Role of the Isoprenyl Tail of Ubiquinone in Reaction with Respiratory Enzymes: Studies with Bovine Heart Mitochondrial Complex I and *Escherichia coli bo*-Type Ubiquinol Oxidase†

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ABSTRACT: The hydrophobic isoprene tail of ubiquinone-2 (Q_2) exihibits binding specificity in redox reactions with bovine heart mitochondrial complex I (Ohshima, M., Miyoshi, H., Sakamoto, K., Takegami, K., Iwata, J., Kuwabara, K., Iwamura, H., and Yagi, T. (1998) *Biochemistry 37*, 6436−6445) and the *Escherichia coli bo*-type ubiquinol oxidase (Sakamoto, K., Miyoshi, H., Takegami, K., Mogi, T., Anraku, Y., and Iwamura, H. (1996) *J. Biol. Chem. 271*, 29897−29902). To identify the structural factor(s) of the diprenyl tail of Q_2 governing the specific interaction with these enzymes, we synthesized a series of novel Q_2 analogues in which only one of the structural factors of the diprenyl tail was systematically modified. In bovine complex I, the presence of the methyl branch and the π-electron system in the first isoprene unit are responsible for high-affinity binding of Q_2 to the ubiquinone reduction site, which results in a low K_m and k_{cat} values of Q_2 reduction. The position of the methyl group in the tail is strictly recognized by the enzyme. In contrast to complex I, in *bo*-type ubiquinol oxidase, either of the two π-electron systems in the tail is required for high-affinity binding of Q_2H_2 to the enzyme, while the presence of the methyl branch and the location of the π-electron systems are not strictly recognized by the enzyme. We concluded that the role of the ubiquinone tail is not simply the enhancement of the hydrophobicity of the molecule and that molecular recognition of the tail by the quinone redox site differs among the respiratory enzymes.

Ubiquinones function as mobile mediators for electron transfer and proton translocation between redox enzymes in mitochondrial and bacterial respiratory systems (*1*). Therefore, they are regarded as functional elements in respiratory systems. In the bacterial photosynthetic reaction center, extensive physicochemical studies of the quinone redox sites have been performed on the basis of the atomic structures of the protein and bound cofactors (*2*−*4*). Alternatively, as shown for mitochondrial succinate−cytochrome *c* oxidoreductase (*5*−*7*), the structural and functional features of the ubiquinone redox site were probed with a systematic set of ubiquinone analogues. Nevertheless, our knowledge concerning the binding and redox properties of ubiquinone in most quinone redox enzymes is still not sufficient.

Ubiquinone is an amphiphilic molecule that is composed of the polar benzoquinone ring and a hydrophobic alkyl tail. The ring moiety is directly involved in redox reactions. Nonempirical molecular orbital calculation demonstrated that the conformation of the methoxy groups in the 2 and 3 positions of the ring affects the electrical potential of the oxidized form of the ubiquinone or semiquinone radical through conformational interconversion (*8*). On the other hand, the tail seems to increase the hydrophobicity of the ubiquinone molecule to facilitate lateral diffusion in biomembranes (*9*). In fact, the electron-accepting and -donating efficiencies of ubiquinone-2 (Q_2)[1] and 6-*n*-decyl-5-methyl-2,3-dimethoxy-1,4-benzoquinone (DB), which have alkyl tails with the same number of carbon atoms (i.e., diprenyl versus *n*-decyl), were completely identical in succinate−cytochrome *c* oxidoreductase (*5*). This indicated that the

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[1] Abbreviations: complex I, mitochondrial NADH−ubiquinone oxidoreductase; cytochrome *bo*, *bo*-type ubiquinol oxidase from *Escherichia coli*; DB, 6-*n*-decyl-5-methyl-2,3-dimethoxy-1,4-benzoquinone; DMF, dimethylformamide; Q, oxidized form of ubiquinone; QH_2, reduced form of ubiquinone; Q_1, ubiquinone-1, Q_2, ubiquinone-2; Q_{10}, endogenous ubiquinone-10; r.t., room temperature; THF, tetrahydrofuran; SMP, submitochondrial particles.

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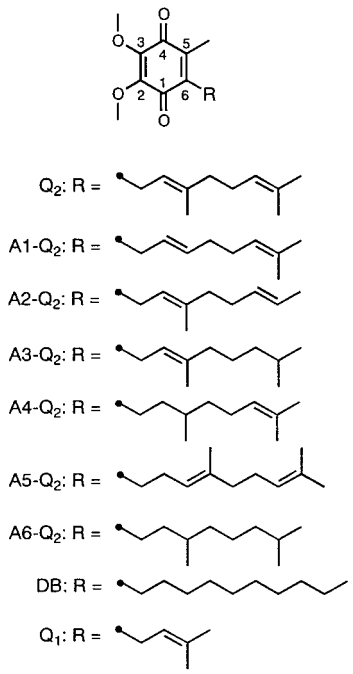


FIGURE 1: Structure of Q_2 analogues used in this study.

native isoprene structure does not contribute to substrate specificity in the redox reactions.

Warncke et al. (*10*) studied the quinone redox sites (Q_A and Q_B) in the photosynthetic reaction center from *Rhodobacter sphaeroides* with short-chain ubiquinone analogues and concluded on the basis of the binding free energy and electron-transfer rate that the native isoprene tail increases binding specificity at the Q_A and Q_B sites more than the saturated alkyl tail. In *bo*-type ubiquinol oxidase from *Escherichia coli* (cytochrome *bo*), Welter et al. (*11*) showed that the electron-donating activities of Q analogues with saturated *n*-alkyl side chain vary significantly depending upon the total number of carbon atoms. We have shown that the K_m values of the Q_2H_2 analogues are much smaller than those of the corresponding DBH_2 analogues with cytochrome *bo*, irrespective of the substitution pattern in the quinol ring (*12*). Furthermore, it is well-known that the V_{max} value of Q_2 is much lower than those of Q_1 and DB in bovine heart mitochondrial NADH−ubiquinone oxidoreductase (complex I) (*13*, *14*), although the side chain of Q_2 is part of an endogenous ubiquinone (Q_{10}). Recently, we suggested that this phenomenon arose primarily due to severe product inhibition by the reduced form (Q_2H_2) under turnover conditions and that the diprenyl tail is responsible for the high−affinity binding of Q_2H_2 to the ubiquinone reduction site (*15*). In these studies, however, the structural factors required for the specific interactions of the isoprene tail with the ubiquinone redox sites remains to be studied.

To identify the structural factors of the diprenyl tail which determine the specific interactions with bovine complex I and cytochrome *bo*, we synthesized a novel series of the Q_2 analogues in which only one of the structural factors of the diprenyl group was systematically modified (e.g., A1-Q_2, A2-Q_2, A3-Q_2, A4-Q_2 and A5-Q_2, in Figure 1). We found that the presence of the methyl branch and the π-electron system in the first isoprene unit are responsible for an increase in the binding affinity of Q_2 to bovine complex I, resulting in the low k_{cat} and K_m values. In cytochrome *bo*, the presence of either of the two π-electron systems enhanced the binding affinity. Thus, our studies demonstrated for the first time the structural factor governing the specific binding of the isoprene tail to the ubiquinone redox enzymes.

MATERIALS AND METHODS

Materials. 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). A6-Q_2 and DB were synthesized by a previous method (*12*). Other chemicals were commercial products of analytical grade.

Synthesis. A1-Q_2, A3-Q_2, A4-Q_2, and A5-Q_2 were synthesized according to the schemes shown in Figure 2. The synthetic compounds were characterized by ^{1}H NMR spectra (Bruker ARX-300) and elemental analyses for C and H, within an error of ±0.3%.

6-[(E)-7-Methylocta-2,6-dienyl]-2,3-dimethoxy-5-methyl-1,4-benzoquinone (A1-Q_2). *n*-Butyllithium (17.5 mL of a 1.6 M solution in hexane) was added over 30 min under N_2 to a solution of 1,2,3,4-tetramethoxy-5-methylbenzene (5 g, 24 mmol) in 50 mL of hexane containing *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TMEDA) (5 g) at 0 °C. The mixture was stirred for an additional 30 min, followed by the addition of THF (250 mL). After the mixture was cooled to −78 °C, CuI (0.23 g) and a solution of *trans*-1,4-dichloro-2-butene in THF (50 mL) were added dropwise, and the mixture was slowly warmed to room temperature (r.t.). The reaction mixture was then quenched with saturated aqueous NH_4Cl; Et_2O was added; and the organic layer was separated, washed with H_2O and brine, and dried ($MgSO_4$). The solvent was evaporated in vacuo, and the crude product was purified by column chromatography (silica gel, hexane/AcOEt = 9:1) to give 6-[(*E*)-4-chloro-2-butenyl]-1,2,3,4-tetramethoxy-5-methylbenzene (1.9 g, 27%).

Sodium benzenesulfinate (1.2 g, 7.2 mmol) and prenyl bromide (0.9 g, 6.0 mmol) were dissolved in anhydrous DMF (20 mL). The mixture was stirred for 20 h at r.t. and then hydrolyzed with H_2O. The aqueous layer was extracted with Et_2O. The combined organic layers were washed with brine, dried ($MgSO_4$), and evaporated in vacuo. The crude product was purified by column chromatography (silica gel, hexane/AcOEt = 8:2) to give 1-benzenesulfonyl-3-methyl-2-butene (1.0 g, 78%).

A solution of 1-benzenesulfonyl-3-methyl-2-butene (0.33 g, 1.5 mmol) and 6-[(*E*)-4-chloro-2-butenyl]-1,2,3,4-tetramethoxy-5-methylbenzene (0.41 g, 1.4 mmol) in a mixture of THF−DMF (9:1, 20 mL) was cooled to −20 °C, and potassium *tert*-butoxide (0.2 g, 1.8 mmol) was added under an N_2 atmosphere. The mixture was stirred for 1 h at −20 °C and then for 1 h at r.t. A saturated aqueous NH_4Cl solution was added, the aqueous layer was extracted with Et_2O; and the organic extract was washed with brine, dried ($MgSO_4$), and evaporated in vacuo. The crude product was purified by chromatography (silica gel, hexane/AcOEt = 8:2) to give 6-[(*E*)-5-benzenesulfonyl-7-methylocta-2,6-dienyl]-1,2,3,4-tetramethoxy-5-methylbenzene (0.51 g, 75%).

To a solution of $Pd(AcO)_2$ (22 mg, 0.1 mmol), LiCl (8.5 mg, 0.2 mmol), and 1,3-bis(diphenylphosphino)propane (dppp) (41 mg, 0.1 mmol) in anhydrous THF (20 mL) was

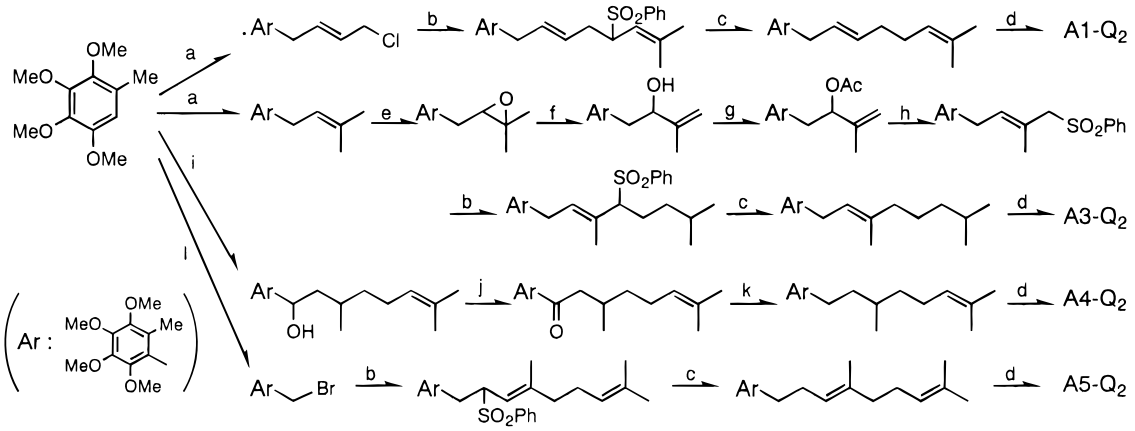


FIGURE 2: Synthetic procedures: (a) (i) *n*-BuLi/TMEDA in hexane at 0 °C, (ii) allyl halide and CuI (cat.) in THF at −78 °C; (b) KOBu-*t* in THF/DMF at −20 °C to r.t.; (c) (i) $Pd(AcO)_2$, LiCl, and dppp in THF, (ii) $LiBHEt_3$ at 0 °C; (d) $Ce(NH_4)_2(NO_3)_6$ in MeCN/H_2O at 0 °C; (e) *m*-CPBA in CH_2Cl_2 at 0 °C; (f) $Al(OPr\text{-}i)_3$ in toluene at reflux; (g) acetyl chloride and diethylaniline in toluene at r.t.; (h) $Pd(PPh_3)_4$ and $PhSO_2Na$ in THF−MeOH at r.t.; (i) (i) *n*-BuLi/TMEDA in hexane at 0 °C, (ii) citronellal in THF at 0 °C; (j) (i) oxalyl chloride and DMSO in CH_2Cl_2 at −60 °C, (ii) triethylamine; (k) $AlCl_3$ and *tert*-butylamine−borane in CH_2Cl_2 at 0 °C; (l) paraformaldehyde and NaBr in AcOH−H_2SO_4 at 85 °C.

added 6-[(*E*)-5-benzenesulfonyl-7-methylocta-2,6-dienyl]-1,2,3,4-tetramethoxy-5-methylbenzene (0.98 g, 2 mmol), and the mixture was cooled to 0 °C (*16*). A solution of $LiBHEt_3$ (4.4 mL of a 1 M solution in THF) was added over 2 h. The mixture was stirred for an additional 3 h; poured into Et_2O (200 mL); washed with a 1 M aqueous solution of NaCN (50 mL), water, and brine; dried ($MgSO_4$); and evaporated in vacuo. The crude product was purified by column chromatography (silica gel, hexane/AcOEt = 8:2) to give 6-[(*E*)-7-methylocta-2,6-dienyl]-1,2,3,4-tetramethoxy-5-methylbenzene (0.57 g, 85%).

To a solution of 6-[(*E*)-7-methylocta-2,6-dienyl]-1,2,3,4-tetramethoxy-5-methylbenzene (0.57 g, 1.7 mmol) and 2,6-pyridinedicarboxylic acid (0.7 g, 4.3 mmol) in a mixture of MeCN−H_2O (7:3, 10 mL) at 0 °C under N_2 was added dropwise a cooled (0 °C) solution of ceric ammonium nitrate (2.4 g, 4.3 mmol) in a mixture of MeCN−H_2O (1:1, 10 mL). The mixture was stirred for 30 min at 0 °C and then for 10 min at r.t. The reaction mixture was hydrolyzed with H_2O, extracted with Et_2O, washed with brine, dried ($MgSO_4$), and evaporated in vacuo. The crude product was purified by chromatography (silica gel, hexane/AcOEt = 9:1) to give A1-Q_2 (0.48 g, 92%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.57 (s, 3H, =C−CH_3), 1.67 (s, 3H, =C−CH_3), 2.00 (m, 4H, $2CH_2$), 2.01 (s, 3H, Ar−CH_3), 3.16 (d, J = 6.2 Hz, 2H, Ar−CH_2), 3.99 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 5.06 (m, 1H, −CH=), 5.32 (dt, J = 6.3, 15.3 Hz, 1H, −CH=), 5.45 (m, 1H, −CH=). Anal. Calcd for $C_{18}H_{24}O_4$: C, 71.03; H, 7.95. Found: C, 71.29; H, 8.02.

6-[(E)-3,7-Dimethyl-2-octenyl]-2,3-dimethoxy-5-methyl-1,4-benzoquinone (A3-Q_2). A solution of *m*-chloroperoxybenzoic acid (1.9 g, 8.8 mmol) in anhydrous CH_2Cl_2 (30 mL) was added dropwise over 15 min to a cooled (0 °C) solution of 6-(3-methyl-2-butenyl)-1,2,3,4-tetramethoxy-5-methylbenzene (2.2 g, 7.8 mmol) in CH_2Cl_2 (30 mL) (*17*). After 1 h of stirring at r.t., the mixture was filtered through a Celite layer, and the solvent was evaporated in vacuo to give crude product 6-(2,3-epoxy-3-methylbutyl)-1,2,3,4-tetramethoxy-5-methylbenzene (2.3 g, 99%).

6-(2,3-Epoxy-3-methylbutyl)-1,2,3,4-tetramethoxy-5-methylbenzene (2.3 g, 7.8 mmol) was added to a solution of $Al(OPr\text{-}i)_3$ (2.4 g, 12 mmol) in anhydrous toluene (90 mL). The mixture was refluxed overnight, cooled, washed with 2 M HCl and brine, dried ($MgSO_4$), and evaporated in vacuo. The crude product was purified by column chromatography (silica gel, hexane/AcOEt = 8:2) to give 6-(2-hydroxy-3-methyl-3-butenyl)-1,2,3,4-tetramethoxy-5-methylbenzene (1.9 g, 80%).

6-(2-Hydroxy-3-methyl-3-butenyl)-1,2,3,4-tetramethoxy-5-methylbenzene (2.2 g, 7.8 mmol) was dissolved in anhydrous toluene (35 mL). Anhydrous diethylaniline (1.9 mL) and pyridine (0.1 mL) were added, followed by the slow addition of acetyl chloride (1.2 g, 15 mmol). After 3 h of stirring at r.t., the mixture was washed successively with saturated aqueous NH_4Cl, H_2O, and brine; dried ($MgSO_4$); and evaporated in vacuo. The crude product was purified by column chromatography (silica gel, hexane/AcOEt = 8:2) to give 6-(2-acetoxy-3-methyl-3-butenyl)-1,2,3,4-tetramethoxy-5-methylbenzene (2.4 g, 96%).

In an Ar atmosphere, $Pd(PPh_3)_4$ (0.4 g, 5 mol %) was added to a solution of 6-(2-acetoxy-3-methyl-3-butenyl)-1,2,3,4-tetramethoxy-5-methylbenzene (2.4 g, 7.1 mmol) and sodium benzenesulfinate (1.4 g, 7.8 mmol) in a THF−methanol mixture, and stirring was continued until the material disappeared (*18*). The mixture was then hydrolyzed with H_2O and extracted with Et_2O. The combined organic layer was washed with H_2O and brine, dried ($MgSO_4$), and evaporated in vacuo. The crude product was purified by column chromatography (silica gel, hexane/AcOEt = 7:3) to give 6-[(*E*)-4-benzenesulfonyl-3-methyl-2-butenyl)-1,2,3,4-tetramethoxy-5-methylbenzene (1.9 g, 60%).

6-[(*E*)-4-Benzenesulfonyl-3-methyl-2-butenyl)-1,2,3,4-tetramethoxy-5-methylbenzene was subjected to the reaction steps b−d, sequentially, to obtain A3-Q_2: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.85 (d, J = 6.6 Hz, 6H, $2CH_3$), 1.09 (dt, J = 8.9, 7.1 Hz, 2H, CH_2), 1.35 (tt, J = 7.6, 7.1 Hz, 2H, CH_2), 1.50 (m, 1H, CH), 1.72 (d, J = 0.6 Hz, 3H, =C−CH_3), 1.93 (t, J = 7.6 Hz, 2H, =C−CH_2), 2.02 (s, 3H, Ar−CH_3), 3.18 (d, J = 7.0 Hz, 2H, Ar−CH_2), 3.98 (s, 3H, OCH_3), 3.99 (s, 3H, OCH_3), 4.92 (br t, J = 7.0 Hz, 1H, −CH=). Anal. Calcd for $C_{19}H_{28}O_4$: C, 71.22; H, 8.81. Found: C, 71.25; H, 9.08.

6-[(E)-3-Methylocta-2,6-dienyl]-2,3-dimethoxy-5-methyl-1,4-benzoquinone (A2-Q_2). A2-Q_2 was synthesized by the same method as that used for A3-Q_2, except that 2-butenyl chloride (*E:Z* = 7:3) was used instead of prenyl bromide in the reaction step b. The trans and cis isomers of A2-Q_2 were separated by column chromatography (silica gel containing 5% $AgNO_3$, hexane/AcOEt = 20:1): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.61 (d, J = 4.8 Hz, 3H, =C−CH_3), 1.72 (d, J = 0.5 Hz, 3H, =C−CH_3), 1.98−2.10 (m, 4H, CH_2−CH_2), 2.01 (s, 3H, Ar−CH_3), 3.18 (d, J = 7.0 Hz, Ar−CH_2−), 3.98 (s, 3H, OCH_3), 4.00 (s, 3H, OCH_3), 4.92 (br t, J = 7.0 Hz, 1H, −CH=), 5.43−5.30 (m, 1H, −CH=). Anal. Calcd for $C_{18}H_{24}O_4$: C, 71.03; H, 7.95. Found: C, 70.99; H, 7.98.

6-(3,7-Dimethyl-6-octenyl)-2,3-dimethoxy-5-methyl-1,4-benzoquinone (A4-Q_2). *n*-Butyllithium (17.5 mL of a 1.6 M solution in hexane) was added over 30 min under N_2 to a solution of 1,2,3,4-tetramethoxy-5-methylbenzene (5 g, 24 mmol) in 50 mL of hexane containing TMEDA (5 g) at 0 °C. The mixture was stirred for an additional 30 min, followed by the addition of THF (250 mL). After the mixture was cooled to 0 °C, a solution of citronellal in THF (50 mL) was added dropwise and the mixture was stirred for an additional 1 h at 0 °C. The reaction mixture was then quenched with saturated aqueous NH_4Cl; Et_2O was added; and the organic layer was separated, washed with H_2O and brine, and dried ($MgSO_4$). The solvent was evaporated in vacuo, and the crude product was purified by column chromatography (silica gel, hexane/AcOEt = 9:1) to give 6-(1-hydroxy-3,7-dimethyl-6-octenyl)-1,2,3,4-tetramethoxy-5-methylbenzene (5.1 g, 59%).

To a solution of oxalyl chloride (1.2 g, 9.1 mmol) in CH_2Cl_2 (20 mL) cooled to −60 °C was added a solution of DMSO (1.6 g, 20 mmol) in CH_2Cl_2 (4.2 mL) dropwise over ca. 5 min (*19*). Stirring was continued at −60 °C for 10 min followed by the addition of 6-(1-hydroxy-3,7-dimethyl-6-octenyl)-1,2,3,4-tetramethoxy-5-methylbenzene (3.1 g, 8.4 mmol) in CH_2Cl_2 (8.4 mL) over ca. 5 min. The reaction mixture was stirred for 15 min, and anhydrous triethylamine (42 mL) was added over ca. 5 min with stirring at −60 °C. The cooling bath was removed, and H_2O (25 mL) was added at r.t. The mixture was extracted with CH_2Cl_2, washed with brine, dried ($MgSO_4$), and evaporated in vacuo. The crude product was purified by chromatography (silica gel, hexane/AcOEt = 19:1) to give 6-(3,7-dimethyl-6-octenoyl)-1,2,3,4-tetramethoxy-5-methylbenzene (1.5 g, 48%).

To a cold (0 °C), stirred suspension of aluminum chloride (1.6 g, 12 mmol) in CH_2Cl_2 (40 mL) was added *tert*-butylamine−borane (2.1 g, 24 mmol) (*20*). The resulting mixture was stirred at 0 °C for 10 min, and a solution of 6-(3,7-dimethyl-6-octenoyl)-1,2,3,4-tetramethoxy-5-methylbenzene (1.5 g, 4.0 mmol) in CH_2Cl_2 (4 mL) was added. The reaction mixture was stirred at 0 °C for 2 h, and then cold, diluted HCl (0.1 M, 20 mL) was added dropwise. The mixture was extracted with AcOEt. The combined organic layer was washed with 0.1 M HCl and brine, dried ($MgSO_4$), and evaporated in vacuo. The crude product was purified by chromatography (silica gel, hexane/AcOEt = 19:1) to give 6-(3,7-dimethyl-6-octenyl)-1,2,3,4-tetramethoxy-5-methylbenzene (0.16 g, 12%).

6-(3,7-Dimethyl-6-octenyl)-1,2,3,4-tetramethoxy-5-methylbenzene was oxidized by ceric ammonium nitrate to give A4-Q_2: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.95 (d, J = 6.5 Hz, 3H, CH_3), 1.14−1.25 (m, 2H, CH_2), 1.32−1.50 (m, 3H, CH, CH_2), 1.61 (s, 3H, =C−CH_3), 1.68 (s, 3H, =C−CH_3), 1.93−2.04 (m, 5H, Ar−CH_3, =C−CH_2), 2.38−2.52 (m, 2H, Ar−CH_2), 3.99 (s, 6H, OCH_3), 5.09 (br t, J = 7.1 Hz, 1H, −CH=). Anal. Calcd for $C_{19}H_{28}O_4$: C, 71.22; H, 8.81. Found: C, 71.61; H, 8.94.

6-[(E)-4,8-Dimethylnona-3,7-dienyl]-2,3-dimethoxy-5-methyl-1,4-benzoquinone (A5-Q_2). 1,2,3,4-Tetramethoxy-5-methylbenzene (2.7 g, 13 mmol), NaBr (1.7 g, 16.5 mmol), and paraformaldehyde (0.7 g) were suspended in AcOH (0.7 mL) and warmed to 85 °C. A mixture of H_2SO_4 (2.5 mL) and AcOH (1.6 mL) was added dropwise, and stirring was continued for an additional 1 h. After the reaction mixture cooled to r.t., H_2O was added and the mixture was extracted with Et_2O, washed with H_2O and brine, dried ($MgSO_4$), and evaporated in vacuo. The crude product was purified by chromatography (silica gel, hexane/AcOEt = 75:15) to give 2,3,4,5-tetramethoxy-6-methyl-benzylbromide (2 g, 51%).

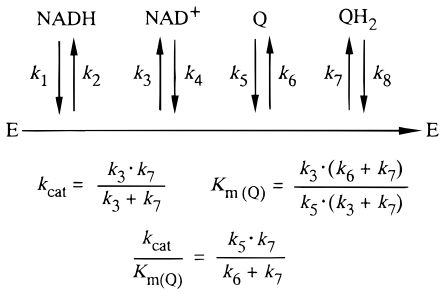
2,3,4,5-Tetramethoxy-6-methyl-benzyl bromide was subjected to reaction steps b−d, sequentially, to obtain A5-Q_2: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 1.58 (d, J = 1.7 Hz, 3H, =C−CH_3), 1.60 (s, 3H, =C−CH_3), 1.68 (d, J = 0.7 Hz, 3H, =C−CH_3), 1.94−2.15 (m, 9H, Ar−CH_3, =C−CH_2), 2.50 (t, J = 7.7 Hz, 2H, Ar−CH_2), 3.99 (s, 6H, OCH_3), 5.08 (br t, J = 6.5 Hz, 1H, −CH=), 5.15 (br t, J = 6.7 Hz, 1H, −CH=). Anal. Calcd for $C_{20}H_{28}O_4$: C, 72.25; H, 8.48. Found: C, 72.13; H, 8.57.

Methods. Bovine heart submitochondrial particles were prepared by the method of Matsuno-Yagi and Hatefi (*21*) and stored in a buffer containing 0.25 M sucrose and 10 mM Tris−HCl (pH 7.4) at −78 °C. NADH−ubiquinone oxidoreductase activity was measured as the rate of NADH oxidation at 30 °C with a Shimadzu UV-3000 spectrophotometer at 340 nm (ϵ = 6200 M^{-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, 0.2 μM MOA−stilbene, and 30 μg/mL of mitochondrial proteins. After equilibration of SMP with the ubiquinone analogues, the reaction was started by the addition of 50 μM NADH. The specific activity of ferricyanide reductase with 2 mM potassium ferricyanide was used to estimate the content of active complex I in submitochondrial particles (*13*). The reduced form of ubiquinone analogues was prepared by the method of Rieske (*22*).

The cytochrome *bo*-type ubiquinol oxidase was purified from *E. coli* strain GO103/pMF02 (*cyo*$^+$ Δ*cyd*/*cyo*$^+$) as described previously (*23*). The purified enzyme in 50 mM Tris−HCl (pH 7.4) containing 0.1% sucrose monolaurate SM-1200 (Mitsubishi-Kagaku Food Co., Tokyo) (buffer A) was stored at −78 °C until use. The Q_8 content was 1.3−2.2 mol of Q_8/ mol of enzyme. The quinol oxidase activity was determined spectrophotometrically using a stirred cuvette in a Shimadzu UV-3000 spectrophotometer. Measurements were performed at 25 °C in 2.5 mL of buffer A containing 0.15 nM enzyme, and the reaction was started by the addition of quinol in an ethanol solution. The oxidase activity was measured by recording the absorbance change at 275 nm with a molar extinction coefficient of 12 300 M^{-1} cm^{-1} (*12*).

The stable conformation of the tail of ubiquinone was studied by the molecular orbital method. Computations were achieved by a semiempirical molecular orbital program package (MOPAC ver. 6.01) with AM1 parametrization (*24*).

Scheme 1



$$k_{cat} = \frac{k_3 \cdot k_7}{k_3 + k_7} \qquad K_{m\,(Q)} = \frac{k_3 \cdot (k_6 + k_7)}{k_5 \cdot (k_3 + k_7)}$$

$$\frac{k_{cat}}{K_{m(Q)}} = \frac{k_5 \cdot k_7}{k_6 + k_7}$$

Initial conformations were constructed using standard bond lengths and angles.

RESULTS AND DISCUSSION

Synthesis of Q_2 Analogues. The V_{max} value of Q_2 in the reaction with bovine heart mitochondrial complex I is much lower than those of DB and Q_1 (*13*, *14*). Accordingly, some structural factors which direct the low turnover number of Q_2 with complex I must be present in the diprenyl tail of Q_2. In studies with cytochrome *bo*, we found that saturation of both double bonds in the diprenyl tail of Q_2 (i.e., A6-Q_2 in this study) resulted in an increase in the K_m value (59 μM), which lies between those for Q_2 (14 μM) and DB (166 μM) (*12*). This difference cannot be attributed to the hydrophobicity of the tail because the isoprene structure is rather less hydrophobic than the saturated alkyl tail with the same number of carbon atoms (*10*, *13*). Our previous study therefore suggested that either or both of the double bonds in the diprenyl tail contribute to the increase in the binding affinity of Q_2H_2 to the quinol oxidation site of cytochrome *bo*.

To identify structural factor(s) in the diprenyl tail for specific interactions with the quinone redox sites, we synthesized a systematic set of novel Q_2 analogues (Figure 1) by synthetic procedures which enabled limited structural modification of only one of the structural factors of the tail. In A1-Q_2 and A2-Q_2, the methyl branch in the first or second isoprene unit was eliminated, respectively. To investigate the role of the π-electron system (first, second, or both of the two double bonds), A3-Q_2, A4-Q_2 and A6-Q_2 were synthesized. In A5-Q_2, one methylene unit was introduced between the ubiquinone ring and the diprenyl group. A1-Q_2, A2-Q_2, A3-Q_2, A4-Q_2 and A5-Q_2 are novel short-chain ubiquinone analogues because the commercially unavailable tail moieties cannot be directly inserted into the quinone ring by conventional synthetic methods (*25*, *26*). The redox potentials of these analogues can be taken as identical to those of Q_2 and DB (100 mV, refs 6 and 7) because the substitution pattern on the quinone ring is identical and there is no conjugation system between the ring and the tail moieties.

Reactions of Q_2 Analogues with Bovine Heart Complex I. The steady-state kinetics of the NADH−Q oxidoreductase activity of complex I is consistent with a ping-pong bi−bi mechanism, whereby the enzyme is first reduced by NADH with release of NAD^+ and then reoxidized by Q with release of QH_2, as shown in Scheme 1 (*13*). It should be mentioned that the electron-accepting activities of all of the Q_2 analogues used were almost completely (>95%) inhibited by 0.1 μM piericidin A or rotenone (data not shown), indicating that these compounds bind to the physiological Q reduction site. The $K_{m(Q)}$, k_{cat}, and second-order rate constant ($k_{cat}/K_{m(Q)}$) values of each Q_2 analogue evaluated from initial velocity measurements are summarized in Table 1. It is notable that althought the k_{cat} value of Q_2 is lower than those of Q_1 and DB, the intrinsic electron-accepting efficiency of Q_2 in terms of $k_{cat}/K_{m(Q)}$ is rather greater than those of the latter two substrates. This means that Q_2 is not necessarily a poor substrate, as described in several reports (*13*−*15*, *27*). Since the reformation of the E−Q complex from E_{ox} and QH_2 in Scheme 1, as well as that of the E−NAD^+ complex from E_{red} and NAD^+ (i.e., the product inhibition), can be practically ignored in the initial velocity measurements, the change in k_{cat} and $K_{m(Q)}$ values of the Q reduction is ascribed to the relative variations of k_5, k_6, and k_7 as shown in Scheme 1. These rate constants are related to the stability of the E−Q complex and the product (QH_2) release from the complex.[2] With respect to Q_2, it could be concluded that the E−Q complex is relatively stable and the product release process is relatively slow compared with A1-Q_2, A4-Q_2, A5-Q_2, DB, and Q_1, as judged from Table 1.

To verify this point, we examined the effects of the Q_2 analogues on the NADH−Q_1 oxidoreductase activity at progressively increased concentrations (Figure 3, closed circles), where the activity was evaluated at [NADH] $\gg$ $K_{m(NADH)}$ and [Q_1] $\gg$ $K_{m(Q1)}$. In the case of Q_2, the NADH oxidation activity was partially inhibited at relatively low concentrations and then it was recovered up to a certain level at higher concentrations. The reactivation can be reasonably ascribed to the electron-accepting activity of Q_2 itself. A similar behavior was observed for the other Q_2 analogues, although the extent of the inhibition and the recovery depended on the structural modification of the Q_2 analogues used. Among the Q_2 analogues, Q_2 and A3-Q_2 with relatively low $K_{m(Q)}$ and k_{cat} values, exhibited strong inhibition effects and limited recovery of the activity. This means that Q_2 and A3-Q_2 compete strongly with Q_1 for occupation of the Q reduction site due to the low $K_{m(Q)}$ (i.e., competitive inhibition), as is consistent with an early study (*13*), and that the limited recovery of the activity is ascribed to low k_{cat}. In contrast, Q_2 analogues with high $K_{m(Q)}$ and k_{cat} values such as A1-Q_2 and A4-Q_2 exhibited weak inhibition effects and high recoveries. On the basis of the above discussion, it could be concluded that the low k_{cat} value of Q_2 is primarily due to stabilization of the E−Q complex or to the high affinity of Q_2 toward the enzyme, resulting in a slowing of the product (Q_2H_2) release.

As pointed out in several reports (*13*−*15*, *27*), the NADH−Q oxidoreductase activity suffers strong inhibition from the reduced product. Because the redox reaction of the quinonoid moiety of the Q_2 analogues would be scarcely affected by the structure of the tail moiety, we expected that the inhibition effects by the reduced Q_2 analogues are related to the substrate affinity to the enzyme (i.e., $K_{m(Q)}$). In this work, the NADH−Q_1 oxidoreductase activity was titrated with the reduced Q_2 analogues. As shown in Figure 3 (closed

[2] To give low $K_{m(Q)}$ and k_{cat} values concurrently, as in the case of Q_2, an increase in k_5 and/or a decrease in k_6 and also a decrease in k_7 far less than k_3 are required. The former and the latter cases are related to the stability of the E−Q complex and the step of product release, respectively, described in the text.

Table 1: Summary of the Kinetic Parameters of Q_2 Analogues in Bovine Complex I and the *E. coli* Cytochrome *bo*[a]

	bovine complex I				*bo*-type ubiquinol oxidase		
	$K_{m(Q)}$ (μM)	k_{cat}[b] (s^{-1})	$k_{cat}/K_{m(Q)}$ (μM^{-1} s^{-1})	I_{50}[c] (μM)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
Q_2	3.0 ± 1.5	109 ± 16	36.5	2.8	18.5 ± 1.2	322 ± 15	17.4
A1-Q_2	17 ± 2.5	234 ± 12	13.8	11	20.0 ± 3.3	245 ± 12	12.3
A2-Q_2	5.1 ± 1.2	192 ± 14	38.4	12	15.2 ± 1.4	295 ± 18	19.4
A3-Q_2	4.2 ± 1.1	102 ± 10	24.3	3.5	24.1 ± 3.0	350 ± 14	14.5
A4-Q_2	11 ± 2.5	203 ± 10	18.5	10	28.0 ± 3.7	333 ± 28	11.9
A5-Q_2	23 ± 3.2	427 ± 26	18.6	11	19.7 ± 2.1	337 ± 13	17.1
A6-Q_2	14 ± 2.1	188 ± 13	13.4	7.2	63.4 ± 4.3	362 ± 8	5.7
DB	14 ± 1.0	255 ± 16	18.2	9.4	152 ± 8	520 ± 33	3.4
Q_1	15 ± 3.1	249 ± 21	23.3	75	60.7 ± 8.3	313 ± 16	5.2

[a] The K_m and V_{max} values were obtained by Lineweaver−Burk plot from at least three independent experiments. [b] The k_{cat} values were obtained by division of the V_{max} by the concentration of complex I, calculated by the ferricyanide reductase assay (see Methods). The concentration of complex I in the SMP preparation was 32 pmol/mg protein. [c] The I_{50} values, defined as the molar concentrations of the reduced form of the Q_2 analogues that decreased NADH−Q_1 oxidoreductase activity to 50% of the control activity, were averaged from two independent experiments.

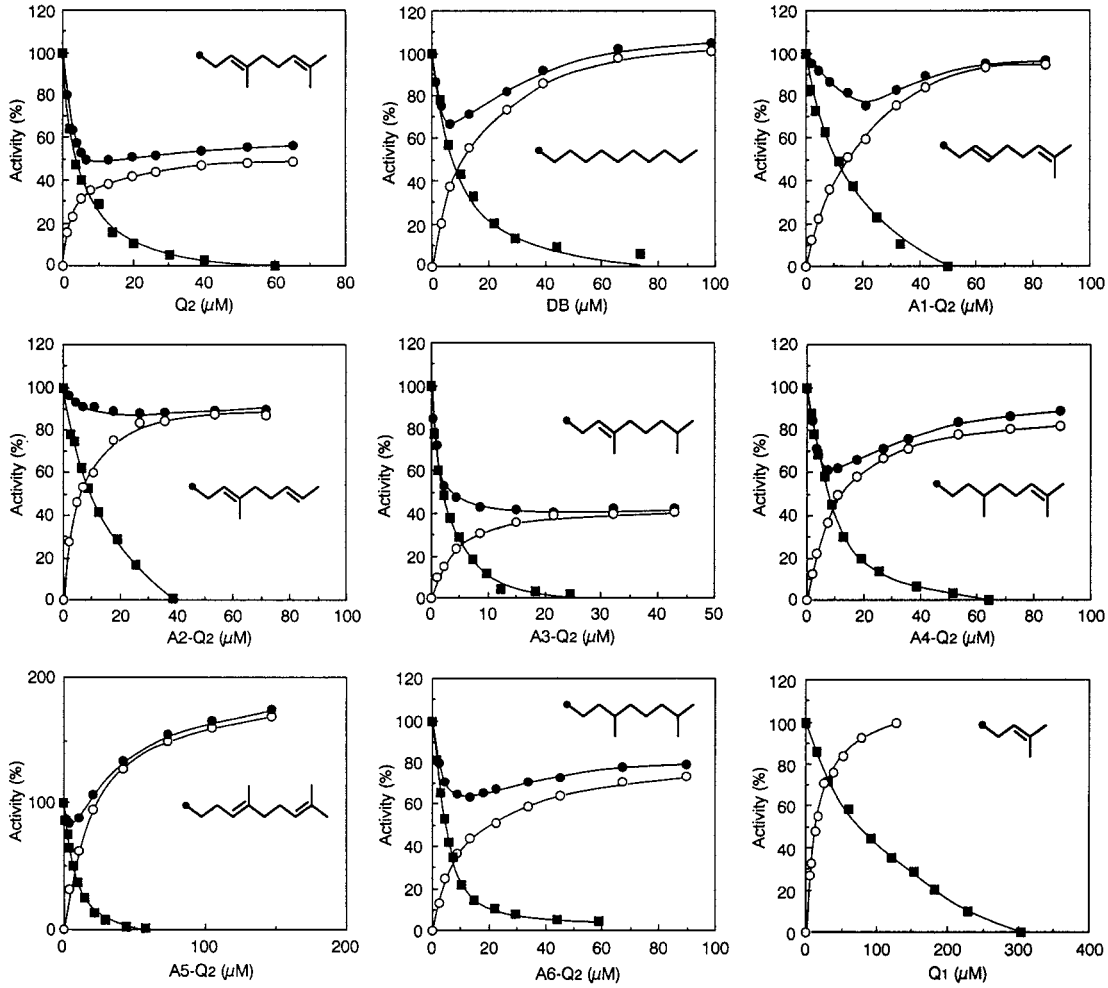


FIGURE 3: Effects of the Q_2 analogues and their reduced form (QH_2) on the reduction of Q_1 in the NADH−Q_1 oxidoreductase assay. The solid circles and squares show the effects of the oxidized and reduced forms, respectively. The control electron-transfer activity in the NADH−Q_1 assay was 0.49 μmol NADH oxidized/min/mg protein, and this value was taken as 100% activity. NADH−Q oxidoreductase activity of each analogue is shown by open circles. In these cases also, the above NADH−Q_1 oxidoreductase activity was taken as 100% activity. The reaction was started by adding 50 μM NADH after the submitochondrial particles had equilibrated for 30 s with Q_1 (50 μM) and the indicated concentrations of the Q_2 analogues.

squares), the activity decreased with increasing concentrations of the reduced Q_2 analogues and was fully inhibited at higher concentrations. The inhibitory ability was tentatively expressed as the concentration of the reduced Q_2 analogues required to decrease the activity to 50% (I_{50}), and summarized in Table 1. The reduced form of Q_2 and A3-Q_2 with low $K_{m(Q)}$ exhibited a strong inhibitory effect on the NADH−Q_1 oxidoreductase activity, as compared with the other Q_2 analogues with high $K_{m(Q)}$. These results support the idea that both the affinity of the oxidized form and the inhibition by the reduced form are governed by some specific structural factors of the diprenyl tail of the Q_2 analogues.

To identify the structural factors of the diprenyl tail that are responsible for the high-affinity binding of Q_2 to the

enzyme, we focused our attention on the kinetic parameters of the Q_2 analogues in Table 1. Because A3-Q_2 was very close to Q_2 from the kinetic point of view, it can be concluded that the π-electron system of the second isoprene unit does not have a significant role in the high binding affinity of Q_2 to the enzyme. The k_{cat} value of A2-Q_2 was larger than that of Q_2, though $K_{m(Q)}$ was slightly increased. Considering that the intrinsic electron-accepting ability of A2-Q_2 in terms of $k_{cat}/K_{m(Q)}$ was similar to that of Q_2, it is likely that the methyl branch in the second isoprene unit is not essential to efficiency as a substrate.

In contrast, the elimination of the methyl branch in the first isoprene unit (A1-Q_2) resulted in significant increases in $K_{m(Q)}$ and k_{cat}. Therefore, the presence of the methyl group is responsible for the high-affinity binding of Q_2 to the enzyme. Interestingly, elongation between the diprenyl tail and the quinone ring by the insertion of one methylene unit (A5-Q_2) markedly increased $K_{m(Q)}$ and k_{cat} values. This indicates either that the position of the methyl group in the first isoprene unit is strictly recognized by the enzyme or that the elongation between the diprenyl unit and the ring moiety allows this unit to take an alternative conformation. This is in marked contrast to a very loose recognition toward the quinone ring moiety; complex I can accommodate exogenous Qs possessing bulky alkoxy groups such as propoxy and butoxy at the 2 and/or 3 positions (*15*, *28*, *29*).

Furthermore, the elimination of the π-electron system in the first isoprene unit (A4-Q_2 or A6-Q_2) also resulted in significant increases in $K_{m(Q)}$ and k_{cat}. The $k_{cat}/K_{m(Q)}$ values of these substrates were about half or less than that of Q_2. The double bond at the first isoprene unit seems to be important in maintaining a specific conformation of this unit, which may in turn allow the methyl group in the first isoprene unit to interact favorably with the enzyme. Since both Q_1 and Q_{10} (the endogenous Q in bovine mitochondria) elicit high k_{cat} values (*13*−*15*, *27*), the remaining part of the polyprenyl tail would contribute to regulation of the local structure (or conformation) of the first isoprene unit.

Role of the First Isoprene Unit in Tail−*Protein Interactions in Complex I.* Warncke et al. (*10*) have shown that the first and second isoprene units of ubiquinone contribute binding specificity at the Q_A and Q_B sites in the photosynthetic reaction center of *R. sphaeroides* and that Q_1 and Q_2 can bind more tightly to these sites than analogues with saturated alkyl tails with the same number of carbon atoms. Possible enthalpic interactions of the π-electron systems in the polyprenyl tail of the bound ubiquinone with the Q_A site do not seem evident in the atomic structure of the photosynthetic reaction center of *R. sphaeroides* (*4*). Consideration of the integrated torsion potential energy of the Q_A site-bound isoprene and saturated alkyl tail structures suggested that the conformational energy of the tail in its protein-bound state is the dominant force in the specific binding of ubiquinone with the native isoprene unit to the Q_A and Q_B sites (*4*).[3]

[3] This could explain the difference in binding specificity between diprenyl (i.e., Q_2) and dimethyloctyl tails of 10 carbon atoms. However, it is not applicable to the difference between monoprenyl (i.e., Q_1) and methylbutyl tails of five carbon atoms, because the difference in the integrated torsion potential energy of the first isoprene moiety is rather small (approximately 1.5 kcal/mol) between the latter pair of tails (see Figure 11 in ref 10).

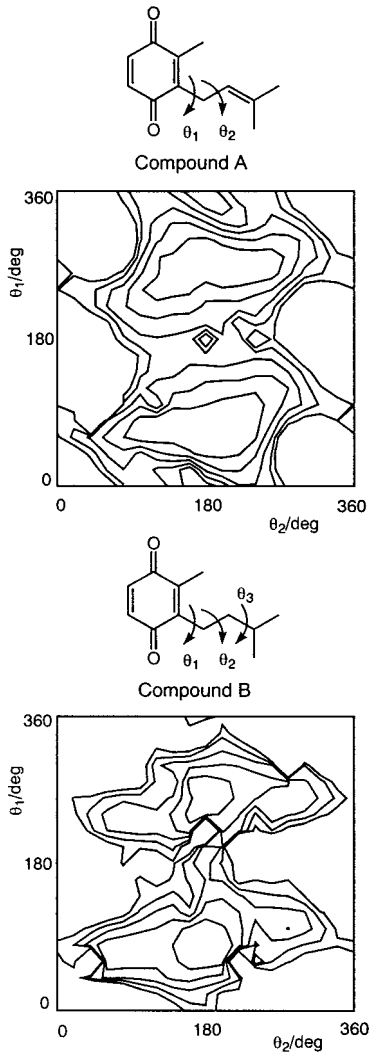


FIGURE 4: Conformational energy diagrams of compounds A and B. The contours represent 1, 2, 3, 4, or 5 kcal/mol above the global minimum for each molecule. The most stable conformations were $\theta_1 = 71.4°$ and $\theta_2 = 121.8°$ and $\theta_1 = 88.8°$ and $\theta_2 = 178.3°$ for compounds A and B, respectively.

Although discussion based on the protein structure or the tail conformation in the protein-bound state is unfeasible for complex I at present, we examined the conformational properties of the tail structures for compounds A and B as model compounds of Q_2 and A4-Q_2, respectively (Figure 4). In each model compound, θ_1 and θ_2 were rotated by steps of 20°, and the total energy was calculated for each conformation. The planar structures depicted in Figure 4 correspond to the conformation of $\theta_1 = 0°$ and $\theta_2 = 180°$. The θ_3 in compound B was optimized in each conformation. The ubiquinone molecule was most stable when the tail was almost perpendicular to the quinone ring plane (i.e., θ_1 is around 90° or 270°) (Figure 4), consistent with electron paramagnetic resonance and electron and nuclear double resonance spectroscopic studies on short-chain plastosemiquinone and ubisemiquinone radicals in organic solvent and protein environments (*30*).

Molecular orbital calculation demonstrated that rotational freedom of the tails of compounds A and B was not markedly different, although the tail of compound B seems to be slightly restrained because of its nonplanar structure and the presence of two additional protons. Assuming that the

positions (or conformations) of the remaining parts of the bound tails of Q_2 and A4-Q_2 are identical, the conformational property of the first isoprene unit would not be responsible for a difference in the binding affinity. Therefore, the π-electron system of the first isoprene unit must alter the interaction of the methyl branch with the substrate binding site.

Reaction of Q_2 Analogues with Cytochrome bo. We also examined the reactions of the Q_2H_2 analogues with cytochrome *bo* (Table 1). The k_{cat}/K_m values of Q_2H_2 analogues were comparable with the exception of A6-Q_2H_2 and DBH_2, which have no π-electron system in their tails. The K_m values of Q_2H_2 (18.5 μM) and A6-Q_2H_2 (63.4 μM) suggest that either or both of the two double bonds in the diprenyl tail contribute to the high-affinity binding to the enzyme. Limited saturation of the double bonds in the diprenyl tail resulted in a slight increase in the K_m values (Q_2H_2 versus A3-Q_2H_2 and A4-Q_2H_2), although the values were significantly smaller than that of A6-Q_2H_2. These results indicate that one of the two double bonds in the tail enhances the binding affinity, although the presence of both double bonds is preferred for high-affinity binding. Comparison of A1-Q_2H_2 and A2-Q_2H_2 with Q_2H_2 revealed that a methyl branch located in the first or second isoprene unit, respectively, has no significant role in the binding affinity to the enzyme. In contrast to complex I, the kinetic parameters of A5-Q_2H_2 were almost identical to those of Q_2H_2 despite the increase in distance of the π-electron system from the quinone ring by one carbon atom. Accordingly, although the number and location of the π-electron system in the tail moiety are not so strictly recognized by cytochrome *bo*, one of the two π-electron systems in the diprenyl unit is required for binding affinity at the quinol oxidation site.

In conclusion, this study demonstrated the structural factors directing the specific interactions of the isoprene tail with the ubiquinone redox enzymes. In bovine complex I, the methyl branch and the π-electron system in the first isoprene unit were responsible for the high-affinity binding of Q_2 to the Q reduction site, resulting in a decrease in k_{cat} and K_m values. In cytochrome *bo*, one of the double bonds in the diprenyl tail enhances the binding affinity to the quinol oxidation site. Molecular recognition of the isoprene tail at the quinone redox site differs significantly among the respiratory enzymes.

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