 3. (A) Membrane preparations from *E. coli*. (B) Membrane (○) and purified cytochrome *bd* (●) preparations from *A. vinelandii*. Data points are overlayed with curves calculated using eq 4 and the parameters shown in Table 3.

Table 3: Kinetic Parameters of Cytochrome *bd* from *A. vinelandii* and *E. coli* Respiring on Ubiquinol-1 (plus DTT) in 0.1 M Phosphate Buffer (pH 7.9)[a]

	E. coli	A. vinelandii	
	membrane-bound *bd*	membrane-bound *bd*	purified *bd*
$K_{m(O_2)}$ (μM)	1.75 ± 0.13	4.10 ± 0.34	
K_{mQ} (μM)	42 ± 14	219 ± 73	
$K_{iq1}K_{mq2}$ (μM²)	213 ± 63	5134 ± 3323	
V_{max} [nmol of O_2 s^{-1} (mg of protein)$^{-1}$]	75 ± 13	1038 ± 539	4669 ± 1600
turnover number (s^{-1})	469 ± 81	706 ± 366	1151 ± 394

[a] There was no significant difference in K_m values for membrane-bound and purified enzyme from *A. vinelandii*. Turnover numbers are expressed in terms of moles of O_2 reduced per mole of enzyme per second.

enzyme (Hill *et al.*, 1994) and also with measurements of the corresponding value for cytochrome *c* oxidase of 1×10^8 M^{-1} s^{-1} (Greenwood & Gibson, 1967; Petersen *et al.*, 1976).

Kinetic analysis by itself cannot reveal the details of the catalytic mechanism of an enzyme, but it can eliminate some schemes and place constraints on other possible mechanisms. In the present case, it is clear that a simple sequential mechanism of substrate binding is not compatible with the results of the primary plots of S/*v* against S. These are linear and intercept on the S/*v* axis, suggesting either ping-pong or some form of mixed reaction sequence. A further refinement can be made by noting that the secondary plots (of the slopes from the primary plots against the reciprocal

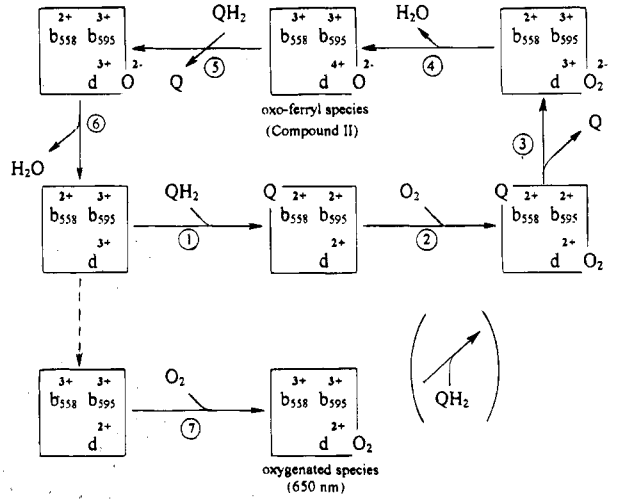


FIGURE 6: Proposed catalytic cycle of cytochrome *bd* based on the present kinetic analysis. Under steady state conditions, the cycle proceeds via steps 1−6. In the absence of reducing substrate, a stable oxygenated species is formed (step 7).

of the respective substrate concentrations) are nonlinear. This nonlinearity is compatible with the formation of a kinetically significant ternary complex as outlined in scheme 3.2. With these kinetic constraints in mind, we suggest a possible catalytic mechanism for cytochrome *bd* which is shown in Figure 6.

Under normal turnover conditions, a molecule of ubiquinol binds to the one-electron-reduced enzyme (step 1). Binding of oxygen (step 2) immediately occurs on reduction of heme *d*, followed by release of ubiquinone and reduction of molecular oxygen to peroxide (step 3). Scission of the oxygen−oxygen bond and release of one molecule of water is followed by the formation of an oxoferryl form of the enzyme (step 4). The presence of a reducing equivalent in the enzyme after step 3 facilitates this reaction (in the catalytic mechanism of peroxidases, the formation of the "compound II" oxoferryl species requires the oxidation of the porphyrin ring or a neighboring aromatic amino acid side chain). A second molecule of ubiquinol can then bind (step 5) and transfer a further two electrons to the enzyme. Release of water leaves the enzyme in a one-electron-reduced form to start the cycle again (step 6). The cycle is kinetically described by the second of the two mechanisms (scheme 3.2), starting with the binding of a ubiquinol molecule to the one-electron-reduced enzyme (denoted as B binding to F in the formal mechanism) followed by oxygen binding as substrate C. Product release and establishment of form E allows binding of the second ubiquinol molecule (denoted as A in the formal mechanism).

The suggested scheme does make some definite predictions. One is that, under turnover conditions, ubiquinol binds to the one-electron-reduced enzyme before oxygen. Since oxygen would be expected to bind immediately to any reduced heme *d*, this implies that reduction of heme *d* by heme *b*-558 in the one-electron-reduced enzyme is slow compared with the binding of ubiquinol. Subsequent reduction of heme *d* would be facilitated by a branched electron transfer pathway, such that electrons could be delivered directly from ubiquinol to heme *b*-595 or heme *d*. Indeed, some evidence for a branched pathway from ubiqinol has been reported for cytochrome *bd* (Jünemann & Wrigglesworth, 1994). The consequence of the present scheme is

that the so-called oxygenated form, identified by an absorption band at 650 nm in the aerobically oxidized enzyme, is not an intermediate in the catalytic cycle. However, in the absence of ubiquinol, heme *d* would eventually be reduced by the one reducing equivalent remaining in the enzyme and a stable oxygenated species would be formed (step 7). This is the form found in preparations of the enzyme purified under aerobic conditions in the absence of reducing substrate.

A second prediction of the present scheme is that, following oxygen binding, there is a rapid conversion to an oxoferryl species, Fe^{IV}=O, equivalent to compound II of the peroxidases. The presence of three reducing equivalents in the enzyme when molecular oxygen binds (step 2) means that formation of the ferryl state can occur without the formation of any cationic radical as found in compound I of catalase and peroxidases. Aerobic reoxidation of samples of reduced cytochrome *bd*, or photolysis of CO-ligated fully reduced cytochrome *bd* (Hill *et al.*, 1994), rapidly results in the formation of a species with an absorption maximum at 680 nm in the visible spectrum. Previously, this had been ascribed to the formation of a coordinated peroxide on heme *d* (Poole & Williams, 1988). However, resonance Raman signals from dithionite-reduced enzyme reacted with mixed isotope oxygen are characteristic of an Fe^{IV}=O stretching mode rather than a peroxo structure (Kahlow *et al.*, 1991), consistent with the scheme shown in Figure 6. In the absence of ubiquinol, this species would be stabilized which would explain why the aerobic visible spectrum of most purified preparations of the enzyme shows a shoulder around 680 nm.

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