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Origin of selective inhibition of mitochondrial complex I by pyridinium-type inhibitor MP-24

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Abstract

Positively charged pyridiniums are unique inhibitors to probe the structural and functional properties of the ubiquinone reduction site of bovine heart mitochondrial complex I. In this study, we synthesized a series of neutral as well as pyridinium analogues of MP-24 (*N*-methyl-4-[2-methyl-2-(*p-tert*-butylbenzyl)propyl]pyridinium), a selective inhibitor of one of the two proposed binding sites of these pyridinium-type inhibitors of complex I (H. Miyoshi et al., J. Biol. Chem. 273 (1998) 17368–17374), to elucidate the origin of its selectivity. Inhibitory potencies of all neutral and pyridinium analogues with tetraphenylboron (TPB^-), which forms an ion-pair with pyridiniums, were comparable, although the degrees of selective inhibition by pyridiniums without TPB^- were entirely different. In contrast to MP-24, the dose–response curves of nonselective pyridiniums and all neutral analogues were not affected by incubation conditions. These results strongly suggested that the process of the inhibitor passage to the binding sites is responsible for the selective inhibition.

Keywords: Mitochondrial complex I; Respiratory chain; Pyridinium inhibitor; Structure–activity relationship

1. Introduction

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]. There are a variety of inhibitors of mitochondrial complex I, and with the exception of a few which inhibit electron input into the enzyme [3,4] all inhibitors act at or close to the ubiquinone reduction site [5,6]. Among the inhibitors, positively charged neurotoxic *N*-methyl-4-phenylpyridinium (MPP^+) and its alkyl analogues exhibit unique inhibitory behavior with bovine heart mitochondrial complex I [7]. Singer and colleagues [7–11] suggested that MPP^+ analogues are bound at two sites in the enzyme, one accessible to 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

Abbreviations: complex I, NADH–ubiquinone oxidoreductase; DPI, diphenylene-iodonium; I_{75}/I_{25} ratio, an index of the biphasic nature of the dose–response curve, wherein I_{75} and I_{25} are the molar concentrations which give 75% and 25% inhibition of the enzyme activity, respectively, without TPB^-; I_{50} ($+TPB^-$), a molar concentration to give 50% inhibition in the presence of 2 μM TPB^-; I_{50} ($-TPB^-$), a molar concentration to give 50% inhibition in the absence of TPB^-; MPP^+, *N*-methyl-4-phenylpyridinium; Q_1, ubiquinone-1; SMP, submitochondrial particles; TPB^-, tetraphenylboron

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for complete inhibition. This should be helpful in elucidating the terminal electron transfer step in complex I and seems to be consistent with the existence of two EPR-detectable species of complex I-associated ubisemiquinones [12,13]. However, recent radioligand binding assays suggested that ordinary complex I inhibitors share a common large binding domain in complex I [6].

We previously synthesized a series of MPP^+ analogues which are much more potent than the original MPP^+ and demonstrated that the presence of hydrophobic counter-anion tetraphenylboron (TPB^-) potentiates the inhibition by MPP^+ analogues differently depending upon the molar ratio of TPB^- to the inhibitors [14]. In the presence of a catalytic amount of TPB^-, the inhibitory potency of MPP^+ analogues was markedly enhanced, and the extent of inhibition was almost complete. The presence of an excess amount of TPB^- partially reactivated the enzyme activity, and the inhibition was partly saturated ($\sim$50%). This complicated inhibitory behavior could be explained by the dual binding sites model [7], which supposes quite different hydrophobic natures of the two sites and/or their environments.

If there are two distinct binding sites of MPP^+ analogues in bovine complex I, there should be specific inhibitors which act selectively at one of the two proposed binding sites since it is unlikely that the structural properties of the two sites are completely identical. We recently synthesized MP-24 (*N*-methyl-4-[2-methyl-2-(*p-tert*-butylbenzyl)propyl]pyridinium), Fig. 1) [15]. This inhibitor showed a remarkably biphasic dose–response curve for the inhibition of NADH–Q_1 oxidoreductase activity in the absence of TPB^-, making this compound the first selective inhibitor of the two sites [15]. On the basis of the observation that even slight structural modification of MP-24 markedly affected the extent of the biphasic nature of the dose–response curve, we suggested that the selective inhibition by MP-24 is closely related to its structural specificity [15].

For use of MP-24 as a probe to examine the mechanism of the terminal electron transfer step of complex I and develop further selective inhibitors, several points concerning the mechanism of its inhibitory effect remain to be elucidated; particularly the origin of the selective inhibition. For this purpose, neutral analogues of MP-24 would be helpful since the effect of a positive charge on the inhibitor passage to the binding sites can be negligible. In the present study, we investigated the origin of the selective inhibition of MP-24 by comparing its inhibitory effects with those of newly synthesized neutral as well as pyridinium analogues of MP-24.

2. Materials and methods

2.1. Materials

MP-6, MP-17, MP-24, MP-25 and MP-26 were synthesized according to the previously described methods [14,15]. PMP-6, PMP-17, PMP-24, PMP-25 and PMP-26 were the precursors of the corresponding *N*-methyl pyridiniums. MP-29 was prepared by *N*-ethylation of PMP-24 with ethyl trifluoromethanesulfonate in dry CH_2Cl_2. THMP-24 and THMP-29 were prepared by reduction of MP-24 and MP-29, respectively, with $NaBH_4$ in ethanol [16]. The structures of these compounds were characterized by ^1H NMR (Bruker AC-300) and elemental analyses for carbon, hydrogen and nitrogen, within an error of $\pm$0.3%. Bullatacin was generously provided by Dr. J.L. McLaughlin (Purdue University). Other chemicals were commercial products of analytical grade.

2.2. Methods

Bovine heart submitochondrial particles (SMP) were prepared by the method of Matsuno-Yagi and Hatefi [17] and stored in a buffer containing 0.25 M sucrose and 10 mM Tris/HCl (pH 7.4) at −78°C. NADH–Q_1 oxidoreductase activity was measured as the rate of NADH oxidation with a Shimadzu UV-3000 at 340 nm ($\varepsilon = 6.2\ mM^{-1}\,cm^{-1}$). 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 the final mitochondrial protein concentration was 30 μg/ml. Unless otherwise noted, the reaction was started by adding 50 μM NADH after incubation of SMP with inhibitor for 4 min at 30°C. Q_1 was used as an electron acceptor since this substrate yields high reaction rate with linear kinetics [18]. The experimental conditions for investigation of the effects of incubation temper-

ature or incubation period on the inhibition are described in the figure legends.

3. Results and discussion

MP-24 showed a marked biphasic dose–response curve for inhibition of NADH–Q_1 oxidoreductase activity (Fig. 2), making this compound the first selective inhibitor of the two sites [15]. The selectivity of MP-24 in terms of the I_{75}/I_{25} ratio was >690 (Table 1). The complete inhibition by MP-24 without TPB^- could not be determined because of solubility limit above $\sim$400 μM. Such a marked biphasic nature of the dose–response curve has not been reported previously for complex I inhibitors. However, complete inhibition was readily achieved at low concentrations of MP-24 (<10 μM) in the presence of 2 μM TPB^- (Fig. 2). As TPB^- increases pyridinium concentration in the membrane lipid phase due to ion-pair formation and facilitates pyridinium passage through the hydrophobic barrier to the binding site, the site that is readily blocked by low concentrations of MP-24 without TPB^- would be the hydrophilic binding site [7,14,15].

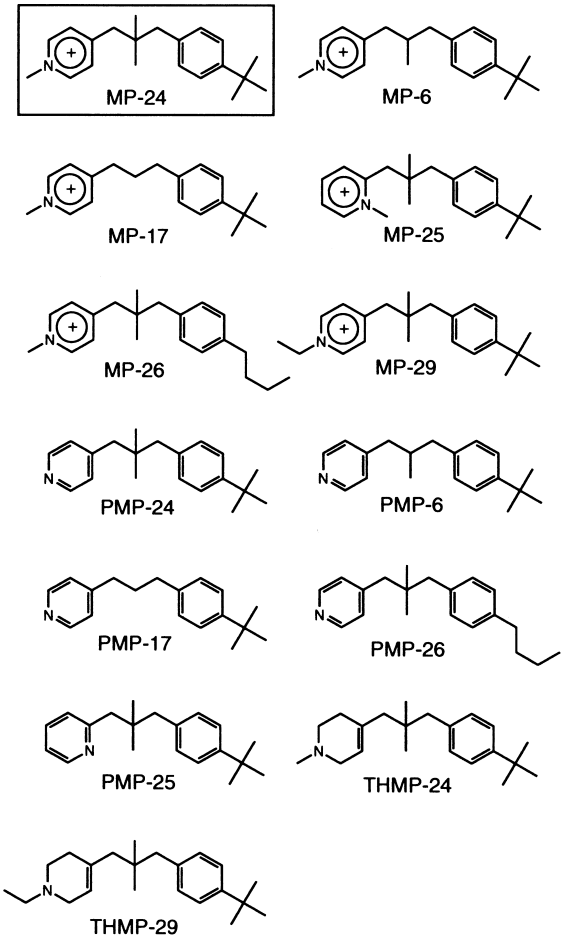

Fig. 1. Structures of pyridinium-type inhibitors and their neutral analogues synthesized in this study.

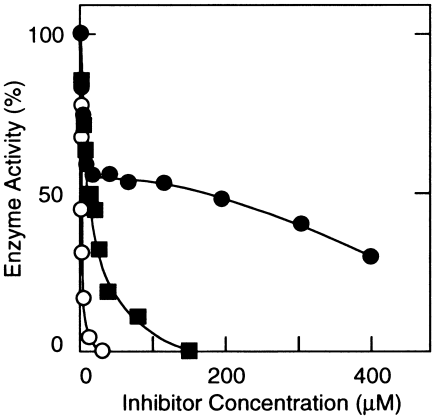

Fig. 2. Inhibition of NADH–Q_1 oxidoreductase activity by MP-24 and MP-29. The reaction medium contained 50 mM potassium phosphate (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 2 mM KCN, 0.4 μM antimycin A, and a final mitochondrial protein concentration of 30 μg/ml. The reaction was started by adding 50 μM NADH after incubation of SMP with the indicated concentrations of inhibitors for 4 min at 30°C. The effects of MP-24 and MP-29 in the absence of TPB^- are shown by closed circles and squares, respectively. The effect of MP-24 in the presence of 2 μM TPB^- is also shown (open circles). The control enzyme activity was 0.62 μmol NADH oxidized per min/mg of protein. The extent of inhibition by 0.1 μM piericidin A was taken as 100%.

The previous observation that the two components of total enzyme activity exhibiting different sensitivities to MP-24 (without TPB^-) showed markedly different pH dependencies (Fig. 7 in [15]) suggested that MP-24 interacts with the two distinct binding sites in complex I. To verify this and also to confirm whether the apparent partial saturation of the inhibition by MP-24 is indeed due to that of occupation of the binding site (probably the hydrophilic site), we examined double inhibitor titrations in combination of MP-24 and bullatacin (rolliniastatin-2), the most potent inhibitor of the ubiquinone reduction site of bovine heart mitochondrial complex I [6,19,20]. The titration curve of bullatacin alone was linear when exogenous ubiquinone (Q_1) was used as an electron

Table 1
Summary of the inhibition of complex I activity by pyridinium-type inhibitors and their neutral forms

Inhibitors	I_{75}/I_{25}	I_{25}^{a} (μM)	I_{50} (+TPB$^-$)[b] (μM)	I_{50} (−TPB$^-$)[c] (μM)
MP-24	>690[d]	0.58	0.30	ND
MP-6	207	0.83	0.33	ND
MP-17	19	0.93	0.72	6.3
MP-25	14	2.1	2.3	9.5
MP-26	152	0.67	0.34	ND
MP-29	38	0.61	0.18	6.1
PMP-24	4.6	–	ND	0.063
PMP-6	5.0	–	ND	0.065
PMP-17	6.3	–	ND	0.075
PMP-25	3.8	–	ND	1.95
PMP-26	5.6	–	ND	0.052
THMP-24	5.2	–	ND	0.073
THMP-29	6.5	–	ND	0.029

The I_{75}/I_{25} ratio was used as an index of the biphasic nature of the dose–response curve, where I_{75} and I_{25} are the molar concentrations which gave 75% and 25% inhibition of the control NADH–Q_1 oxidoreductase activity, respectively, without TPB$^-$. The I_{50} (+TPB$^-$) and I_{50} (−TPB$^-$) values, i.e., the molar concentrations which gave 50% inhibition, were obtained with and without 2 μM TPB$^-$, respectively. The values are averages from at least two independent measurements.

[a]The I_{25} values (without TPB$^-$) of pyridiniums are listed since the values can be regarded as indexes of inhibitor sensitivity of the hydrophilic binding site.

[b]ND, not determined because the I_{50} values of the neutral analogues were identical to those obtained without TPB$^-$.

[c]ND, not determined since the I_{50} values of MP-24, MP-6 and MP-26 could not be accurately estimated due to relatively large experimental error in independent experiments. This is because 50% inhibition by these compounds was observed in the plateau region of biphasic dose–response curves.

[d]The value could not be determined because this inhibitor did not elicit 75% inhibition up to the solubility limit (∼400 μM). It should be noted that the value is highly affected by incubation conditions [15].

acceptor, and complete inhibition was attained at about 1.8 nM (Fig. 3, see also [20]). In the presence of 10 or 100 μM MP-24 which elicited 50∼60% inhibition, complete inhibition by bullatacin was achieved at around 0.9 nM irrespective of MP-24 concentration. As a reference, titration by bullatacin was carried out in combination with diphenyleneiodonium (DPI), which inhibits *electron input* into the enzyme [4]. In the presence of 2.5 μM DPI which elicits about 50% inhibition, complete inhibition by bullatacin was attained at an identical concentration with that obtained in the absence of DPI (i.e., about 1.8 nM). These results showed that the extent of inhibition by MP-24 and that of occupation of the binding sites are comparable. This result along with the previous findings described above [15] suggested that MP-24 interacts with two distinct binding sites in complex I and the partial saturation of the inhibition by MP-24 is due to that of occupation of the binding site.

The extent of selective inhibition by MP-24 in

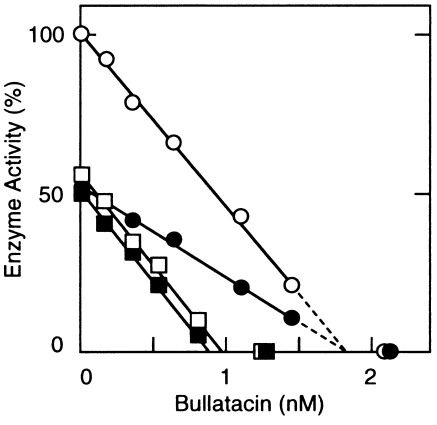

Fig. 3. Inhibition of NADH–Q_1 oxidoreductase activity by bullatacin. The experimental conditions were the same as those described in the legend to Fig. 2. Titration by bullatacin alone is shown by open circles. Titration by bullatacin was performed in the presence of 10 μM MP-24 (open squares), 100 μM MP-24 (closed squares) or 2.5 μM DPI (closed circles).

terms of I_{75}/I_{25} ratio was markedly diminished even by slight structural modification (Table 1). As an example, the titration curve of MP-29 (without TPB^-), which possesses *N*-ethyl group in place of *N*-methyl group of MP-24, is shown in Fig. 2 (closed squares). Judging from the structural dependency of the selective inhibition revealed using a wide variety of pyridiniums [14,15], it is evident that hydrophobicity of the inhibitors is not a determining factor of the extent of selective inhibition. Irrespective of wide variations in I_{75}/I_{25} ratio, inhibitory potencies of the pyridiniums in the presence of 2 μM TPB^-, in terms of I_{50} ($+TPB^-$), were comparable except for that of MP-25 (Table 1)[1]. As TPB^- facilitates pyridinium passage through the hydrophobic barrier to the binding sites [7,14,15], it is unlikely that a difference in the intrinsic inhibitor sensitivities between the two binding sites is responsible for the observed selective inhibition.

To verify this notion, we compared the inhibitory potencies of neutral forms of the pyridiniums to exclude disadvantageous effects of a positive charge on the inhibitor passage through the hydrophobic barrier to the binding sites. It should be mentioned that semiempirical molecular orbital calculation study with AM1 parameterization showed that stable conformation of the neutral analogues of MP-24 (i.e., PMP-24 and THMP-24) is identical to that of MP-24. The dose–response curves of the neutral analogues showed no biphasic nature, as indicated by their I_{75}/I_{25} ratios (Table 1). The inhibitory potencies of the neutral analogues in terms of the I_{50} ($-TPB^-$) values were comparable except for that of PMP-25 (Table 1) [1], although the selectivities were entirely different among the corresponding pyridiniums. The relatively poor structural dependency of the inhibitory potencies may reflect the large cavity-like structure of the ubiquinone reduction domain of the enzyme [6,21]. Moreover, although the inhibitory potencies of the neutral analogues (I_{50} ($-TPB^-$)) were greater than those of corresponding pyridiniums with 2 μM TPB^- (I_{50} ($+TPB^-$)), structural dependencies of the two potencies were comparable. For instance, *para*-substituted derivatives (MP-24, PMP-24) were much more potent than *ortho*-substituted derivatives (MP-25, PMP-25), and *N*-ethyl derivatives (MP-29, THMP-29) were slightly more potent than *N*-methyl derivatives (MP-24, THMP-24). Thus, these results indicated not only that the difference in the intrinsic inhibitor sensitivities between the two binding sites is not pronounced, but also that structural specificity of the inhibitor sensitivities at the binding sites is not associated with that of the observed selective inhibition. Therefore, the possibility that a difference in the intrinsic MP-24 sensitivities between the two binding sites is responsible for the observed selective inhibition could be excluded.

On the other hand, we previously reported that with longer incubation periods or higher incubation temperatures of SMP with MP-24, the biphasic titration curve of this inhibitor becomes less distinct, suggesting that access of MP-24 to the hydrophobic binding site is promoted by prolongation of incubation and/or increasing incubation temperature [15]. In this sense, the selectivity of interest is highly dependent on incubation conditions. Murphy et al. [11] showed that partitioning of pyridiniums possessing hydrophobic substituent(s) into submitochondrial particles is established more rapidly than the development of inhibition. On the basis of these observations, we proposed that the level of the energetic barrier involving inhibitor passage to the binding sites after partitioning into the membrane is responsible for the observed selectivity [15].

If this is the case, the titration curves of both nonselective pyridiniums and all the neutral analogues of MP-24 would not vary with different incubation conditions. We therefore examined the titration curves of MP-17, MP-25, PMP-24, and THMP-24 under various incubation periods (i.e., for 4, 10 or 30 min at 30°C) or temperatures (i.e., at 20°C, 25°C or 30°C for 4 min incubation). As expected, their titration curves were not affected by the incubation conditions, as shown in Fig. 4 taking the effect of incubation temperature on inhibition by MP-17 as an example. Titration by MP-24 under the same

[1] The activities of *ortho*-substituted derivatives (MP-25 and PMP-25) were less potent than those of the corresponding *para*-substituted derivatives (MP-24 and PMP-24, respectively), probably because the position of the nitrogen atom in the heterocycle is important for interaction with the binding site. The marked loss of inhibitory potency of [2-methyl-2-(*p*-*tert*-butylbenzyl)propyl]benzene (I_{50} ($-TPB^-$), 20 μM), which possesses a benzene ring in place of a pyridine ring of PMP-24, supported this suggestion.

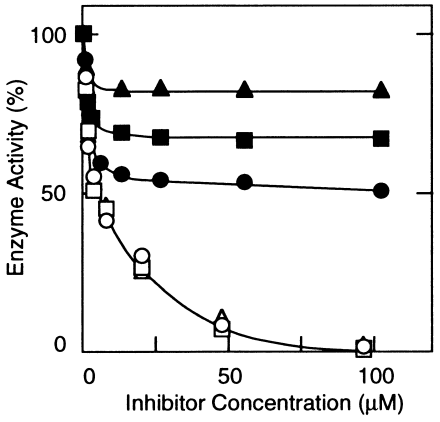

Fig. 4. Inhibition of NADH–Q_1 oxidoreductase activity under various incubation conditions. The experimental conditions were the same as those described in the legend to Fig. 2, except for incubation temperature. Inhibition by MP-17 (open symbols) was determined after incubation for 4 min at 20°C (triangles), 25°C (squares) or 30°C (circles). As references, titration by MP-24 (closed symbols) was also performed under the same experimental conditions.

experimental conditions is also shown in Fig. 4 as a reference. Complete inhibition by MP-17 was attained at less than 10 μM in the presence of 2 μM TPB^- irrespective of incubation temperature (data not shown). In contrast, MP-24 did not show complete inhibition even with 2 μM TPB^- at 20°C, although complete inhibition was readily attained at less than 10 μM at 30°C (Fig. 2). These observations indicated that the energetic barrier preventing access of MP-24 to the binding sites is significantly higher than that of nonselective pyridinium MP-17.

Based on the present observations, we concluded that the process of the inhibitor passage to the binding sites is responsible for the selective inhibition by MP-24. Similar notion had been proposed to explain the complicated inhibitory behavior of an original MPP^+ [7]. The high structural specificity required for the selectivity would be closely related to the level of the energetic barrier preventing access of the inhibitor to the binding sites.

The present results were discussed on the basis of heterogeneity of the two binding sites of pyridinium-type inhibitors, which was originally proposed by Singer's group [7–11]. Recently, from the radioligand binding assay [6] and the photoaffinity labeling study [24], it was claimed that ordinary complex I inhibitors share a common binding domain with partially overlapping sites, although the stoichiometry of inhibitor binding relative to the domain was not precisely defined in the studies. If there is only one inhibitor binding domain in complex I, the complexity of inhibitory action of MP-24 would have to be interpreted by another scenario. For instance, two molecules of MP-24 per one large binding domain may be required for complete inhibition of the enzyme activity, as suggested for E-β-methoxyacrylate inhibitors at center P of cytochrome bc_1 complex [22,23]. The binding of the first molecule of this charged inhibitor may induce conformational change of the enzyme, which results in significant decrease in the binding affinity of the second inhibitor molecule to the domain. Even in this case, the high structural specificity of MP-24 including the existence of a positive charge could be closely related to this phenomenon.

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