Characterization and Functional Role of the Q_H Site of *bo*-Type Ubiquinol Oxidase from *Escherichia coli*†

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ABSTRACT: Cytochrome *bo* is a four-subunit terminal ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli* that vectorially translocates protons not only via directed protolytic reactions but also via proton pumping. Previously, we postulated that a bound quinone in the high-affinity quinone binding site (Q_H) mediates electron transfer from the low-affinity quinol oxidation site (Q_L) in subunit II to low-spin heme *b* in subunit I as an electron gate and a transient electron reservoir [Sato-Watanabe, M., Mogi, T., Ogura, T., Kitagawa, T., Miyoshi, H., Iwamura, H., and Anraku, Y. (1994b) *J. Biol. Chem.* **269**, 28908−28912]. In the present study, we carried out screening of ubiquinone analogues using a bound ubiquinone-free enzyme (ΔUbiA[1]) that has been isolated from a ubiquinone biosynthesis mutant, and identified PC24 (2-chloro-4,6-dinitrophenol), PC32 (2,6-dibromo-4-cyanophenol), and PC52 (2-isopropyl-5-methyl-4,6-dinitrophenol) as potent Q_H site inhibitors. PC15 (2,6-dichloro-4-nitrophenol) and PC16 (2,6-dichloro-4-dicyanovinylphenol), potent Q_L site inhibitors, did not exhibit such a selective inhibition of the Q_H site. Binding studies using the air-oxidized ΔUbiA enzyme showed that PC32 and PC52 have 4- to 7-fold higher affinity than ubiquinone-1. Reconstitution of the Q_H site with PC32 and PC52 resulted in a decrease of the apparent V_{max} value to 1/7 and 1/3, respectively, of the control activity. These findings suggest that structural features of the Q_L and Q_H sites are different, and provide further support for the involvement of the Q_H site in intramolecular electron transfer and facile oxidation of quinols at the Q_L site.

Cytochrome *bo* is one of three terminal ubiquinol oxidases in the aerobic respiratory chain of *Escherichia coli* that is expressed predominantly under highly aerated growth conditions (1). It catalyzes the two-electron oxidation of quinols at the periplasmic side of subunit II (2; Sato-Watanabe et al., unpublished results) and the four-electron reduction of molecular oxygen to water at the heme-copper binuclear center in subunit I (3, 4). The enzyme belongs to the heme-copper respiratory oxidase superfamily (5), and vectorially translocates protons not only via directed protolytic reactions but also via proton pumping mechanism (6).

Subunit I binds all the redox metal centers, low-spin heme *b*, high-spin heme *o* and Cu_B, and serves as a reaction center for proton pumping and dioxygen reduction (3, 4). Photoaffinity cross-linking studies using an azido-quinone derivative (2) and analysis of quinone analogue-resistant mutations (Sato-Watanabe et al., unpublished results) showed that a low-affinity quinol oxidation site (Q_L) resides in subunit II. Subunits III and IV are required for assembly of the redox metal centers in subunit I (7−9) but not involved in catalytic functions (10).

Cytochrome bc_1 complex (quinol:cytochrome *c* oxidoreductase; Q_o and Q_i; 11,12), photosynthetic reaction center (Q_A and Q_B; 13,14), and Complex II (succinate:quinone oxidoreductase and quinol:fumarate oxidoreductase; Q_s pair; 15,16), which utilize quinols as electron carriers, all contain two quinone/quinol binding sites. Because electron donors for cytochrome *bo* are hydrophobic quinols, there must be an electron gate that connects two-electron, two-proton redox components with one-electron-transfer system (i.e., heme irons). Previously, we demonstrated the presence of the high-affinity quinone binding site (Q_H) in cytochrome *bo*, and showed that the Q_H site is close to both the Q_L site and low-spin heme *b* (17). Subsequently, potentiometric studies showed that the bound quinone at the Q_H site lowers midpoint potential of low-spin heme *b* by 20−25 mV, and can be stabilized as ubisemiquinone radical during the catalytic cycle (18). The bound quinone undergoes double reduction followed by protonation but does not leave from the Q_H site. These properties suggest that a unique mechanism for the

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[1] Abbreviations: BQ, 1,4-benzoquinone; DMBQ, 2,6-dimethyl-1,4-benzoquinone; ΔUbiA, the wild type cytochrome *bo* isolated from a ubiquinone-deficient mutant; HQNO, heptyl hydroxyquinoline-*N*-oxide; Q_n, ubiquinone-n; Q_nH_2, ubiquinol-n; Q_H, a high-affinity quinone binding site; Q_L, a low-affinity quinol oxidation site; TX100 enzyme, the wild-type cytochrome *bo* purified in Triton X-100.

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quinol oxidation is operative in bacterial quinol oxidases, and the Q_H site mediates electron transfer from the Q_L site to low-spin heme *b*, not only as an electron gate but also a transient electron reservoir (18).

In the present study, we explored structural features of the Q_H site through screening of the potent Q_H site inhibitors using a bound quinone-free enzyme isolated from a ubiquinone deficient mutant (Δ*ubiA*), and demonstrated that these compounds have higher binding affinities than ubiquinone-1 (Q_1) and inhibit oxidase activity in a noncompetitive manner when introduced in the Q_H site. These observations support the previous proposal that the bound quinone at the Q_H site mediates electron transfer from the Q_L site to low-spin heme *b* (18).

MATERIALS AND METHODS

Chemicals. 2,6-Dimethyl-1,4-benzoquinone (DMBQ) was obtained from Tokyo Kasei Company (Tokyo). Ubiquinone-1 (Q_1) was a generous gift from Dr. S. Ohsono of Eisai Company Ltd. (Tsukuba). Substituted phenols (19−21) and capsaicins (22) were synthesized as described previously. Sucrose monolaurate SM-1200 was purchased from Mitsubishi-Kagaku Foods Company (Tokyo). Other chemicals are commercial products of analytical grade (17,23).

Purification of Cytochrome bo. The wild-type enzyme with a bound Q_8 was isolated from a cytochrome *bd*-deficient mutant GO103 (*cyo*$^+$ Δ*cyd*::Km^r; 24) harboring a single copy vector pMFO2 (*cyo*$^+$ Ap^r; 7), and stored in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate. The ΔUbiA enzyme, which is free from any bound ubiquinones, was purified from a ubiquinone biosynthesis mutant MU1227 (*cyo*$^+$ *cyd*$^+$ Δ*ubiA*::Cm^r; 25) harboring pMFO4 (*cyo*$^+$ Ap^r; 26). The TX100 enzyme was similarly prepared by purification in Triton X-100, and stored in 50 mM Tris-HCl (pH 7.4) containing 0.1% Triton X-100. Concentrations of the enzymes were estimated from heme content (27).

Quinol Oxidase Assay. Ubiquinol-1 (Q_1H_2) oxidase activity was determined spectroscopically at 25 °C as described previously (23). The reaction mixture contains 1 nM enzyme, 50 mM Tris-HCl (pH 7.4), and 0.1% sucrose monolaurate, and the reaction was started by addition of Q_1H_2 at a final concentration of 50 μM. The activity was calculated with a molar extinction coefficient of 12 250 at 275 nm for an oxidized form of ubiquinones (28). Effects of quinone analogues were examined after preincubation of the enzyme at 25 °C for 1 min. Double-reciprocal plot analysis was carried out at final Q_1H_2 concentrations from 25 to 150 μM. The I_{50} values, defined as the inhibitor concentrations that decrease the oxidase activity to 50% of the control activity, were determined graphically.

Determination of K_d Values of Quinone Analogues. The ΔUbiA enzyme (2.5 nmol) in 1 mL of 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate was incubated overnight on ice with quinone analogues at final concentrations of 0.5 to 20 μM. The wild-type enzyme was treated similarly with quinone analogues at final concentrations of 10 to 200 μM. Absolute spectra of the air-oxidized enzymes were recorded with a Shimadzu UV-3000 spectrophotometer (Shimadzu Company, Kyoto). Digital outputs were processed as described previously (26), and quinone analogue-bound *minus* unbound difference spectra were calculated.

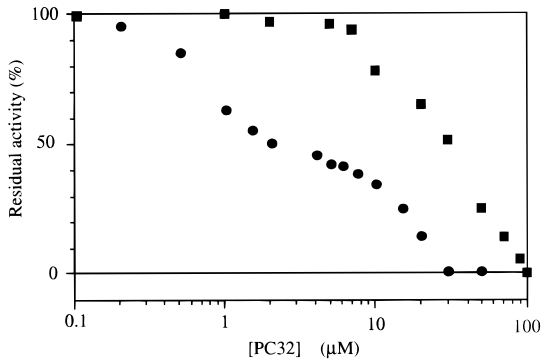

FIGURE 1: Effect of PC32 on Q_1H_2 oxidase activity of the wild-type and ΔUbiA enzymes. The wild-type (square) and ΔUbiA (circle) enzymes were preincubated with PC32 at final concentrations as indicated for 1 and 10 min, respectively. Oxidase activity was determined in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate with Q_1H_2 at a final concentration of 50 μM. The control values of the wild-type and ΔUbiA enzymes are 32 and 29 μmol Q_1H_2 oxidized/nmol enzyme/min, respectively.

The K_d values of quinone analogues at the Q_H site were determined by double-reciprocal plot analysis of $Abs_{406-422}$ versus the concentrations of quinone analogues.

Reconstitution of the Q_H Site. The ΔUbiA enzyme (25 nmol) in 2 mL of 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate was incubated overnight on ice with quinone analogues at the final concentration of 1 mM. The enzyme was incubated with 40% poly(ethylene glycol) 4000 for 30 min on ice, and recovered by centrifugation at 100 000 × *g* and at 4 °C for 1 h. Excess amount of the precipitant was removed by dialysis of the supernatant against 2 L of the same buffer at 4 °C for 2 days with two changes.

RESULTS

Screening of Potent Q_H Site Inhibitors. To probe structural features of the Q_H site, we examined effects of quinone-related inhibitors on Q_1H_2 oxidase activity of the ΔUbiA enzyme where the Q_H site is freely accessible to exogenous ligands (17). We determined the concentrations of substituted phenols and ubiquinone analogues required for 50% inhibition of the activity (I_{50}) (Tables 1 and 2). Among 28 substituted phenols examined, the I_{50} values of the ΔUbiA enzyme for PC24, PC32, and PC52 were 5- to 10-times smaller than those of the wild type enzyme (Table 1). In quinone analogues, capsaicins more strongly inhibited the ΔUbiA enzyme (Table 2). These compounds are potential Q_H site inhibitors that exert their effects on the quinol oxidation through specific binding to the Q_H site of the ΔUbiA enzyme. In fact, we were able to observe a biphasic concentration dependence of the residual activity on PC32 (Figure 1) and PC52 (data not shown). The low-affinity components showed the I_{50} values comparable to the I_{50} values of the wild-type enzyme. The high-affinity components, therefore, are attributable to the binding of quinone analogues to the Q_H site. Incomplete inhibition observed for the ΔUbiA enzyme at the lower concentrations is likely due to dynamic binding equilibrium and to insufficient preincubation of the enzyme with the inhibitors to attain the equilibrium state. Prolonged incubation (i.e., 10 min) seemed to result in the exchange of the bound Q_8 of the wild-type enzyme with the high-affinity inhibitors (data not shown).

Table 1: Concentrations of Substituted Phenols Required for 50% Inhibition of Q_1H_2 Oxidase Activity of the Wild-Type (WT) and ΔUbiA Enzymes

	substituting group				I_{50} (mM)		Ratio
compound	X_2	X_4	X_6	X_5	WT	ΔUbiA	WT/ΔUbiA
PC11	Cl	H	Cl		173	107	1.6
PC12	Cl	Cl	Cl		105	151	0.70
PC13	Cl	CN	Cl		15	12	1.3
PC15	Cl	NO_2	Cl		9.6	16	0.60
PC16	Cl	$CH{=}C(CN)_2$	Cl		4.5	11	0.41
PC23	I	NO_2	I		19	23	0.83
PC24	Cl	NO_2	NO_2		23	4.5	5.1
PC25	Cl	Br	NO_2		170	164	1.0
PC27	CH_3	NO_2	CH_3		72	56	1.3
PC32	Br	CN	Br		36	4.8	7.5
PC33	I	CN	I		25	72	0.35
PC34	Cl	CN	Br		11	12	0.92
PC35	Cl	CN	I		35	13	2.7
PC36	Cl	CN	H		22	45	0.49
PC42	Br	$CH{=}C(CN)_2$	Br		37	43	0.86
PC43	I	$CH{=}C(CN)_2$	I		74	121	0.61
PC44	Br	$CH{=}C(CN)_2$	CH_3O		68	36	1.9
PC45	I	$CH{=}C(CN)_2$	CH_3O		36	37	0.97
PC46	Cl	$CH{=}C(CN)_2$	CH_3		149	153	0.97
PC51	$CH(CH)_2$	NO_2	NO_2		57	54	1.1
PC52	$CH(CH)_2$	NO_2	NO_2	CH_3	74	7.2	10
PC53	$CH(CH_3)CH_2CH_3$	NO_2	NO_2		61	42	1.5
PC54	$CH_3CH(CH_3)_2$	NO_2	NO_2		58	78	0.74
PC62	$CH(CH_3)CH_2CH_3$	NO_2	$CH(CH_3)CH_2CH_3$		154	164	0.94
PC63	$CH(CH_3)CH_2CH_3$	NO_2	CH_3O		35	81	0.43
PC64	Cl	H	NO_2		58	143	0.41
PC66	Br	Br	NO_2		69	64	1.1
PC67	I	COOH	I		76	153	0.50

Table 2: Concentrations of Quinone Analogues Required for 50% Inhibition of Q_1H_2 Oxidase Activity of the Wild-Type (WT) and ΔUbiA Enzymes

	I_{50} (mM)		Ratio
Compound	WT	ΔUbiA	WT/ΔUbiA
BQ	29	22	1.3
methyl BQ	6.8	41	0.17
2,6-dichloro-BQ	8.0	21	0.38
2,6-dimethyl BQ	0.73	25	0.03
2,5-dichloro BQ	21	15	1.4
2,5-dimethyl BQ	3.7	39	0.09
tetramethyl BQ	320	415	0.77
ubiquinone-0	83	64	1.3
piericidin A	3.4	3.5	0.97
HQNO	1.7	1.1	1.5
antimycin A	56	367	0.15
DCMU	172	334	0.51
fumigacin	240	130	1.9
capsaicin (CAP12)	400	170	2.4
capsaicin (CAP13)	460	100	4.6

In contrast, the I_{50} values of the potent Q_L site inhibitors (i.e., PC15 and PC16) showed insignificant difference. HQNO (heptyl hydroxyquinoline-*N*-oxide), which mimics the ubisemiquinone radical, did not exhibit selective inhibition of the Q_H site (Table 2). Methyl BQ, 2,6-dimethyl BQ, 2,5-dimethyl BQ, and antimycin A showed the I_{50} ratios of <0.5 (Tables 1 and 2), indicating that the ΔUbiA enzyme was more resistant to these quinone analogues. This result can be explained by structural perturbation of the Q_L and Q_H sites in the ΔUbiA enzyme that has been synthesized in the ubiquinone biosynthesis mutant (17). In conclusion, molecular recognition of the Q_H site is different from that of the Q_L site.

Binding of Potent Q_H Site Inhibitors to the ΔUbiA *Enzyme*. As reported previously (17), the Soret peak of the air-oxidized ΔUbiA enzyme is 3 nm red-shifted relative to the peak value of the wild-type enzyme, and it can be restored by addition of excess amount of Q_1. Upon addition of PC24, PC32, and PC52 to the ΔUbiA enzyme, the Soret peak was also shifted from 412 to 409 nm (data not shown). Quinone analogue bound *minus* unbound difference spectra showed a peak at 406 nm and a trough at 422 nm, and change in the absorbance difference $Abs_{406-422}$ exhibited a saturation behavior (Figure 2). The wild-type enzyme showed a similar spectral change to a much smaller extent (~6%). Double-reciprocal plot analysis of $Abs_{406-422}$ versus the concentration of quinone analogues yielded a linear correlation, and the K_d values for Q_1, PC24, PC32, and PC52 were determined to be 2.1, 20, 0.3, and 0.5 μM, respectively (Figure 3). Unexpectedly, at the air-oxidized state, the binding affinity of PC24 was much weaker than that of Q_1.

Effect of Bound Substituted Phenols at the Q_H Site on Quinol Oxidase Activity. Subsequently, we reconstituted the Q_H site of the ΔUbiA enzyme with either PC32 or PC52, and examined their effects on the kinetic parameters of Q_1H_2 oxidase activity. Absolute spectra of the ΔUbiA and reconstituted enzymes were recorded in the presence and absence of 1 mM Q_1, and the reconstitution yields were estimated to be 93 and 98%, respectively, based on the absorbance change of $Abs_{406-422}$ in the PC32- and PC52-reconstituted enzymes relative to the control value in the ΔUbiA enzyme. Double-reciprocal plot analysis demonstrated that the apparent V_{max} values of the PC32- and PC52-reconstituted enzymes were reduced to about 1/7 and 1/3, respectively, of the ΔUbiA enzyme (Figure 4). In contrast, the apparent K_m value for Q_1H_2 changed only slightly by the bound substituted phenols (i.e., 65 to 54 μM). Variations of the K_m values of the ΔUbiA enzymes from the wild-type

Table 3: Properties of the Q_8-Bound and Q_8-Free Enzymes[a]

preparation	Q_8 content (mol/mol enzyme)	abs. maximum (nm)		Q_1H_2 oxidase	
		-Q_1	+Q_1	K_m (μM)	V_{max} (μmol/nmol enzyme/min)
Q_8-bound	1.03	409	409	47	26.7
Q_8-free					
(ΔUbiA)	<0.01	412	409	65	23.5
(TX100)	0.13	410	406	26	9.1
				138	21.4

[a] The Q_8-free enzyme (TX100) was purified in the presence of Triton X-100 in place of sucrose monolaurate, and stored in 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate. The absorption maximum of the air-oxidized enzymes was recorded before and after overnight incubation of the enzymes with 1 mM Q_1. Q_8 content and Q_1H_2 oxidase activity were determined as described in *Materials and Methods*. Average values of two independent preparations are shown.

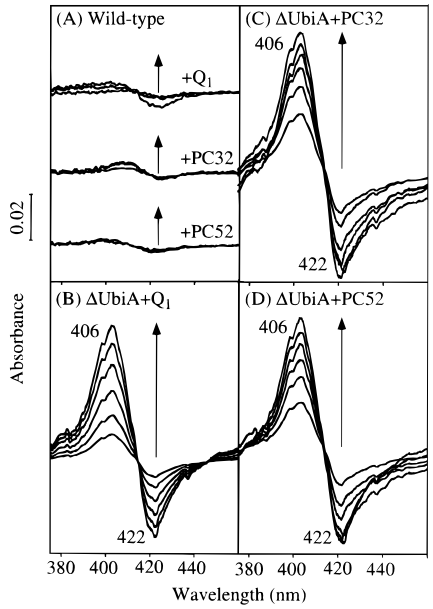

FIGURE 2: Effect of quinone analogues on the Soret peak of the air-oxidized wild-type and ΔUbiA enzymes. The wild-type and ΔUbiA enzymes (2.5 μM) were incubated overnight on ice with quinone analogues at final concentrations of 10, 100, and 200 μM and 0.5, 1, 2, 4, 8, and 20 μM, respectively. Absolute spectra of the air-oxidized enzyme were recorded at room temperature, and analogue-bound *minus* unbound difference spectra were calculated.

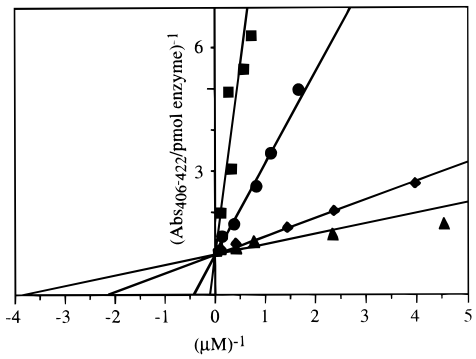

FIGURE 3: Double-reciprocal plot analysis of binding of quinone analogues to the Q_H site of the ΔUbiA enzyme. Changes in absorbance difference ($Abs_{406-422}$) induced by addition of Q_1 (circle), PC24 (square), PC32 (triangle), and PC52 (diamond) to the ΔUbiA enzyme were plotted against concentrations of free quinone analogues.

control (Table 3) (17) indicate the importance of a farnesyl side-chain of ubiquinols in binding into the Q_H site. A 0.5:5 nM mixture of the wild-type and PC32-reconstituted enzymes showed the activity comparable to 1 nM wild-type enzyme

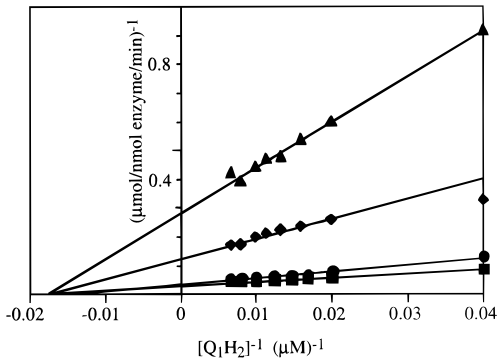

FIGURE 4: Kinetic analysis of Q_1H_2 oxidase activities of the ΔUbiA enzyme reconstituted with either PC32 or PC52. Oxidase activity of the wild-type (square) and ΔUbiA (circle) enzymes at a final concentration of 1 nM and of the ΔUbiA enzyme reconstituted with either PC32 (triangle) or PC52 (diamond) at a final concentration of 4 nM were determined in the presence of Q_1H_2 at final concentrations of 25−150 μM.

(data not shown), indicating that the free substituted phenol, which may be present in detergent micelles or released from the reconstituted enzymes, is not responsible for the decrease of turnover rate in the reconstituted enzymes. Thus, noncompetitive inhibition of Q_1H_2 oxidase activity by the bound substituted phenols strongly suggest that the bound ubiquinone at the Q_H site is involved in electron transfer from the Q_L site to low-spin heme *b*. However, it is not clear why the V_{max} value of the PC52-reconstituted enzyme was reduced to only 1/3 of the control activity. During quinol oxidase assay, the reduced enzyme may release the bound PC52 at the enzyme concentration (1 nM) well below the K_d value of PC52 (0.5 μM), and bind free Q_1 or Q_1H_2 molecules in the reaction mixture.

Effects of Removal of Bound Q_8 on Spectroscopic and Enzymatic Properties of Cytochrome bo. The bound Q_8-free wild-type enzyme can be prepared by isolation from the ubiquinone biosynthesis mutant Δ*ubiA* using sucrose monolaurate (17) or by purification in the presence of Triton X-100, one of stronger nonionic detergents (10, 29, 30). We reported previously that the former preparation (sucrose monolaurate-purified ΔUbiA enzyme) completely lacks the bound Q_8 and shows perturbations of low-spin heme *b* and the Q_L site (17, 18). The absence of the bound Q_8 in both preparations induced red-shifts of the Soret peak of the air-oxidized enzymes that can be reversed by incubation of the enzyme with the excess amount of added Q_1 (Table 3). Furthermore, the latter preparation (TX100 enzyme) reproducibly showed a peculiar concentration dependence in the Soret peak change (data not shown) and a biphasic Q_1H_2

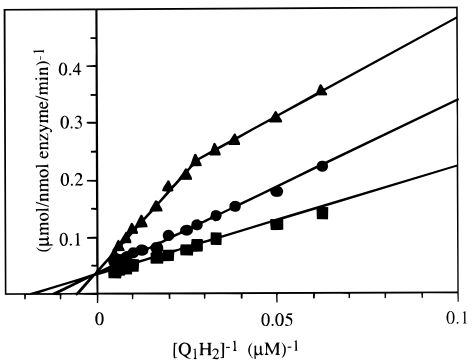

FIGURE 5: Kinetic analysis of Q_1H_2 oxidase activity of the ΔUbiA enzyme (circle) and the wild-type enzyme purified in sucrose monolaurate (square) or Triton X-100 (triangle).

oxidation kinetics with K_m values of 26 and 138 μM (Table 3, Figure 5). The TX100 enzyme has been reported to have the Soret peak maximum and steady-state kinetics similar to the Q_8-bound enzyme (30); however, our data suggest that Triton X-100 not only removes a tightly bound Q_8 from cytochrome *bo* but concomitantly alters the enzymatic properties.

DISCUSSION

Structural Features of the Q_H Site. Screening of a series of substituted phenols and ubiquinone analogues using the bound Q_8-free (ΔUbiA) enzyme identified PC24, PC32, and PC52 as the potent Q_H site inhibitors. Binding studies with the air-oxidized ΔUbiA enzyme demonstrated that PC32 and PC52 have 4- to 7-fold higher affinity for the Q_H site than the oxidized form of substrate, Q_1. The potent Q_L site inhibitors such as PC15 and PC16 (23) did not show such a selective inhibition of the Q_H site. Furthermore, the ΔUbiA enzyme, which has been folded in vivo in the absence of Q_8 molecules, became less sensitive to methyl BQ, 2,6-dimethyl BQ, 2,5-dimethyl BQ, and antimycin A than the wild-type enzyme. This result is probably due to structural perturbation of the nearby Q_L site (17). Therefore, it is concluded that molecular recognition by two quinone/quinol binding sites in cytochrome *bo* is different. However, unlike the Q_L site inhibitors (23), it is difficult to identify structural factors of substituted phenols required for specific binding to the Q_H site because of limited numbers of available structurally related compounds. Nevertheless, in 4,6-dinitrophenols, a 2-chloro derivative (PC24) showed the highest inhibitory activity, but 2-alkyl derivatives, potent inhibitors for mammalian NADH dehydrogenase and cytochrome bc_1 complex (20, 31), are not effective for the Q_H site except 2-isopropyl-5-methyl derivative (PC52).

Functional Role of the Q_H Site. The proximity of the Q_H site to low-spin heme *b* in subunit I has been shown by optical and resonance Raman spectroscopic studies (17) (see Figure 6). Small perturbation of the Q_L site in the ΔUbiA enzyme indicates that the Q_H site is also close to the Q_L site in subunit II (17; unpublished results). Potentiometric studies on the wild-type enzyme with a single bound Q_8 molecule showed that the bound quinone at the Q_H site can be stabilized as the ubisemiquinone radical and undergoes double reduction followed by protonation (18). Similar behaviors of the semiquinone radical in the presence of excess amount of Q_1 have been reported (32). These findings

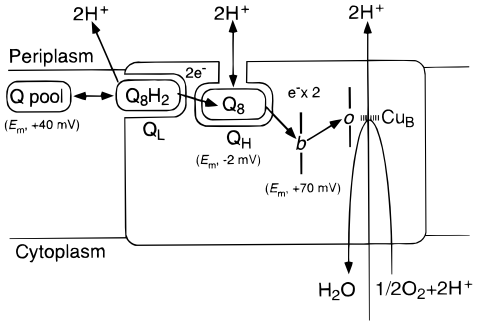

FIGURE 6: Schematic model of cytochrome *bo* showing relative locations of two quinone/quinol binding sites and three redox metal centers. See details in text.

are inconsistent with the idea that the Q_L site stabilizes ubisemiquinone during enzyme turnover. Rather, the Q_H site appears to function as a transient electron reservoir and an electron gate that ensures sequential one-electron transfer from ubiquinols, two-electron donor, to low-spin heme *b*, one-electron redox component (Figure 6). Rapid two-electron transfer from ubiquinol in the Q_L site, to the bound quinone in the Q_H site facilitates dynamic equilibrium with membrane ubiquinol pool and prevents formation of ubisemiquinone at the Q_L site which may produce oxygen radical at the protein surface. Transient appearance of ubisemiquinone during the oxidation of the potentiometrically poised enzyme (18) and noncompetitive inhibition of Q_1H_2 oxidase activity by PC32 (and PC52) introduced in the Q_H site (this study) support our proposal that the bound quinone is an intrinsic electron-transfer component in the heme-copper terminal quinol oxidases and mediates electron transfer from the Q_L site to low-spin heme *b* (33, 34). Thus, the fully reduced enzyme can complete the four-electron reduction of molecular oxygen to water by providing the fourth electron from the bound quinol, like a binuclear purple copper center (Cu_A) of cytochrome *c* oxidase (35). Recent flow flash studies on the reaction of the fully reduced enzyme with molecular oxygen supported electron transfer from the bound quinol to low-spin heme *b* (29, 36).

The presence of a tightly bound ubiquinone in cytochrome *bo* from *E. coli* (17, 29, 30, 36) and cytochrome ba_3 from *Paracoccus denitrificans* (37) indicates the functional importance of the Q_H site in bacterial quinol oxidases. In contrast to our proposal, Musser et al. (38, 39) postulated that the proton motive Q cycle is operative in cytochrome *bo* for vectorial translocation of protons. The bound Q_8 can be removed from cytochrome *bo* by stronger detergents such as Triton X-100 (29, 30). However, unlike the Q_o and Q_i sites of cytochrome bc_1 complex, the Q_H site is not in dynamic equilibrium with membrane quinol pool (17). This property ensures the enzyme from abortive catalytic cycle due to release of either ubisemiquinone or ubiquinol from the Q_H site. Furthermore, full reduction of the bound quinone at the Q_H site (18, 36) indicates that cooperation of two quinone/quinol binding sites in cytochrome *bo* is carried out in a manner different from photosynthetic reaction center (i.e., Q_A and Q_B sites).

In conclusion, cytochrome *bo* contains two quinone/quinol binding sites with different binding affinity, and a unique redox mechanism is operative in a cooperative manner between the Q_L and Q_H sites to carry out the oxidation of

quinols in subunit II and one-electron transfer to the metal centers in subunit I (Figure 6).

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