J. Biochem. 125, 138-142 (1999)

Characterization of the Ubiquinol Oxidation Sites in Cytochromes *bo* and *bd* from *Escherichia coli* using Aurachin C Analogues[1]

Hideto Miyoshi,*,[2] Kazuhiro Takegami,* Kimitoshi Sakamoto,* Tatsushi Mogi,† and Hajime Iwamura*

**Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606-8502; and †Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033*

Received September 9, 1998; accepted October 2, 1998

Natural aurachin C is the most potent inhibitor of oxidation of ubiquinols by cytochromes *bo* and *bd* from *Escherichia coli*. To probe the structural properties of the substrate oxidation site in the ubiquinol oxidases, we synthesized a systematic set of aurachin C analogues (*N*-hydroxy-4-quinolone derivatives) and examined how their structure affects their activity towards cytochromes *bo* and *bd*, which are structurally unrelated. We found that the presence of the 3-methyl group of the 2-*n*-decyl and 2-*n*-undecyl derivatives increased the inhibitory potency towards both enzymes, probably due to a local steric congestion that allows favorable interaction of the alkyl tail with the enzyme. Increase in the chain length of the 3-alkyl tail of the 2-*n*-undecyl derivatives decreased the inhibitory potency only in cytochrome *bo*, indicating that the binding site for the alkyl tails of cytochrome *bo* is smaller than that of cytochrome *bd*. Based on these findings, we discuss the differences in the molecular mechanism of substrate oxidation by these two terminal ubiquinol oxidases.

Key words: aurachin C, cytochrome *bd*, cytochrome *bo*, terminal oxidase, ubiquinone.

Cytochrome *bo* is a four-subunit ubiquinol oxidase in the aerobic respiratory chain of *Escherichia coli*, and belongs to a superfamily of the heme-copper terminal oxidases (*1* for a recent review). The enzyme catalyzes the four-electron reduction of dioxygen with two molecules of ubiquinol-8 (Q_8H_2) and can establish a proton electrochemical gradient across the cytoplasmic membrane not only *via* scalar protolytic reactions but also *via* a proton pump mechanism (*2*). Cytochrome *bd*, an alternative two-subunit ubiquinol oxidase, is structurally unrelated to cytochrome *bo* (*1*) and does not pump protons (*2*).

Subunit I of cytochrome *bo* binds all the redox metal centers, low-spin heme *b*, high-spin heme *o*, and a copper ion (Cu_B), and serves as a reaction field for redox-coupled proton pumping (*1*). Subunit II does not contain a redox metal center, but photoaffinity crosslinking studies with an azido-ubiquinone (*3, 4*) and mapping of quinone-related inhibitor-resistant mutations (*5*) demonstrated the presence of the substrate oxidation site in its C-terminal hydrophilic domain. The oxidation of ubiquinols by cytochrome *bo* proceeds through the cooperation of two ubiquinol/ubiquinone binding sites: a low-affinity ubiquinol oxidation site (Q_L) that is in dynamic equilibrium with the membrane quinone pool (*6*); and a high-affinity ubiquinone binding site (Q_H) that is close to both the Q_L site and low-spin heme *b* and mediates intramolecular electron transfer (*7-11*). Sato-Watanabe *et al.* (*7-9*) postulated that the Q_H site serves as a transient electron reservoir for two-electron supply from the Q_L site and gates electron flux, allowing sequential one-electron transfer from the Q_L site to heme *b*.

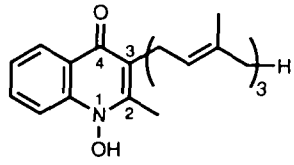
In contrast, cytochrome *bd* does not require a tightly bound Q_8 for oxidation of substrates (*1*). Q_8H_2 molecules are oxidized at a site close to low-spin heme b_{558} at the periplasmic surface of subunit I (*1*). Electrons are then transferred to heme *d* through high-spin heme b_{595}, both of which are assumed to be present at an interface between subunits I and II (*1*). Thus, the molecular mechanism for two-electron oxidation of ubiquinols by cytochrome *bd* may not be the same as that of cytochrome *bo*.

Specific inhibitors are useful tools for probing structural and functional properties of the active center of redox proteins. Several inhibitors (*e.g.*, hydroxyquinoline-*N*-oxide (HQNO), piericidin A, and undecylhydroxydioxobenzothiazole (UHDBT)) are known to act at or close to the Q_L site of both cytochromes *bo* and *bd* with μM-order K_i values (*1, 12*). Previously, we carried out structure-function studies on the Q_L site of cytochromes *bo* and *bd* using a series of benzoquinones and substituted phenols and found that their Q_L sites exhibit similar structural prop-

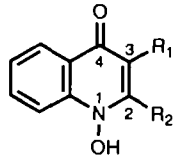
[1] This work was supported in part by Grants-in-aid for Scientific Research on Priority Areas (10129213 to HM and 08249106 to TM) and for Scientific Research (C) (08660136 to HM) from the Ministry of Education, Science, Sports and Culture of Japan.

[2] To whom correspondence should be addressed. Fax: +81-75-753-6408, E-mail: miyoshi@kais.kyoto-u.ac.jp

Abbreviations: AC, aurachin C; DMBQ, 2,6-dimethyl-1,4-benzoquinone; PC15, 2,6-dichloro-4-nitrophenol; PC16, 2,6-dichloro-4-dicyanovinylphenol; Q_n, ubiquinone-*n*; Q_nH_2, ubiquinol-*n*; Q_H, a high-affinity ubiquinone binding site; Q_L, a low-affinity ubiquinol oxidation site; RT, room temperature; THF, tetrahydrofuran.



Natural Aurachin C



AC0-10; R_1 = H, R_2 = n-$(CH_2)_9CH_3$

AC1-10; R_1 = CH_3, R_2 = n-$(CH_2)_9CH_3$

AC10-1; R_1 = n-$(CH_2)_9CH_3$, R_2 = CH_3

AC0-11; R_1 = H, R_2 = n-$(CH_2)_{10}CH_3$

AC1-11; R_1 = CH_3, R_2 = n-$(CH_2)_{10}CH_3$

AC2-11; R_1 = CH_2CH_3, R_2 = n-$(CH_2)_{10}CH_3$

AC3-11; R_1 = n-$(CH_2)_2CH_3$, R_2 = n-$(CH_2)_{10}CH_3$

AC4-11; R_1 = n-$(CH_2)_3CH_3$, R_2 = n-$(CH_2)_{10}CH_3$

Fig. 1. **Structures of aurachin C analogues studied in this work.**

erties, even though these two ubiquinol oxidases are structurally unrelated (*6*). Rich and colleagues (*13*) studied the effects of natural aurachin C (AC), which was discovered as the quinone redox site inhibitor of cytochrome bc_1 complex (*14*), and its analogues with long alkyl tails [3-n-$(CH_2)_nCH_3$-2-CH_3 derivatives where n=7, 9 and 11, or 2-n-$(CH_2)_nCH_3$ derivatives where n=7, 9, 10, and 11; *cf.* Fig. 1] on the *E. coli* ubiquinol oxidases and showed that these compounds are the most potent inhibitors for the Q_L site of ubiquinol oxidases (K_i, 10^{-8} M).

In the present study, to probe structural features of the Q_L site of the *E. coli* cytochromes *bo* and *bd*, we extended the work of Meunier *et al.* (*13*) and synthesized a new set of AC analogues in which either CH_3 or n-$(CH_2)_9CH_3$ was introduced at R_1 and R_2 (AC0-10, AC1-10, and AC10-1), or R_1 of the 2-n-undecyl AC was changed from CH_3 to n-$(CH_2)_nCH_3$ (AC1-11 to AC4-11) (Fig. 1). Structure-activity studies with these inhibitors showed that the Q_L sites of these two oxidases were in fact different in terms of recognition of the AC analogues.

MATERIALS AND METHODS

Synthesis of Aurachin C Analogue, N-Hydroxy-2-n-decyl-3-methyl-4-quinolone (AC1-10)—AC1-10 was synthesized as illustrated in Fig. 2. Aniline (18.6 g, 200 mmol), ethyl acetoacetate (26.0 g, 200 mmol), $MgSO_4$ (72.2 g, 600 mmol), and a catalytic amount of concentrated HCl were vigorously stirred in 300 ml of toluene at room temperature (RT) for 24 h. After removing $MgSO_4$ by filtration, the solvent was removed *in vacuo*. The crude product was purified by column chromatography (silica gel, ethyl acetate/hexane = 1:4) to give ethyl 3-anilinocrotonate in a 63% yield.

To a solution of ethyl 3-anilinocrotonate (10 g, 49 mmol) in 100 ml of *m*-xylene was added metal Na (1.2 g, 50 mmol) at 0°C under N_2, and the mixture was stirred for 12 h. To the mixture were added KI (1.0 g) and bromomethane (5.2 g, 55 mmol), and the reaction mixture was refluxed at 120°C for 8 h. After removal of a precipitated material by filtration, the crude product was purified by column chromatography (silica gel, ethyl acetate:hexane = 1:9) to give ethyl 3-anilino-2-methylcrotonate in a 55% yield.

Ethyl 3-anilino-2-methylcrotonate (6.6 g, 30 mmol) and biphenyl (20 g, 130 mmol) were refluxed in 200 ml of diphenylether at 200°C for 30 min. After the reaction mixture had cooled to RT, 300 ml of hexane was added and stirred for 30 min. Precipitated material was separated by filtration and washed with hexane. The crude product was purified by recrystalization from methanol to give 2,3-dimethyl-4-quinolone in a 43% yield.

To a solution of lithium diisopropylamine (11 mmol) in 200 ml of tetrahydrofuran (THF) was added 2,3-dimethyl-4-quinolone (0.9 g, 5 mmol) at 0°C and stirred at RT for 1 h. To the mixture, 1-bromononane (1.0 g, 5 mmol) and KI (1.0 g) were added and stirred at RT for 8 h. The reaction mixture was extracted with diethylether and washed with brine. The organic phase was dried over $MgSO_4$ and evaporated. The crude product was purified by recrystalization from methanol to give 2-n-decyl-3-methyl-4-quinolone in a 65% yield.

To a solution of lithium diisopropylamine (7.5 mmol) in 100 ml of THF was added hexamethylphosphoric triamide (1.9 g, 7.5 mmol) slowly at 0°C, and the mixture was stirred for 15 min. To the mixture was added 2-n-decyl-3-methyl-4-quinolone (1.5 g, 5 mmol), and the mixture was stirred at RT for 1 h. Ethyl chloroformate (1.1 g, 10 mmol) was added to the mixture and stirred for 6 h. After addition of 200 ml of saturated Na_2CO_3 solution, the organic phase was extracted by diethylether and washed with brine. The organic solvent was evaporated to give crude product ester in a 85% yield.

To a solution of the ester (1.1 g, 3 mmol) in 10 ml of chloroform was added slowly *m*-chloroperoxybenzoic acid (0.62 g, 3.6 mmol) in 5 ml of chloroform, and the mixture was stirred at RT for 1 h. After addition of 200 ml of saturated Na_2CO_3 solution, the organic phase was extracted by chloroform and washed with saturated Na_2CO_3 solution (3 times). The organic solvent was evaporated to give crude N-oxide in a 93% yield.

To a solution of the N-oxide (1.1 g, 3 mmol) in 200 ml of ethanol was added 50 ml of 1 M NaOH slowly, and stirred at RT for 1 h. After acidification by adding 3 M HCl (pH 5), the solvent was removed *in vacuo*. The crude product was purified by recrystalization from ethanol to give N-hydroxy-2-n-decyl-3-methyl-4-quinolone (AC1-10) in a quantitative yield. ^{1}H NMR (CD_3OD, 300 MHz) δ 0.89 (t, J=6.4 Hz, 3H, CH_3), 1.30-1.41 (m, 12 H, CH_2), 1.49 (m, 2H, CH_2), 1.71 (m, 2H, CH_2), 2.21 (s, 3H, $ArCH_3$), 3.03 (t, J=7.9 Hz, 2H, $ArCH_2$), 7.42 (t, J=6.9 Hz, 1H, ArH), 7.73 (t, J=7.9 Hz, 1H, ArH), 7.96 (d, J=8.3 Hz, 1H, ArH), 8.28 (d, J=8.3 Hz, 1H, ArH). FAB-MS (m/e), 316 $(M+H)^+$.

Synthesis of Other AC Derivatives—AC0-10 and AC0-11 (Fig. 1) were synthesized by the same method used for AC1-10, except that the reaction step b was omitted.

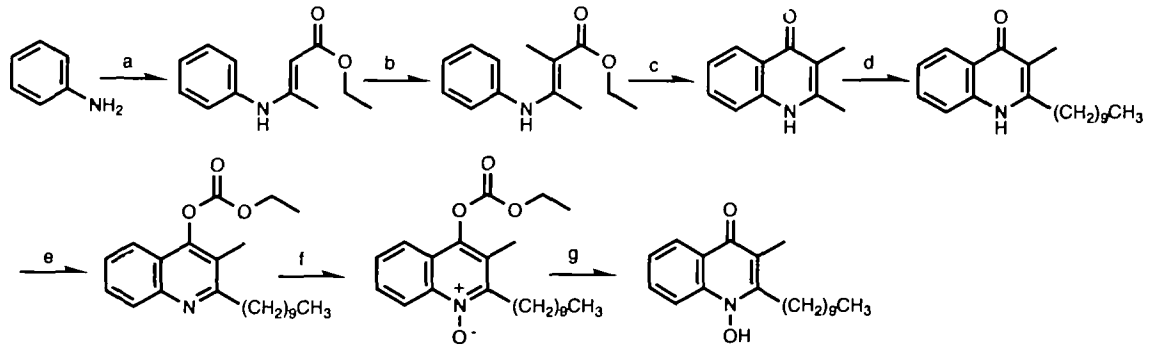


Fig. 2. **Synthetic procedure for AC1-10.** (a) ethyl acetoacetate, $MgSO_4$, HCl in toluene, (b) (1) Na in *m*-xylene, (2) iodomethane, KI, (c) biphenyl in diphenylether, (d) (1) lithium diisopropylamine in THF at −78°C, (2) 1-bromononane, KI, (e) (1) lithium diisopropylamine, hexaethylphosphorous triamide in THF at −78°C, (2) ethyl chloroformate, (f) *m*-chloroperoxybenzoic acid in $CHCl_3$, (g) (1) 1 M NaOH, (2) 3 M HCl.

AC0-10; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.89 (t, J=6.6 Hz, 3H, CH_3), 1.09-1.25 (m, 16 H, CH_2), 2.36 (m, 2H, $ArCH_2$), 5.94 (s, H, ArH), 7.35 (t, J=7.5 Hz, 1H, ArH), 7.66 (t, J=7.5 Hz, 1H, ArH), 8.21 (m, 2H, ArH). FAB-MS (*m/e*), 302 $(M+H)^+$. AC0-11; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.89 (t, J=6.4 Hz, 3H, CH_3), 1.08-1.42 (m, 18 H, CH_2), 2.34 (m, 2H, $ArCH_2$), 5.91 (s, 1H, ArH), 7.35 (t, J=7.6 Hz, 1H, ArH), 7.66 (t, J=7.4 Hz, 1H, ArH), 8.21 (m, 2H, ArH). FAB-MS (*m/e*), 316 $(M+H)^+$.

AC1-11, AC2-11, AC3-11, and AC4-11 (Fig. 1) were synthesized by the same method used for AC1-10, except that corresponding 1-bromoalkanes and 1-bromodecane were used in place of bromomethane and 1-bromononane in the reaction steps b and d, respectively. AC1-11: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, J=6.3 Hz, 3H, CH_3), 1.19-1.26 (m, 14 H, CH_2), 1.43 (m, 2H, CH_2), 1.63 (m, 2H, CH_2), 2.10 (s, 3H, $ArCH_3$), 3.00 (t, J=7.6 Hz, 2H, $ArCH_2$), 7.30 (m, 1H, ArH), 7.57 (t, J=7.8 Hz, 1H, ArH), 8.07 (d, J=8.5 Hz, 1H, ArH), 8.26 (d, J=8.3 Hz, 1H, ArH). FAB-MS (*m/e*), 330 $(M)^+$. AC2-11: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.88 (t, J=6.3 Hz, 3H, CH_3), 1.17-1.26 (m, 14 H, CH_2), 1.41 (m, 2H, CH_2), 1.64 (m, 2H, CH_2), 2.51 (q, J=6.8 Hz, 2H, $ArCH_2$), 2.83 (t, J=7.4 Hz, 2H, $ArCH_2$), 6.89 (t, J=7.6 Hz, 1H, ArH), 7.21 (t, J=7.9 Hz, 1H, ArH), 7.77 (d, J=8.6 Hz, 1H, ArH), 7.92 (d, J=8.1 Hz, 1H, ArH). FAB-MS (*m/e*), 344 $(M+H)^+$. AC3-11: ^{1}H NMR (CD_3OD, 300 MHz) δ 0.89 (t, J=6.5 Hz, 3H, CH_3), 1.09 (t, J=7.3 Hz, 3H, CH_3), 1.30-1.45 (m, 14 H, CH_2), 1.60 (m, 2H, CH_2), 1.66 (m, 2H, CH_2), 1.80 (m, 2H, CH_2), 2.89 (t, J=8.0 Hz, 2H, $ArCH_2$), 3.25 (t, J=8.2 Hz, 2H, $ArCH_2$), 7.84 (t, J=7.0 Hz, 1H, ArH), 8.09 (t, J=7.0 Hz, 1H, ArH), 8.34 (d, J=8.7 Hz, 1H, ArH), 8.48 (d, J=8.5 Hz, 1H, ArH). MS (*m/e*), 357 $(M)^+$. AC4-11: ^{1}H NMR (CD_3OD, 300 MHz) δ 0.94 (m, 6H, CH_3), 1.30-1.49 (m, 20H, CH_2), 1.74 (m, 2H, CH_2), 2.68 (m, 2H, CH_2), 2.98 (m, 2H, CH_2), 7.41 (m, 1H, ArH), 7.74 (m, 1H, ArH), 7.93 (m, 1H, ArH), 8.29 (m, 1H, ArH). MS (*m/e*), 372 $(M+H)^+$.

AC10-1 (Fig. 1) was synthesized by the same method used for AC1-10, except that 1-bromodecane was used in place of bromomethane in the reaction step b, and the reaction step d was omitted. ^{1}H NMR (CD_3OD, 300 MHz) δ 0.89 (t, J=6.5 Hz, 3H, CH_3), 1.28-1.50 (m, 16H, CH_2), 2.62 (s, 3H, $ArCH_3$), 2.71 (t, J=7.0 Hz, 2H, $ArCH_2$), 7.40 (m, 1H, ArH), 7.73 (m, 1H, ArH), 7.96 (d, J=8.6 Hz, 1H, ArH), 8.28 (d, J=8.1 Hz, 1H, ArH). FAB-MS (*m/e*), 316 $(M+H)^+$.

Preparations of Enzymes and Crude Membranes—Cytochromes *bo* and *bd* were isolated from the overproducing *E. coli* strains GO103/pMFO2 (*cyo*$^+$ *Δcyd/cyo*$^+$ Amp^r) (*15*) and GR84N/pNG2 (*cyo*$^+$ *cydA2/cyd*$^+$ Tet^r) (*16*), respectively, as described previously (*15, 17*). Crude membranes were isolated from the wild-type and mutant ST2592/pMFO4 (*Δcyo Δcyd/cyo*$^+$), as described previously (*5*).

Ubiquinol Oxidase Assay—Ubiquinol oxidase activity was determined spectrophotometrically with a Shimadzu UV-3000 spectrophotometer using an extinction coefficient of 12,300 at 275 nm (*18*). Q_1 and Q_2 were synthesized as described previously (*18*), and their reduced forms were prepared by the method of Rieske (*19*). Measurements were performed at 25°C in 2.5 ml of 50 mM Tris-HCl (pH 7.4) containing 0.1% sucrose monolaurate SM-1200 (Mitsubishi Kagaku Foods, Tokyo). The final concentrations of the purified cytochromes *bo* and *bd* were 0.15 and 1.0 nM, respectively. Crude membranes of the cytochrome *bo* mutants were used at a final concentration of 3 μg/ml. After 2 min of preincubation with various concentrations of the inhibitors, the reaction with cytochrome *bo* was started by the addition of 10 μM Q_2H_2 in ethanolic solution. For the reaction with cytochrome *bd*, 100 μM Q_1H_2 was used as a substrate since Q_2H_2 exerts severe product inhibition (*18*).

RESULTS AND DISCUSSION

Inhibition of Wild-Type Cytochrome bo—The inhibitory potencies of a systematic set of AC analogues in terms of the I_{50} values, defined as the inhibitor concentrations required for 50% reduction of the control oxidase activity, were determined with the purified wild-type cytochrome *bo* (Table I). Among the compounds examined in this study, AC1-10 is the most potent competitive inhibitor of Q_2H_2 oxidation. The K_i value of AC1-10 was estimated to be 9.8 (±0.9) nM from Dixon plot (not shown), which is comparable to that of natural AC (7 nM in Ref. *13*).

Comparisons of AC0-10 and AC1-10 and of AC0-11 and AC1-11 (*i.e.*, R_1=H *vs.* CH_3) showed that the presence of a methyl group as R_1 increased the inhibitory potency by about 5-fold. This is probably due to steric congestion arising from the methyl group, which allows the hydrophobic alkyl tail to interact favorably with the enzyme. A similar steric effect of a neighboring methyl group on the

TABLE I. **Inhibition of the purified wild-type cytochromes *bo* and *bd* by AC analogues.**

Compound	Cytochrome *bo* I_{50} (nM)[a]	Cytochrome *bd* I_{50} (μM)[a]
AC0-10	62	2.0
AC1-10	13	0.28
AC10-1	34	0.72
AC0-11	81	2.1
AC1-11	17	0.35
AC2-11	46	0.44
AC3-11	120	0.46
AC4-11	144	0.34

[a]The I_{50} values for Q_2H_2 or Q_1H_2 oxidation are averages from two independent measurements.

TABLE II. **Inhibition of the ubiquinone-related inhibitor-resistant mutants of cytochrome *bo* by AC1-10, AC10-1, and AC0-10.**

Mutant	I_{50} (nM)[a]		
	AC1-10	AC10-1	AC0-10
Wild-type	12	27	65
PC15-S1	9.6	24	69
PC15-S4	11	22	66
PC15-S5 (Ser258Asn)[b]	10	25	63
PC15-S7	11	33	94
PC16-S6 (Glu233His)	12	31	66
PC16-S8	11	23	87
PC16-S10	11	24	57
DMBQ-S3 (His284Glu)	19	38	116
DMBQ-S4	9.1	27	65
DMBQ-S5 (Phe281Ser)	12	27	93

[a]The I_{50} values for Q_2H_2 oxidation by crude membranes are averages from two independent measurements. [b]The point mutations identified in Ref. 5 are indicated.

conformation of the alkyl tail was discussed for the ubiquinone molecule (*20, 21*). Interchange of the methyl and *n*-decyl groups at positions R_1 and R_2 resulted in only 2.6-fold decrease in the inhibitory potency (AC1-10 *vs.* AC10-1). Further, the inhibitory potency of *n*-undecyl AC ($R_2 = n\text{-}(CH_2)_{10}CH_3$) decreased with increase in the bulkiness of R_1 substituent (AC1-11 *vs.* AC2-11, AC3-11, and AC4-11). Accordingly, a binding pocket for side chains can accommodate a single longer alkyl tail at either R_1 or R_2, but does not have enough capacity to accept two longer alkyl chains.

Inhibition of Mutant Cytochrome bo—Recently, we have isolated and characterized spontaneous mutants resistant to ubiquinone-related inhibitors, 2,6-dimethyl-1,4-benzoquinone (DMBQ), 2,6-dichloro-4-nitrophenol (PC15), and 2,6-dichloro-4-dicyanovinylphenol (PC16) (*5*). The 25 mutations isolated are all localized in the C-terminal hydrophilic domain of subunit II (Pro96-His315). Mapping of representative mutations indicates that the ubiquinol oxidase-specific (Qox) domain and/or the Cu_A end of the cupredoxin fold provide the Q_L site, whose location in the three-dimensional structure is reasonable to mediate electron transfer to low-spin heme *b* in subunit I (*5*). Since these mutants can grow aerobically with endogenous Q_8H_2, the profiles of electron-donating activities of Q_1H_2 and the Q_2H_2 analogues, in which the methoxy groups at the 2- and/or 3-positions are replaced by ethoxy groups, are the same in the wild-type and mutant enzymes (*5*). Thus, the binding sites for these competitive inhibitors (K_i, 0.3-3 μM; Refs. *5* and *6*) partially overlap the Q_L site.

To probe whether the quinone analogue-resistant mutations affect a binding site for AC, the most potent competitive inhibitor (K_i, 10 nM), we examined the effects of AC0-10, AC1-10, and AC10-1 on the Q_2H_2 oxidase activities of wild-type and ten mutant membrane preparations (Table II). The I_{50} values for AC10-1 and AC0-10 are 2-3- and 6-9-fold higher than that for AC1-10, indicating that the inhibitory effects of the AC analogues on the mutant enzymes are similar to those on the wild-type enzyme, irrespective of differences in structures of the selecting compounds. This result indicates that the binding site for the AC ring partially overlaps the Q_L site, as previously found for DMBQ and substituted phenols (*5, 6*).

Inhibition of Wild-Type Cytochrome bd—The inhibitory potencies of the same set of AC analogues were examined in the oxidation of Q_1H_2 by the purified wild-type cytochrome *bd* (Table I). Removal of the methyl group from the position R_1 (*i.e.*, AC0-10 *vs.* AC1-10 and AC0-11 *vs.* AC1-11) resulted in similar decrease in the inhibitory potencies for cytochrome *bd*. However, in contrast to cytochrome *bo*, the bulkiness of the R_1 substituent (AC1-11 *vs.* AC2-11, AC3-11, and AC4-11) did not affect the inhibitory potency. This indicates that the binding pocket for the AC alkyl chain of cytochrome *bd* is significantly larger than that of cytochrome *bo* and that the quinolone ring of AC mainly determines the binding affinity to the enzyme. Meunier *et al.* (*13*) reported that the removal of a hydroxy group from the quinolone ring of AC (*i.e.*, aurachin D) decreases the inhibitory potency by two orders of magnitude in cytochrome *bo*, but not in cytochrome *bd*. We confirmed this phenomenon using AC10-1 and AC1-10 (data not shown), indicating the difference in a binding pocket surrounding the =NOH moiety between the two ubiquinol oxidases. This feature may be related to stabilization of a ubisemiquinone radical at the ubiquinol oxidation site of cytochrome *bd* (*22*) and to the sequential one-electron transfer from the site to low-spin heme b_{558}.

Conclusion—We carried out structure-activity studies on the AC analogues, the most potent competitive inhibitors of the *E. coli* cytochromes *bo* and *bd* (*13*), and showed that the 3-methyl group increases the inhibitory potency of the 2-*n*-decy and 2-*n*-undecyl AC analogues in both enzymes, even though they are structurally unrelated (*1*). This effect can be ascribed to the local steric congestion around the 3-methyl group. We also showed that the binding pocket for the alkyl tails of the AC analogues is larger in cytochrome *bd* than in cytochrome *bo*, although the previous studies (*6, 18*) did not identify significant structural differences in the Q_L site. In contrast to cytochrome *bo*, which requires a tightly bound Q_8 for fascile oxidation of ubiquinols at the Q_L site and for the one-electron transfer to the metal center (*7-9*), a molecular mechanism of the substrate oxidation by cytochrome *bd* is poorly understood. Detailed structure-function studies on cytochrome *bd* using the AC analogues may provide a clue to understanding the unique mechanism operative in cytochrome *bd*.

We would like to thank R.B. Gennis for the *E. coli* strains GO103 and GR84N/pNG2 and sending us a manuscript prior to publication.

REFERENCES

1. 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
2. Puustinen, A., Finel, M., Haltia, T., Gennis, R.B., and Wikström, M. (1991) Properties of the two terminal oxidases of *Escherichia coli*. *Biochemistry* **30**, 3936-3942
3. Welter, R., Gu, L.-Q., Yu, L., Yu, C.-A., Rumbley, J., and Gennis, R.B. (1994) Identification of the ubiquinone-binding site in the cytochrome bo_3-ubiquinol oxidase of *Escherichia coli*. *J. Biol. Chem.* **269**, 28834-28838
4. Tsatsos, P.H., Raynolds, K., Nickels, E.F., He, D.-Y., Yu, C.-A., and Gennis, R.B. (1998) Using matrix assisted laser desorption ionization mass spectroscopy to map the quinol binding site of cytochrome bo_3 from *Escherichia coli*. *Biochemistry* **37**, 9884-9888
5. Sato-Watanabe, M., Mogi, T., Sakamoto, K., Miyoshi, H., and Anraku, Y. (1998) Isolation and characterizations of quinone analogue-resistant mutants of *bo*-type ubiquinol oxidase from *Escherichia coli*. *Biochemistry* **37**, 12744-12752
6. Sato-Watanabe, M., Mogi, T., Miyoshi, H., Iwamura, H., Matsushita, K., Adachi, O., and Anraku, Y. (1994) Structure-function studies on the ubiquinol oxidation site of the cytochrome *bo* complex from *Escherichia coli* using *p*-benzoquinones and substituted phenols. *J. Biol. Chem.* **269**, 28899-28907
7. 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
8. Sato-Watanabe, M., Itoh, S., Mogi, T., Matsuura, K., Miyoshi, H., and Anraku, Y. (1995) Stabilization of a semiquinone radical at the high affinity quinone binding site of the *Escherichia coli* *bo*-type ubiquinol oxidase. *FEBS Lett.* **374**, 265-269
9. Sato-Watanabe, M., Mogi, T., Miyoshi, H., and Anraku, Y. (1998) Characterization and functional role of the Q_H site of *bo*-type ubiquinol oxidase from *Escherichia coli*. *Biochemistry* **37**, 5356-5361
10. Svensson-Ek, M., Thomas, J.W., Gennis, R.B., Nilsson, T., and Brzezinski, P. (1996) Kinetics of electron and proton transfer during the reaction of wild type and helix VI mutants of cytochrome bo_3 with oxygen. *Biochemistry* **35**, 13673-13680
11. Puustinen, A., Verkhovsky, M.I., Morgan, J.E., Belevich, N.P., and Wikström, M. (1996) Reaction of the *Escherichia coli* quinol oxidase cytochrome bo_3 with dioxygen: The role of a bound ubiquinone molecule. *Proc. Natl. Acad. Sci. USA* **93**, 1545-1548
12. Sato-Watanabe, M., Mogi, T., Miyoshi, H., and Anraku, Y. (1998) Cooperation of two quinone binding sites in the oxidation of substrates by cytochrome *bo* in *Oxygen Homeostasis and Its Dynamics* (Ishimura, Y., Shimada, H., and Suematsu, M., eds.), pp. 24-32, Springer-Verlag, Tokyo
13. Meunier, B., Madgwick, S.A., Reil, E., Oettmeier, W., and Rich, P.R. (1995) New inhibitors of the quinol oxidation sites of bacterial cytochromes *bo* and *bd*. *Biochemistry* **34**, 1076-1083
14. Kunze, B., Höfle, G., and Reichenbach, H. (1987) The aurachins, new quinoline antibiotics from myxobacteria: Production, physicochemical and biological properties. *J. Antibiot.* **40**, 258-265
15. Tsubaki, M., Mogi, T., Anraku, Y., and Hori, H. (1993) Structure of heme-copper binuclear center of the cytochrome *bo* complex of *Escherichia coli*: EPR and Fourier-transform infrared spectroscopic studies. *Biochemistry* **32**, 6065-6072
16. Green, G.N., Kranz, J.E., and Gennis, R.B. (1984) Cloning of the *cyd* locus coding for the cytochrome *d* complex of *Escherichia coli*. *Gene* **32**, 99-106
17. 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
18. 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. *J. Biol. Chem.* **271**, 29897-29902
19. 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
20. Sakamoto, K., Miyoshi, H., Matsushita, K., Nakagawa, M., Ikeda, J., Ohshima, M., Adachi, O., Akagi, T., and Iwamura, H. (1996) Comparison of the structural features of ubiquinone reduction sites between glucose dehydrogenase in *Escherichia coli* and bovine heart mitochondrial complex I. *Eur. J. Biochem.* **237**, 128-135
21. Zheng, M. and Dismukes, G.C. (1996) The conformation of the isoprenyl chain relative to the semiquinone head in the primary electron acceptor (Q_A) of higher plant PS II (plastosemiquinone) differs from that in bacterial reaction centers (ubisemiquinone or menasemiquinone) by *ca.* 90°. *Biochemistry* **35**, 8955-8963
22. Hastings, 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