J. Biochem. **139**, 779–788 (2006)
doi:10.1093/jb/mvj087

Kinetic Mechanism of Quinol Oxidation by Cytochrome *bd* Studied with Ubiquinone-2 Analogs

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Received December 9, 2005; accepted March 1, 2006

Cytochrome *bd* is a heterodimeric terminal ubiquinol oxidase of *Escherichia coli* under microaerophilic growth conditions. The oxidase activity shows sigmoidal concentration-dependence with low concentrations of ubiquinols, and a marked substrate inhibition with high concentrations of ubiquinol-2 analogs [Sakamoto, K., Miyoshi, H., Takegami, K., Mogi, T., Anraku, Y., and Iwamura H. (1996) *J. Biol. Chem.* 271, 29897–29902]. Kinetic analysis of the oxidation of the ubiquinol-2 analogs, where the 2- or 3-methoxy group has been substituted with an azido or ethoxy group, suggested that its peculiar enzyme kinetics can be explained by a modified *ping-pong bi-bi* mechanism with the formation of inactive binary complex FS in the one-electron reduced oxygenated state and inactive ternary complex $(E_2S)S^n$ on the oxidation of the second quinol molecule. Structure-function studies on the ubiquinol-2 analogs suggested that the 6-diprenyl group and the 3-methoxy group on the quinone ring are involved in the substrate inhibition. We also found that oxidized forms of ubiquinone-2 analogs served as weak noncompetitive inhibitors. These results indicate that the mechanism for the substrate oxidation by cytochrome *bd* is different from that of the heme-copper terminal quinol oxidase and is tightly coupled to dioxygen reduction chemistry.

Key words: cytochrome *bd*, quinol oxidase, quinone analog, respiratory chain, steady state kinetics.

Abbreviations: Q_1, 2,3-dimethoxy-5-methyl-6-*n*-monoprenyl-1,4-benzoquinone (ubiquinone-1); Q_2, 2,3-dimethoxy-5-methyl-6-*n*-diprenyl-1,4-benzoquinone (ubiquinone-2); 2-amino- or 3-amino-Q_2, 2-amino or 3-amino derivative of Q_2 (isorhodoquinone-2 and rhodoquinone-2, respectively); 2-azido-Q_2 and 3-azido-Q_2, 2-azido and 3-azido derivatives of Q_2 (azidoquinone-2); Q_nH_2, a reduced form of Q_n; HQNO, 2-heptyl-hydroxyquinoline-*N*-oxide.

Cytochrome *bd* (CydAB) is a hetero-dimeric ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli*, and is predominantly expressed under microaerophilic growth conditions (*1–3* for reviews). It catalyzes dioxygen reduction with two molecules of ubiquinol-8 (Q_8H_2)[1], leading to the release of four protons from quinols into the periplasm. Through a putative proton channel, four protons used for dioxygen reduction are taken up from the cytoplasm and delivered to the dioxygen reduction site at the periplasmic side of the cytoplasmic membrane (*4*). During dioxygen reduction, cytochrome *bd* generates an electrochemical proton gradient (ΔpH and membrane potential) across the membrane through apparent transmembrane movement of four chemical protons (*5–7*). In contrast to cytochrome *bo*, an alternative oxidase under highly aerated growth conditions, cytochrome *bd* has no proton pumping activity and does not belong to the heme-copper terminal oxidase superfamily.

On the basis of spectroscopic and ligand binding studies, three distinct redox metal centers have been identified as heme b_{558}, heme b_{595}, and heme *d* (*8* for a recent review). Topological analysis suggested that all the hemes are located at the periplasmic side of transmembrane helices (*4*). Heme b_{558} is a low-spin protoheme IX, and is ligated by His^{186} (helix V) and Met^{393} (helix VII) of subunit I (CydA) (*9*). Heme b_{558} is in close proximity to the quinol oxidation site and serves as an electron acceptor for quinols (*10*, *11*). Heme b_{595} is a high-spin protoheme IX bound to His^{19} (helix I) of subunit I (*9*), and mediates electron transfer from heme b_{558} to heme *d* (*12–15*). Heme *d*, where dioxygen is reduced to water is a high-spin chlorin bound to an unidentified nitrogenous ligand (*16–18*), and forms a di-heme binuclear center with heme b_{595} (*18*, *19*). Heme *d* exhibits extremely high affinity for dioxygen (*19–21*), and in the air-oxidized state heme *d* mainly exists in an oxygenated form [Fe(II)-O_2; 80%>] (*22–24*) and partly an oxoferryl form [Fe(IV)=O] (*25*).

Photoaffinity cross-linking studies with 2-methyl-3-azido-5-methoxy-6-(3,7-dimethyl [^{3}H]octyl)-1,4-benzoquinone

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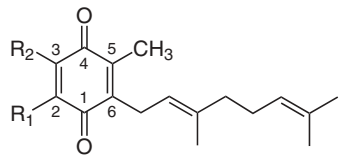
(*26*) and the decrease in the oxidase activity upon either binding of monoclonal antibodies to 252-KLAAIEAEWET-262 of subunit I (*27*, *28*) or proteolytic cleavage of subunit I with trypsin (Tyr^{290}) or chymotrypsin (Arg^{298}) (*29*, *30*) indicate the presence of a quinol oxidation site in loop VI/VII (Q-loop) of subunit I. Currently, no atomic structure for cytochrome *bd* is available, and the structure of the quinol oxidation site remains unknown.

Cytochrome *bd* has been isolated from γ-proteobacteria (*5*, *31–35*), and the steady state kinetics has been interpreted as being of a simple Michaelis type. The K_m values estimated for Q_1H_2 are 0.04–0.28 mM (*5*, *26*, *31–36*), and the K_m value for Q_2H_2 has been reported to be 0.05 mM for the *E. coli* enzyme (*26*). Electron paramagnetic resonance studies on the semiquinone anion (*37*) and inhibitor-binding studies (*38*) are consistent with the presence of a single quinol/quinone-binding site in cytochrome *bd*. To probe the structural features of the quinone/quinol binding sites in respiratory enzymes, we have synthesized a series of Q_2 analogues in which one of the alkyl groups on the quinone ring has been replaced with another alkyl group (*39–41*). Contrary to previous reports (*5*, *26*, *31–36*), our careful kinetic analysis with a wide range of substrate concentrations revealed that the oxidase activity of cytochrome *bd* shows sigmoidal concentration-dependence with low concentrations of both Q_1H_2 and Q_2H_2, and that it shows marked substrate inhibition with Q_2H_2 analogs and decylbenzoquinols at high concentrations (*39*). Unlike cytochrome *bo*, the isolated cytochrome *bd* does not contain a tightly bound Q_8 for the oxidation of substrates supplied from the quinol pool in the membrane. Such a structural difference at the quinol oxidation site and the unusual stability of dioxygen reduction intermediates (*22–25*) would affect the kinetic mechanism underlying the quinol oxidation by cytochrome *bd*-type quinol oxidase.

In this study, we examined the peculiar enzyme kinetics with Q_2H_2 analogs, in which the 2- or 3-methoxy group on the quinone ring had been substituted with an azido or ethoxy group (Fig. 1), and postulated a modified *ping-pong bi-bi* mechanism with the formation of an inactive binary complex, FS, in the one-electron reduced oxygenated state and an inactive ternary complex, $(E_2S)S^n$, on the oxidation of the second quinol molecule. Such a mechanism would be tightly coupled to dioxygen reduction chemistry, and different from that of the heme-copper terminal quinol oxidase, cytochrome *bo* (*38–40*).



Q_2 : R_1 = R_2 = OCH_3
2-Azido-Q_2 : R_1 = N_3, R_2 = OCH_3
2-Amino-Q_2 : R_1 = NH_2, R_2 = OCH_3
2-Ethoxy-Q_2 : R_1 = OCH_2CH_3, R_2 = OCH_3
3-Azido-Q_2 : R_1 = OCH_3, R_2 = N_3
3-Amino-Q_2 : R_1 = OCH_3, R_2 = NH_2
3-Ethoxy-Q_2 : R_1 = OCH_3, R_2 = OCH_2CH_3

Fig. 1. **Structures of Q_2 analogs used in this study.**

MATERIALS AND METHODS

Syntheses of Q_2 Analogues—The synthesis of 2-ethoxy-Q_2 (2-ethoxy-3-methoxy-5-methyl-6-*n*-diprenyl-1,4-benzoquinone) and 3-ethoxy-Q_2 (2-methoxy-3-ethoxy-5-methyl-6-*n*-diprenyl-1,4-benzoquinone) was described previously (*39*). 2-azido-Q_2, 3-azido-Q_2, 2-amino-Q_2 (isorhodoquinone-2), and 3-amino-Q_2 (rhodoquinone-2) were prepared according to Sakamoto *et al*. (*41*). Q_1 was a kind gift from Eisai Co. (Tokyo, Japan). Reduced forms of ubiquinones were prepared by the method of Rieske (*42*).

Purification of Cytochrome bd—The enzyme was isolated from cytochrome *bd*–overproducing strain GR84N/pNG2 (*43*), a generous gift from R. B. Gennis (Univ. of Illinois), as described previously (*14*). The concentration of the enzyme was calculated from the Soret absorption of the air-oxidized form by using a millimolar extinction coefficient of 223 mM^{-1} cm^{-1} (*44*). The purified enzyme in 50 mM potassium phosphate (pH 6.8) containing 0.1% sucrose monolaurate SM-1200 (Mitsubishi-Kagaku Foods Co., Tokyo) was stored at –80°C until use.

Quinol Oxidase Assay—The enzyme activity was determined spectrophotometrically. The reaction mixture (1 ml) comprised 50 mM Tris-HCl (pH 7.3), 0.1% sucrose monolaurate, and 40 nM cytochrome *bd*. The reaction was started by the addition of a quinol in an ethanol solution at a final concentration of 0.14 mM. The enzyme activity was determined at 25°C by recording the absorbance change at 278 nm for Q_2H_2, 2-ethoxy-Q_2H_2 and 3-ethoxy-Q_2H_2, and at 287 nm for 2-azido-Q_2H_2 and 3-azido-Q_2H_2 with a JASCO V-550 UV/Vis spectrophotometer (*39*). Calculation of the steady state reaction rate and data analysis were carried out with Mathematica (Wolfram Research, Inc.) and Origin ver. 7 (OriginLab, MA), respectively.

RESULTS

Kinetic Analysis of the Oxidation of Q_2H_2 Analogs by Cytochrome bd—We examined spectrophotometrically the concentration-dependence of the electron-donating activity of Q_2H_2 analogs and found sigmoidal behavior with low concentrations of all substrates examined in this study (Fig. 2). Because of the auto-oxidation of low-potential quinols under aerobic assay conditions, we did not study the oxidation of rhodoquinols (2- or 3-amino-Q_2H_2). In contrast to Q_1H_2, a shorter isoprenyl tail analog, all the Q_2H_2 analogs showed substrate inhibition at higher concentrations. Partial relief of the substrate inhibition for 3-azido- and 3-ethoxy-Q_2H_2 (Fig. 2, D and F) indicates that in addition to the 6-diprenyl group the 3-methoxy group is partly involved in substrate inhibition. Because of its poor solubility in buffer, we are unable to carry out kinetic analysis with the native substrate, Q_8H_2. In a similar concentration range for Q_1H_2 and Q_2H_2 analogs, *bo*-type heme-copper quinol oxidase, the alternative *E. coli* terminal oxidase under highly aerated growth conditions, never exhibits such sigmoidality or substrate inhibition (*39*, *40*). Thus, such a peculiar kinetics is a unique feature of *bd*-type quinol oxidase.

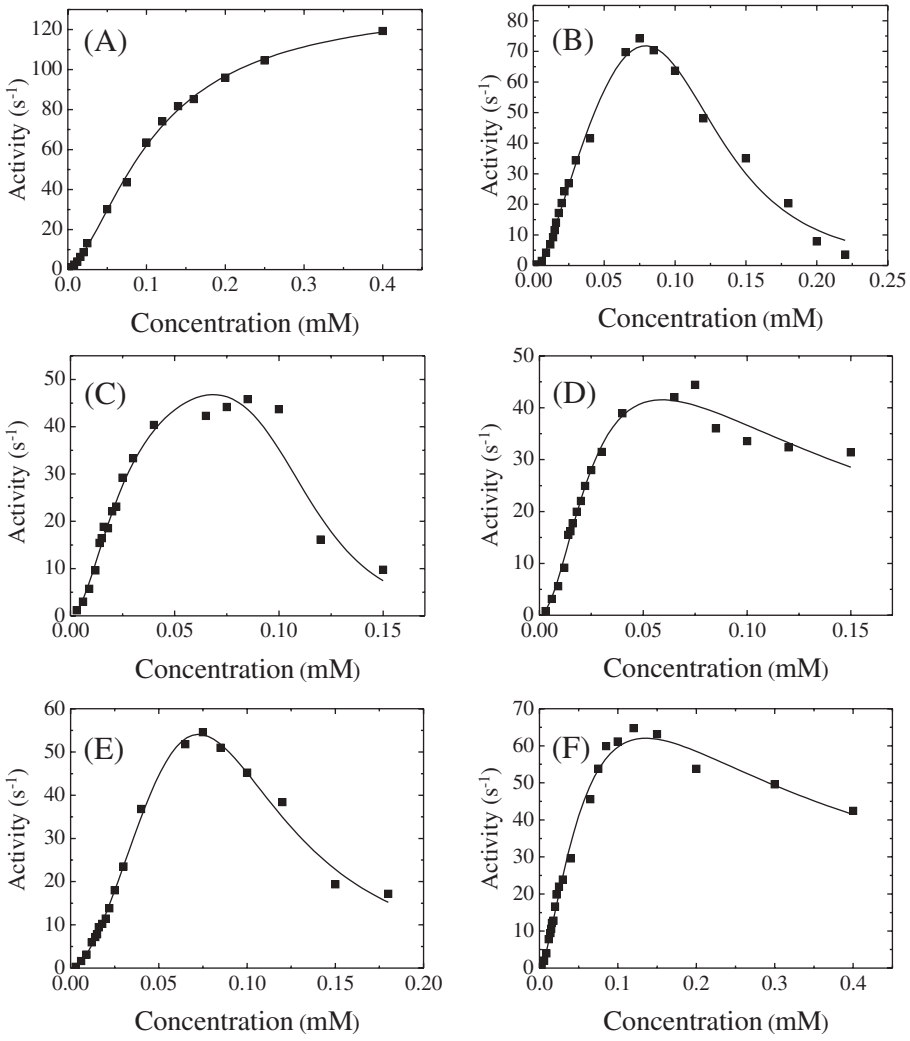


Fig. 2. **Concentration dependence of the oxidation of Q_2H_2 analogs by cytochrome *bd*.** The oxidation of ubiquinols with the indicated concentrations of Q_1H_2 (A), Q_2H_2 (B), 2-azido-Q_2H_2 (C), 3-azido-Q_2H_2 (D), 2-ethoxy-Q_2H_2 (E), and 3-ethoxy-Q_2H_2 (F) was measured as described under "MATERIALS AND METHODS."

On dioxygen reduction by cytochrome *bd*, the one-electron–reduced oxygenated form and oxoferryl (–1) intermediate are unusually stable (*22–25*), so the oxidation of two quinol molecules would be tightly coupled to the four-electron reduction of dioxygen (Fig. 3A). As a simple and plausible scheme to express the observed positive cooperativity with one substrate-binding site, we propose Scheme 1. In Scheme 1, E_1 and E_2 denote the one-electron reduced, oxygenated form [(a) in Fig. 3], and oxoferryl-1 intermediate [(c) in Fig. 3], respectively. Although seemingly complicated, Scheme 1 is a modified *ping-pong bi-bi* mechanism into which inactive conformers termed "F (*45*)" and "FS" have been introduced. F is likely a one-electron reduced, oxygenated form without labile Met ligation for heme b_{558} (*11*). Without the conformers F and FS, the simple *ping-pong bi-bi* mechanism cannot exhibit positive cooperativity.

Assuming a rapid equilibrium, the steady state reaction rate was calculated based on Scheme 1 using Mathematica. The rate equation is a complicated function of rate constants (k_1 to k_{12}) and the total enzyme concentration (E_t), and their physical meanings are difficult to grasp. For clarity, we arrange the rate equation as Eq. 1. Parameters K_{m1}, K_{m2}, V_{max1}, and V_{max2} are assigned based on their dimensions (*46*, *47*), and are not defined for the specific kinetic step in Scheme 1.

$$v = \frac{V_{max1}K_{m2}[S] + V_{max2}[S]^2}{K_{m1}K_{m2} + K_{m2}[S] + [S]^2} \quad (1)$$

When [S] >> 1 mM, Eq.1 can be approximated as $v \approx V_{max2}$ [S]/(K_{m2} + [S]). On the other hand, when $[S]^2$ << [S] with a low substrate concentration ([S] << 1 mM), Eq.1 can be approximated as $v \approx V_{max1}$ [S]/(K_{m1} + [S]). Roughly speaking, V_{max2} is the maximum velocity of the quinol oxidation. The larger one of K_{m1} or K_{m2} approximately corresponds to the substrate concentration, which gives the half maximum velocity when there is no substrate inhibition (*e.g.*, with Q_1H_2) or inhibitors.

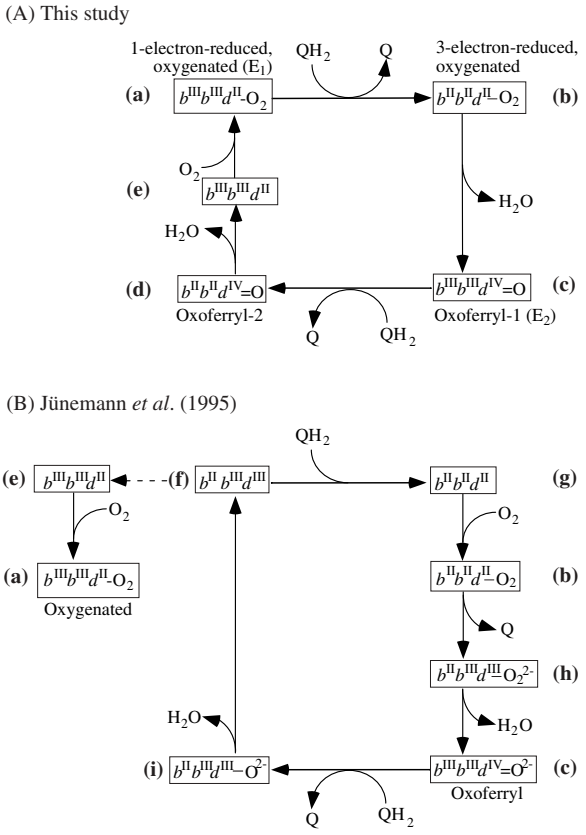
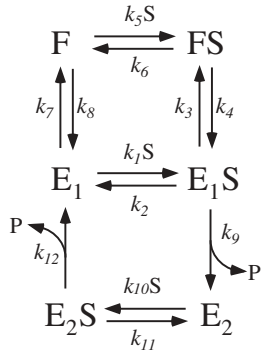
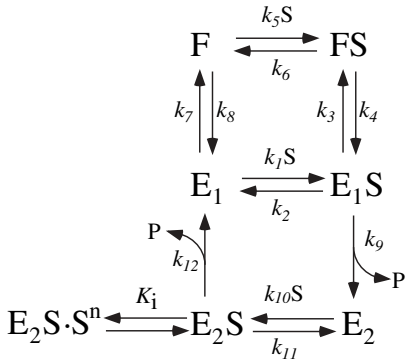


Fig. 3. **Kinetic model for the dioxygen reduction by cytochrome *bd*.** (A) This study, and (B) Fig. 6 of Jünemann *et al*. (32).



Scheme 1

In the cases of all Q_2H_2 analogs examined in this study, we observed uncompetitive inhibition by substrates at higher concentrations. Although we assume a single catalytic site in cytochrome *bd*, substrate inhibition seems to require the formation of a ternary complex, $(E_2S)S^n$, on the oxidation of the second ubiquinol molecule (Scheme 2). The absence of substrate inhibition with Q_1H_2 indicates that the binding site for substrate inhibition should recognize the diprenyl unit of the Q_2H_2 analogs, and would comprise part of the substrate-binding pocket for the quinol oxidation site, allowing the cooperative interactions. The rate equation can be expressed as Eq. 2, where



Scheme 2

the apparent inhibition constant $K_i'^n = k_{12}K_i^n/V_{max2}$, and "$n$" indicates the Hill coefficient.

$$v = \frac{V_{max1}K_{m2}[S] + V_{max2}[S]^2}{K_{m1}K_{m2} + K_{m2}[S] + [S]^2 + \left(\left(\frac{V_{max1}K_{m2}}{V_{max2}}\right)[S] + [S]^2\right)\left(\frac{[S]}{K_i'}\right)^n} \quad (2)$$

If we assume inhibitory-binding of n·S to enzyme species E_1S in addition to E_2S in Scheme 2, very similar expression is obtained (not shown, see Eq. 3). For fitting with actual data sets, all the parameters were assumed to be positive, and statistical weighting was applied when necessary for stable convergence. The results of fitting calculation for V_{max1} converged to zero or a very small number. Therefore, we assume $V_{max1} = 0$, and Eq. 2 can be modified as Eq. 2′,

$$v = \frac{V_{max2}[S]^2}{K_{m1}K_m^2 + K_{m2}[S] + \left(1 + \left(\frac{[S]}{K_i'}\right)^n\right)[S]^2} \quad (2')$$

The kinetic parameters for the ubiquinol oxidation were recalculated with Eq. 2′ using Origin (solid lines in Fig. 2 and Table 1). The apparent K_{m1} and K_{m2} values for Q_1H_2 (0.073 and 0.077 mM, respectively) and Q_2H_2 (0.047 and 0.042 mM, respectively) are comparable to 0.04–0.28 mM (*5*, *26*, *31–36*) and 0.05 mM (*26*), respectively, which have been obtained by assuming simple Michaelis-type kinetics. The value n (Hill coefficient) for the substrate inhibition showed variation with different substrates. Although the meaning of n is not clear, the values faithfully reflect the observed inhibition curves in Fig. 2. The K_{m1} and K_{m2} values for native type substrates (Q_1H_2 and Q_2H_2) are very similar, thus Eq. 2′ for the oxidation of Q_1H_2 could be further simplified to Eq. 2″.

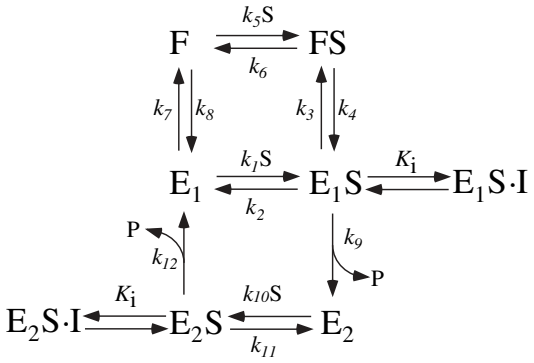
$$v = \frac{V_{max}[S]^2}{K_m^2 + K_m[S] + [S]^2} \quad (2'')$$

For reactions of the wild type cytochrome *bd* and eleven Q-loop mutants with Q_1H_2, we obtained reasonable fitting for kinetic data with Eq. 2″ (*i.e.*, R values of around 0.997), and identified key residues involved in the binding and oxidation of quinol molecules (T. Mogi *et al*., unpublished results).

As found for cytochrome *bo* (*39*), increasing length of the 6-isoprenyl group on the quinone ring appears to increase the binding affinity to cytochrome *bd*. The constants for substrate inhibition are estimated to be around 0.1 mM,

Table 1. **Kinetic parameters for the oxidation of Q_2H_2 analogs by cytochrome *bd*.**

Substrate	K_{m1} (mM)	K_{m2} (mM)	V_{max2} (s^{-1})	K_i' (mM)	*n*
Q_1H_2	0.073	0.077	147	–	–
Q_2H_2	0.047	0.042	85	0.125	4.0
2-Ethoxy-Q_2H_2	0.044	0.223	464	0.050	2.6
3-Ethoxy-Q_2H_2	0.016	0.145	219	0.099	0.99
2-Azido-Q_2H_2	0.073	0.007	60	0.108	6.2
3-Azido-Q_2H_2	0.084	0.008	69	0.135	1.5



Scheme 3

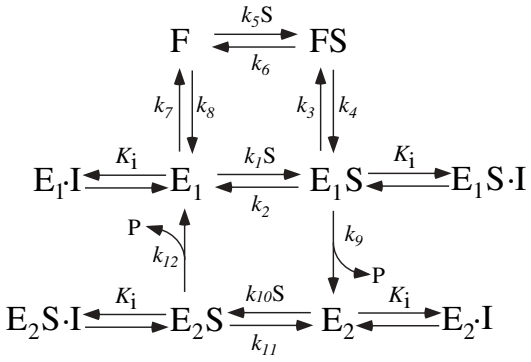
this being consistent with our assumption that binding of the 6-diprenyl group causes substrate inhibition. Also, substitutions of the 3-methoxy group decrease the Hill coefficient and may weaken protein-substrate interactions.

Comparison of the kinetic parameters suggested the presence of a linear correlation between the K_{m2} and V_{max2} values (data not shown) for Q_1H_2, Q_2H_2 and ethoxy derivatives of Q_2H_2. Lowering of the affinity for substrates increases the V_{max2} value. Similar to cytochrome *bo* (*39*), the 2-methoxy group appears to contribute more greatly to protein–quinone interactions at the quinol oxidation site than the 3-methoxy group. In contrast, substitution of both the 2- and 3-methoxy group with an azido group resulted in small changes in the K_{m1} and V_{max2} values, and a large decrease in the K_{m2} value, suggesting that the binding pockets for the 2- and 3-substituent groups may be spacious enough to accommodate a rod-like azido group. This finding proved that both azido derivatives are efficient substrates for the enzyme, and these azidoquinols were used for the photo-affinity labeling study for the identification of the quinol oxidation site in cytochrome *bd* (*48*).

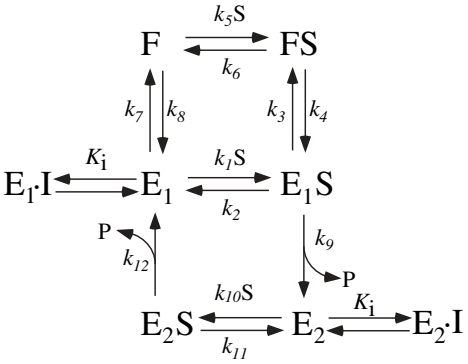
Inhibition of the Q_1H_2 Oxidation by the Oxidized Forms of Q_2 Analogs—When an uncompetitive inhibitor (I) binds to enzyme species E_1S and E_2S with a dissociation constant, K_i (Scheme 3), the rate equation can be expressed as Eq. 3, where $K_i' = K_i/(1/k_9 + 1/k_{12})V_{max2}$ and $V_{max1} = 0$.

$$v = \frac{V_{max2}[S]^2}{K_{m1}K_{m2} + K_{m2}[S] + \left(1 + \left(\frac{[I]}{K_i'}\right)\right)[S]^2} \quad (3)$$

When a noncompetitive inhibitor (I) binds to enzyme species E_1, E_1S, E_2, and E_2S with a dissociation constant, K_i (Scheme 4), the rate equation can be expressed as Eq. 4, where $K_i' = (1 - k_3\,k_{12}/(k_9 + k_{12})(k_4 + 2k_6))\,K_i$ and $V_{max1} = 0$.



Scheme 4



Scheme 5

For simplicity, E_1 and $E_1{\cdot}I$, E_1S and $E_1S{\cdot}I$, E_2 and $E_2{\cdot}I$, E_2S and $E_2S{\cdot}I$ are assumed to be in rapid equilibrium, and interconversion between $E_1{\cdot}I$ and $E_1S{\cdot}I$, and between $E_2{\cdot}I$ and $E_2S{\cdot}I$ on binding or dissociation of S was ignored.

$$v = \frac{V_{max2}[S]^2}{K_{m1}K_{m2} + K_{m2}[S] + [S]^2 + (\alpha' + \beta'[S] + [S]^2)\left(\frac{[I]}{K_i'}\right)} \quad (4)$$

Here, α' and β' can be expressed by the rate constants ($k_1 \sim k_{12}$).

When a competitive inhibitor (I) binds to enzyme species E_1 and E_2 with a dissociation constant, K_i (Scheme 5), the rate equation can be expressed as Eq. 5, where $K_i' = K_i/\{(k_1k_9(k_{11} + k_{12}) + k_{10}k_{12}(k_2 + k_9))(k_4 + 2k_6) + 2k_3k_{10}k_{12}(k_4 + k_6)\}/\{k_1(-k_3k_{12} + (k_9 + k_{12})(k_4 + 2k_6))\}$ and $V_{max1} = 0$.

$$v = \frac{V_{max2}[S]^2}{K_{m1}K_{m2} + K_{m2}[S] + [S]^2 + (\delta' + [S])\left(\frac{[I]}{K_i'}\right)} \quad (5)$$

Here, δ' can be expressed by the rate constants ($k_1 \sim k_{12}$).

Competitive inhibition (Eq. 5) can be discerned from uncompetitive (Eq. 3) or noncompetitive (Eq. 4) inhibition, because competitive inhibition does not alter the maximum velocity, and a $1/v$ *vs.* 1/[S] plot with or without a competitive inhibitor intersects on the vertical axes. Discrimination between uncompetitive and noncompetitive inhibition is difficult at a glance. But in the case of Eq. 3, we can see that the reaction velocity with or without an uncompetitive inhibitor coincides where [S] is zero. This means that the

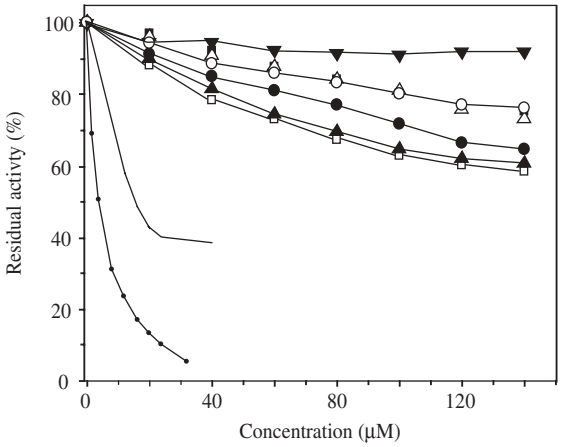


Fig. 4. **Concentration dependence of the oxidation of Q_1H_2 by cytochrome *bd* in the presence of inhibitors and the oxidized forms of Q_2 analogs.** The ubiquinol oxidase activity was measured with 0.17 mM Q_1H_2 in the presence of HQNO (solid circles), antimycin A (*), Q_2 (inverted solid triangles), 2-azido-Q_2 (solid squares), 3-azido-Q_2 (open squares), 2-ethoxy-Q_2 (solid circles), 3-ethoxy-Q_2 (open circles), 2-amino-Q_2 (solid triangles), and 3-amino-Q_2 (open triangles).

velocity is also zero, and the 1/*v vs*. 1/[S] plot with or without an uncompetitive inhibitor virtually does not intersect in a finite range. On the other hand, in the case of Eq. 4, a 1/*v vs*. 1/[S] plot with or without a noncompetitive inhibitor intersects where $(\alpha' + \beta'[S] + [S]^2)$ is zero. It may not always true that $\alpha' + \beta'\ [S] + [S]^2 = 0$ has a solution of real number, but it may be said that when a plot with or without an inhibitor intersects on a 1/*v vs.* 1/[S] plot and the maximum velocity is attenuated by the inhibitor, Eq. 4 should be selected rather than Eq. 3.

We examined the effects of the oxidized forms of Q_2 analogs on the oxidation of Q_1H_2 at 0.17 mM and found that they are weak inhibitors (Fig. 4). Among them, the inhibition by Q_2 was negligibly low and no further analysis was performed. In contrast to HQNO, the inhibition by antimycin A and the Q_2 analogs appears to reach some level of equilibration. 1/*v vs*. 1/[S] double reciprocal plot analysis in the presence of HQNO showed an intercept on the y-axis, indicating that HQNO serves as a competitive inhibitor with a K_i value of 3 μM (Fig. 5A). On 1/*v vs*. 1/[S] plot analysis in the presence of various concentrations of inhibitors, antimycin A and all Q_2 analogs used in this study were found to be noncompetitive inhibitors (Fig. 5, B and C).

The inhibition constants for antimycin A and the Q_2 analogs were estimated by means of 1/*v vs.* [I] plots (Fig. 6), but are relative values because K_i' is expressed as $K_i/\{(k_9 + k_{12})(k_4 + 2k_6)\}/\{-k_3\ k_{12} + (k_9 + k_{12})(k_4 + 2k_6)\}$. Since it is practically impossible to determine each of the rate constants, we are unable to determine the K_i value. The K_i' values for the oxidized forms are in the order of antimycin A < 2-amino-Q_2 < 3-azido-Q_2 < 2-ethoxy-Q_2 < 3-ethoxy-Q_2, < 2-azido-Q_2, 3-amino-Q_2 (cTable 2). In addition to the size, the orientation of the substituting groups relative to the quinone plane and the effect on the redox potential (*e.g.*, –150 mV decrease for 2- and 3-amino-Q_2) would affect the inhibitory potency of the Q_2 analogs. The results for the ethoxy-Q_2 analogs suggest that in contrast to

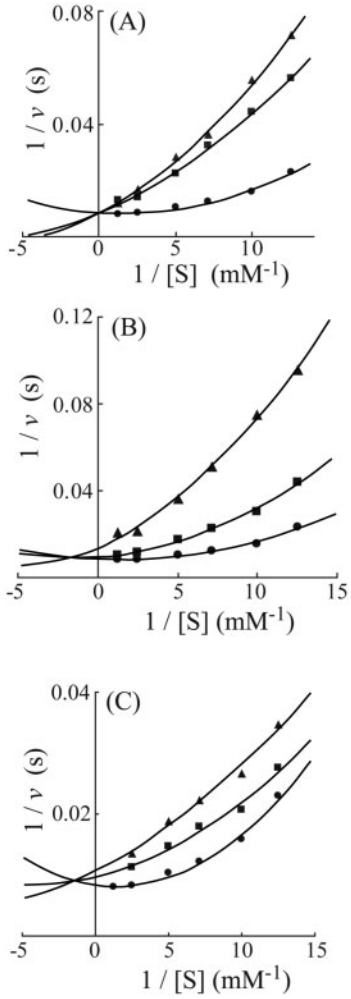


Fig. 5. **Double-reciprocal plot analysis of the inhibition of Q_1H_2 oxidation by Q_2 analogs.** Q_1H_2 oxidase activity was measured in the presence of HQNO (A) at the final concentration at 0 (solid circles), 8 (solid squares), and 16 μM (solid triangles); antimycin A (B) at 0 (solid circles), 4 (solid squares), and 16 μM (solid triangles); and 2-amino-Q_2 (C) at 0 (solid circles), 10 (solid squares), and 40 μM (solid triangles).

cytochrome *bo* the binding pocket more strictly recognizes a methoxy group at the 3-position than the 2-position.

DISCUSSION

Enzyme Kinetics of Cytochrome bd—The oxidation of quinols by cytochrome *bd* from *E. coli*, *Azotobacter vinelandii*, *Klebsiella pneumoniae* and *Photobacterium phosphoreum* has been interpreted as being of the simple Michaelis-Menten type for Q_1H_2 and Q_2H_2 (*5*, *26*, *31–36*). Electron paramagnetic resonance studies on the semiquinone anion (*37*), inhibitor-binding studies (*38*), and photoaffinity labeling studies with azidoquinols (*48*) are consistent with the presence of a single quinol oxidation site in cytochrome *bd*. Unlike cytochrome *bo* (*49*, *50*), the purified cytochrome *bd* does not contain a tightly bound ubiquinone-8. Sequence analysis showed the presence of only 20 conserved residues in subunit I and 2 in subunit II (*51*), which are not enough

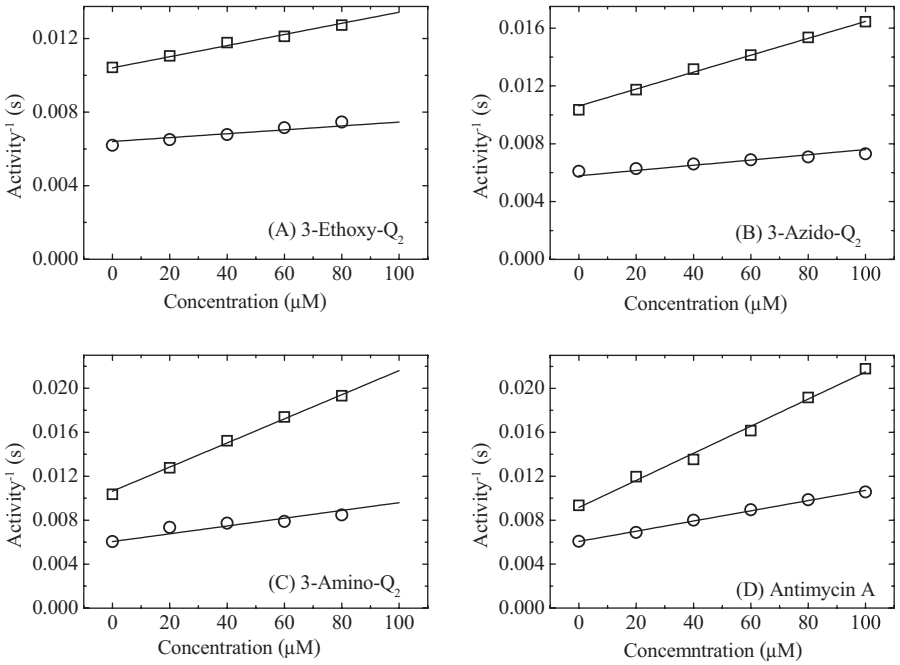


Fig. 6. **1/*v vs.* inhibitor concentration plots for 3-ethoxy- (A), 3-azido- (B), 3-amino-Q_2 (C), and antimycin A (D) in the presence of 0.14 (squares) and 0.28 (circles) mM Q_1H_2.**

Table 2. **Inhibition constants for the Q_1H_2 oxidation by Q_2 analogs.**

Inhibitor	K_i'* (mM*)	Inhibition mode
Antimycin A	46	Non-competitive
2-Azido-Q_2	244	Non-competitive
2-Ethoxy-Q_2	159	Non-competitive
2-Amino-Q_2	60	Non-competitive
3-Azido-Q_2	113	Non-competitive
3-Ethoxy-Q_2	214	Non-competitive
3-Amino-Q_2	245	Non-competitive

*$K_i' = (1 - k_3\, k_{12}/(k_9 + k_{12})(k_4 + 2k_6))\, K_i$.

to form the multiple quinol/quinone binding sites. However, careful analysis of the electron-donating activity of ubiquinols revealed the sigmoidal concentration dependence and remarkable substrate inhibition with the Q_2H_2 analogs and decylbenzoquinols (*39*). Such sigmoidal behavior has been reported for the oxidation of dimethylnaphthoquinol and menaquinol-3 by cytochrome *bd* isolated from a Gram-positive bacterium, *Corynebacterium glutamicum* (*52*). Substrate inhibition by quinol/quinone is also known for the cytochrome bc_1 complex with 2,3-dimethoxy-6-decyl-1,4-benzoquinol, and for succinate dehydrogenase with mono- and diethoxy analogs of decylbenzoquinone (*53*, *54*). Although the catalytic site is assumed to be one in these respiratory complexes, the kinetic mechanism cannot be of the simple Michaelis-Menten-type.

We examined the kinetic properties of the oxidation of Q_2H_2 analogs by cytochrome *bd* with a wide range of substrate concentrations, and found that the substrate oxidation by cytochrome *bd* can be explained by a modified *ping-pong bi-bi* mechanism (Scheme 2). To control aerobic respiration under oxygen-limiting microaerophilic growth conditions, the oxidation of quinols should be tightly coupled to dioxygen reduction. In our kinetic model, the presence of the inactive form (F) and the inactive binary complex (FS) can account for the sigmoidality of the oxidation of Q_1H_2 and the Q_2H_2 analogs. Since cytochrome *bd* is a monomeric oxidase complex (CydAB) with a single quinol oxidation site (*11*, *37*, *38*, *48*), the sigmoidality cannot be ascribed to the positive cooperativity on multiple substrate binding to oligomeric enzymes. In general Met ligation is weaker than His ligation, so the inactive conformer F could be a one-electron reduced, oxygenated form without labile Met ligation for heme b_{558}. Potentiometric analysis of the *Azotobacter* cytochrome *bd* has revealed two E_m values for heme b_{558} (60 and 125 mV), consistent with the presence of two populations of heme b_{558} (*11*), the primary electron acceptor for quinols.

In the *E. coli* membrane, the 6-polyprenyl tail on the quinone ring serves as an anchor to the lipid bilayer. Ubiquinol-8 (or menaquinol-8) molecules are allowed to diffuse laterally within the membrane, and electrons are transferred from the C_1-OH and/or C_4-OH group(s) on the quinone ring to the quinol oxidation site, which is located at the periplasmic interface of terminal oxidases. Such an orientation of quinol molecules in the membrane controls interactions with the quinol oxidation site. With the purified enzyme within detergent micelles, we found substrate inhibition by ubiquinol analogs with a 6-diprenyl or 6-decyl group (*39*). An excess amount of Q_1H_2 was unable to suppress the substrate inhibition by Q_2H_2, therefore, the inhibition mode cannot be competitive. The random access of quinol molecules to the quinol oxidation site cannot be prevented in the purified enzyme, and multiple binding of the longer 6-diprenyl group of excess Q_2H_2 to a large hydrophobic pocket near or within the quinol oxidation site of

the oxoferryl-1 intermediate would result in the formation of the inactive ternary complex $(E_2S)S^n$ (Scheme 2 and Fig. 3). Such partially overlapping binding of quinone analogs to the binding site has been reported for the Q_B site of photosynthetic reaction center (*55*, *56*) and the quinone reduction site of the proton-translocating NADH dehydrogenase complex (*57*). Estimation of the number of bound quinols involved in substrate inhibition is practically not possible. Because of the hydrophobic nature of substrates and *bd*-type oxidase, experiments involving detergent micelles make it difficult to discriminate substrates bound to the enzyme from the majority of substrates partitioned into detergent micelles. Furthermore, upon mixing with the enzyme, quinols will be fully oxidized within a few minutes, and thus cannot be kept as reduced forms during extraction procedures.

The meaning of the Hill coefficient is currently unknown. The Hill coefficient may represent the strength of interaction between the polyprenyl tail and the binding site. It is also possible that substrates could bind to a large hydrophobic pocket in the vicinity of the quinol oxidation site. The ability to oxidize both ubiquinol and naphthoquinols (*i.e.*, menaquinol), and the relatively high K_m (~0.08 mM) value for Q_2H_2 (Table 1) are consistent with the presence of a large substrate-binding pocket in cytochrome *bd*.

Cytochrome *bd* is widely distributed from Archaea to Eubacteria as one of the terminal oxidases, and can be divided into three groups with different substrate specificities: ubiquinols (α-, β-, and γ-proteobacteria), plastoquinols (cyanobacteria), or menaquinols (other bacteria). Such plasticity of the low-affinity quinol oxidation site in cytochrome *bd* may be one of the possible causes of substrate inhibition. In addition, our results suggest that the recognition of the 2- and 3-methoxy groups on the quinone ring by the quinol oxidation site may differ depending on the redox states of the substrates (Tables 1 and 2).

Dioxygen Reduction Mechanism of Cytochrome bd—In the heme-copper terminal oxidases, aa_3-type cytochrome *c* oxidase binds heme *a*, and the heme a_3-Cu_B binuclear center in subunit I and Cu_A in subunit II, whereas *E. coli* cytochrome *bo*-type ubiquinol oxidase binds heme *b*, the heme *o*-Cu_B binuclear center, and a tightly bound Q_8 at the Q_H site in subunit I (*49*, *50*). Thus, electrons present in the fully reduced enzymes can complete the four-electron reduction of dioxygen. A conserved tyrosine, that is crosslinked to one of the Cu_B ligand histidines, is present in the vicinity of the dioxygen reduction site, and facilitates the O-O bond scission as an acid-base catalyst through hydrogen transfer (*58*, *59*). Cytochrome *bd* does not belong to the heme-copper terminal oxidase superfamily, and binds only three redox metal centers, heme b_{558}, b_{595} and *d*, in subunit I. The lack of the fourth redox center and/or the crosslinked His-Tyr couple in the dioxygen reduction site suggest(s) that the dioxygen reduction mechanism of cytochrome *bd* is different from that of the heme-copper terminal oxidases. Cytochrome *bd* exhibits extremely high affinity for dioxygen (*19–21*), and heme *d* mainly exists in the oxygenated (d^{II}-O_2) and oxoferryl (d^{IV}=O) forms in the air-oxidized state (*22–25*). Here we propose a dioxygen reduction mechanism for cytochrome *bd* (Fig. 3A), which includes the one-electron-reduced oxygenated form [b_{558}^{III}, b_{595}^{III}, d^{II}-O_2; (a) (E_1 in reaction Schemes)] and the oxidized oxoferryl form [b_{558}^{III}, b_{595}^{III}, d^{IV}=O; oxoferryl-1 (c) (E_2 in reaction Schemes)] as two stable intermediates in the catalytic cycle. Upon two-electron reduction with the first quinol molecule, the fully reduced, oxygenated form (oxy intermediate) will be quickly transformed to the oxidized oxoferryl form (oxoferryl-1). Reduction with the second quinol yields the reduced oxoferryl form (oxoferryl-2), which decays to the one-electron reduced form [b_{558}^{III}, b_{595}^{III}, d^{II} (e)] through the hydroxy intermediate [b_{558}^{III}, b_{595}^{III}, d^{III}-OH (not shown in Fig. 3)]. The one-electron reduced form is immediately transformed to the one-electron oxygenated form [b_{558}^{III}, b_{595}^{III}, d^{II}-O_2 (a)], because cytochrome *bd* is designed as a thermodynamic trap for dioxygen and the rate of dioxygen binding to ferrous heme *d* is extremely fast (2×10^9 M^{-1} s^{-1}) (*11*). In the kinetic model of Jünemann *et al*. (*32*) (Fig. 3B), they place intermediates (a) and (e) in the non-catalytic branch, and the reaction starts from the one-electron reduced form [b_{558}^{II}, b_{595}^{III}, d^{III} (f)]. Since the binding of quinols is a diffusion-controlled slower process, intermediate (f) should be converted to intermediate (e) through intramolecular electron transfer within 10 μs (*14*) before it accepts electrons from quinols. Our kinetic model revealed the roles of stable intermediates (a) and (c) of the as-prepared enzyme in the catalytic mechanism. We hope that our findings and kinetic model will facilitate our understanding of the simple energy transduction mechanism of cytochrome *bd*-type quinol oxidase.

This work was supported in part by the 21st Century COE (Center of Elegance) Program (Y. M.), JST (M. Y. and T. M.), and a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (#15380083 to H. M.). We thank Dr. N. Sone (JST, ERATO) for the valuable comments, Dr. R. B. Gennis (Univ. Illinois) for the *E. coli* strain GR84N/pNG2, and Eisai Co. (Tokyo, Japan) for Q_1.

REFERENCES

1. Ingledew, W.J. and Poole, R.K. (1984) The respiratory chain of *Escherichia coli*. *Microbiol. Rev*. **48**, 222–271
2. Jünemann, S. (1997) Cytochrome *bd* terminal oxidase. *Biochim. Biophys. Acta* **1321**, 107–127
3. Mogi, T., Tsubaki, M., Hori, H., Miyoshi, H., Nakamura, H., and Anraku, Y. (1998) Two terminal quinol oxidase families in *Escherichia coli*: Variations on molecular machinery for dioxygen reduction. *J. Biochem. Mol. Biol. Biophys.* **2**, 79–110
4. Zhang, J., Barquera, B., and Gennis, R.B. (2004) Gene fusions with β-lactamase show that subunit I of the cytochrome *bd* quinol oxidase from *E. coli* has nine transmembrane helices with the O_2 reactive site near the periplasmic surface. *FEBS Lett.* **561**, 58–62
5. Kita, K., Konishi, K., and Anraku, Y. (1984) Terminal oxidases of *Escherichia coli* aerobic respiratory chain. II. Purification and properties of cytochrome b_{558}-*d* complex from cells grown with limited oxygen and evidence of branched electron-carrying systems. *J. Biol. Chem*. **259**, 3375–3381
6. Miller, M.J. and Gennis, R.B. (1985) The cytochrome *d* complex is a coupling site in the aerobic respiratory chain of *Escherichia coli*. *J. Biol. Chem*. **260**, 14003–14008
7. Jasaitis, A., Borisov, V.B., Belevich, N.P., Morgan, J.E., Konstantinov, A.A., and Verkohsky, M.I. (2000) Electrogenic reactions of cytochrome *bd*. *Biochemistry* **39**, 13800–13809
8. Tsubaki, M., Hori, H., and Mogi, T. (2000) Probing molecular structure of dioxygen reduction site of bacterial quinol oxidases

through ligand binding to the redox metal centers. *J. Inorg. Biochem*. **82**, 19–25
9. Fang, G.H., Lin, R.J., and Gennis, R.B. (1989) Location of heme axial ligands in the cytochrome *d* terminal oxidase complex of *Escherichia coli* determined by site-directed mutagenesis. *J. Biol. Chem*. **264**, 8026–8032
10. Jünemann, S. and Wrigglesworth, J.M. (1994) Antimycin inhibition of the cytochrome *bd* complex from *Azotobacter vinelandii* indicates the presence of a branched electron transfer pathway for the oxidation of ubiquinol. *FEBS Lett.* **345**, 198–202
11. Jünemann, S., Wrigglesworth, J.M., and Rich, P.R. (1997) Effects of decyl-aurachin D and reversed electron transfer in cytochrome *bd*. *Biochemistry* **36**, 9323–9331
12. Poole, R.K. and Williams, H.D. (1987) Proposal that the function of the membrane-bound cytochrome a_1-like haemoprotein (cytochrome *b*-595) in *Escherichia coli* is a direct electron donation to cytochrome *d*. *FEBS Lett.* **217**, 49–52
13. Hill, B.C., Hill, J.J., and Gennis, R.B. (1994) The room temperature reaction of carbon monoxide and oxygen with the cytochrome *bd* quinol oxidase from *Escherichia coli*. *Biochemistry* **33**, 15110–15115
14. Kobayashi, K., Tagawa, S., and Mogi, T. (1999) Pulse radiolysis studies on electron transfer processes in cytochrome *bd*-type ubiquinol oxidase from *Escherichia coli*. *Biochemistry* **38**, 5913–5917
15. Zhang, J., Hellwig, P., Osborne, J.P., Huang, H., Moenne-Loccoz, P., Konstantinov, A.A., and Gennis, R.B. (2001) Site-directed mutation of the highly conserved region near the Q-loop of the cytochrome *bd* quinol oxidase from *Escherichia coli* specifically perturbs heme b_{595}. *Biochemistry* **40**, 8548–8556
16. Hirota, S., Mogi, T., Ogura, T., Anraku, Y., Gennis, R. B., and Kitagawa, T. (1995) Resonance Raman study on axial ligands of heme irons in cytochrome *bd*-type ubiquinol oxidase from *Escherichia coli*. *Biospectroscopy* **1**, 305–311
17. Sun, J., Kahlow, M.A., Kaysser, T.M., Osborne, J., Hill, J.J., Rohlfs, R.J., Hille, R., Gennis, R.B., and Loehr, T.M. (1996) Resonance Raman spectroscopic identification of a histidine ligand of b_{595} and the nature of the ligation of chlorin *d* in the fully reduced *Escherichia coli* cytochrome *bd* oxidase. *Biochemistry* **35**, 2403–2412
18. Hori, H., Tsubaki, M., Mogi, T., and Anraku, Y. (1996) EPR study of NO complex of *bd*-type ubiquinol oxidase from *Escherichia coli*. The proximal ligand of heme *d* is a nitrogenous amino acid residue. *J. Biol. Chem.* **271**, 9254–9258
19. Hill, J.J., Alben, J.O., and Gennis, R.B. (1993) Spectroscopic evidence for a heme-heme binuclear center in the cytochrome *bd* ubiquinol oxidase from *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **90**, 5863–5867
20. D'Mello, R., Hill, S., and Poole, R.K. (1996) The cytochrome *bd* quinol oxidase in *Escherichia coli* has an extremely high oxygen affinity and two oxygen-binding haems: implications for regulation of activity *in vivo* by oxygen inhibition. *Microbiology* **142**, 755–763
21. Rice, C.W. and Hempling, W.P. (1978) Oxygen-limited continuous culture and respiratory energy-conservation in *Escherichia coli*. *J. Bacteriol.* **134**, 115–124
22. Poole, R.K., Salmon, I., and Chance, B. (1983) Formation of the 680 nm-absorbing form of the cytochrome *bd* oxidase complex of *Escherichia coli* by reaction of hydrogen peroxide with the ferric form. *FEBS Lett.* **231**, 243–246
23. Hata, A., Kirino, Y., Matsuura, K., Itoh, S., Hiyama, T., Konishi, K., Kita, K., and Anraku, Y. (1985) Assignment of ESR signals of *Escherichia coli* terminal oxidase complexes. *Biochim. Biophys. Acta* **810**, 62–72
24. Kahlow, M.A., Loehr, T.M., Zuberi, T.M., and Gennis, R.B. (1993) The oxygenated complex of cytochrome *d* terminal oxidase: direct evidence for iron-oxygen coordination in a chlorin-containing enzyme by resonance Raman spectroscopy. *J. Am. Chem. Soc.* **115**, 5845–5846
25. Lorence, R.M. and Gennis, R. B. (1989) Spectroscopic and quantitative analysis of the oxygenated and peroxy states of the purified cytochrome *d* complex of *Escherichia coli*. *J. Biol. Chem.* **264**, 7135–7140
26. Yang, F.D., Yu, L., Yu, C.A., Lorence, R.M., and Gennis, R.B. (1986) Use of an azido-ubiquinone derivative to identify subunit I as the ubiquinol binding site of the cytochrome *d* terminal oxidase complex of *Escherichia coli*. *J. Biol. Chem*. **261**, 14987–14990
27. Kranz, R.G. and Gennis, R.B. (1984) Characterization of the cytochrome *d* terminal oxidase complex of *Escherichia coli* using polyclonal and monoclonal antibodies. *J. Biol. Chem*. **259**, 7998–8003
28. Dueweke, T.J. and Gennis, R.B. (1990) Epitopes of monoclonal antibodies which inhibit ubiquinol oxidase activity of *Escherichia coli* cytochrome *d* complex localize functional domain. *J. Biol. Chem*. **265**, 4273–4277
29. Lorence, R.M., Carter, K., Gennis, R.B., Matsushita, K., and Kaback, H.R. (1988) Trypsin proteolysis of the cytochrome *d* complex of *Escherichia coli* selectively inhibits ubiquinol oxidase activity while not affecting *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine oxidase activity. *J. Biol. Chem*. **263**, 5271–5276
30. Dueweke, T.J. and Gennis, R.B. (1991) Proteolysis of the cytochrome *d* complex with trypsin and chymotrypsin localizes a quinol oxidase domain. *Biochemistry* **30**, 3401–3406
31. Miller, M.J. and Gennis, R.B. (1983) The purification and characterization of the cytochrome *d* terminal oxidase complex of the *Escherichia coli* aerobic respiratory chain. *J. Biol. Chem*. **260**, 9159–9165
32. Jünemann, S., Butterworth, P.J., and Wrigglesworth, J.M. (1995) A suggested mechanism for the catalytic cycle of cytochrome *bd* terminal oxidase based on kinetic analysis. *Biochemistry* **34**, 14861–14867
33. Kolonay, J.F., Jr., Moshiri, F., Gennis, R.B., Kaysser, T.M., and Maier, R.J. (1994) Purification and characterization of the cytochrome *bd* complex from *Azotobacter vinelandii*-Comparison to the complex from *Escherichia coli*. *J. Bacteriol*. **176**, 4177–4181
34. Smith, A., Hill, S., and Anthony, C. (1990) The purification, characterization and role of the *d*-type cytochrome oxidase of *Klebsiella pneumoniae* during nitrogen fixation. *J. Gen. Microbiol*. **136**, 171–180
35. Konishi, K., Ouchi, M., Kita, K., Horikoshi, I. (1986) Purification and properties of a cytochrome b_{560}-*d* complex, a terminal oxidase of the aerobic respiratory chain of *Photobacterium phosphoreum*. *J. Biochem*. **99**, 1227–1236
36. Lorence, R.M., Miller, M.J., Borochov, A., Faiman-Weinberg, R., and Gennis, R.B. (1984) Effects of pH and detergent on the kinetic and electrochemical properties of the purified cytochrome *d* terminal oxidase complex of *Escherichia coli*. *Biochim. Biophys. Acta* **790**, 148–153
37. Hasting, S.F., Kaysser, T.M., Jiang, F., Salerno, J.C., Gennis, R.B., and Ingledew, W.J. (1998) Identification of a stable semiquinone intermediate in the purified and membrane bound ubiquinol oxidase cytochrome *bd* from *Escherichia coli*. *Eur. J. Biochem*. **255**, 317–323
38. Meunier, B., Madgwick, S.A., Reil, E., Oettemeier, W., and Rich, P.R. (1995) New inhibitors of the quinol oxidation sites of bacterial cytochromes *bo* and *bd*. *Biochemistry* **34**, 1076–1083
39. Sakamoto, K., Miyoshi, H., Takegami, K., Mogi, T., Anraku, Y., and Iwamura H. (1996) Probing substrate binding site of the *Escherichia coli* quinol oxidases using synthetic ubiquinol analogues based upon their electron-donating efficiency. *J. Biol. Chem.* **271**, 29897–29902
40. Sakamoto, K., Miyoshi, H., Ohshima, M., Kuwabara, K., Kano, K., Akagi, T., Mogi, T., and Iwamura, H. (1998) Role of isoprenyl tail of ubiquinone in reaction with respiratory enzymes: Studies with bovine heart mitochondrial complex I

and *Escherichia coli bo*-type ubiquinol oxidase. *Biochemistry* **37**, 15106–15113
41. Sakamoto, K., Nomura, K., and Miyoshi, H. (2002) Synthesis and electron-transfer activity of azido ubiquinone-2. *J. Pesticide Sci.* **27**, 147–149
42. Rieske, J.S. (1967) Preparation and properties of reduced coenzyme Q-cytochrome *c* reductase (Complex III of the respiratory chain). *Methods Enzymol.* **10**, 239–245
43. Green, G.N., Kranz, R.G., Lorence, R.M., and Gennis, R.B. (1984) Identification of subunit I as the cytochrome b_{558} component of the cytochrome *d* terminal oxidase complex of *Escherichia coli*. *J. Biol. Chem.* **259**, 7994–7997
44. Tsubaki, M., Hori, H., Mogi, T., and Anraku, Y. (1995) Cyanide-binding site of *bd*-type ubiquinol oxidase from *Escherichia coli*. *J. Biol. Chem.* **270**, 28565–28569
45. Dixon, M. and Webb, E.C. (1979) *Enzymes* (3 ed.), Longman Group Ltd.
46. Muneyuki, E., Odaka, M., and Yoshida, M. (1997) A single mutation at the catalytic site of TF_1-$\alpha_3\beta_3\gamma$ complex switches the kinetics of ATP hydrolysis from negative to positive cooperativity. *FEBS Lett.* **413**, 55–59
47. Yasuda, R., Noji, H., Kinosita, K., Jr., and Yoshida, M. (1998) F_1-ATPase is a highly efficient molecular motor that rotates with discrete 120° steps. *Cell* **93**, 1117–1124
48. Matsumoto, Y., Murai, M., Fujita, D., Sakamoto, K., Miyoshi, H., Yoshida, M., and Mogi, T. (2006) Mass spectrometric analysis of the ubiquinol-binding site in cytochrome *bd* from *Escherichia coli*. *J. Biol. Chem.* **281**, 1905–1912
49. Sato-Watanabe, M., Mogi, T., Ogura, T., Kitagawa, T., Miyoshi, H., Iwamura, H. and Anraku, Y. (1994) Identification of a novel quinone binding site in the cytochrome *bo* complex from *Escherichia coli*. *J. Biol. Chem.* **269**, 28908–28912
50. Sato-Watanabe, M., Mogi, T., Miyoshi, H. and Anraku, Y. (1998) Characterization and functional role of the Q_H site of *bo*-type quinol oxidase from *Escherichia coli*. *Biochemistry* **37**, 5356–5361
51. Osborne, J.P., and Gennis, R.B. (1999) Sequence analysis of cytochrome *bd* oxidase suggests a revised topology for subunit I. *Bichim. Biophys. Acta* **1410**, 32–50
52. Kusumoto, K., Sakiyama, M., Sakamoto, J., Noguchi S., and Sone, N. (2000) Menaquinol oxidase activity and primary structure of cytochrome *bd* from the amino-acid fermenting bacterium *Corynebacterium glutamicum*. *Arch. Microbiol.* **173**, 3990–397
53. He, D.-Y., Yu, L., and Yu, C.-A. (1994) Protein ubiquinone interaction. Synthesis and biological properties of 5-alkyl ubiquinone derivatives. *J. Biol. Chem.* **269**, 27885–27888
54. He, D.-Y., Gu, L.-Q., Yu, L., and Yu, C.-A. (1994) Protein-ubiquinone interaction: synthesis and biological properties of ethoxy ubiquinone derivatives. *Biochemistry* **33**, 880–884
55. Sinning, I. (1992) Herbicide binding in the bacterial photosynthetic reaction center. *Trends Biochem. Sci*. **17**, 150–154
56. Lancaster, C.R.T., and Michel, H. (1999) Refined crystal structures of reaction centers from *Rhodopseudomonas viridis* in complexes with the herbicide atrazine and two chiral atrazine derivatives also lead to a new model of the bound carotenoid. *J. Mol. Biol.* **286**, 883–898
57. Okun, J., Lümmen, P., and Brandt, U. (1999) Three classes of inhibitors share a common binding domain in mitochondrial complex I (NADH:ubiquinone oxidoreductase. *J. Biol. Chem.* **274**, 2625–2630
58. Proshlyakov, D.A., Pressler, M.A. and Babcock, G.T. (1998) Dioxygen activation and bond cleavage by mixed-valence cytochrome *c* oxidase. *Proc. Natl. Acad. Sci. USA* **95**, 8020–8025
59. Uchida, T., Mogi, T., and Kitagawa, T. (2000) Resonance Raman studies of oxo intermediates in the reaction of pulsed cytochrome *bo* with hydrogen peroxide. *Biochemistry* **39**, 6669–6678