Probing the Ubiquinone Reduction Site of Mitochondrial Complex I Using Novel Cationic Inhibitors

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A wide variety of N-methylpyridinium and quinolinium cationic inhibitors of mitochondrial complex I was synthesized to develop potent and specific inhibitors acting selectively at one of the two proposed ubiquinone binding sites of this enzyme (Gluck, M. R., Krueger, M. J., Ramsay, R. R., Sabin, S. O., Singer, T. P., and Nicklas, W. J. (1994) J. Biol. Chem. 269, 3167-3174). N-Methyl-2-n-dodecyl-3-methylquinolinium (MQ18) inhibited electron transfer of complex I at under µM order regardless of whether exogenous or endogenous ubiquinone was used as an electron acceptor. The presence of tetraphenylboron (TPB) potentiated the inhibition by MQ18 in a different way depending upon the molar ratio of TPB to MQ18. In the presence of a catalytic amount of TPB, the inhibitory potency of MQ18 was remarkably enhanced, and the extent of inhibition was almost complete. The presence of equimolar TPB partially reactivated the enzyme activity, and the inhibition was saturated at an incomplete level (∼50%). These results are explained by the proposed dual binding sites model for ubiquinone (cited above). The inhibition behavior of MQ18 for proton pumping activity was similar to that for electron transfer activity. The good correlation of the inhibition behavior for the two activities indicates that both ubiquinone binding sites contribute to redox-driven proton pumping. On the other hand, N-methyl-4-[2-methyl-3-(p-tert-butylphenyl)]propylpyridinium (MP6) without TPB brought about approximately 50% inhibition at 5 µM, but the inhibition reached a plateau at this level over a wide range of concentrations. Almost complete inhibition was readily obtained at low concentrations of MP6 in the presence of TPB+. Thus MP6 appears to be a selective inhibitor of one of the two ubiquinone binding sites. With a combined use of MP6 and 2,3-diethoxy-5-methyl-6-geranyl-1,4-benzoquinone, we also provided kinetic evidence for the existence of two ubiquinone binding sites.

Mitochondrial NADH-ubiquinone oxidoreductase (complex I) is a large enzyme that catalyzes the oxidation of NADH by ubiquinone coupled to proton translocation across the inner membrane (1, 2). Due to the enormous complexity of this enzyme, little is known about the pathway of the electron(s) and the mechanism of proton pumping. There is a wide variety of inhibitors of mitochondrial complex I (3). Except rhein (4) and diphenyleneiodonium (5) which inhibit electron input into the enzyme, all inhibitors act at or close to the ubiquinone reduction site (i.e. so-called “rotenone site”) (3). Among the inhibitors, positively charged neurotoxic N-methylphenylpyridinium (MPP+) and its analogues exhibit unique inhibitory action (6), although their inhibitory potencies are much poorer than those of classical potent inhibitors like piericidin A and rotenone. A series of studies on the inhibition mechanism of MPP+ analogues by Singer and colleagues (6-9) have suggested that MPP+ analogues are bound at two sites, one being accessible to the relatively hydrophilic inhibitors (termed the “hydrophilic site”) and one shielded by a hydrophobic barrier on the enzyme (the “hydrophobic site”), and that occupation of both sites is required for complete inhibition. This notion is supported by the existence of two EPR-detectable species of complex I-associated ubisemiquinones (10) and by circumstantial evidence derived from studies on other types of complex I inhibitors (11-14). Thus MPP+ analogues are useful probes with which to characterize the structural and mechanistic features of the ubiquinone reduction site of complex I.

However, MPP+ analogues synthesized to date have certain limitations when they are used as a complex I inhibitor. For instance, the inhibition by MPP+ analogues requires very high concentrations (approximate mM order) compared with classical inhibitors, and there has been no specific inhibitor that acts selectively at one of the two proposed binding sites. In particular, the latter point seems to be unusual since if indeed there are two distinct binding sites, it is unlikely that their structural properties are completely identical. In addition, considering that proton pumping machinery would be close to the ubiquinone binding site which is a part of the membrane-embedded segment of the enzyme (15-17), it remained to be defined whether the two MPP+ (inevitably ubiquinone) binding sites contribute to proton pumping. To overcome these problems and advance the usefulness of MPP+ analogues, potent and specific inhibitors acting selectively at one of the two MPP+ binding sites are earnestly required.

In the present study, we synthesized a wide variety of N-methylpyridinium and quinolinium cationic inhibitors (Fig. 1) to develop such a candidate. In searching through our compound sets, we found some very potent and unique inhibitors. Analysis of the inhibition behaviors of these compounds provided strong support for the existence of the two ubiquinone binding sites in complex I and revealed that both sites contribute to redox-driven proton pumping. Moreover, this study iden...
Novel Cationic Inhibitors of Mitochondrial Complex I

Antimycin A and oligomycin were purchased from Sigma. Pyranine (8-hydroxy-1,3,6-pyrene trisulfonate) was obtained from Molecular Probes. MOA-stilbene was provided by Aburahi Laboratories, Shionogi Co., Ltd. (Shiga, Japan). Q1 and Q2 were a generous gift from Eisai Co. (Tokyo, Japan). Piericidin A was generously provided by Dr. Shigeo Yoshida (RIKEN, Japan). Diethoxy-Q2 (2,3-diethoxy-5-methyl-6-geranyl-1,4-benzoquinone) was from a previous sample (18). Other chemicals were commercial products of analytical grade.

**EXPERIMENTAL PROCEDURES**

**Materials**

All synthetic compounds were characterized by 1H NMR spectra (Bruker ARX-300) and elemental analyses for C, H, and N, within an error of ±0.3%.

**Syntheses**

**MP1**—To a solution of commercially available 2-methyl-3-(p-tolyl)benzaldehyde (2.2 g, 10.8 mmol) in 15 ml of methanol, NaBH₄ (0.5 g, 13.2 mmol) was added, and the reaction mixture was stirred at room temperature for 30 min. After the solvent was removed in vacuo, the residue was extracted by Et₂O and washed with brine to give 2-methyl-3-(p-tolyl)benzaldehyde (2.2 g, 10.8 mmol) of 25% yield. In 20 ml of THF under N₂, 1-bromomethyl-3-(p-tolyl)methylpropene (0.7 g, 6.26 mmol) was stirred with metal Mg to give Grignard reagent. To a 5-m1 solution of pyridine (0.24 g, 2.6 mmol) in CH₂Cl₂, tert-butylmethyldisilyl trifluoromethanesulfonate (0.69 g, 2.6 mmol) (19) was added, and the mixture was stirred at room temperature for 2 h. Purification by silica gel column chromatography (ethyl acetate/hexane = 1:9) gave 1-bromomethyl-3-(p-tolyl)methylpropene in 25% yield. In 20 ml of THF under N₂, 1-bromomethyl-3-(p-tolyl)methylpropene (0.7 g, 6.26 mmol) was stirred with metal Mg to give Grignard reagent. To a 5-m1 solution of pyridine (0.24 g, 2.6 mmol) in CH₂Cl₂, tert-butylmethyldisilyl trifluoromethanesulfonate (0.69 g, 2.6 mmol) (19) was added, and the mixture was stirred at room temperature for 2 h. Purification by silica gel column chromatography (ethyl acetate/hexane = 1:9) gave 1-bromomethyl-3-(p-tolyl)methylpropene in 25% yield. In 20 ml of THF under N₂, 1-bromomethyl-3-(p-tolyl)methylpropene (0.7 g, 6.26 mmol) was stirred with metal Mg to give Grignard reagent. To a 5-m1 solution of pyridine (0.24 g, 2.6 mmol) in CH₂Cl₂, tert-butylmethyldisilyl trifluoromethanesulfonate (0.69 g, 2.6 mmol) (19) was added, and the mixture was stirred at room temperature for 2 h. Purification by silica gel column chromatography (ethyl acetate/hexane = 1:9) gave 1-bromomethyl-3-(p-tolyl)methylpropene in 25% yield. In 20 ml of THF under N₂, 1-bromomethyl-3-(p-tolyl)methylpropene (0.7 g, 6.26 mmol) was stirred with metal Mg to give Grignard reagent. To a 5-m1 solution of pyridine (0.24 g, 2.6 mmol) in CH₂Cl₂, tert-butylmethyldisilyl trifluoromethanesulfonate (0.69 g, 2.6 mmol) (19) was added, and the mixture was stirred at room temperature for 2 h. Purification by silica gel column chromatography (ethyl acetate/hexane = 1:9) gave 1-bromomethyl-3-(p-tolyl)methylpropene in 25% yield. In 20 ml of THF under N₂, 1-bromomethyl-3-(p-tolyl)methylpropene (0.7 g, 6.26 mmol) was stirred with metal Mg to give Grignard reagent. To a 5-m1 solution of pyridine (0.24 g, 2.6 mmol) in CH₂Cl₂, tert-butylmethyldisilyl trifluoromethanesulfonate (0.69 g, 2.6 mmol) (19) was added, and the mixture was stirred at room temperature for 2 h. Purification by silica gel column chromatography (ethyl acetate/hexane = 1:9) gave 1-bromomethyl-3-(p-tolyl)methylpropene in 25% yield. In 20 ml of THF under N₂, 1-bromomethyl-3-(p-tolyl)methylpropene (0.7 g, 6.26 mmol) was stirred with metal Mg to give Grignard reagent. To a 5-m1 solution of pyridine (0.24 g, 2.6 mmol) in CH₂Cl₂, tert-butylmethyldisilyl trifluoromethanesulfonate (0.69 g, 2.6 mmol) (19) was added, and the mixture was stirred at room temperature for 2 h. Purification by silica gel column chromatography (ethyl acetate/hexane = 1:9) gave 1-bromomethyl-3-(p-tolyl)methylpropene in 25% yield. In 20 ml of THF under N₂, 1-bromomethyl-3-(p-tolyl)methylpropene (0.7 g, 6.26 mmol) was stirred with metal Mg to give Grignard reagent. To a 5-m1 solution of pyridine (0.24 g, 2.6 mmol) in CH₂Cl₂, tert-butylmethyldisilyl trifluoromethanesulfonate (0.69 g, 2.6 mmol) (19) was added, and the mixture was stirred at room temperature for 2 h. Purification by silica gel column chromatography (ethyl acetate/hexane = 1:9) gave 1-bromomethyl-3-(p-tolyl)methylpropene in 25% yield. In 20 ml of THF under N₂, 1-bromomethyl-3-(p-tolyl)methylpropene (0.7 g, 6.26 mmol) was stirred with metal Mg to give Grignard reagent. To a 5-m1 solution of pyridine (0.24 g, 2.6 mmol) in CH₂Cl₂, tert-butylmethyldisilyl trifluoromethanesulfonate (0.69 g, 2.6 mmol) (19) was added, and the mixture was stirred at room temperature for 2 h. Purification by silica gel column chromatography (ethyl acetate/hexane = 1:9) gave 1-bromomethyl-3-(p-tolyl)methylpropene in 25% yield. In 20 ml of THF under N₂, 1-bromomethyl-3-(p-tolyl)methylpropene (0.7 g, 6.26 mmol) was stirred with metal Mg to give Grignard reagent. To a 5-m1 solution of pyridine (0.24 g, 2.6 mmol) in CH₂Cl₂, tert-butylmethyldisilyl trifluoromethanesulfonate (0.69 g, 2.6 mmol) (19) was added, and the mixture was stirred at room temperature for 2 h. Purification by silica gel column chromatography (ethyl acetate/hexane = 1:9) gave 1-bromomethyl-3-(p-tolyl)methylpropene in 25% yield.
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(s, 3H, N-CH₃), 7.07 (d, J = 8.2, 2H), 7.31 (d, J = 8.2, 2H), 7.66 (d, J = 6.6, 2H), 8.70 (d, J = 6.6, 2H).

$$C_{D_2H_8N_2}CF_5SO_3$$

Calculated: C 58.45  H 6.54  N 3.25
Found: C 58.17  H 6.41  N 3.19

Synthesis of MP7 (MP10)−4-Chloropyridine (3.0 g, 20.0 mmol) and metal Na (0.5 g, 22.0 mmol) was stirred in undeacnol (10.0 g, 60.0 mmol) at 100 °C for 8 h. The reaction mixture was extracted by Et₂O and washed with brine. The organic phase was dried over MgSO₄ and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 3:17) to give MP7 in a 60% yield.

$$MP10: m.p. 65 \degree C, 1H NMR (CDCl₃, 300 MHz) 0.96 (t, J = 7.3, 2H), 1.11 (t, J = 7.3, 2H), 1.46 (m, 2H), 1.58 (m, 2H), 1.77 (m, 2H), 1.83 (m, 2H), 2.93 (t, J = 7.7, 2H), 3.40 (m, 2H), 4.65 (s, 3H, N-CH₃), 7.58 (t, J = 7.3, 1H), 8.07 (dd, J = 7.3, 9.5, 1H), 8.10 (d, J = 7.3, 1H), 8.39 (d, J = 9.5, 1H), 8.60 (s, 1H).

$$C_{D_2H_8N_2}CF_5SO_3$$

Calculated: C 56.28  H 6.46  N 3.45
Found: C 56.17  H 6.38  N 3.22

MP13 and MP18 were synthesized by the same method used for MQ14. 3-Methylpyridinium was reacted with n-propyl brodine and n-dodecyl brodine for the preparation of MQ13 and MQ18, respectively. MP13: m.p. 125–126 °C, 1H NMR (CDCl₃, 300 MHz) 0.93 (t, J = 7.0, 3H), 1.44 (m, 2H), 1.56 (m, 2H), 1.71 (m, 2H), 1.74 (m, 2H), 2.97 (m, 2H), 3.10 (s, 3H), 4.60 (s, 3H, N-CH₃), 7.84 (t, J = 7.5, 1H), 8.07 (dd, J = 7.5, 9.0, 1H), 8.10 (d, J = 7.5, 1H), 8.33 (d, J = 9.0, 1H), 8.56 (s, 1H).

$$C_{D_2H_8N_2}CF_5SO_3$$

Calculated: C 54.10  H 5.88  N 3.71
Found: C 54.03  H 5.90  N 3.42

MQ18: m.p. 140–141 °C, 1H NMR (CDCl₃, 300 MHz) 0.88 (t, J = 6.5, 3H), 1.27–1.39 (m, 16H), 1.61 (m, 4H), 2.71 (s, 3H), 3.40 (t, J = 7.9, 2H), 4.62 (s, 3H, N-CH₃), 7.83 (t, J = 9.4, 1H), 8.06 (m, 2H), 8.35 (d, J = 9.4, 1H), 8.62 (s, 1H).

$$C_{D_2H_8N_2}CF_5SO_3$$

Calculated: C 60.61  H 7.63  N 2.94
Found: C 60.48  H 7.66  N 2.92

MQ 12 was prepared by reacting methyl lithium and 3-n-propylquinoiline which was synthesized from 3-methylquinoline and n-butyl bromide by the same method as that for MQ14. 1H NMR (CDCl₃, 300 MHz) 0.94 (t, J = 7.0, 3H), 1.45 (m, 2H), 1.55 (m, 2H), 1.71 (m, 2H), 2.69 (s, 3H), 3.37 (m, 2H), 4.59 (s, 3H, N-CH₃), 7.51 (t, J = 7.6, 1H), 8.04 (dd, J = 7.6, 9.0, 1H), 8.09 (d, J = 7.6, 1H), 8.34 (d, J = 9.0, 1H), 8.68 (s, 1H).

$$C_{D_2H_8N_2}CF_5SO_3$$

Calculated: C 54.10  H 5.88  N 3.71
Found: C 54.63  H 5.86  N 3.67

MQ19 was prepared by reacting methyl lithium with 3-dodecyquinoline which was derived from a reaction of 3-methylquinoline and n-undecyl bromide according to the method for MQ14. 1H NMR (CDCl₃, 300 MHz) 0.88 (t, J = 6.5, 3H), 1.27–1.43 (m, 15H), 1.73 (m, 2H), 2.96 (t, J = 7.8, 2H), 3.11 (s, 3H), 4.61 (s, 3H, N-CH₃), 7.54 (t, J = 8.8, 1H), 8.06 (m, 2H), 8.33 (d, J = 8.8, 1H), 8.54 (s, 1H).

$$C_{D_2H_8N_2}CF_5SO_3$$

Calculated: C 60.61  H 7.63  N 2.94
Found: C 60.33  H 7.75  N 2.89

Synthesis of MQ15—To a solution of 5-nonanone (21 g, 148 mmol) in 15 ml of methanol, NaBH₄ (5.6 g, 148 mmol) was added slowly at 0 °C and the mixture was stirred for 1 h. After the reaction mixture was extracted by Et₂O and washed with brine, removal of Et₂O gave 5-nonanol in a quantitative yield. To a solution of 5-nonanone (5.0 g, 34.7 mmol) in 15 ml of pyridine, p-toluensulfonyl chloride (7.3 g, 38.4 mmol) was added dropwise, and the mixture was stirred at room temperature for 24 h. The reaction mixture was extracted by Et₂O and washed with brine. The organic phase was dried over MgSO₄ and evaporated. The crude product was purified by silica gel column chromatography (ethyl acetate/hexane = 3:17) to give a 12-dihydro-2-n-pentyl-3-n-propylquinoline in a 62% yield. 12-Dihydro-2-n-pentyl-3-n-propylquinoline was oxidized by stirring with 10% palladium on carbon in methanol with a catalytic amount of HCl at room temperature for 3 h. The reaction mixture was extracted by Et₂O and washed with brine. The product (3-n-propylquinoline) was isolated by silica gel chromatography (ethyl acetate/hexane = 3:17) to give MQ15 in a 43% yield; m.p. 99 °C. 1H NMR (CDCl₃, 300 MHz) 0.96 (t, J = 7.3, 3H), 1.11 (t, J = 7.3, 1H), 1.46 (m, 2H), 1.58 (m, 2H), 1.77 (m, 1H), 1.83 (m, 2H), 2.93 (t, J = 7.7, 2H), 3.40 (2H), 4.65 (s, 3H, N-CH₃), 7.58 (t, J = 7.3, 1H), 8.08 (dd, J = 7.3, 9.5, 1H), 8.10 (d, J = 7.3, 1H), 8.39 (d, J = 9.5, 1H), 8.60 (s, 1H).
Novel Cationic Inhibitors of Mitochondrial Complex I

Acetate/hexane (1:20) to give 1-n-butylpentyl p-toluenesulfonate. To a solution of NaBr (3.1 g, 2.6 mmol) in 10 ml of water, 1-n-butylpentyl p-toluenesulfonate (7.8 g, 2.6 mmol) and a catalytic amount of tetra-n-decylammonium bromide were added, and the mixture was refluxed at 100 °C for 24 h. The reaction mixture was extracted by EtOAc and washed with brine to give 5-bromononane in a 66% yield. To a solution of commercially available 2-methylquinoline (1.0 g, 7.0 mmol) in 10 ml of THF, 4.8 ml of 1.6 M n-butyl lithium (7.7 mmol) was added dropwise at −20 °C, and the mixture was stirred for 1 h. To the reaction mixture, 5-bromononane (1.45 g, 7.0 mmol) was added, and the mixture was stirred at room temperature for 24 h. After the reaction mixture was extracted by EtOAc and washed with brine, the organic phase was dried over MgSO4 and evaporated. The crude final product was isolated by silica gel column chromatography (ethyl acetate/hexane 1:20) to give 1-[(dodecylammonium bromide was added, and the mixture was refluxed at 20 °C, and the mixture was stirred for 1 h. To the reaction mixture, the reaction medium contained 0.25 M sucrose, 50 mM KCl, 10 mM MgSO4, 50 mM Tris-HCl (pH 7.4). Unless otherwise noted, SMP were incubated with reacting pyridine or quinoline analogues with methyl trifluoromethane-sulfonate. To a solution of NaBr (3.1 g, 2.6 mmol) in 10 ml of water, 1-pyrenine in the internal compartment were containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4) at 78 °C. The proton pumping activity of complex I was determined with SMP containing pyranine by monitoring changes in the fluorescence intensity of pyranine, which reflects acidification in the internal compartment of SMP, at 510 nm (excited at 460 nm) with a Shimadzu RF-5000 spectrophotometer at 25 °C. The reaction medium and final mitochondrial protein concentration were the same as those for the electron transfer experiments, except that the reaction medium contained 0.2 μM oligomycin and 0.2 μM valinomycin.

Effect of TPB on the Inhibition by MPP+ Analogues—The inhibition of NADH-Q1 oxidoreductase activity by newly synthesized pyridinium and quinolinium derivatives was examined using SMP. Throughout this study, Q1 was mainly used as an electron acceptor since this quinone is considered to be an ideal exogenous substrate for complex I assay (23). The I50 values obtained by inhibitor alone are listed in Table I with that of MPP+ as control. The inhibitory potencies varied widely depending upon structure. The N-methylpyridiniums possessing an undecyloxy group (MP7, MP8, and MP9) appeared to be potent inhibitors regardless of substitution positions on the pyridinium ring. Replacement of a methyl group of MP7 by an ethyl group retained the activity (MP1 versus MP10). The inhibitory potencies of these compounds were much stronger than those of original MPP+ and its simple alkyl analogues (6, 8). Replacing the pyridinium ring by the quinolinium ring slightly enhanced the activity (MP1 versus MQ11, and MP7 versus MQ17). In this case, as well, variation in substitution position did not significantly affect the activity (MQ18 versus MQ19). These results along with the fact that N-homopyridiniums retain activity (24) indicated that the steric requirement for the binding of MPP+ analogues to complex I is not so severe. This conclusion is consistent with the notion that the ubiquinone reduction site is spacious enough to accommodate a variety of structurally different inhibitors in a dissimilar manner, as claimed from the studies on other types of complex I.

### Methods

Bovine heart submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi (22) using a sonication medium containing 0.25 M sucrose, 1 mM succinate, 1.5 mM ATP, 10 mM MgCl2, 10 mM MnCl2, and 10 mM Tris-HCl (pH 7.4) and stored in a buffer containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4) at 78 °C. SMP containing a pH probe pyranine in the internal compartment were prepared as for ordinary SMP, except that the sonication medium contained 1 mM pyranine. After sonicated particles were collected by centrifugation in a Beckman type 50Ti rotor for 45 min at 43,000 rpm, the pellet was washed twice in buffer A containing 0.25 M sucrose, 50 mM KCl, and 10 mM Tris-HCl (pH 7.4) and suspended by homogenization in the same buffer at 30–40 mg of protein/ml. Untrapped pyranine was removed by gel filtration on Sephadex G-25 equilibrated with buffer A. The filtration was done at 4 °C, and the particles were collected by centrifugation as described above. The sediments were finally suspended in buffer A.

The NADH oxidase activity was followed spectrophotometrically with a Shimadzu UV-3000 at 340 nm (ε = 8.2 lM cm−1) at 25 °C. The reaction medium contained 0.25 M sucrose, 1 mM MgCl2, and 50 mM phosphate buffer (pH 7.4). The final mitochondrial protein concentration was 30 μg of protein/ml. The reaction was started by adding 50 μM NADH. The NADH-Q1 oxidoreductase activity was determined following NADH oxidation at 25 °C in the same reaction medium in the presence of 50 μM Q1, 0.2 μM antimycin A, 0.2 μM MOA-stibene, and 2 mM KCN. For the assays with SMP containing pyranine, the reaction medium contained 0.25 M sucrose, 50 mM KCl, 10 mM MgSO4, 50 μM Q1, 0.2 μM antimycin A, 0.2 μM MOA-stibene, 2 mM KCN, and 30 mM Tris-HCl (pH 7.4). Unless otherwise noted, SMP were incubated with inhibitors for 5 min before starting the reaction. When the incubation time was extended to 30 or 120 min, SMP and a definite concentration of inhibitor were preincubated in test tubes at 5 ml of total volume with

### Table I

| Compounds  | A, I50 (μM) without TPB | B, I50 (μM) with TPB | A/B  
|------------|--------------------------|----------------------|------|
| MP1        | 4700  
| MP2        | 1900  
| MP3        | 360   
| MP4        | 25    
| MP5        | 1500  
| MP6        | 5.2   
| MP7        | 1.9   
| MP8        | 1.5   
| MP9        | 2.8   
| MP10       | 1.1   
| MQ11       | 820   
| MQ12       | 280   
| MQ13       | 550   
| MQ14       | 260   
| MQ15       | 52    
| MQ16       | 13    
| MQ17       | 1.2   
| MQ18       | 0.73  
| MQ19       | 0.61  

a The figures in parentheses are the concentration of TPB.

b The ratio of I50 value (without TPB) to I50 value (with TPB).

c N-Methylpyridinium.

d The relative inhibition (%) was ~40% at this concentration.

e The relative inhibition (%) was ~30% at this concentration.

f The relative inhibition was saturated at about 50%, as described in the text.

30 μg of protein/ml on ice. The test tubes were put into a thermostatic water bath (25 °C) 5 min before starting the reaction to revert the temperature to 25 °C, and a 2.5-ml aliquot was placed in a spectrometer cuvette.

### RESULTS

The experimental results are listed in Table I with that of MPP+ as control. The inhibitory potencies varied widely depending upon structure. The N-methylpyridiniums possessing an undecyloxy group (MP7, MP8, and MP9) appeared to be potent inhibitors regardless of substitution positions on the pyridinium ring. Replacement of a methyl group of MP7 by an ethyl group retained the activity (MP1 versus MP10). The inhibitory potencies of these compounds were much stronger than those of original MPP+ and its simple alkyl analogues (6, 8). Replacing the pyridinium ring by the quinolinium ring slightly enhanced the activity (MP1 versus MQ11, and MP7 versus MQ17). In this case, as well, variation in substitution position did not significantly affect the activity (MQ18 versus MQ19). These results along with the fact that N-homopyridiniums retain activity (24) indicated that the steric requirement for the binding of MPP+ analogues to complex I is not so severe. This conclusion is consistent with the notion that the ubiquinone reduction site is spacious enough to accommodate a variety of structurally different inhibitors in a dissimilar manner, as claimed from the studies on other types of complex I.
The inhibitory potency of MPP⁺ analogues is potentiated in the presence of TPB⁻ (7). This potentiation effect would be accounted for first by an increase in MPP⁺ concentration in the membrane lipid phase due to an ion pair formation and second by facilitating MPP⁺ passage through the hydrophobic barrier to the binding site (6). Gluck et al. (6) have claimed that the extent of the potentiation effect diminishes as the hydrophobicity of MPP⁺ analogues increases since hydrophobic inhibitors are capable of reaching the hydrophobic site without the assistance of TPB⁻. However, it seems reasonable to assume that even if the hydrophobicity of some MPP⁺ analogues is fairly large, partitioning into or passage through the hydrophobic membrane environment of positively charged compounds would be supported energetically by the formation of a neutral ion pair. In fact, Sayre et al. (27) showed that the inhibitory potencies of fairly hydrophobic MPP⁺ analogues are also potentiated in the presence of 1–5 μM TPB⁻. The uncertainty reported by Gluck et al. (6) may be because they examined the potentiation effect of TPB⁻ at a definite high concentration of TPB⁻ (10 μM). The concentration of TPB⁻ was in excess relative to that of some MPP⁺ analogues under their experimental conditions. As discussed in the literature (6) and also below, an excess amount of TPB⁻ relative to MPP⁺ analogues complicates the inhibition behavior of the inhibitors and, to begin with, the inhibition by TPB⁻ itself is unavoidable at 10 μM.

To evaluate the potentiation effect of TPB⁻, we determined the inhibitory potency of our compounds in terms of IC₅₀ in the presence of various concentrations of TPB⁻ (Table I). The concentration of TPB⁻ was set much lower than that of the inhibitor. The upper concentration limit of TPB⁻ was set at 2 μM for the assays of relatively weak inhibitors since the inhibition by TPB⁻ itself was not negligible above this concentration. It is clear that a catalytic amount of TPB⁻ potentiated the inhibition of all test compounds regardless of their hydrophobicity. The IC₅₀ values of some compounds were below the μM level (for instance, MQ17–19). These potent compounds elicited no inhibition against the succinate oxidase activity in SMP at least up to 400 μM, indicating that they are specific inhibitors of complex I.

**Correlation between Electron Transfer Inhibition and Proton Pumping Inhibition**—The inhibitory action of MQ18, which appeared to be one of the most potent inhibitors among the compounds synthesized in this study, was examined closely using Q₁ as an electron acceptor. The electrically neutral form compounds synthesized in this study, was examined closely. The inhibitory action of MQ18, which was examined closely, appeared to be one of the most potent inhibitors among the inhibitors (3, 25, 26).

The inhibition by MQ18 of NADH-Q₁ oxidoreductase activity (A) and of proton pumping activity (B) was investigated at 25 °C. The reaction was started by adding 50 μM NADH after preincubation of SMP with the inhibitor for 5 min. The final mitochondrial protein concentration was 30 μg of protein/ml for both experiments. TPB⁻ concentrations were as follows: ○, 0 nM; ●, 50 nM; ■, 2 μM. The control electron transfer activity was 0.45 μmol of NADH oxidized per min/mg protein.

**FIG. 2. Effect of TPB⁻ on the inhibition behavior of MQ18.** The inhibition by MQ18 of NADH-Q₁ oxidoreductase activity (A) and of proton pumping activity (B) was investigated at 25 °C. The reaction was started by adding 50 μM NADH after preincubation of SMP with the inhibitor for 5 min. The final mitochondrial protein concentration was 30 μg of protein/ml for both experiments. TPB⁻ concentrations were as follows: ○, 0 nM; ●, 50 nM; ■, 2 μM. The control electron transfer activity was 0.45 μmol of NADH oxidized per min/mg protein.
internal compartment by the addition of 50 μM NADH (plus 50 μM Q₁). This quenching was completely prevented in the presence of 50 nM piericidin A or rotenone (data not shown), indicating that the fluorescence quenching is well coupled to the proton pumping activity of complex I. The inhibition curves for the inhibition by MQ18 of proton pumping activity in terms of fluorescence quenching under the same experimental conditions as those for the electron transfer experiment in Fig. 2A. It is clear that inhibition behavior of MQ18 for the proton pumping activity is similar to that for the electron transfer activity. The good correlation indicated that both the MQ18 binding sites contribute to proton pumping.

Selective Inhibitor of One of the Two Ubiquinone Binding Sites—The two binding sites model for MPP⁺ (or ubiquinone) successfully explains the complicated inhibition behavior of the potent cationic inhibitors. Then, if there are indeed two binding sites, it is reasonable to assume that the sensitivities of the two sites to inhibition by some inhibitors are different because of a difference in their structural natures. In other words, certain inhibitors should act at one of the two sites with a high selectivity. However, no such inhibitor has been reported to date. In this sense, the inhibition behavior of MP6 is noteworthy.

As shown in Fig. 3, MP6 without TPB⁻ brought about approximately 50% inhibition of NADH-Q₁ oxidoreductase activity at ~5 μM, but the inhibition reached a plateau at this level over a wide range of concentrations. Weak inhibition was again observed above ~80 μM, and a maximum inhibition (>90%) was obtained only when the concentration of the inhibitor rose to about 200 μM. This saturation was not due to the slow inhibition process observed for original MPP⁺ since elongation of preincubation time of SMP with MP6 from 5 min to 30 or 120 min did not affect the dose-response curve at all (Fig. 3), indicating that the inhibition by MP6 alone is established fairly rapidly. In contrast, almost complete inhibition (>90%) was readily obtained at low concentrations of MP6 in the presence of 0.5 μM TPB⁻ (Fig. 3). Similar inhibitory behavior of MP6 was observed when the inhibition of the proton pumping activity was investigated, except that the inhibition without TPB⁻ was saturated at about 30% of the control quenching (data not shown). Although the relative prevention of fluorescence quenching seems to be slightly poorer than the relative inhibition of electron transfer activity, quantitative comparison of the effect of the inhibitor on proton pumping and electron transfer activities is not practical under the experimental conditions, as mentioned above.

The saturation of inhibition became somewhat ambiguous depending upon the ubiquinone (Q₁₀, Q₂, or diethoxy-Q₂ (2,3-dioctyloxy-5-methyl-6-geranyl-1,4-benzoquinone, see Ref. 18)) used as an electron acceptor, although the dose-response curves were still clearly biphasic (Fig. 4). The presence of TPB⁻-attained almost complete inhibition (>90%) at low concentrations of MP6 regardless of the ubiquinones used (data not shown). Thus MP6 appears to have clear selectivity against the two ubiquinone binding sites. The site blocked by low concentrations of MP6 without TPB⁻ will be the hydrophilic site. The presence of TPB⁻ made the selectivity of MP6 inhibition ambiguous probably by facilitating MP6 passage through the hydrophobic barrier to the remaining site.

Kinetic Evidence for Two Ubiquinone Reduction Sites—The above consideration leads to the further question of whether the two kinetic components of ubiquinone reduction can be distinguished in the presence of MP6 at concentrations exhibiting the inhibition of only one of the two binding sites. If the affinities for an exogenous ubiquinone are markedly different between the two sites, separation of a mixture of two kinetic components would be possible. We therefore examined the kinetics of exogenous ubiquinone reduction in the presence of 5 μM MP6. With Q₁ or Q₂ as an electron acceptor, precise separation of the two kinetic components was unsuccessful probably due to similar affinities of the two sites to the substrate. However, the kinetic analysis for diethoxy-Q₂ reduction was noteworthy for the following reasons.

The bulky diethoxy-Q₂ accepts electrons from the physiological site in complex I because when this quinone was used as an electron acceptor, both the proton pumping, detected by membrane potential changes using oxonol VI (29), and the electron transfer activities were almost completely (>90%) inhibited by 50 nM piericidin A or rotenone. The [S] versus rate plot for diethoxy-Q₂ is shown in Fig. 5A, and the kinetic data were

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2 Under the present experimental conditions, it is not definitive that the extent of fluorescence quenching is linearly related to the stoichiometry of pumped protons. Therefore, it is also not clear whether the relative prevention of fluorescence quenching directly corresponds to that of proton pumping activity. However, the inhibition behavior of MQ18 for electron transfer and proton pumping activities can be discussed in a qualitative way.

3 H. Miyoshi and M. Ohshima, unpublished data.
Fig. 5. Effect of MP6 on the kinetics of NADH oxidation by diethoxy-Q$_2$ in SMP. The reaction conditions were the same as in the legend to Fig. 2. A, MP6-sensitive activity (○) is the difference between the total rate (●) and the rate in the presence of 5 μM MP6 (□). B is an Eadie-Hofstee plot of the kinetic data shown in A. The lines were fit by linear regression. For MP6-sensitive component, the six data points in low substrate concentrations were not included in the regression analysis because of relatively large experimental errors.

replotted in an Eadie-Hofstee plot (30) in Fig. 5B. For convenience, the diethoxy-Q$_2$ reduction activity in the presence of 5 μM MP6 is referred to as MP6-resistant reduction, and the difference between the total and MP6-resistant reduction activities is termed MP6-sensitive reduction. The total activity clearly showed two components (Fig. 5B). The MP6-sensitive and resistant diethoxy-Q$_2$ reduction had an apparent $K_m$ of 70 and 6 μM, respectively. At low diethoxy-Q$_2$ concentrations, the total rate and MP6-resistant component showed similar affinity for diethoxy-Q$_2$ (10 and 6 μM, respectively). At high diethoxy-Q$_2$ concentrations, the $K_m$ value for the total activity (31 μM) was close to the arithmetic mean of the $K_m$ values for the two components (i.e. $(70 + 6)/2 = 38$ μM). Although a statistical significance of linear regression of the MP6-sensitive component was not so high because of relatively large experimental errors, the important results are that the activities at the two ubiquinone reduction sites could be distinguished and that their affinities for diethoxy-Q$_2$ differed by a factor of about 10.

**DISCUSSION**

We synthesized novel potent N-methylpyridinium and quinolinium cationic inhibitors of mitochondrial complex I. The effects of TPB$^-$ on the inhibition behavior of these inhibitors (i.e. potentiation and reversal of inhibition) could be explained by the dual binding sites model which was proposed originally for MPP$^+$ and inevitably for ubiquinone supposing a difference in the hydrophobic nature of the two sites and/or their environment (6). The remarkable biphasic nature of the dose-response curves of MP6 likely reflects a significant difference in sensitivity to the inhibitor between the two sites. The similar inhibition behaviors of the potent inhibitors for electron transfer and proton pumping activities indicated that both ubiquinone reduction sites contribute to redox-driven proton pumping. The combined use of a selective inhibitor MP6 and diethoxy-Q$_2$ provided for the first time, kinetic evidence indicating that the affinity for an exogenous ubiquinone significantly differs between the two ubiquinone reduction sites. Thus, the present study provided strong evidence for the existence of two ubiquinone reduction sites and offers a new approach to the design synthesis of ideal selective inhibitors of the ubiquinone reduction sites in complex I.

The inhibition of NADH-ubiquinone oxidoreductase activity by MP6 alone was saturated at a partial inhibition level. In particular, when Q$_1$ was used as an electron acceptor, the plateau phase in the dose-response curve continued over a wide range of inhibitor concentrations. The site that was readily blocked by low concentrations of MP6 would be the hydrophilic binding site since almost complete inhibition was brought about at the same concentration range of MP6 in the presence of TPB$^-$. However, the range of the plateau phase varied depending upon the ubiquinone used. This may be because the manner of binding or reduction of the ubiquinone analogues in the hydrophobic site differs because of structural differences of the ubiquinones, resulting in differences in the sensitivity to inhibition by MP6. This notion also explains why two kinetic components of ubiquinone reduction were not always distinguishable irrespective of the ubiquinones used, because the difference in the affinities of the two sites to the ubiquinone varies with ubiquinone structure, and is not necessarily large enough to be separated. Concerning the structural nature of the ubiquinone binding sites, it is notable that bulky diethoxy-Q$_2$ could serve as a good electron acceptor from the physiological site of complex I. This specificity in complex I is conspicuous because diethoxy-Q$_2$ and its reduced form proved to be very poor substrates in studies with other electron transfer enzymes such as bovine heart mitochondrial complexes II and III (31), glucose dehydrogenase (25), and terminal ubiquinol oxidases (18) in *Escherichia coli*. This fact supports our previous conclusion that the ubiquinone reduction site in complex I is spacious enough to accommodate bulky exogenous ubiquinones and a variety of structurally different inhibitors in a dissimilar manner (25).

Vinogradov et al. (10) have indicated the existence of two distinct species of complex I-associated ubisemiquinones by EPR spectroscopy. The existence of two ubisemiquinones itself seems to be consistent with the dual binding sites model for ubiquinone. However, they have interpreted the data as evidence supporting the energy-coupled vectorial dismutation between bound paired ubisemiquinones (10, 32). This model has been proposed for the terminal electron transfer between Fe-S cluster N-2 and the ubiquinone pool and assumes two ubisemiquinone molecules bound at spatially separated and environmentally different sites. These two models are, however, based on essentially different ideas concerning a functional relation between the two ubiquinone binding sites. Although the dual binding sites model supported by the present study provides no interpretation for the stoichiometry of proton transfer at present, this model premises two functionally independent ubiquinone binding sites; therefore, a selective inhibition of one of the two sites is possible, as was actually the case. In contrast, the latter model assumes tightly coupled electron and/or proton transfer between the two ubisemiquinones (32).

*H. Miyoshi and M. Ohshima, unpublished data. Similar results have been reported in Ref. 31.*
It therefore seems unlikely that a redox reaction accompanied by one of the two ubisemiquinones is disturbed by inhibitors with a high selectivity. To draw a conclusion on the functional relation between the two ubiquinone binding sites, further experimental data are needed. The fact that energy-coupled ubiquinone reduction can be selectively blocked under certain conditions will be suggestive in elucidating the terminal electron transfer step in complex I.

A consensus on the ubisemiquinone as the obligatory intermediate during the electron transfer from NADH to the ubiquinone pool has been reached (10, 33). In addition, it is now certain that there are two ubiquinone reduction sites (or two distinct ubisemiquinones) in complex I. The functional and spatial relations of the two ubiquinone binding sites remain to be elucidated. To solve these problems, synthetic development of very potent and completely selective inhibitors of one of the two sites is very helpful. Although MP6 is not the completely selective inhibitor in the strict sense, this compound would be a worthy lead compound for synthesis of such an ideal inhibitor. Further structural modification of MP6 is currently underway in our laboratory.

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