Characterization of the Ubiquinone Reduction Site of Mitochondrial Complex I Using Bulky Synthetic Ubiquinones†

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ABSTRACT: A wide variety of alkyl derivatives of Q_2 (6-geranyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone) and DB (6-*n*-decyl-2,3-dimethoxy-5-methyl-1,4-benzoquinone), in which methoxy groups of the 2- and/or 3-positions of the quinone ring were replaced by other bulky alkoxy groups from ethoxy to butoxy, were prepared by novel synthetic procedures. Electron-accepting activities of the bulky quinones were investigated with bovine heart mitochondrial complex I and its counterpart of *Paracoccus denitrificans* (NDH-1) to elucidate structural and functional features of the quinone reduction site of the enzymes. The bulky quinone analogues served as sufficient electron acceptors from the physiological quinone reduction site of bovine complex I. Considering the very poor activities of even the ethoxy derivatives as substrates for other respiratory enzymes such as mitochondrial complexes II and III [He, D. Y., Gu, L. Q., Yu, L., and Yu, C. A. (1994) *Biochemistry* 33, 880−884], this result indicated that the quinone reduction site of bovine complex I is spacious enough to accommodate bulky exogenous substrates. In contrast to bovine complex I, bulky quinone analogues served as poor electron acceptors with *Paracoccus* NDH-1. These observations indicated that bovine complex I recognizes the substrate structure with poor specificity. The substituent effects in the 2- and 3-positions of the quinone ring on the electron-transfer activity with bovine complex I differed significantly between Q_2 and DB series despite having the same total number of carbon atoms in the side chain. The inhibitory effect involving Q_2 due to its geranyl side chain was markedly diminished by structural modifications of the quinone ring moiety. These findings indicate that the side chain plays a specific role in the redox reaction and that the quinone ring and side-chain moieties contribute interdependently to binding interaction. Moreover, structural dependency of the proton-pumping activity of the quinone analogues was comparable to that of the electron-transfer activity with bovine complex I, indicating that the mechanism of redox-driven proton-pumping does not differ depending upon the substrate structure.

Mitochondrial NADH-ubiquinone (Q) oxidoreductase (complex I)[1] is a large enzyme that catalyzes the oxidation of NADH by Q coupled to proton translocation across the inner membrane (*1*, *2*). Despite recent progress in structural studies of the mammalian (*2*) and *Neurospora crassa* (*3*) enzymes and their simpler bacterial counterparts (*4*), little is known about the pathway of the electron(s) and the mechanism of proton-pumping due to the enormous complexity of this enzyme. Nevertheless, a consensus on the ubisemiquinone as the obligatory intermediate during the electron-transfer from NADH to Q pool has been reached (*5*, *6*). There seems to be general agreement on the proton-pumping stoichiometry of $4H^+/2e^-$ (*7*−*9*). In addition, several studies on specific inhibitors of bovine complex I (*10*, *11*) have suggested the existence of more than one Q reduction site in the enzyme, while the number of sites remains to be defined precisely.

To elucidate the structural and functional properties of the Q reduction site in bovine heart mitochondrial complex I, understanding the structural requirements for Q in the redox reaction is important. A molecular orbital calculation study demonstrated that conformation of the methoxy groups in the 2- and/or 3-positions of the quinone ring affects electrical potentials of the oxidized form of quinone or semiquinone radical through conformer interconversion (*12*). Therefore, binding manner of the methoxy groups to the protein environment is expected to impose a significant influence on the Q redox reaction. This view affords a strategical basis for structure/electron-transfer activity analyses of a series of Q analogues.

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[1] Abbreviations: complex I, mitochondrial NADH-ubiquinone oxidoreductase; complex II, succinate-ubiquinone oxidoreductase; complex III, ubiquinol-cytochrome *c* oxidoreductase; DB, 6-*n*-decyl-5-methyl-2,3-dimethoxy-1,4-benzoquinone; NDH-1, bacterial proton-pumping NADH-ubiquinone oxidoreductase; PB, 6-*n*-pentyl-5-methyl-2,3-dimethoxy-1,4-benzoquinone; Q, oxidized form of ubiquinone; QH_2, reduced form of ubiquinone; Q_0, 2,3-dimethoxy-5-methyl-1,4-benzoquinone; Q_1, ubiquinone-1; Q_2, ubiquinone-2; Q_{10}, endogenous ubiquinone-10.

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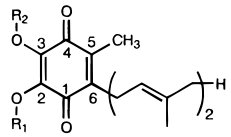
Structure/electron-transfer activity studies of various synthetic Q derivatives, in which both the quinone ring and the side-chain moieties are modified, have been performed by Yu and colleagues with bovine heart mitochondrial complexes II and III (*13*−*15*). Structural modifications of Q derivatives were limited to only the side-chain moiety in previous structure/activity studies of complex I (*16*−*20*). On the basis of kinetic studies of Q analogues with various short side chains, Lenaz and colleagues (*16, 19*) showed that the endogenous Q pool is not required for electron-transfer to exogenous short-chain Q analogues, and that Q_1 (ubiquinone-1) is the best exogenous substrate for bovine complex I. On the other hand, Degli Esposti and colleagues (*18, 20*) suggested that the redox-driven proton-pumping efficiencies of exogenous Qs vary depending upon hydrophobicity of the side chain. It is noteworthy that both research groups reported that Q_2 (ubiquinone-2) is a poor substrate despite its native isoprene tail structure (i.e., a geranyl group) (*18, 19*), although the reason for this remains to be elucidated. In these previous studies, however, the effect of structural modification in the quinone ring moiety on the electron-accepting activity and its alteration depending upon the side-chain structure were not defined because of limited structural variation of the Q analogues used.

We have recently developed synthetic procedures which enable selective transformation of either the 2- or 3-methoxy group to the corresponding ethoxy group (*21, 22*). Then, using 2- or 3-monoethoxy and 2,3-diethoxy derivatives of Q_2 and DB (2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone), we showed that these bulky Q analogues can retain electron-accepting ability from the physiological site in bovine complex I (*11, 21*). As these ethoxy derivatives are very poor substrates for mitochondrial complexes II and III (*14*), this finding suggests that the Q reduction site in complex I is spacious enough to accommodate bulky exogenous Q analogues. This is supported by the results of structure/activity studies on complex I inhibitors such as capsaicins (*23*), rotenones (*24*), and *N*-methylpyridiniums (*11, 25*), which revealed that the essential structural factors of these inhibitors for the inhibitory action are rather ambiguous and that a variety of structurally different derivatives of the inhibitors bind to the Q reduction site in a dissimilar manner depending upon structural modifications.

To further characterize the Q reduction site in bovine complex I, in particular to determine how much bulky Q derivatives can retain the electron-accepting activity and also elucidate the effects of side-chain structure on recognition of the quinone ring moiety by the enzyme, we synthesized further bulky Q_2 and DB analogues (Figure 1) and investigated their electron-accepting activities. As the proton-pumping machinery would be close to the Q binding site of the enzyme (*26*−*28*), we investigated the correlation between electron-accepting and proton-pumping activities of a variety of Q analogues to gain insight into the mechanism of redox-driven proton-pumping in complex I. Moreover, since proton-pumping NADH-Q oxidoreductase (NDH-1) of *Paracoccus denitrificans* is thought to be a useful system for studying the structure and mechanism of action of mitochondrial complex I (*29*), we compared profiles of the structure/activity relationship of the Q analogues between bovine complex I and *Paracoccus* NDH-1. This report is the first detailed structure/activity analysis of Q analogues with proton-pumping NADH-Q oxidoreductase in which Q structures were widely and systematically modified.



Q_2 : $R_1 = R_2 = CH_3$

2-EtO-Q_2 : $R_1 = C_2H_5$, $R_2 = CH_3$

3-EtO-Q_2 : $R_1 = CH_3$, $R_2 = C_2H_5$

2,3-(EtO)$_2$-Q_2 : $R_1 = R_2 = C_2H_5$

2-*n*-PrO-Q_2 : $R_1 = n\text{-}C_3H_7$, $R_2 = CH_3$

2-*i*-PrO-Q_2 : $R_1 = i\text{-}C_3H_7$, $R_2 = CH_3$

3-*n*-PrO-Q_2 : $R_1 = CH_3$, $R_2 = n\text{-}C_3H_5$

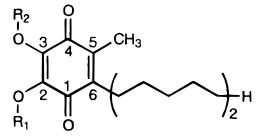
3-*i*-PrO-Q_2 : $R_1 = CH_3$, $R_2 = i\text{-}C_3H_7$

2,3-(*n*-PrO)$_2$-Q_2 : $R_1 = R_2 = n\text{-}C_3H_7$

2-*n*-BuO-Q_2 : $R_1 = n\text{-}C_4H_9$, $R_2 = CH_3$

3-*n*-BuO-Q_2 : $R_1 = CH_3$, $R_2 = n\text{-}C_4H_9$

3-*i*-BuO-Q_2 : $R_1 = CH_3$, $R_2 = i\text{-}C_4H_9$



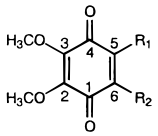
DB : $R_1 = R_2 = CH_3$

2-EtO-DB : $R_1 = C_2H_5$, $R_2 = CH_3$

3-EtO-DB : $R_1 = CH_3$, $R_2 = C_2H_5$

2,3-(EtO)$_2$-DB : $R_1 = R_2 = C_2H_5$

3-*n*-PrO-DB : $R_1 = CH_3$, $R_2 = n\text{-}C_3H_5$



PB : $R_1 = CH_3$, $R_2 = C_5H_{11}$

5-H-PB : $R_1 = H$, $R_2 = C_6H_{13}$

5-H-6-(α-Me)-PB : $R_1 = H$, $R_2 = \overset{\alpha}{C}H(CH_3)(CH_2)_3CH_3$

5-H-6-(β-Me)-PB : $R_1 = H$, $R_2 = CH_2\overset{\beta}{C}H(CH_3)(CH_2)_2CH_3$

5-H-6-(γ-Me)-PB : $R_1 = H$, $R_2 = (CH_2)_2\overset{\gamma}{C}H(CH_3)CH_2CH_3$

5-H-6-(δ-Me)-PB : $R_1 = H$, $R_2 = (CH_2)_3\overset{\delta}{C}H(CH_3)CH_3$

FIGURE 1: Structure of Q_2, DB, and PB analogues studied in this study.

EXPERIMENTAL PROCEDURES

Materials. Antimycin A, nigericin, and oligomycin were purchased from Sigma. Oxonol VI was obtained from Molecular Probes. MOA-stilbene was provided by Aburahi Laboratories, Shionogi Co., Ltd. (Shiga, Japan). Q_1 and Q_2 were generous gifts from Eisai Co. (Tokyo, Japan). Piericidin A was generously provided by Dr. Shigeo Yoshida (RIKEN, Japan). The ethoxy derivatives of Q_2 and DB and PB derivatives were from previous samples (*21, 22*). Other chemicals were commercial products of analytical grade.

Synthesis. Direct replacement of the methoxy groups of Q_2 (or DB) by another alkoxide gives a mixture of two monoalkoxy derivatives (i.e., 2-methoxy-3-alkoxy and 2-alkoxy-3-methoxy derivatives). Since these two derivatives cannot be separated due to their similar chromatographic natures (*14, 21*), the two derivatives have to be synthesized

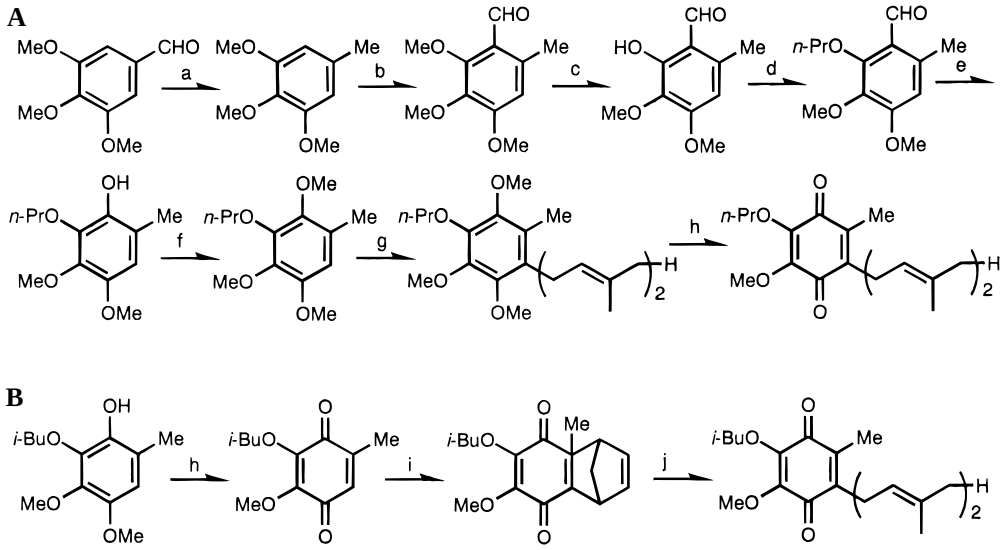


FIGURE 2: Synthetic procedures. (a) H_2NNH_2, KOH in ethyleneglycol, (b) *N*-methylformanilide, $POCl_3$, (c) $AlCl_3$ in benzene, (d) *n*-propyliodide, K_2CO_3 in acetone, (e) (i) *m*-chloroperoxylbenzoic acid in ethyl acetate, (ii) NaOH in dioxane/H_2O, (f) Me_2SO_4, (g) (i) *n*-BuLi/*N*,*N*,*N*′*N*′-tetramethylethylenediamine in hexane under N_2 at 0 °C, (ii) geranylbromide, CuI (cat) in tetrahydrofurane at −78 °C, (h) $Ce(NH_4)_2(NO_3)_6$ in CH_3CN/H_2O under N_2 at 0 °C, (i) cyclopentadiene/CH_2Cl_2, (j) (i) KOBu-*t*/Et_2O at −40 °C and geranylbromide at −70 °C to room temperature, (ii) reflux in toluene at 80 °C for 3 h.

independently by different synthetic routes. We have developed synthetic methods which enable selective preparation of two monoethoxy Q analogues (2-methoxy-3-ethoxy and 2-ethoxy-3-methoxy derivatives) (*21*). However, since the previous method for synthesis of 2-methoxy-3-ethoxy derivative is limited to the preparation of this ethoxy compound and is not available for other 2-methoxy-3-alkoxy compounds, we developed new synthetic methods for a variety of 2-methoxy-3-alkoxy-Q analogues as shown in Figure 2. All synthetic compounds were characterized by ^{1}H NMR (Bruker ARX-300) and elemental analyses for C and H within an error of ±0.3%.

Synthesis of 2-Monoalkoxy Analogues of Q_2. 2-Monoalkoxy analogues of Q_2 (2-*n*-PrO-Q_2, 2-*i*-PrO-Q_2 and 2-*n*-BuO-Q_2) were synthesized by introducing a geranyl group (*22*) into corresponding 2-alkoxy-3-methoxy-5-methyl-1,4-benzoquinones, which were prepared according to the previous method for preparation of 2-EtO-Q_2 (scheme 1 in ref *21*), but using corresponding alkylbromide in the presence of a catalytic amount of KI in place of diethyl sulfate. 2-*n*-PrO-Q_2; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.00 (t, J = 7.4 Hz, 3H, $CH_2C\mathbf{H}_3$), 1.57 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 1.73 (s, 3H, CH_3), 1.73−1.84 (m, 2H, CH_2), 2.00 (s, 3H, $ArCH_3$), 1.94−2.05 (m, 4H, $2CH_2$), 3.18 (d, J = 11.6 Hz, 2H, $ArCH_2$), 3.98 (s, 3H, OCH_3), 4.12 (t, J = 11.2 Hz, 2H, $OC\mathbf{H}_2CH_3$), 4.98 (m, 1H, −CH=), 5.03 (m, 1H, −CH=). Anal. Calcd for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.82; H, 8.85. 2-*i*-PrO-Q_2; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.31 [d, J = 6.9 Hz, 6H, $CH(C\mathbf{H}_3)_2$], 1.59 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 1.73 (s, 3H, CH_3), 2.00 (s, 3H, $ArCH_3$), 1.94−2.08 (m, 4H, $2CH_2$), 3.18 (d, J = 11.6 Hz, 2H, $ArCH_2$−), 3.99 (s, 3H, OCH_3), 4.73 [m, 1H, $OC\mathbf{H}(CH_3)_2$], 4.93 (m, 1H, −CH=), 5.03 (m, 1H, −CH=). Anal. Calcd for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.86; H, 8.90. 2-*n*-BuO-Q_2; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.96 (t, J = 7.4 Hz, 3H, $CH_2C\mathbf{H}_3$), 1.46 (m, 2H, CH_2), 1.57 (s, 3H, CH_3), 1.63−1.78 (m, 2H, CH_2), 1.65 (s, 3H, CH_3), 1.70 (br s, 3H, CH_3), 1.94−2.06 (m, 4H, $2CH_2$), 2.01 (s, 3H, $ArCH_3$), 3.18 (d, J = 7.0 Hz, 2H, $ArCH_2$), 3.99 (s, 3H, OCH_3), 4.16 (t, J = 6.7 Hz, 2H, OCH_2), 4.94 (m, 1H, −CH=), 5.04 (m, 1H, −CH=). Anal. Calcd for $C_{22}H_{32}O_4$: C, 73.30; H, 8.95. Found: C, 73.09; H, 9.24.

Synthesis of 3-n-Propoxy-Q_2 (Figure 2A). To a solution of commercially available 3,4,5-trimethoxybenzaldehyde (25.0 g, 127.4 mmol) in 120 mL of ethyleneglycol were added hydrazinemonohydrate (19.1 g, 382.2 mmol) and KOH (21.4 g, 382.2 mmol), and the reaction mixture was stirred at 150 °C for 3 h. The mixture was extracted with benzene and washed with brine. The organic phase was dried over $MgSO_4$ and evaporated to give 1,2,3-trimethoxy-5-methylbenzene in quantitative yield.

To a mixture of 1,2,3-trimethoxy-5-methylbenzene (23.0 g, 126.1 mmol) and *N*-methylformanilide (25.6 g, 189.2 mmol) was added phosphorousoxychloride (29.0 g, 189.2 mmol) dropwise at room temperature, and the mixture was stirred for 1 day and heated at 40 °C for 1 h. The reaction mixture was extracted by Et_2O and washed with brine. The organic phase was dried over $MgSO_4$ and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 1:4) to give 2,3,4-trimethoxy-6-methylbenzaldehyde in a 79% yield.

To a solution of 2,3,4-trimethoxy-6-methylbenzaldehyde (19.0 g, 90.4 mmol) in 200 mL of benzene was added aluminum chloride (13.3 g, 99.4 mmol), and the reaction mixture was stirred at 50 °C for 8 h. To this mixture was added 1 L of water, 400 mL of benzene, and 50 mL of 12 M HCl, and the mixture was stirred for 8 h. The reaction mixture was extracted with Et_2O, and then the organic phase was extracted with 5% aqueous NaOH. After acidification by addition of 12 M HCl, the aqueous phase was extracted with Et_2O and washed with brine. The organic phase was dried over $MgSO_4$ and evaporated to give 2-hydroxy-3,4-dimethoxy-6-methylbenzaldehyde in a 76% yield.

To a solution of 2-hydroxy-3,4-dimethoxy-6-methylbenzaldehyde (5.0 g, 29.0 mmol) in 100 mL of acetone were added *n*-propyliodide (6.0 g, 34.8 mmol) and K_2CO_3 (8.1 g, 58.0 mmol), and the reaction mixture was refluxed for 8 h. After acidification by addition of 1 M HCl, the mixture was

extracted with Et_2O and washed with brine. The organic phase was dried over $MgSO_4$ and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 1:9) to give 3,4-dimethoxy-6-methyl-2-*n*-propoxybenzaldehyde (3.3 g) in a 55% yield.

To a solution of 3,4-dimethoxy-6-methyl-2-*n*-propoxybenzaldehyde (3.3 g, 13.9 mmol) in 200 mL of ethyl acetate was added *m*-chloroperoxybenzoic acid (3.3 g, 15.3 mmol), and the reaction mixture was stirred at room temperature for 16 h. Activated potassium fluoride (2.2 g) was added, and the mixture was stirred for 1 h. The mixture was filtrated through Celite layer, and the solvent was removed in vacuo to give crude 1-formyloxy-3,4-dimethoxy-6-methyl-2-*n*-propoxybenzene. To the crude product were added 12 mL of dioxane and 12 mL of 3 M NaOH, and the reaction mixture was stirred for 1 h. To this mixture was added 3.5 mL dimethyl sulfate followed by stirring for 8 h. The reaction mixture was extracted with Et_2O and washed with brine. The organic phase was dried over $MgSO_4$ and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 1:20) to give 1,2,4-trimethoxy-5-methyl-3-*n*-propoxybenzene (2.1 g) in a 63% yield.

To a solution of 1,2,4-trimethoxy-5-methyl-3-*n*-propoxybenzene (2.1 g, 8.3 mmol) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (1.7 g) in 20 mL of hexane was added 7.3 mL of 1.6 M *n*-butyllithium (11.6 mmol) slowly at 0 °C under N_2, and the mixture was stirred for 30 min. This reaction mixture was supplemented with 60 mL of THF and cooled to −78 °C. To this mixture was added CuI (0.08 g, 0.4 mmol), and geranylbromide (1.8 g, 8.3 mmol) in 20 mL of THF was then added slowly. The reaction mixture was slowly warmed to 0 °C and stirred for 2 h. Then, the reaction was quenched with saturated aqueous NH_4Cl. The mixture was extracted with ethyl acetate and washed with brine. The organic phase was dried over $MgSO_4$ and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetae/hexane = 1:20) to give 1-geranyl-2,3,5-trimethoxy-6-methyl-4-*n*-propylbenzene (1.0 g) in a 32% yield.

To a solution of 1-geranyl-2,3,5-trimethoxy-6-methyl-4-*n*-propylbenzene (1.0 g, 2.6 mmol) and 2,6-pyridinedicarboxylic acid (1.1 g, 6.5 mmol) in 15 mL of acetonitrile:water mixture (7:3) at 0 °C under N_2 was added dropwise a cooled (0 °C) solution of ceric ammonium nitrate (3.5 g, 6.5 mol) in 15 mL of acetonitrile:water mixture (1:1) and stirred for 30 min at 0 °C and then for 10 min at room temperature. The reaction mixture was extracted with Et_2O and washed with brine. The organic phase was dried over $MgSO_4$ and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 1:20) to give 3-*n*-propoxy-Q_2 (2-methoxy-5-methyl-3-*n*-propoxy-1,4-benzoquinone) in a 50% yield. 3-*n*-PrO-Q_2; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.00 (t, J = 7.4 Hz, 3H, CH_3), 1.57 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 1.72 (s, 3H, $-CH_3$), 1.73−1.81 (m, 2H, $OCH_2C\mathbf{H}_2$), 2.00 (s, 3H, $ArCH_3$), 1.95−2.06 (m, 4H, $2CH_2$), 3.18 (d, J = 11.7 Hz, 2H, CH_2), 4.00 (s, 3H, OCH_3), 4.11 (t, J = 11.2 Hz, 2H, $OC\mathbf{H}_2CH_3$), 4.92 (m, 1H, −CH=), 5.03 (m, 1H, −CH=). Anal. Calcd for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.64; H, 8.66.

3-*i*-PrO-Q_2 was synthesized by the same method using *i*-propylbromide (plus KI) in place of *n*-propyliodide in reaction step d in Figure 2A. 3-*i*-PrO-Q2; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.32 [d, J = 10.3 Hz, 6H, $CH(C\mathbf{H}_3)_2$], 1.58 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 1.73 (s, 3H, CH_3), 2.00 (s, 3H, $ArCH_3$), 1.94−2.06 (m, 4H, $2CH_2$), 3.18 (d, J = 11.7 Hz, 2H, CH_2), 4.00 (s, 3H, OCH_3), 4.71 [m, 1H, $OC\mathbf{H}(CH_3)_2$], 4.74 (m, 1H, −CH=), 5.03 (m, 1H, −CH=). Anal. Calcd for $C_{21}H_{30}O_4$: C, 72.80; H, 8.73. Found: C, 72.61; H, 8.73.

Synthesis of 3-i-butoxy-Q₂ (Figure 2B). The above method for the preparation of 3-*n*-propoxy-Q_2 was not applied to that of 3-*i*-butoxy-Q_2 because of low yield in methylation (reaction step f) of 1-*i*-butoxy-2-hydroxy-5,6-dimethoxy-3-methylbenzene, probably due to steric hindrance. Therefore, the procedure shown in Figure 2B was used. 1-*i*-Butoxy-2-hydroxy-5,6-dimethoxy-3-methylbenzene prepared according to the above method was oxidized by ceric ammonium nitrate to give 3-*i*-butoxy-2-methoxy-5-methyl-1,4-benzoquinone, as described above. Then, introduction of a geranyl group into this quinone was carried out through Diels−Alder-type adduct as reported previously (*22*). 3-*i*-BuO-Q_2; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.00 [d, J = 6.7 Hz, 6H, $CH(C\mathbf{H}_3)_2$], 1.57 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 1.73 (s, 3H, CH_3), 1.97−2.12 [m, 5H, $2CH_2$, $C\mathbf{H}(CH_3)_2$], 2.00 (s, 3H, $ArCH_3$), 3.18 (d, J = 7.0 Hz, 2H, $ArCH_2$), 3.91 (d, J = 6.6 Hz, 2H, OCH_2), 3.98 (s, 3H, CH_3), 4.93 (m, 1H, −CH=), 5.03 (m, 1H, −CH=). Anal. Calcd for $C_{22}H_{32}O_4$: C, 73.30; H, 8.95. Found: C, 73.06; H, 8.99.

3-*n*-BuO-Q_2 and 3-*n*-PrO-DB were synthesized by the same method. 3-*n*-BuO-Q_2; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.96 (t, J = 7.3 Hz, 3H, $CH_2C\mathbf{H}_3$), 1.47 (m, 2H, $-CH_2-$), 1.63 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 1.70−1.78 (m, 2H, $-CH_2-$), 1.73 (s, 3H, CH_3), 1.94−2.06 (m, 4H, $2CH_2$), 2.00 (s, 3H, $ArCH_3$), 3.20 (d, J = 7.0 Hz, 2H, $ArCH_2$), 3.99 (s, 3H, OCH_3), 4.14 (t, J = 6.7 Hz, 2H, OCH_2), 4.93 (m, 1H, −CH=), 5.03 (m, 1H, −CH=). Anal. Calcd for $C_{22}H_{32}O_4$: C, 73.30; H, 8.95. Found: C, 73.19; H, 9.14. 3-*n*-PrO-DB; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.88 (t, J = 6.7 Hz, 3H, $CH_2C\mathbf{H}_3$), 1.00 (t, J = 7.4 Hz, 3H, $CH_2C\mathbf{H}_3$), 1.20−1.45 (m, 16H, $-CH_2-$), 1.77 (m, 2H, $C\mathbf{H}_2CH_3$), 2.00 (s, 3H, $ArCH_3$), 2.44 (m, 2H, $ArCH_2$), 4.00 (s, 3H, OCH_3), 4.11 (t, J = 6.7 Hz, 2H, OCH_2). Anal. Calcd for $C_{21}H_{34}O_4$: C, 71.96; H, 9.78. Found: C, 71.78; H, 9.92.

Synthesis of 2,3-(n-PrO)₂-Q₂. 6,7-Dichloro-1,4,4a,8a-tetrahydro-4a-methyl-1,4-methanonaphthalene-5,8-dione (*30*) and sodium propoxide were reacted in propanol to give 1,4,-4a,8a-tetrahydro-6,7-di-*n*-propoxy-4a-methyl-1,4-methanonaphthalene-5,8-dione. Introduction of a geranyl group into this compound and transformation to 2,3-(*n*-PrO)$_2$-Q_2 were carried out by the previously reported method (*22*). 2,3-(*n*-PrO)$_2$-Q_2; ^{1}H NMR ($CDCl_3$, 300 MHz): δ 1.00 (t, J = 8.0 Hz, 6H, $CH_2C\mathbf{H}_3$), 1.57 (s, 3H, CH_3), 1.65 (s, 3H, CH_3), 1.73 (s, 3H, CH_3), 1.67−1.82 (m, 4H, $OCH_2C\mathbf{H}_2$), 2.00 (s, 3H, $ArCH_3$), 1.94−2.06 (m, 4H, $2CH_2$), 3.18 (d, J = 7.0 Hz, 2H, $ArCH_2$), 4.12 (m, 4H, $OC\mathbf{H}_2CH_2$), 4.91−4.96 (m, 1H, −CH=), 5.01−5.05 (m, 1H, −CH=). Although the elemental analysis was not done because of limited product, no contamination was determined by ^{1}H NMR.

Methods. Bovine heart submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi (*31*) and stored in a buffer containing 0.25 M sucrose and 10 mM Tris/HCl (pH 7.4) at −78 °C. NADH-Q oxidoreductase activity was measured as the rate of NADH oxidation at 30 °C with a Shimadzu UV-3000 at 340 nm

($\epsilon = 6.2$ mM^{-1} cm^{-1}). The reaction medium contained 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM KCN, 0.2 μM antimycin A, and 0.2 μM MOA-stilbene, and the final mitochondrial protein concentration was 30 μg/mL. The reaction was started by adding 50 μM NADH after SMP were equilibrated with Q analogues. The experimental conditions for investigation of product inhibition are described in detail in the figure legends. The reduced forms of Q analogues were prepared by the method of Rieske (*32*).

The membrane potential generated by NADH oxidation was measured at 30 °C by following the absorbance changes of oxonol VI at 630−601 nm (*18*) with Shimadzu UV-3000 in a dual wavelengths mode. The reaction medium consisted of 50 mM Tricine/NaOH (pH 7.4), 0.2 M sucrose, 3 mM KCN, 2.5 mM $MgCl_2$, 10 nM nigericin, 0.3 μM oligomycin, 0.4 μM antimycin A, 0.1 μM MOA-stilbene, and 3 μM oxonol VI. The final mitochondrial protein concentration was 110 μg/mL. The reaction was started by adding 200 μM NADH after SMP were equilibrated with Q analogues.

P. denitrificans membranes were prepared as described previously (*33*). NADH-Q oxidoreductase activity was measured at 30 °C as described above. The reaction medium contained 50 mM potassium phosphate (pH 7.4), 1 mM EDTA, and 10 mM KCN.

RESULTS

Structure/Electron-Accepting Activity Relationship of Q Analogues with Bovine Complex I. The kinetic parameters in terms of apparent K_m and V_{max} values of Q_2, DB, and PB analogues are listed in Table 1. Redox potentials of these Qs are almost identical (*14*, *15*). Although the electron-transfer activities of some of DB and PB analogues have been studied previously (*21*), they were reexamined in the present study under the same experimental conditions to avoid possible experimental differences due to different SMP preparations and to compare their activities with those determined with *Paracoccus* NDH-1. All of the Q analogues accepted electrons from the physiological site in complex I irrespective of the efficiency as an electron acceptor because the electron-transfer activities of these compounds were almost completely (>90%) inhibited by 0.1 μM piericidin A or rotenone (data not shown). In a preliminary experiment, we also confirmed that the electron-transfer activity of a very hydrophilic Q_0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone) was inhibited just by about 30% under the same experimental conditions. The large inhibitor-insensitive activity of Q_0 is due to electron flow at a site upstream of the physiological site (*7*, *19*).

The ethoxy analogues of Q_2 (2-EtO-Q_2, 3-EtO-Q_2, and 2,3-$(EtO)_2$-Q_2) appeared to be better electron acceptors than Q_2, with V_{max} values about twice that of Q_2. This result indicated not only that even bulky analogues can serve as efficient electron acceptors, but also that the inhibition involving Q_2 reported previously (*19*) was markedly reduced, as described in the following section. In contrast, the V_{max} values of 2-EtO-DB and 2,3-$(EtO)_2$-DB were decreased considerably compared to that of DB. Thus, the structural dependency of the ethoxy analogues of DB differs from that of Q_2 analogues. This indicated that the manner of binding of the quinone ring moiety to the enzyme is affected by the side-chain structure (geranyl versus *n*-decyl groups), although the total number of carbon atoms is identical between the two series. It is therefore likely that the side chain plays a specific role in the binding interaction depending upon its structural specificity, rather than simply increasing the hydrophobicity of the molecule.

Table 1: Summary of Kinetic Parameters of Synthetic Q Analogues[a]

	bovine complex I		*Paracoccus* NDH-1	
quinones	K_m (μM)	V_{max} (μmol/min/mg)	K_m (μM)	V_{max} (μmol/min/mg)
Q_2	2	0.21	5	0.21
2-EtO-Q_2	4	0.45	8	0.22
3-EtO-Q_2	4	0.43	17	0.20
2,3-$(EtO)_2$-Q_2	5	0.42	5	0.10
2-*n*-PrO-Q_2	15	0.58	2	0.08[b]
2-*i*-PrO-Q_2	6	0.28	2	0.07[b]
3-*n*-PrO-Q_2	16	0.60	3	0.10[b]
3-*i*-PrO-Q_2	10	0.36	2	0.07[b]
2,3-(*n*-PrO)$_2$-Q_2	[c]		[c]	
2-*n*-BuO-Q_2	3	0.18	[c]	
3-*n*-BuO-Q_2	2	0.20	[c]	
3-*i*-BuO-Q_2	1	0.11	[c]	
DB	6	0.45	[c]	
2-EtO-DB	4	0.32	[c]	
3-EtO-DB	6	0.41	[c]	
2,3-$(EtO)_2$-DB	9	0.28	[c]	
3-*n*-PrO-DB	10	0.31	[c]	
PB	15	0.60	121	0.88
5-H−PB	8	0.69	5	0.49
5-H-6-(α-Me)-PB	3	0.49	26	0.80
5-H-6-(*β*-Me)-PB	3	0.35	3	0.44
5-H-6-(*γ*-Me)-PB	3	0.39	6	0.47
5-H-6-(*δ*-Me)-PB	7	0.65	6	0.46

[a] The K_m and V_{max} values were determined by Lineweaver-Burk analysis. The values were averaged from at least two independent measurements. [b] The kinetic parameters were estimated by extrapolation of the linear part of the Lineweaver-Burk plot since significant substrate inhibition was observed at concentrations of added Q above ~10 μM. [c] The kinetic parameters could not be estimated due to very poor activity. The reaction rate was less than 0.03 μmol/min/mg of protein even when the concentration of the substrate was increased up to 100 μM.

Four monopropoxy Q_2 analogues possessing *n*- or *i*-propoxy groups at either the 2- or the 3-position were synthesized. The steric hindrance of the *i*-propoxy group is greater than that of *n*-propyl group despite having the same number of carbon atoms (*34*). The V_{max} value of 2-*n*-PrO-Q_2 was greater than that of 2-EtO-Q_2, whereas the K_m value was increased. The V_{max} value of 2-*i*-PrO-Q_2 was significantly smaller than that of 2-*n*-PrO-Q_2 and similar to that of Q_2. This might be because more bulky 2-*i*-propoxy group is unfavorable for redox reaction due to steric congestion arising from the binding site, but the flexible *n*-propoxy group can avoid steric congestion. A similar tendency was observed for a pair of 3-*n*-PrO-Q_2 and 3-*i*-PrO-Q_2, while the V_{max} value of 3-*i*-PrO-Q_2 was larger than that of 2-*i*-PrO-Q_2. This result suggested that the 2-position of the quinone ring is recognized more strictly than the 3-position by the enzyme. In contrast to the case of Q_2 analogues, 3-*n*-PrO-DB was a poorer electron acceptor than 3-EtO-DB. Thus, the Q_2 and DB series showed opposite effects of steric bulkiness in the 3-position on the activity. This result also indicated that recognition of the quinone ring moiety by the enzyme differs depending upon the side-chain structure.

The electron-accepting efficiencies of further bulky *n*-butoxy analogues (2-*n*-BuO-Q_2 and 3-*n*-BuO-Q_2) were much poorer than those of *n*-propoxy analogues irrespective of substitution position on the quinone ring and almost identical to that of Q_2. Although the *n*-butoxy group is also flexible, this long alkyl substituent might no longer be free from steric congestion arising from the binding environment. The V_{max} value of 3-*i*-BuO-Q_2 was significantly smaller than that of 3-*n*-BuO-Q_2, consistent with the case of the pair of 3-*i*-PrO-Q_2 and 3-*n*-PrO-Q_2. The efficiency of 2,3-(*n*-PrO)$_2$-Q_2, the most bulky substrate examined in this study, was much poorer than that of Q_2. This compound did not actually serve as an electron acceptor. Supposing that the binding environment of quinone ring moiety is formed by cavity-like structure which enables stabilization of a semiquinone radical, it is reasonable that there are limitations in bulkiness of the quinone ring moiety to fit into the cavity.

Although the above interpretation of the experimental data would be based on mixed kinetic properties if there are indeed two distinct Q reduction sites in bovine complex I (*10*, *11*), the important result is that the Q reduction site(s) is actually spacious enough to accommodate bulky exogenous Q and that recognition of the quinone ring moiety by the enzyme is significantly affected by the side-chain structure. The latter finding indicates that the quinone ring and the side-chain moieties contribute interdependently to binding interaction.

Effects of Structural Modification on Inhibitory Action Involving Q_2. The electron-accepting ability of Q_2 is much poorer than those of DB and Q_1, derivatives possessing a side chain of 10 carbon atoms and of one isoprene unit, respectively (*18*, *19*). Fato et al. (*19*) suggested that this is because the side chain of Q_2 (i.e., geranyl group) is not correctly positioned at the active site for optimal electron-transfer, although a geranyl group itself is a part of the isoprene tail of endogenous Q_{10}. Thus, the investigation of unusual action of Q_2 is interesting to obtain insight into the binding manner of short-chain Q to the enzyme.

The steady-state kinetics of NADH-Q oxidoreductase activity of complex I are consistent with a ping-pong bi-bi mechanism (*19*), whereby the enzyme is first reduced by NADH with release of NAD^+ and then reoxidized by Q with release of QH_2. The same mechanism was suggested using ferricyanide and DCIP (2,6-dichlorophenolindophenol) as electron acceptors (*35*). Since first (NADH) and second (Q) substrates bind reversibly at different sites of the enzyme, this kinetic mechanism is generally supposed to suffer product inhibition; that is, the presence of a reduced form of Q inhibits the enzyme reaction in a competitive manner against an oxidized form. As shown in Figure 3, Q_1H_2 and 3-*n*-PrO-Q_2H_2 actually inhibited NADH-Q_1 and NADH-3-*n*-PrO-Q_2 oxidoreductase activities, respectively, in a concentration-dependent manner. In these cases, the mechanism of inhibition by the reduced form of Q was revealed to be competitive against its oxidized form, as shown in the inset of Figure 3 taking Q_1H_2 as an example. In addition, if the catalytic site of exogenous Qs is identical irrespective of different types of Q, product inhibition should occur between them. Fato et al. (*19*) indeed observed competitive inhibition of NADH-Q_1 oxidoreductase activity by Q_2H_2, although they did not interpret this observation from the viewpoint of product inhibition. It is therefore reasonable to consider that

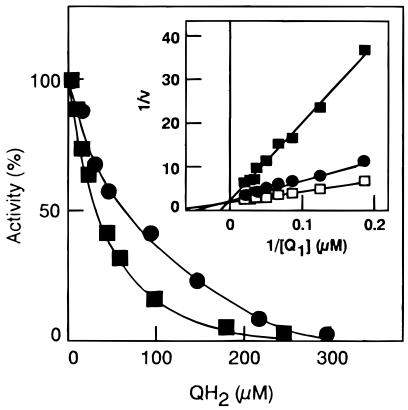


FIGURE 3: The inhibition of NADH-Q oxidoreductase activity by a reduced form of Q. NADH-Q_1 (●) and NADH-3-*n*-PrO-Q_2 (■) oxidoreductase activities were determined in the presence of the indicated concentrations of Q_1H_2 and 3-*n*-PrO-Q_2H_2, respectively. The reaction medium (30 °C) contained 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM KCN, 0.2 μM antimycin A, and 0.2 μM MOA-stilbene, the final mitochondrial protein concentration being 30 μg/mL. The reaction was started by adding 50 μM NADH after SMP were equilibrated with Q and indicated concentrations of its reduced form for 30 s. The concentration of Q_1 and 3-*n*-PrO-Q_2 was 50 μM. The control enzyme activities were 0.48 and 0.52 μmol of NADH oxidized/min/mg of protein for $Q_1$and 3-*n*-PrO-Q_2, respectively. The inset shows a double-reciplocal plot of NADH-Q_1 oxidoreductase activity in the presence of 0 M (□), 25 μM (●), or 100 μM (■) of Q_1H_2.

apparent poor electron-transfer activity of Q_2 is primarily due to its severe product inhibition under turnover conditions, but not poor intrinsic ability as an electron acceptor, and that structural specificity of the geranyl side chain of Q_2 is related to product inhibition.

The present observation that bulky Q_2 analogues such as 2,3-(EtO)$_2$-Q_2 and 3-*n*-PrO-Q_2 exhibit much higher activity than Q_2 suggests that the inhibition involving Q_2 is significantly diminished by structural modification of the quinone ring moiety. To confirm this, we examined the effects of bulky Q_2 analogues on Q_1 reduction activity in NADH-Q_1 oxidoreductase assay. Inhibition by Q_2 was examined as a reference (Figure 4A). When NADH-Q_1 oxidoreductase activity was titrated with increasing concentrations of Q_2H_2, the activity decreased as the concentration of Q_2H_2 increased (closed circles). The enzyme activity was fully inhibited in the presence of about 40 μM Q_2H_2. The extent of inhibition by the oxidized form of Q_2 was once saturated at about 10 μM, whereas the enzyme activity recovered slightly at higher concentrations of Q_2 (closed squares). A previous study (*19*) overlooked this recovery because of the limited concentration range of Q_2 examined. Considering that Q_2 is an electron acceptor, albeit a poor acceptor, the "inhibitory" form might be the reduced form which was rapidly converted when the enzyme reaction started. Reactivation of the enzyme activity can be accounted for by the electron-accepting activity of Q_2 itself since the electron-transfer rate in the presence of high concentrations of Q_2 almost corresponded to that obtained with Q_2 alone as an electron acceptor (open circles).

3-*n*-PrO-Q_2 slightly inhibited Q_1 reduction in NADH-Q_1 assay at lower concentrations (<10 μM), but the enzyme reaction rate increased over the control rate at higher concentrations (Figure 4B, closed squares) and was slightly higher than that obtained with 3-*n*-PrO-Q_2 alone (open circles). Similarly to the case of Q_2, it is likely that the slight

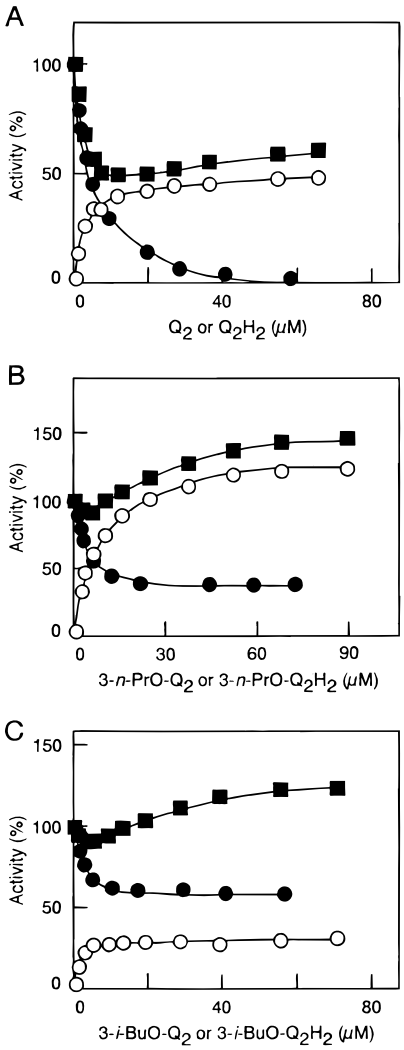


FIGURE 4: The effects of Q analogues and their reduced form (QH_2) on Q_1 reduction in NADH-Q_1 oxidoreductase assay. (A) Q_2 (■), Q_2H_2 (●). (B) 3-*n*-PrO-Q_2 (■), 3-*n*-PrO-Q_2H_2 (●). (C) 3-*i*-BuO-Q_2 (■), 3-*i*-BuO-Q_2H_2 (●). The control electron-transfer activity in NADH-Q_1 assay was 0.49 μmol of NADH oxidized/ min/mg of protein, and this value was taken as the 100% activity. As a reference, NADH-Q_2, NADH-3-*n*-PrO-Q_2 and NADH-3-*i*-Bu-Q_2 oxidoreductase activities are shown in panels *A*−*C*, respectively, by open circles (○). In these case also, the above NADH-Q_1 oxidoreductase activity was taken as the 100% activity. The reaction medium (30 °C) contained 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM KCN, 0.2 μM antimycin A, and 0.2 μM MOA-stilbene, the final mitochondrial protein concentration being 30 μg/mL. The reaction was started by adding 50 μM NADH after SMP were equilibrated with Q_1 (50 μM) and indicated concentrations of Q_2 analogues for 30 s.

inhibition at low concentrations of 3-*n*-PrO-Q_2 was elicited by its reduced form, and the enhancement of enzyme reaction rate was due to electron-transfer activity of 3-*n*-PrO-Q_2 itself. Unexpectedly, the extent of inhibition by 3-*n*-PrO-Q_2H_2 was saturated at about 60% above 20 μM (closed circles), though NADH-3-*n*-PrO-Q_2 oxidoreductase activity was fully inhibited by 3-*n*-PrO-Q_2H_2, as shown in Figure 3. Both forms of 2,3-$(EtO)_2$-Q_2 showed similar effects on Q_1 reduction in NADH-Q_1 assay with those by 3-*n*-PrO-Q_2 (data not shown).

The effects of 3-*i*-BuO-Q_2, an apparently poorer substrate than Q_2, on Q_1 reduction in the NADH-Q_1 assay were also examined (Figure 4C). Despite the much poorer electron-transfer activity of 3-*i*-BuO-Q_2, the effects of both forms of this compound were similar to those of 3-*n*-PrO-Q_2, while the electron-transfer rate in the presence of 3-*i*-BuO-Q_2 (closed squares) was much higher than that obtained with 3-*i*-BuO-Q_2 alone (open circles). These findings suggested that the apparently poor electron-transfer activity of 3-*i*-BuO-Q_2 is due to its poor intrinsic ability as an electron acceptor owing to its bulkiness, but not to severe product inhibition. Although, why the production inhibition by the bulky Q_2 analogues in NADH-Q_1 assay is incomplete is not fully understood, it is suggested that the binding site of the bulky Q_2 analogues differs somewhat from that of Q_1. It is anyhow notable that the severe inhibitory action involving Q_2 was markedly diminished by structural modifications of the ring moiety. This finding also supports the above conclusion that the quinone ring and side-chain moieties contribute interdependently to binding interaction. In other words, the manner of interaction of the quinone ring moiety to the enzyme affects that of the side chain.

Correlation between Electron-Transfer and Proton-Pumping Activities of Q_2 Analogues. The sensitivity of exogenous Q reduction in complex I to inhibitors such as piericidin A and rotenone has been generally thought to be an index of electron-transfer from the physiological site (*7*, *19*), which is coupled to proton-pumping function. It is therefore certain that all of the Q_2 and DB analogues accept electron from the physiological site since their electron-transfer activities were almost completely (>90%) inhibited by 0.1 μM piericidin A or rotenone. To obtain further support for this notion, we determined proton-pumping activities of Q_2 analogues by monitoring formation of membrane potential across SMP membranes using oxonol VI (inside positive) and compared them with the electron-transfer activities from the viewpoint of the structure/activity relationship. This comparison would be important to obtain insight into the mechanism of proton-pumping in complex I since the possibility that the mechanism varies depending upon exogenous Q structure has not been necessarily ruled out. Although no such phenomenon has been known to date for other respiratory enzymes, it is also true that there is no hard experimental evidence which rules out this possibility.

In preliminary experiments, we confirmed that, in the presence of 0.1 μM piericidin A or rotenone, the extent of oxonol signal at 630−601 nm reflecting formation of membrane potential does not change from the level before energization irrespective of the exogenous Q used, indicating that the signal response well coupled to the proton-pumping activity of complex I.[2] Figure 5 shows the titration of height of maximum oxonol VI signal with increasing concentrations of Q, with Q_2, 2,3-$(EtO)_2$-Q_2 and 3-*n*-PrO-Q_2 as examples. The increase in the height of a sharp transient signal of oxonol VI was saturated at a certain concentration range of exogenous Q, and after saturation, the signal was broadened with further increases in concentration of Q since a dynamic equilibrium was reached where the proton translocation rate of the enzyme was equal to the passive backward proton

[2] We appreciate that the absorbance change of oxonol VI may not be a linear function of membrane potential. However, since the aim of this experiment was to investigate whether the electron-transfer and proton-pumping activities of Q_2 analogues are comparable from the viewpoint of structural dependency of the two activities, the oxonol VI response was regarded as an indicator of membrane potential changes.

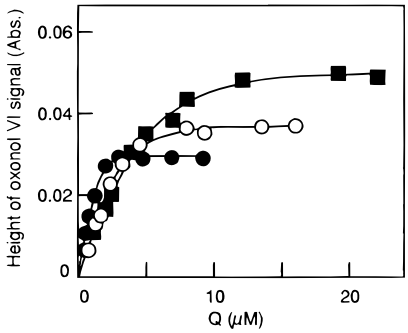


FIGURE 5: Formation of membrane potential by NADH-Q oxidoreductase activity. The height of the maximum oxonol VI signal (630−601 nm), expressed in absorbance units, was determined at various concentrations of Q_2 (●), 2,3-$(EtO)_2$-Q_2 (○), or 3-*n*-PrO-Q_2 (■) at 30 °C. The reaction medium consisted of 50 mM Tricine/NaOH (pH 7.4), 0.2 M sucrose, 3 mM KCN, 2.5 mM $MgCl_2$, 10 nM nigericin, 0.3 μM oligomycin, 0.4 μM antimycin A, 0.1 μM MOA-stilbene, and 3 μM oxonol VI. The final mitochondrial protein concentration was 110 μg/mL. The reaction was started by adding 200 μM NADH.

current. Therefore, the apparent saturation of the increase in signal height did not indicate that of the rate of proton-pumping activity and appeared before the saturation of electron-transfer activity. This was confirmed by the titration of the two activities with increasing concentrations of NADH in NADH oxidase assay under the same experimental conditions, except that the inhibitors of complexes III and IV were omitted (data not shown).

The order of proton-pumping efficiencies in terms of the maximum signal height [3-*n*-PrO-Q_2 > 2,3-$(EtO)_2$-Q_2 > Q_2] was identical to that of electron-accepting efficiencies in terms of V_{max} (Table 1). Saturation of the increase in signal height was observed at lower concentrations in the order of Q_2, 2,3-$(EtO)_2$-Q_2 and 3-*n*-PrO-Q_2, which is identical to the order of K_m values for their electron-transfer activity. Although the titrations of typical three Q_2 analogues are illustrated in Figure 5 for the sake of clarity, structural dependency of the proton-pumping activity was reproducibly comparable to that of the electron-transfer activity for other Q_2 analogues. This indicated that the redox reaction of the Q_2 analogues is well coupled to the proton-pumping irrespective of wide structural variations. As support of this conclusion, it should be mentioned that the proton-pumping activity of hydrophilic substrate Q_0, which is believed to accept electrons, in a large part, at a site upstream the physiological site, was much poorer than that expected from its electron-transfer activity (data not shown).

Structure/Electron-Accepting Activity Relationship of Q Analogues with Paracoccus NDH-1. We also investigated the electron-accepting activities of the above Q analogues with proton-pumping NADH-Q oxidoreductase (NDH-1) of *P. denitrificans* membranes (Table 1). The kinetic parameters of all of the DB series could not be evaluated precisely because of their very poor activities. Their electron-accepting activities were less than 0.03 μmol/min/mg of protein even when added up to about 100 μM. Therefore, the *n*-decyl group appeared to be unfavorable as the side chain of Q with *Paracoccus* enzyme.

The K_m value of Q_2 analogues increased in the order of $Q_2 \leq$ 2-EtO-Q_2 < 3-EtO-Q_2, while V_{max} values were almost identical. The V_{max} value of 2,3-$(EtO)_2$-Q_2 was about half that of Q_2. These results indicated that steric bulkiness in the 2- and 3-positions is unfavorable for the electron-accepting activity with *Paracoccus* NDH-1. In support of this notion, the activities of monopropoxy analogues of Q_2 were much poorer than that of Q_2 irrespective of the substituted position. The activities of more bulky analogues were less than 0.03 μmol/min/mg of protein even at high concentrations. There are two possible reasons for the very poor activities of the bulky Q_2 analogues; the analogues may not be able to occupy the Q reduction site due to steric congestion, or they may occupy the site but not serve as a sufficient electron acceptor. We therefore measured the rate of Q_2 reduction in NADH-Q_2 oxidoreductase assay in the presence of various concentrations of 3-*i*-Bu-Q_2 or 2,3-(*n*-PrO)$_2$-Q_2. The Q_2 reduction was inhibited in a concentration-dependent manner, and complete inhibition was attained at above ∼80 μM of these substrates (data not shown). The inhibition pattern was competitive against Q_2. These findings strongly suggest that the bulky Q_2 analogues occupy the Q reduction site (probably partially), but they are not correctly positioned for redox reaction due to steric congestion.

The PB analogues are useful to examine the effects of conformation of the side chain on the electron-accepting activity for the following reasons. Molecular orbital calculations have indicated that the existence of a methyl group in the 5-position (as in the case of PB) or a methyl branch at the α-position in the side chain [as in the case of 5-H-6-(α-Me)-PB] significantly reduced rotational freedom of the side chain (*21*). In the presence of either of these methyl groups, the Q molecule is energetically most stable when the side chain is almost perpendicular to the Q ring plane. This notion regarding the effect of the 5-methyl group on conformation of the side chain was experimentally supported by EPR and ENDOR spectroscopic studies on short-chain plastosemiquinone and ubisemiquinone radicals in organic solvent and protein environments (*36*). The flexibility of the side chain is not reduced in the presence of a methyl branch anywhere other than the α-position (*21*).

The effects of conformational changes of the side chain on the electron-accepting activity with *Paracoccus* NDH-1 are noteworthy. Comparing the activities among the PB analogues, it is clear that apparent affinities of PB and 5-H-6-(α-Me)-PB, in which the side chains are almost perpendicular to the ring plane, markedly decreased, whereas the V_{max} value increased. The electron-accepting activities of other PB analogues were almost identical irrespective of the position of the attached methyl group. This finding indicated that conformation of the side chain significantly affects the electron-accepting activity with *Paracoccus* NDH-1. Thus, considering the important role of the side chain moiety in the enzyme reaction, the very poor activities of the DB series described above might be due to some sort of unfavorable interaction of the *n*-decyl side chain with the binding environment.

We have reported the structure/activity relationship of PB series with bovine complex I previously (*21*). However, to compare the structure/activity profiles between the two enzymes, we will briefly summarize the results (Table 1). The affinity and maximum reaction rate of 5-H-PB are higher than those of PB (Table 1), indicating that the lack of a 5-methyl group is favorable for the electron-accepting activity. Although the electron-accepting activities of other

PB analogues differ slightly depending upon the position of the attached methyl group, conformational change of the side chain does not significantly affect the electron-accepting activity.

DISCUSSION

Alkyl derivatives of short-chain Qs, in which the native substituents of the quinone ring are replaced by other alkyl groups, are useful to probe the structural natures of the substrate-binding site of Q redox respiratory enzymes since this type of structural modification inevitably alters the molecular shape while minimizing changes in the redox properties of the molecule (*14, 21*). In other words, such structural modification enables separation of the steric and electronic effects of the substituents on the redox activity. We have reported synthetic procedures which enable chemical modifications of the substituents at all positions in the quinone ring to other alkoxy or alkyl groups (*21, 22*). However, the previous method for transformation of the 3-methoxy group was limited only to a corresponding ethoxy group. To overcome this limitation, we developed general procedures to enable transformation of the 3-methoxy group to any other alkoxy group. It is therefore now feasible to prepare any alkyl derivative of short-chain Qs by these synthetic procedures.

Several experimental approaches have suggested that there are more than one Q catalytic site in bovine complex I (for recent review, see ref *37*). On the basis of studies of inhibition mechanism of pyridinium-type inhibitors, we previously proposed that there are two distinct physiological Q reduction sites in bovine heart mitochondrial complex I, and a mixture of two kinetic components of 2,3-$(EtO)_2$-Q_2 reduction can be distinguished by a curved Eadie-Hofstee plot (*38*) of kinetic data with combined use of MP6, a specific pyridinium-type inhibitor of one of the two proposed sites (*11*). The incomplete product inhibition observed in the present study by combined use of different types of Q_2 analogues is difficult to explain by the one catalytic site model for exogenous Q. Therefore, if there are indeed two Q catalytic sites, it is ideal to separate the two kinetic components for a variety of Q analogues and compare the structural factors required for electron-transfer activity between the two sites to elucidate the structural nature of each of the sites. In this study, we tried to separate a mixture of two kinetic components of newly synthesized bulky Q analogues by the previous procedure (*11*), whereas no clear separation succeeded except 2,3-$(EtO)_2$-Q_2. This is probably not only because affinities of the two sites to the Q analogues are similar, or at least not sufficiently different to allow separation, but also because MP6 is not a completely selective inhibitor of one of the two sites in the strict sense (*11*). Thus, comparison of the struture/activity profiles of Qs between the two sites was impractical, and consequently, our discussion is based on apparently mixed functions of the two sites.

However, the present study clearly demonstrated that the Q reduction site of bovine complex I is actually spacious enough to accommodate bulky exogenous Q. In other words, this enzyme recognizes the quinone ring moiety very loosely. This structural specificity in complex I is extremely unusual because even ethoxy derivatives of Q_2 (or Q_2H_2) and DB (or DBH_2) proved to be very poor substrates in studies with other electron-transfer enzymes such as bovine heart mitochondrial complexes II and III (*14*), glucose dehydrogenase (*21*), and terminal ubiquinol oxidases (*22*) in *Escherichia coli*. As for the propoxy derivatives, no electron-transfer activity was observed with these enzymes.[3] On the basis of time-resolved fluorescence measurement of fluorescent probes which compete for the binding site of Q in complex I, Ahmed and Krishnamoorthy (*39*) suggested that the Q-binding site has a high level of segmental mobility, which enables efficient rotational motion of the bulky fluorescent ligands. The large binding cavity of Q in complex I would be a requirement for such a high degree of flexibility in this region. In comparison with bovine complex I, *Paracoccus* NDH-1 recognizes the substrate structure in a more strict sense. For instance, replacement of one of the two methoxy groups of Q_2 with a propoxy group resulted in considerable reduction of the electron-accepting activity. Therefore, the structural properties described above are specific for bovine complex I and not common features for all proton-pumping NADH-Q oxidoreductases. Thus, the results suggest that Q-binding sites are somewhat different between the bovine complex I and *Paracoccus* NDH-1 in terms of substrate specificity. According to Singer (*40*), repeated washing with bovine serum albumin removes all unspecifically bound rotenone along with some specifically bound rotenone, resulting in partial restoration of NADH oxidase activity of bovine complex I. By contrast, Stouthamer and colleagues (*41*) reported that the inhibition of the *Paracoccus* NDH-1 activity by rotenone can be completely reversed by washing the membranes with a medium containing bovine serum albumin. These may support the above notion.

Furthermore, the present study revealed that recognition of the quinone ring moiety by bovine complex I is significantly affected by the side-chain structure, indicating that the quinone ring and the side-chain moieties contribute interdependently to binding interaction with the enzyme. In support of this notion, inhibitory property involving Q_2, which is thought to be due to the presence of a geranyl side chain (*19*), was markedly diminished by structural modifications of the *quinone ring* moiety. This is in marked contrast to the case of terminal ubiquinol oxidases in *E. coli*, in which binding of the quinone ring moiety is not affected by the side-chain structure (*22*). Concerning the role of the side chain, Warncke et al. (*42*) reported that native isoprenoid tail increases the binding affinity of Q through a specific interaction at Q_A and Q_B sites of the reaction center of *Rhodobacter sphaeroides*. On the other hand, Yu et al. (*13*) reported that electron-accepting and -donating activities of Q_2 and DB are completely identical in mitochondrial succinate-cytochrome *c* oxidoreductase. Thus, the role of the side chain in redox reaction might differ between different respiratory redox proteins.

As stated in the introductory portion of this paper, information regarding the mechanism of energy-coupled electron-transfer in complex I is very limited. Even the H^+/e^- stoichiometory is still uncertain, and values reported ($H^+/e^- \geq 2$) are too high to rationalize on the basis of a

[3] M. Ohshima, H. Miyoshi, and K. Sakamoto, unpublished data.

mechanism in which a single electron-transfer reaction would be coupled to the translocation of one proton in analogy to the Q-cycle hypothesis of complex III (*43*). On the other hand, there is virtually no hard experimental evidence from which to infer the possibility that the mechanism (or stoichiometery) of proton-pumping varies depending upon substrate structures. Degli Esposti and colleagues (*18*, *20*) reported that electron-accepting and proton-pumping activities of exogenous Q analogues, which possess different tail structures but an identical substitution pattern of the quinone ring, are not comparable. To examine the above possibility, however, comparison of the two activities among the compounds used by these authors is not necessarily adequate not only because the structure of the quinone ring moiety is not varied, but also because less hydrophobic Q analogues interact incompletely with the physiological Q reduction site (*18*), making the comparison very complicated. To overcome these limitations and considering the structural specificity of the Q reduction site in bovine complex I revealed in this study, Q analogues synthesized here should be good probes to examine this issue. On the basis of comparison of structure/activity profiles for electron-accepting and proton-pumping activities, it was revealed that the two activities are comparable irrespective of wide structural variations of the Qs. This finding strongly suggests that the proton-pumping mechanism is identical or at least not significantly different, regardless of the substrate structure.

In conclusion, in the present study, we developed novel procedures for the synthesis of any alkyl derivative of short-chain Q. Using a variety of Qs obtained by these procedures, it was demonstrated that the Q reduction site in bovine complex I is sufficiently spacious to accommodate a bulky substrate and that the quinone ring and side-chain moieties contribute interdependently to binding interaction. The mechanism of redox-driven proton-pumping of the enzyme does not differ significantly depending upon the substrate structure.

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