Journal of Bioenergetics and Biomembranes, Vol. 33, No. 3, 2001

Probing the Ubiquinone Reduction Site in Bovine Mitochondrial Complex I Using a Series of Synthetic Ubiquinones and Inhibitors

Hideto Miyoshi[1]

Studies of the structure–activity relationships of ubiquinones and specific inhibitors are helpful to probe the structural and functional features of the ubiquinone reduction site of bovine heart mitochondrial complex I. Bulky exogenous short-chain ubiquinones serve as sufficient electron acceptors from the physiological ubiquinone reduction site of bovine complex I. This feature is in marked contrast to other respiratory enzymes such as mitochondrial complexes II and III. For various complex I inhibitors, including the most potent inhibitors, acetogenins, the essential structural factors that markedly affect the inhibitory potency are not necessarily obvious. Thus, the loose recognition by the enzyme of substrate and inhibitor structures may reflect the large cavitylike structure of the ubiquinone (or inhibitor) binding domain in the enzyme. On the other hand, several phenomena are difficult to explain by a simple one-catalytic site model for ubiquinone.

KEY WORDS: Complex I; respiratory enzymes; ubiquinone; acetogenin; structure–activity relationship.

STRUCTURE–ACTIVITY STUDY OF SYNTHETIC SHORT-CHAIN UBIQUINONES

Ubiquinones (Qs) function as mobile mediators for electron transfer and proton translocation between redox enzymes in mitochondrial and bacterial respiratory systems. Therefore, they are regarded as functional elements in the respiratory systems. Q is an amphiphilic molecule composed of a polar 1,4-benzoquinone ring and a hydrophobic isoprenyl tail. The ring moiety is directly involved in redox reactions. Molecular orbital calculations demonstrated that the conformations of the methoxy groups in the 2- and 3-positions of the ring affects electrical potential of the oxidized form of Q or semiquinone radical through conformational interconversion (Robinson and Kahn, 1990; Burie *et al.*, 1997). Therefore, the manner of binding of the methoxy groups to the protein environment is expected to significantly influence the Q redox reaction. On the other hand, the tail seems to increase the hydrophobicity of the Q molecule to facilitate lateral diffusion in biomembranes (Lenaz and Degli Esposti, 1985). In the bacterial photosynthetic reaction center, extensive physicochemical studies of the Q redox sites have been performed on the basis of the atomic structures of the protein and bound cofactors (*e.g.*, Graige *et al.*, 1998; Rabenstein *et al.*, 2000), whereas our knowledge concerning the binding and redox properties of Q in most respiratory enzymes including complex I is still not sufficient.

To probe the structural and functional features of the Q reduction site in bovine complex I, a structure–activity relationship study using a series of alkyl derivatives of short-chain Qs, in which the native substituents of the quinone ring are replaced by other alkyl groups, is helpful since this type of structural modification inevitably alters the molecular shape while minimizing changes in the redox properties of the molecule (He *et al.*, 1994). Such structural modifications enable separation of the steric and electronic effects of the substituents on the redox activity. Synthetic procedures that enable chemical modifications of the substituents at all positions in the quinone ring to other alkyl or alkoxyl groups have been established (Sakamoto *et al.*, 1996a; Ohshima *et al.*, 1998).

[1] Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kita-shirakawa, Sakyo-ku, Kyoto 606-8502, Japan. e-mail: miyoshi@kais.kyoto-u.ac.jp

0145-479X/01/0600-0223$19.50/0

Bulky alkoxy derivatives of Q_2 (ubiquinone-2) and DB (2,3-dimethoxy-5-methyl-6-*n*-decyl-1, 4-benzoquinone), where the methoxy groups in the 2- and/or 3-positions are replaced by other alkoxy groups, such as ethoxy, propoxy, and butoxy groups (Fig. 1), served as sufficient electron acceptors from the physiological Q-reduction site in bovine complex I (Ohshima *et al.*, 1998). This observation is in marked contrast to other respiratory enzymes, such as mitochondrial complexes II and III (He *et al.*, 1994), cytochrome bo_3 (Sakamoto *et al.*, 1996b), glucose dehydrogenase in *E. coli* (Sakamoto *et al.*, 1996a), and alcohol dehydrogenases of acetic acid bacteria (Matsushita *et al.*, 1999). In these enzymes, even monoethoxy derivatives of Q_2 (or Q_2H_2) and DB (or DBH_2) were very poor substrates, indicating that the structural requirements for the methoxy groups in the 2- and 3-positions are stringent. Structural modification of the 2- and 3-positions affected the electron-accepting ability in similar way, indicating that steric congestion surrounding both methoxy groups is comparable in bovine complex I. In addition, the presence of a methyl group in the 5-position was not crucial for the electron-accepting activity (Sakamoto *et al.*, 1996a). Thus, bovine complex I recognizes the quinone ring moiety very loosely. This is probably because the Q reduction site of the enzyme is sufficiently spacious to accommodate bulky exogenous substrate.

The steady-state kinetics of NADH-Q oxidoreductase activity of complex I are 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 (Fato *et al.*, 1996). The same mechanism was suggested using ferricyanide and DCIP (2,6-dichlorophenolindophenol) as electron acceptors Dooijewaard and Slater, 1976). Since first (NADH) and second (Q) substrates bind reversibly at different sites of the enzyme, this kinetic mechanism is generally supposed to suffer product inhibition; that is, the presence of a reduced form of Q inhibits the enzyme reaction in a competitive manner against an oxidized form. In fact, NADH-Q oxidoreductase activity for various Q_2 analogs shown in Fig. 1 was inhibited by its reduced form in a concentration-dependent manner and was completely blocked at high concentrations (Ohshima *et al.*, 1998). In these cases, the mechanism of inhibition by the reduced form of Q was competitive against its oxidized form. Interestingly, the extent of product inhibition by the reduced form of a *different* type of Q was saturated at 40–60%. This incomplete product inhibition by combined use of different Q_2 analogs is difficult to explain by a one-catalytic site model for exogenous short-chain Q (Ohshima *et al.*, 1998).

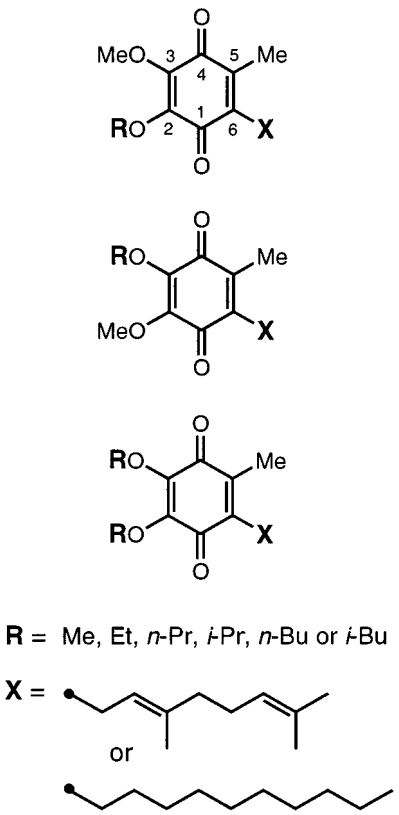

Fig. 1. Structures of Q_2 and DB analogs in which methoxy group(s) at the 2- and/or 3-positions were replaced by other bulky alkoxy groups.

Only limited information is available regarding the mechanism of energy-coupled electron transfer in complex I. Even the H^+/e^- stoichiometry is still uncertain and values reported ($H^+/e^- \geq 2$) are too high to rationalize on the basis of a mechanism in which a single electron-transfer reaction is coupled to the translocation of one proton in analogy to the Q-cycle model of complex III (Brandt, 1997). On the other hand, there is virtually no hard experimental evidence from which to infer the possibility that the mechanism (or stoichiometry) of proton pumping varies depending upon substrate structures. Degli Esposti and colleagues reported that electron accepting and proton pumping activities of exogenous Q analogs, which possess different tail structures but an identical substitution pattern of the quinone ring, are not comparable (Degli Esposti *et al.*, 1996; Helfenbaum *et al.*, 1997). To examine the above possibility, however, comparison of the two activities among the compounds used by these authors is not necessarily adequate not only because the structure of the quinone ring moiety is not varied, but also because less hydrophobic Q analogs interact incompletely with the physiological Q reduction site, making the comparison very complicated. To overcome these limitations

and considering the structural specificity of the Q reduction site in bovine complex I described above, Q_2 analogs listed in Fig. 1 should be good probes to examine this issue. On the basis of comparison of structure–activity profiles for electron-accepting and proton-pumping activities of the Q_2 analogs, it was revealed that the two activities are comparable irrespective of wide structural variations of the quinone ring moiety (Ohshima *et al.*, 1998). This finding indicated that the proton-pumping mechanism is identical, or at least not significantly different, regardless of the substrate structure.

The V_{max} (or k_{cat}) value of Q_2 in the reaction with bovine complex I is much lower than those of DB and Q_1 (Fato *et al.*, 1996; Degli Esposti *et al.*, 1996). It is, however, noteworthy that the intrinsic electron-accepting efficiency of Q_2 in terms of k_{cat}/K_m is rather greater than those of the latter two substrates (Sakamoto *et al.*, 1998). This means that Q_2 is not necessarily a poor substrate, as described in several reports (Estornell *et al.*, 1993; Fato *et al.*, 1996; Degli Esposti *et al.*, 1996). The low V_{max} value of Q_2 is primarily due to stabilization of the enzyme–Q complex or to the high affinity of Q_2 toward the enzyme, resulting in slowdown of the product (Q_2H_2) release (Sakamoto *et al.*, 1998). However, the difference between Q_2 and DB cannot be attributed to the hydrophobicity of the tail, since the isoprene structure is rather less hydrophobic than the saturated alkyl tail with the same number of carbon atoms (Warncke *et al.*, 1994; Fato *et al.*, 1996).

To identify the structural factor(s) of the diprenyl tail, which is responsible for the high-affinity binding of Q_2 to the enzyme, we performed a structure–activity study using a systematic set of synthetic Q_2 analogs (Fig. 2), where only one of the structural factors of the diprenyl tail was modified in a limited way (Sakamoto *et al.*, 1998). This study clearly showed that 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 Q-reduction site, resulting in low K_m and k_{cat} values of Q_2 reduction. It is, however, unclear whether the enthalpic interaction of the first isoprene unit with the binding environment or conformational energy of the isoprene unit in its protein-bound state is the dominant force in the specific binding of Q_2 to the enzyme, since discussion based on the protein structure or the tail conformation in the protein-bound state is unfeasible for complex I at present.

With regard to the role of the isoprenyl chain of Q, Yu *et al.* (1985) reported that electron-accepting and -donating activities of Q_2 (or Q_2H_2) and DB (or DBH_2) are completely identical in mitochondrial succinate-cytochrome *c* oxidoreductase. On the other hand, a specific

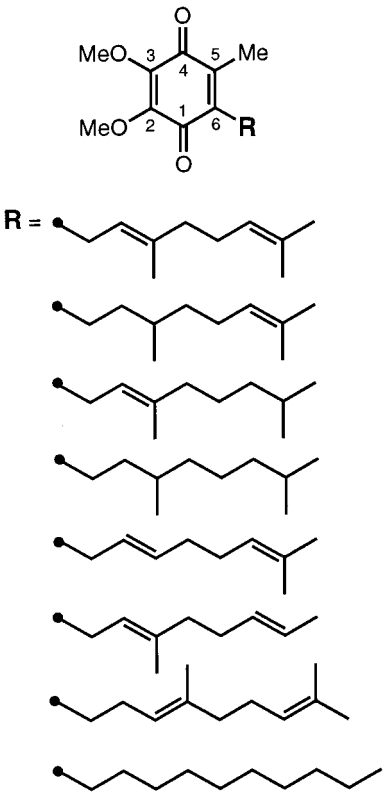

Fig. 2. Structures of Q_2 analogs in which only one of the structural factors of the diprenyl tail was systematically modified.

role of diprenyl tail of Q_2 was reported for bacterial photosynthetic reaction center (Warncke *et al.*, 1994) and *E. coli* cytochrome bo_3 (Sakamoto *et al.*, 1998). It is, therefore, concluded that the role of the isoprenyl tail is not simply to enhance the hydrophobicity of the molecule and molecular recognition of the tail by the Q redox site differs among the respiratory enzymes. Some specific interaction between the isoprenyl tail and its binding environment may be associated with the flip motion of the quinone ring around the tail suggested for Q_B in bacterial photosynthetic reaction center (Lancaster and Michel, 1997) and Q_o site in complex III (Crofts *et al.*, 1999).

ESSENTIAL STRUCTURAL FACTORS OF COMPLEX I INHIBITORS

With the exception of rhein (Kean *et al.*, 1971) and diphenyleneiodonium (Majander *et al.*, 1994), which inhibit electron input into complex I, all inhibitors act at or close to the Q-reduction site (the so-called "rotenone site"). Detailed study of the inhibitory action of specific complex I inhibitors is important to elucidate the structural and functional features of the terminal electron transfer

step of this enzyme. As the first step toward this purpose, identification of the crucial structural factors of the inhibitors including the active conformation required for potent inhibition would be very useful, as recently revealed for antimycin A binding to Q_i site in complex III (Kim *et al.*, 1999), wherein the crucial structural factors of antimycin A and their interaction with the enzyme suggested by structure–activity relationship study (Miyoshi *et al.*, 1995) were almost corroborated by an X-ray crystallographic study.

On the basis of structure–activity relationship studies of various complex I inhibitors (e.g., Ueno *et al.*, 1994; Satoh *et al.*, 1996; Miyoshi *et al.*, 1997, 1998a), the author noted in a previous review that in contrast to complex III inhibitors, essential structural factors of complex I inhibitors that markedly affect the inhibitory potency are not necessarily obvious (Miyoshi, 1998). For instance, only the pyridinol hydroxy group is undoubtedly essential for the action of piericidin A. Other functional groups on the pyridinol ring are indeed important to maintain its very high potency, but do not determine its inhibitory effects. The bent form of the rotenone molecule is a very important structural factor, whereas the two methoxy groups in the A ring, 12-C=O group in the C ring, and presence of the E ring itself are not essential for potent inhibition. Particularly for capsaicins and pyridinium-type inhibitors, it is not easy to define the crucial structural properties important for inhibition, except hydrophobicity of the molecule. These findings along with the observation that a wide variety of structurally different inhibitors act at common binding domain in complex I (Friedrich *et al.*, 1994; Okun *et al.*, 1999; Schuler *et al.*, 1999) suggest that this domain is a large cavitylike structure that enables occupation by a variety of inhibitors in a dissimilar manner depending on their structural specificity, in analogy with different types of Q_o center inhibitors of complex III (Kim *et al.*, 1998). In this sense, the manner of binding of complex I inhibitors might not follow the so-called "key and keyhole" relation in which multiple functional groups of the inhibitors are thought to interact tightly and strictly with their binding environment in a similar manner.

Interestingly, based on the structure–activity studies performed in our laboratory, this also appeared to be true for acetogenins, the most potent inhibitors of bovine complex I. The acetogenins are characterized by two functional units, the hydroxylated tetrahydrofuran (THF) and the α,β-unsaturated γ-lactone ring, separated by a long alkyl chain, as shown in Fig. 3, taking bullatacin as an example. These inhibitors act at the terminal electron transfer step of the enzyme (probably Q reduction site), similarly to the usual complex I inhibitors such as piericidin

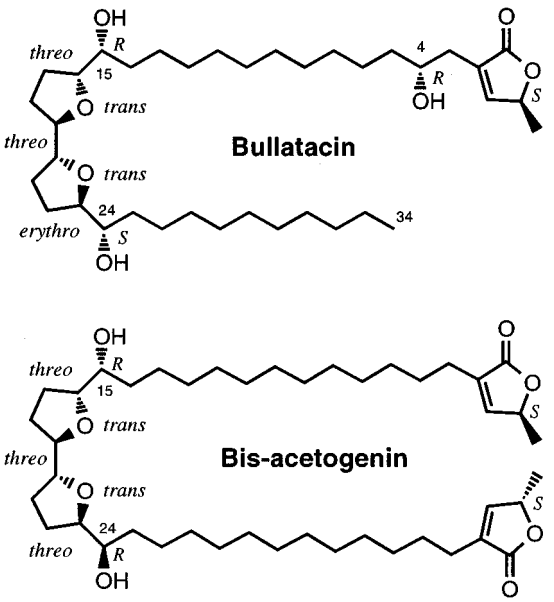

Fig. 3. Structure of bullatacin (the most potent natural acetogenin) and synthetic *bis*-acetogenin described in the text.

A and rotenone (Okun *et al.*, 1999; Schuler *et al.*, 1999); however, structural similarities are not apparent between the acetogenins and these known complex I inhibitors. A systematic set of natural and synthetic acetogenins have been examined for their inhibitory actions with bovine complex I to identify the essential structural factors of these inhibitors (Miyoshi *et al.*, 1998b; Kuwabara *et al.*, 2000; Takada *et al.*, 2000).

The most common structural units of a large number of natural acetogenins, such as adjacent *bis*-tetrahydrofuran (THF) rings, OH groups in the 4- and/or 10-positions of the spacer, and the α,β-unsaturated γ-lactone, were not essential for potent activity. This conclusion is in agreement with the results from other laboratories using natural products (Degli Esposti *et al.*, 1994; Gonzalez *et al.*, 1997; Tormo *et al.*, 1999). It is notable that the γ-lactone ring of some natural acetogenins can be substituted by natural ubiquinone-type 1,4-benzoquinone ring (Hoppen *et al.*, 2000). The stereochemistry surrounding the *bis*-THF rings with flanking OH groups was also unimportant for potent activity (Miyoshi *et al.*, 1998b). This is because the stereochemical difference of the *bis*-THF rings makes little difference in the three-dimensional structure of this moiety, which was corroborated by an exhaustive conformational space search analysis (Miyoshi *et al.*, 1998b). Furthermore, the presence of free OH group(s) in the adjacent *bis*-THF rings was favorable, but not essential, for potent activity. This is probably because high polarity (or hydrophilicity), rather than hydrogen

bond-donating ability, around the *bis*-THF rings is required to retain the inhibitor in the active conformation (Takada *et al.*, 2000). From a molecular viewpoint, the importance of high polarity may be associated with the fact that the putative target subunit of acetogenin in bovine complex I (PSST, Schuler *et al.*, 1999) is hydrophilic protein and contains no transmembrane helixes according to secondary structure prediction (Arizmendi *et al.*, 1992; Finel *et al.*, 1992; Weidner *et al.*, 1993).

Either of the THF and γ-lactone ring moieties alone, which were synthesized individually, exhibited no or very weak inhibitory effects. Combined use of the two ring moieties at various molar ratios showed no synergistic enhancement of the inhibitory potency. These observations indicated that both functional units work efficiently only when they are directly linked by a flexible alkyl spacer (Kuwabara *et al.*, 2000). Interestingly, length of the alkyl spacer proved to be a very important structural factor for the potent activity, the optimal length being approximately 13 carbon atoms (Takada *et al.*, 2000). It is, therefore, strongly suggested that the γ-lactone and THF ring moieties act in a cooperative manner on complex I with the support of some specific conformation of the spacer. That is, a specific conformation of the alkyl spacer could be important for optimal positioning of the two ring units in the enzyme. Taking into account the proposal of fairly large binding domain of inhibitor in complex I as mentioned above, the possibility that both ring moieties occupy different subsites in the same binding domain, in analogy with different types of Q_o center inhibitors of complex III (Kim *et al.*, 1998), may not be excluded. All together, the crucial structural features of acetogenins are unclear, except for the important function of the alkyl spacer.

Based on the results of [^{1}H]NMR spectroscopic and differential scanning calorimetry studies of acetogenins in liposomal membranes, McLaughlin and colleagues proposed a model of an active conformation of these inhibitors in the mitochondrial membrane environment, wherein the THF ring(s) with flanking hydroxy groups resides near the glycerol backbone of phosphatidylcholine irrespective of the number of THF rings and act as hydrophilic anchor at the membrane surface, and the γ-lactone ring directly interacts with the target site of complex I by lateral diffusion in the mitochondrial membrane interior (Shimada *et al.*, 1998). This model is of interest to obtain insight into the topographical distribution of the Q-reaction site in the membrane environment.

If this model is correct, γ-lactone can be regarded as the only reactive species directly interacting with the enzyme and, hence, the structural modification of this moiety is expected to result in a drastic decrease in inhibitory potency. Further, if the γ-lactone ring is the only reactive species, *bis*-acetogenin possessing *two* natural γ-lactone rings connected to the THF ring moiety by flexible alkyl spacers (Fig. 3) would be expected to elicit inhibitory activity about twice as potent as that of ordinary acetogenins because of the presence of two reactive species per molecule. However, wide structural modifications of the γ-lactone moiety, such as deletion of the γ-methyl group and transformation of the γ-lactone ring to a six-membered δ-lactone ring, did not significantly affect the inhibitory potency (Takada *et al.*, 2000). The inhibitory potency of *bis*-acetogenin was identical to that of natural-type acetogenins, such as bullatacin (Kuwabara *et al.*, 2000). These observations do not support the putative essential role of the γ-lactone ring. Moreover, the observation that hydrogen bond-donating ability of OH groups in the adjacent THF ring is not crucial for the potent activity is in disagreement with the supposed important function of these OH groups as hydrophilic anchors at the membrane surface. Thus, structure–activity studies carried out in our laboratory do not support the model of an active conformation of acetogenins proposed by Shimada *et al.*, (1998).

It should be mentioned that a marked decrease (about 10^6-fold) in the cytotoxicity of bullatacin against carcinoma cells due to saturation of the double bond in the α,β-unsaturated γ-lactone ring (i.e., dihydrobullatacin) was one of the most important experimental evidences for the essential role of γ-lactone ring in the model of McLaughlin *et al.*, (Hui *et al.*, 1989; Shimada *et al.*, 1998). However, this observation is in conflict with the recent report that dihydrosquamocin retains very potent cytotoxic activity compared with squamocin against human epidermoid carcinoma cells (KB), African green monkey epithelial cells (VERO), and mouse lymphocytic leukemia cells (L1210) (Queiroz *et al.*, 2000).

INHIBITION BEHAVIOR OF POSITIVELY CHARGED PYRIDINIUM-TYPE INHIBITORS

Among a wide variety of complex I inhibitors, positively charged neurotoxic *N*-methyl-4-phenylpyridinium (MPP^+, Fig. 4) and its analogs exhibit unique inhibitory actions (Gluck *et al.*, 1994). A series of studies on the inhibition mechanism of MPP^+ analogs by Singer and colleagues (Ramsay *et al.*, 1989, 1991; Gluck *et al.*, 1994) suggested that MPP^+ analogs 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

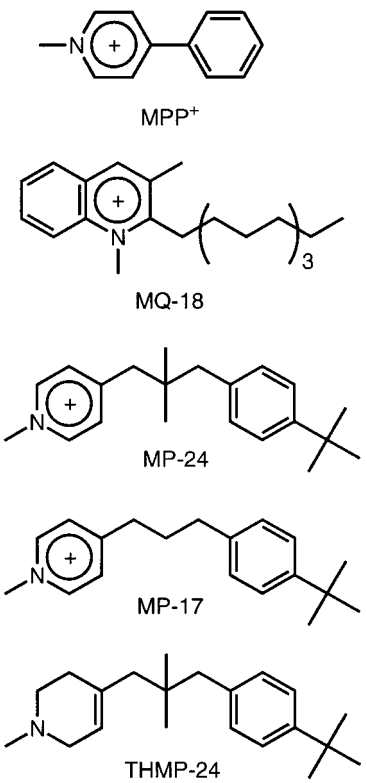

Fig. 4. Structure of pyridinium and quinolinium-type inhibitors described in the text.

for complete inhibition. This seems to be in agreement with the existence of two EPR-detectable species of complex I-associated ubisemiquinones (Vinogradov *et al.*, 1995; Yano *et al.*, 2000) and the circumstantial evidence derived from studies on other types of complex I inhibitors (Gutman *et al.*, 1970; Yagi, 1990; Ueno *et al.*, 1994). Thus, MPP^+ analogs are useful probes with which to characterize the structural and mechanistic features of the Q-reduction site of complex I.

Nevertheless, original MPP^+ analogs have certain limitations when they are used as complex I inhibitors. For instance, the inhibition by MPP^+ analogs requires very high concentrations (ca. mM order) compared to classical potent inhibitors such as piericidin A and rotenone and there have been no specific inhibitors that act selectively at one of the two proposed binding sites. The latter point is particularly unusual since if indeed there are two distinct binding sites, it is unlikely that their structural properties are completely identical. To overcome these problems and advance the usefulness of pyridinium-type inhibitors, potent and specific inhibitors acting selectively at one of the two binding sites are required. We have synthesized a wide variety of *N*-methyl pyridinium and quinolinium cationic inhibitors to develop such an inhibitor.

Some cationic inhibitors such as MQ-18 (*N*-methyl 2-*n*-dodecyl-3-methylquinolinium, Fig. 4) exhibited unique inhibitory behaviors with bovine complex I (Miyoshi *et al.*, 1997). MQ-18 inhibited electron transfer and proton-pumping activity of the enzyme (at under μM order) regardless of whether exogenous or endogenous ubiquinone was used as an electron acceptor. The presence of tetraphenylboron (TPB^-), a counteranion, potentiated the inhibition by MQ-18 in a different way, depending upon the molar ratio of TPB^- to MQ-18. In the presence of a catalytic amount of TPB^-, the inhibitory potency of MQ-18 was markedly enhanced, and the extent of inhibition was almost complete. The presence of equimolar TPB^- potentiated the inhibition more effectively than a catalytic amount of TPB^- at lower concentrations of MQ18, while the inhibition was saturated at an incomplete level (50–60%) at higher concentrations of MQ18. Extensive inhibition resulted when the concentration of the inhibitor was nearly equimolar with TPB^-. These results can be explained by the proposed dual-binding sites model mentioned above, which supposes quite different hydrophobic natures of the two sites and/or their environments (Gluck *et al.*, 1994). The potentiation at the lower concentration range of MQ-18 is due to an increase in the effective concentration of MQ-18 in the membrane lipid phase by ion-pair formation and also to facilitation of inhibitor passage to the hydrophobic binding site. The reversal of the inhibition in the presence of high concentrations of TPB^- is due to a decrease in the effective concentration of the free MQ-18 approaching the hydrophilic site due to an increase in ion-pair formation.

Among a wide variety of the cationic inhibitors, MP-24 {*N*-methyl-4-[2-methyl-2-(*p-tert*-butylbenzyl) propyl]pyridinium; Fig. 4} proved to be a good candidate as a selective inhibitor of one of the two proposed binding sites (Miyoshi *et al.*, 1998a). In the absence of TPB^-, this inhibitor showed 50–60% inhibition at 5 μM in NADH-Q_1 oxidoreductase assay, but the inhibition reached a plateau at this level over a wide range of concentrations. Weak inhibition was again observed above $\sim$150 μM, whereas complete inhibition could not be determined because of solubility limit above $\sim$400 μM. Such a marked biphasic nature of the dose–response curve has not been reported previously for usual complex I inhibitors. On the other hand, complete inhibition was readily obtained at low concentrations of MP-24 ($<$10 μM) in the presence of 2 μM TPB^-. 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 (Gluck *et al.*, 1994), the site that was readily blocked by low concentrations of MP-24 without TPB^- would be the hydrophilic

binding site (Miyoshi *et al.*, 1998a). The residual enzyme activity could be attributable to the hydrophobic site.

Structural modification of MP-24 significantly affected the extent of the biphasic nature of the dose–response curve. For instance, transformation of *para*-substitution on the pyridine ring to *ortho*-substitution and *N*-methyl group to *N*-ethyl group resulted in loss of the biphasic nature of the dose–response curve. Therefore, the selective inhibition by MP-24 is closely related to its structural specificity, indicating that the inhibition is the result of some specific interaction between the inhibitor molecule and the enzyme (or its environment).

An important question is whether the apparent partial saturation of the inhibition of the enzyme activity by MP-24 is indeed due to that of occupation of the binding site (probably the hydrophilic site). Double inhibitor titrations in combination of MP-24 and bullatacin showed that the extent of inhibition by MP-24 and that of occupation of the binding sites are comparable (Iwata *et al.*, 1999). This result, along with the fact that two components of total enzyme activity exhibiting different susceptibilities to MP-24 without TPB^- (i.e., high and low susceptibilities) showed markedly different pH dependencies (Miyoshi *et al.*, 1998a), strongly suggest that MP-24 interacts with two distinct binding sites in complex I and the partial saturation of the inhibition is due to that of occupation of the binding site.

The selectivity of the inhibitory effect is highly dependent on incubation conditions of submitochondrial particles with MP-24 (Miyoshi *et al.*, 1998a). With longer incubation periods at 30°C, the biphasic titration curve of the inhibitor became less distinct, suggesting that access of MP-24 to the hydrophobic binding site is promoted by prolongation of incubation. With 4-min incubation without TPB^-, the relative inhibition was saturated at ∼35, ∼20, and ∼10% at 25, 20, and 15°C, respectively. The presence of TPB^- no longer facilitated complete inhibition at 15 or 20°C. Murphy *et al.* (1995) 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 there is a significant energetic barrier preventing access of the inhibitor to the hydrophilic site as well as the hydrophobic site, although the latter is greater than the former (Miyoshi *et al.*, 1998a). This difference in the energetic barrier could be responsible for the apparent selective inhibition. A similar notion had been proposed to explain the complicated inhibitory behavior of the original MPP^+ (Gluck *et al.*, 1994).

If this is the case, the titration curves of nonselective pyridiniums such as MP-17 (Fig. 4) and all neutral analogs of MP-24, such as THMP-24 (Fig. 4), in which the effect of a positive charge on the inhibitor passage to the binding site is negligible, would not vary with different incubation conditions. Therefore, we examined the titration curves of these analogs under various incubation periods or temperatures (Iwata *et al.*, 1999). As expected, their titration curves were not affected by the incubation conditions, indicating that the energetic barrier preventing access of MP-24 to the binding sites is significantly higher than that of nonselective pyridiniums.

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 molecular basis of how the structural specificity is concerned with ease of inhibitor passage is unclear because of the limited available information on the three-dimensional structure of the enzyme. Nevertheless, as the susceptibility of the hydrophobic site to the inhibition by MP-24 was significantly enhanced as the pH increased (Miyoshi *et al.*, 1998a), it is likely that a positively charged amino residue(s) with pK_a of around neutral pH (probably histidine, cf. Gluck *et al.*, 1994) interferes with access of the inhibitor to the hydrophobic site. Neutralization of the residue(s) by an increase in pH may reduce the energetic barrier of this kinetic process.

Here, we discussed the inhibition behavior of pyridinium-type inhibitors on the basis of heterogeneity of the two binding sites, which was originally proposed by Singer's group (Ramsay *et al.*, 1989, 1991; Gluck *et al.*, 1994). Recently, based on the results of a radioligand binding assay (Okun *et al.*, 1999) and photoaffinity labeling study (Schuler *et al.*, 1999), it was suggested that ordinary complex I inhibitors, including MPP^+, share a common binding domain with partially overlapping sites, although the stoichiometry of inhibitor binding relative to the domain was not precisely defined in these 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. The binding of the first molecule of this charged inhibitor may induce a conformational change of the enzyme, resulting in a 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.

In summary, the results of structure–activity relationship studies on short-chain Qs and various specific inhibitors strongly suggest that the Q (or inhibitor)-binding domain in bovine complex I is a large cavitylike structure

that can be occupied by a wide variety of structurally different inhibitors, as well as bulky Qs, in a dissimilar manner, depending on their structural specificity. This is in agreement with the recent observations that various inhibitors share a common binding domain with partially overlapping sites (Okun *et al.*, 1999; Schuler *et al.* 1999). On the other hand, the incomplete product inhibition by combined use of different Q_2 analogs and the complicated inhibition behavior of pyridinium-type inhibitors are difficult to explain by a simple one-catalytic site model for Q. The existence of two distinct EPR-detectable species of complex I-associated ubisemiquinones (Yano *et al.*, 2000) seems to be consistent with the dual-binding sites model for Q. Precise elucidation of the number and location of Q reaction sites in complex I is required to understand the energy-coupling mechanism of the enzyme.

REFERENCES

Arizmendi, J. M., Runswick, M. J., Skehel, J. M., and Walker, J. E. (1992). *FEBS Lett.* **301**, 237–242.

Brandt, U. (1997). *Biochim. Biophys. Acta* **1318**, 79–91.

Burie, J. R., Boullais, C., Nonella, M., Mioskowski, C., Nebedryk, E., and Breton, J. (1997). *J. Phys. Chem.* **101**, 6607–6617.

Crofts, A. R., Hong, S., Ugulava, N., Barquera, B., Gennis, R., Guergova-Kuras, M., and Berry, E. (1999). *Proc. Natl. Acad. Sci. USA* **96**, 10021–10026.

Degli Esposti, M., Ghelli, A., Ratta, M., Cortes, D., and Estornell, E. (1994). *Biochem. J.* **301**, 161–167.

Degli Esposti, M., Gabrielle, A. N., McMullen, G. L., Ghelli, A., Sparla, F., Benelli, B., Ratta, M., and Linnane, A. W. (1996). *Biochem. J.* **313**, 327–334.

Dooijewaard, G., and Slater, E. C. (1976). *Biochim. Biophys. Acta* **440**, 1–15.

Estornell, E., Fato, R., Pallotti, F., and Lenaz, G. (1993). *FEBS Lett.* **332**, 127–131.

Fato, R., Estornell, E., Di Bernardo, S., Pallotti, F., Castelli, G. P., and Lenaz, G. (1996). *Biochemistry* **35**, 2705–2716.

Finel, M., Skehel, J. M., Albracht, S. P. J., Fearnley, I. M., and Walker, J. E. (1992). *Biochemistry* **31**, 11425–11434.

Friedrich, T., Van Heek, P., Leif, H., Ohnishi, T., Forche, E., Kunze, B., Jansen, Trowitzsch-Kienast, W., Höfle, G., Reichenbach, H., and Weiss, H. (1994). *Eur. J. Biochem.* **219**, 691–698.

Graige, M. S., Feher, G., and Okamura, M. (1998). *Proc. Natl. Acad. Sci. USA* **95**, 11679–11684

Gluck, M. R., Krueger, M. J., Ramsay, R. R., Sablin, S. O., Singer, T. P., and Nicklas, W. J. (1994). *J. Biol. Chem.* **269**, 3167–3174.

Gonzalez, M. C., Tormo, J. R., Bermejo, A., Zafra-Polo, M. C., Estornell, E., and Cortes, D. (1997). *Bioorg. Med. Chem. Lett.* **7**, 1113–1118.

Gutman, M., Singer, T. P., and Casida, J. E. (1970). *J. Biol. Chem.* **245**, 1992–1997.

He, D. Y., Gu, L. Q., Yu, L., and Yu, C. A. (1994). *Biochemistry* **33**, 880–884.

Helfenbaum, L., Ngo, A., Ghelli, A., Linnane, A. W., and Degli Esposti, M. (1997). *J. Bioenerg. Biomembr.* **29**, 71–80.

Hoppen, S., Emde, U., Friedrich, T., Grubert, L., and Koert, U. (2000). *Angew. Chem. Intern. Ed.* **39**, 2099–2102.

Hui, Y. H., Rupprecht, J. K., Lin, Y. M., Anderson, J. E., Smith, D. L., Chang, C. J., and McLaughlin, J. L. (1989). *J. Nat. Prod.* **52**, 463–477.

Iwata, J., Miyoshi, H., and Iwamura, H. (1999). *Biochim. Biophys. Acta* **1413**, 63–69.

Kean, E. A., Gutman, M., and Singer, T. P. (1971). *J. Biol. Chem.* **246**, 2346–2353.

Kim, H., Xia, D., Yu, C. A., Xia, J. Z., Kachurin, A. M., Zhang, L., Yu, L., and Deisenhofer, J. (1998). *Proc. Natl. Acad. Sci. USA* **95**, 8026–8033.

Kim, H., Esser, L., Hossain, M. B., Xia, D., Yu, C. A., Rizo, J., van der Helm, D., and Deisenhofer, J. (1999). *J. Am. Chem. Soc.* **121**, 4902–4903.

Kuwabara, K., Takada, M., Iwata, J., Tatsumoto, K., Sakamoto, K., Iwamura, H., and Miyoshi, H. (2000). *Eur. J. Biochem.* **267**, 2538–2546.

Lancaster, C. R. D., and Michel, H. (1997). *Structure* **5**, 1339–1359.

Lenaz, G., and Degli Esposti, M. (1985). In *Coenzyme Q* (Lenaz, G. ed.), Wiley, Newyork, pp. 83–106.

Majander, A., Finel, M., and Wikström, M. (1994). *J. Biol. Chem.* **269**, 21037–12042.

Matsushita, K., Yakushi, T., Toyama, H., Adachi, O., Miyoshi, H., Tagami, E., and Sakamoto, K. (1999). *Biochim. Biophys. Acta* **1409**, 154–164.

Miyoshi, H. (1998). *Biochim. Biophys. Acta* **1364**, 236–244.

Miyoshi, H., Tokutake, N., Imaeda, Y., Akagi, T., and Iwamura, H. (1995). *Biochim. Biophys. Acta* **1229**, 140–154.

Miyoshi, H., Inoue, M., Okamoto, S., Sakamoto, K., and Iwamura, H. (1997). *J. Biol. Chem.* **272**, 16176–16183.

Miyoshi, H., Iwata, J., Sakamoto, K., Furukawa, H., Takada, M., Iwamura, H., Watanabe, T., and Kodama, Y. (1998a). *J. Biol. Chem.* **273**, 17368–17374.

Miyoshi, H., Ohshima, M., Shimada, H., Akagi, T., Iwamura, H., and McLaughlin, J. L. (1998b). *Biochim. Biophys. Acta* **1365**, 443–452.

Murphy, M. P., Krueger, M. J., Sablin, S. O., Ramsay, R. R., and Singer, T. P. (1995). *Biochem. J.* **306**, 359–365.

Ohshima, M., Miyoshi, H., Sakamoto, K., Takegami, K., Iwata, J., Kuwabara, K., Iwamura, H., and Yagi, T. (1998). *Biochemistry* **37**, 6436–6445.

Okun, J. G., Lümmen, P., and Brandt, U. (1999). *J. Biol. Chem.* **274**, 2625–2630.

Queiroz, E. F., Roblot, F., Duret, P., Figadere, B., Gouyette, A., Laprevote, O., Serani, L., and Hocquemiller, R. (2000). *J. Med. Chem.* **43**, 1604–1610.

Rabenstein, B., Ullmann, G. M., and Knapp, E. W. (2000). *Biochemistry* **39**, 10487–10496.

Ramsay, R. R., Youngster, S. K., Nicklas, W. J., McKeown, K. A., Jin, Y. Z., Heikkila, R. E., and Singer, T. P. (1989). *Proc. Natl. Acad. Sci. USA* **86**, 9168–9172.

Ramsay, R. R., Krueger, M. J., Youngster, S. K., and Singer, T. P. (1991). *Biochem. J.* **273**, 481–484.

Robinson, H. H., and Kahn, S. D. (1990). *J. Amer. Chem. Soc.* **112**, 4728–4731.

Sakamoto, K., Miyoshi, H., Matsushita, K., Nakagawa, M., Ikeda, J., Ohshima, M., Adachi, O., and Iwamura, H. (1996a). *Eur. J. Biochem.* **237**, 128–135.

Sakamoto, K., Miyoshi, H., Takegami, K., Mogi, T., Anraku, Y., and Iwamura, H. (1996b). *J. Biol. Chem.* **271**, 29897–29902.

Sakamoto, K., Miyoshi, H., Ohshima, M., Kuwabara, K., Kano, K., Akagi, T., Mogi, T., and Iwamura, H. (1998). *Biochemistry* **37**, 15106–15113.

Satoh, T., Miyoshi, H., Sakamoto, K., and Iwamura, H. (1996). *Biochim. Biophys. Acta* **1273**, 21–30.

Schuler, F., Yano, T., Di Bernardo, S., Yagi, T., Yankovskaya, V., Singer, T. P., and Casida, J. E. (1999). *Proc. Natl. Acad. Sci. USA* **96**, 4149–4153.

Shimada, H., Grutzner, J. B., Kozlowski, J. F., and McLaughlin, J. L. (1998). *Biochemistry* **37**, 854–866.

Takada, M., Kuwabara, K., Nakato, H., Tanaka, A., Iwamura, H., and Miyoshi, H. (2000). *Biochim. Biophys. Acta* **1460**, 302–310.

Tormo, J. R., Gallardo, T., Aragon, R., Cortes, D., and Estornell, E. (1999). *Chem-Biol. Interact.* **122**, 171–183.

Ueno, H., Miyoshi, H., Ebisui, K., and Iwamura, H. (1994) *Eur. J. Biochem.* **225**, 411–417.
Vinogradov, A. D., Sled, V. D., Burbaev, D. S., Grivennikova, V. G., Moroz, J. A., and Ohnishi, T. (1995). *FEBS Lett.* **370**, 83–87.
Warncke, K., Gunner, M. R., Braun, B. S., Gu, L., Yu, C.-A., Bruce, J. M., and Dutton, P. L. (1994). *Biochemistry* **33**, 7830–7841.
Weidner, V., Geier, S., Ptock, A., Friedrich, T., Leif, H., and Weiss, H. (1993). *J. Mol. Biol.* **233**, 109–122.
Yagi, T. (1990). *Arch. Biochem. Biophys.* **281**, 305–311.
Yano, T., Magnitsky, S., and Ohnishi, T. (2000). *Biochim. Biophy. Acta.* **1459**, 299–304.
Yu, C. A., Gu, L., Lin, Y., and Yu, L. (1985) *Biochemistry* **24**, 3897–3902.