Effects of Phenolic Respiration Inhibitors on Cytochrome bc1 Complex of Rat-liver Mitochondria

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The respiration inhibitory effects of the inhibitory uncouplers, 2,6-diiodo-4-(2,2-dicyanovinyl)phenol and 2,6-dimethoxy-4-(2,2-dicyanovinyl)phenol, the binding site of which is cytochrome bc1 (cyt. bc1) complex, were studied with rat-liver mitochondria. The inhibitory potency of 2,6-diiodo-4-(2,2-dicyanovinyl)phenol and of 2,6-dimethoxy-4-(2,2-dicyanovinyl)phenol was increased and decreased, respectively, by steep dissipation of the transmembrane electrical potential after adding the potent uncoupler, fluazinam, the uncoupling activity of which disappears with time. Changes in the inhibitory potency may have been due to variation of the binding affinity of these compounds to cyt. bc1 complex. Furthermore, the enhancement to the binding affinity of 2,6-diiodo-4-(2,2-dicyanovinyl)phenol was governed by the degree of reduction in the transmembrane electrical potential. These results suggest that the extent of conformational changes of cyt. bc1 complex, which resulted in an enhanced interaction between 2,6-diiodo-4-(2,2-dicyanovinyl)phenol and its binding niche, increased with decreasing transmembrane electrical potential. From examinations of the reduction of cyt. b, it is suggested that the action site of 2,6-diiodo-4-(2,2-dicyanovinyl)phenol may be close to or partially overlapping that of antimycin A.

The mechanistic details of the redox reactions and proton translocation pathways of cytochrome bc1 (cyt. bc1) complex at the molecular level have remained elusive since there is little information available for the definitive structure of this proteinous complex beyond the characteristic amino acid sequence analyses and hydropathy profiles.1–3) Potent and specific inhibitors of cyt. bc1 complex such as antimycin A and myxothiazol have been an important probe in the development of the mechanistic concepts and evolutionary aspects of cyt. bc1 complex.4,5) In this sense, novel inhibitors with peculiar inhibitory characteristics could be invaluable for mechanistic studies on cyt. bc1 complex.

We have already shown that some phenolic uncouplers have respiratory inhibition concurrently with respiratory acceleration due to an uncoupling effect with rat-liver mitochondria.6) The inhibition site of these phenolic inhibitory uncouplers was identified as cyt. bc1 complex,7) which changed conformation due to a change in the mitochondrial energy state.8–10) Moreover, their inhibitory effects were classified into types I—III, depending on the pattern of the change in inhibitory potency observed when the transmembrane electrical potential was steeply dissipated by the potent uncoupler, SF6847.7) Since inhibitory uncouplers have two opposite effects on the respiration chain, i.e., respiratory inhibition and acceleration, changes in their inhibitory potency are readily detectable from those in the apparent respiratory rate that they induce by themselves. The extent of inhibition by type I phenols like 2,4,6-trichlorophenol does not vary with dissipation of the transmembrane electrical potential, but the extent of inhibition by types II and III phenols such as 2,6-dimethoxy-4-(2,2-dicyanovinyl)phenol and 2,6-diiodo-4-(2,2-dicyanovinyl)phenol is decreased and increased, respectively. To explain these patterns of change in the inhibitory potency, we have postulated that changes in the binding affinity of phenolic inhibitors to cyt. bc1 complex may be closely related to the conformational change of this proteinous complex accompanying the steep dissipation of the transmembrane electrical potential.9) Using another potent uncoupler, fluazinam, the uncoupling activity of which disappears with time,10) it was also suggested that changes in the binding affinity of types II and III phenols due to the conformational change of cyt. bc1 complex take place in a reversible and an irreversible manner, respectively.7)

The binding of antimycin A induces conformational changes to cyt. bc1 complex.11,12) It is interesting that the mode of respiratory inhibition of a “simple” inhibitor such as a substituted phenol was also significantly related to the conformational state of the cyt. bc1 complex. In this study, we examined the interactions between types III and II phenol and cyt. bc1 complex in more detail according to the conformational state of this proteinous complex by varying the transmembrane electrical potential or using specific inhibitors of the complex. 2,6-Diiodo-4-(2,2-dicyanovinyl)phenol (compound A) and 2,6-dimethoxy-4-(2,2-dicyanovinyl)phenol (compound B) were used as typical type III and type II phenols, respectively (Fig. 1).

![Figure 1. Structures of the Test Compounds.](image-url)
Materials and Methods

Materials. Antimycin A, myxothiazol, and rotenone were obtained from Sigma. 2,6-Diido-2,2-dicyanovinylphenol (compound A), 2,6-dimethoxy-4-(2,2-dicyanovinyl)phenol (compound B), SF6847 (2,6-di-i- butyl-4-(2,2-dicyanovinyl)phenol), and fluanizam (3-chloro-5-(3-chloro-2,6-dinitro-4-trifluorometylyphenyl)-S-trifluorometyly-pyridylamine) were similar to the samples used previously.1,15 Other reagents were of the purest grade commercially available.

Methods. Mitochondria were isolated from the liver of adult male Wistar rats in a medium containing 230 mM sucrose and 2 mM Tris-HCl (pH 7.4) as described by Myers and Slater.13 The amount of mitochondrial protein was measured by the method of Bradford,14 with bovine serum albumin as the standard. Mitochondrial respiration with 10 mM succinate as the respiration substrate was measured with a Clark-type oxygen electrode at 25°C, the final mitochondrial protein concentration in the medium being 0.7 mg/ml. The incubation medium consisted of a mixture of 200 mM sucrose, 2 mM MgCl₂, 1 mM EDTA, and 2.5 μM rotenone in 2.5 mM potassium phosphate buffer (pH 7.4), the total volume being 2.5 ml.

The transmembrane electrical potential was monitored by the uptake of tetraphenylphosphonium (TPP⁺) from the incubation medium into the mitochondrial matrix. The incubation medium (15 ml) was the same as that used for the respiration experiments. The concentration of TPP⁺ in the incubation medium was monitored continuously with a TPP⁺-sensitive membrane electrode as described by Kamo et al.15 The initial concentration of TPP⁺ in the medium was 10 μM. Because only the change with time of the transmembrane electrical potential was needed, no correction of the amount of TPP⁺ bound to the mitochondrial membrane was made.

The oxidation-reduction status of cytochrome b of the intact mitochondria was measured before and after each treatment with the wavelength pair of 563 and 577 nm.16 The absorbance spectra were measured with a Shimadzu UV3000 spectrophotometer, using a 1 nm bandwidth. The reaction medium was similar to that used for the respiration experiment, except that 1 mM KCN was included. The final mitochondrial protein was 1.4 mg/ml.

Results

Effect of the transmembrane electrical potential on the inhibitory activity of a phenolic inhibitor

The potent uncoupler fluanizam can change the mitochondrial energy state continuously from the uncoupling conditions to state 4, i.e., from certain low transmembrane electrical potential conditions to a higher state. We have shown that the inhibitory potency of type III phenols was enhanced when the transmembrane potential was dissipated by adding fluanizam at the concentration to induce full uncoupling.7 The once-enhanced inhibitory potency by fluanizam was not reduced even after the disappearance of the uncoupling effect of fluanizam. These results suggested that the binding affinity of type III phenols to cyt. bcl complex was enhanced by a conformational change due to steep dissipation of the transmembrane electrical potential, and that the once-enhanced binding affinity was too strong to be reduced reversibly when the uncoupling effect of fluanizam had disappeared.

In this study, we examined changes in the binding affinity of compound A (type III phenol) under various transmembrane electrical potential conditions by the addition of different concentrations of fluanizam. Figures 2A and C show the changes in the transmembrane electrical potential and the respiration rate with time induced by various concentrations of fluanizam as control experiments. The effects of adding various concentrations of fluanizam on the transmembrane electrical potential and on the respiration rate preliminary induced by 6.4 μM of compound A are shown in Figs. 2B and 3B. Through this concentration, compound A induced the apparent maximum respiration rate, which was significantly lower than that induced by the potent uncoupler SF6847.7 The dotted lines in Figs. 2B and D show the response without fluanizam. The respiration rate induced by compound A decreased with increasing concentration of added fluanizam (Fig. 2D). Even after the disappearance of the uncoupling effect of fluanizam, the respiration rate did not return to the level before the addition of fluanizam. The transmembrane electrical potential reduced by compound A was further reduced by the addition of fluanizam, and the extent of reduction of the transmembrane electrical potential depended upon the concentration of fluanizam (Fig. 2B). With gradual disappearance of the uncoupling effect of fluanizam, the once-reduced transmembrane electrical potential recovered gradually, but did not recover.
to the level existing before the addition of fluazinam. The higher the concentration of fluazinam, the lower the final transmembrane electrical potential was. Upon the addition of 20 μM fluazinam, the once-reduced membrane potential did not entirely recover. These results suggest that the inhibitory potency of compound A, i.e., the binding affinity of this compound to cyt. bc1 complex, was significantly magnified with decreasing transmembrane electrical potential in an irreversible manner.

As control experiments, we show the effects of fluazinam on the inhibitory potency of compound B (type II phenol) in Fig. 3. The inhibitory potency of compound B reduced by the addition of fluazinam gradually recovered to the level existing before the addition of fluazinam; that is, the once-reduced binding affinity of compound B to cyt. bc1 complex recovered completely when the uncoupling effect of fluazinam had disappeared. This reversible manner of change in the binding affinity was not dependent on the concentration of fluazinam.

Effects of compounds A and B on the redox state of cytochrome b

The effects of compounds A and B on the redox state of cyt. b caused by succinate are shown in Fig. 4, together with those of antimycin A in the control experiments. Approximately 70% of the dithionite reducible cyt. b was immediately reduced by the addition of antimycin A (62 nM) after succinate (Fig. 4A). The addition of potassium ferricyanide after antimycin A induced extra reduction of cyt. b (Fig. 4A), i.e., the oxidant-induced reduction of cyt. b.5 Compound A (10.5 μM) immediately amplified the reduction of succinate-reduced cyt. b to a level similar to that observed with antimycin A (Fig. 4B). The reduction level of cyt. b was not increased any more by further addition of compound A, at least up to 30 μM. The oxidant-induced reduction of cyt. b was also observed in the presence of compound A (Fig. 4B). The combined addition of myxothiazol and antimycin A or compound A inhibited the oxidant-induced reduction of cyt. b, as shown in Figs. 4C and D, respectively. The addition of antimycin A after compound A did not induce further reduction of cyt. b (Fig. 4E). The results suggest that the cyt. b reducible by antimycin A was almost completely reduced by compound A.

Next, the effects of compound B on the redox state of cyt. b were examined. Compound B (0.20 mM) immediately induced a great reduction of cyt. b (Fig. 4F). Contrary to the effect with compound A, the once-amplified reduction state of cyt. b decreased with time. The final redox state was, however, more reductive than that before the addition of compound B. In analogy with compound A, an oxidant-induced reduction of cyt. b was observed (Fig. 4F). Moreover, the combined addition of myxothiazol and compound B prevented the oxidant-induced reduction of cyt. b (Fig. 4G).

Effect of antimycin A on the inhibitory activity of phenolic inhibitors

Next, we examined the effects of treating mitochondria with antimycin A on the inhibitory potency of compounds A and B. In contrast to the results shown in Fig. 2D, the
respiration rate induced by 2.0 μM of compound A without antimycin A was further accelerated by the addition of 35 nm of SF6847 as shown in Fig. 5. This result is due to the fact that the concentration of compound A (2.0 μM) was lower than that used in the experiment shown in Fig. 2D (6.4 μM); that is, the extent of inhibition by compound A in Fig. 5 was weaker than that observed in Fig. 2D. The respiration rate after the addition of SF6847 significantly decreased with increasing concentration of antimycin A. Within these concentration ranges of antimycin A, the respiration rate induced by compound A was not affected at all. Furthermore, the fully uncoupled respiration rate by SF6847 (35 nm), which was sensitively reduced by the presence of a respiration inhibitor, 6,7 was slightly reduced by 9 nm antimycin A to about 95% of the control. The great decrease in respiration rates, therefore, after the addition of SF6847 in the presence of antimycin A seem not to have been due to the respiratory inhibition by antimycin A itself. These results suggest that the increase in inhibitory potency of compound A resulted from treating cyt. bc1 complex with antimycin A.

In analogy with the case for compound A, the respiration rate induced by compound B was decreased by adding SF6847 in the presence of antimycin A (data not shown). As shown in our previous study7 and in Fig. 3B using fluazinam, the respiration rates induced by compound B were accelerated by the addition of the potent uncoupler, SF6847, in the absence of antimycin A. Therefore, these results suggest that the manner of change in the inhibitory potency of compound B accompanied with steep dissipation of the transmembrane electrical potential were dependent on the existence of antimycin A.

**Discussion**

Cyt. bc1 complex has been shown in experiments to undergo a conformational change due to change in the mitochondrial energy state2,8 and the presence of specific inhibitors of this protein complex such as antimycin A.11,12 In this study, we examined the effects on the binding affinity of compound A (type III phenol) and compound B (type II phenol) with cyt. bc1 complex accompanying a steep reduction in the transmembrane electrical potential by using various concentrations of fluazinam (Figs. 2 and 3). The results shown in Figs. 2 and 3 strongly suggest that the extent of conformational change of cyt. bc1 complex, which resulted in a change in the binding affinity of the inhibitor, increased with decreasing transmembrane electrical potential. The variation in the binding affinity of compounds B and A to cyt. bc1 complex were reversible and irreversible, respectively, according to the change in conformational state of this protein complex. The interaction between compound A and its binding niche may be so strong that this compound would not completely lose the once-enhanced binding affinity even when the uncoupling effect of fluazinam had disappeared. The extent of this remaining binding affinity of compound A after the disappearance of the uncoupling effect of fluazinam was governed by the extent of conformational change to reflect the level of the transmembrane electrical potential as reduced by fluazinam. The variation in binding affinity of compound B was reversible regardless of the level of the transmembrane electrical potential.

Compound A had the obvious characteristics of a Q site inhibitor of cyt. bc1 complex, as was observed with antimycin A; i.e., amplifying the reduction state of cyt. b by succinate, and the oxidant-induced reduction of cyt. b. 5 The difference in the effect on cyt. b reduction between compound A and antimycin A was only that the amplified reduction state of cyt. b by compound A was slightly oxidized by the addition of myxothiazol (Figs. 4C and D). As this result is not fully understood yet, it is not clear whether or not the binding site of compound A was entirely the same as that of antimycin A. However, the action sites of compound A and antimycin A might be close to or partially overlapping each other.

Compound B elicited effects apparently similar to compound A on the redox state of cyt. b; that is, amplifying the reduction state of cyt. b and oxidant-induced reduction (Figs. 4F and G). However, the kinetics for the reduction of cyt. b were significantly different between these two inhibitors. The redox state of cyt. b, in fact, changed with time after the addition of compound B (Figs. 4F and G). At present, we have no appropriate explanation for the relationship between this fact and the kinetics of cyt. b reduction. It is obvious, however, that differences in the manner of interaction with the binding niche, including reduction of cyt. b, between compounds A and B may be reflected in the difference of inhibitory pattern accompanying the dissipation of the transmembrane electrical potential.

The great difference in interaction with the binding niche on cyt. bc1 complex between compounds A and B was due to the physicochemical properties of the ortho substituents. Methoxy derivatives of both inhibitors, which lack the phenolic OH group, did not have any inhibitory activity at all (data not shown). The phenolic OH group is, therefore, essential for their inhibitory activity. The pK<sub>A</sub> value, K<sub>a</sub> being the acid dissociation constant in water, of compounds A and B was 3.31 and 6.50, respectively, compound A being much more acidic than compound B. Besides the acidity of the compounds, the hydrophobic and steric properties of ortho substituents were also very different between these two inhibitors. Such a large difference in the physicochemical properties of ortho substituents may have been significantly reflected in the interaction between the phenolic OH group and the residue of the binding niche.

Treating mitochondria with antimycin A significantly enhanced the inhibitory potency of both compound A and R when the transmembrane electrical potential was dissipated by the addition of SF6847 (Fig. 5). These facts suggest that the manner of change in the interaction between phenolic inhibitors and cyt. bc1 complex accompanying a dissipation of the transmembrane electrical potential may have been different, depending on whether antimycin A was there or not. In other words, when the transmembrane electrical potential was steeply dissipated by SF6847, the presence of antimycin A may have induced conformational changes to cyt. bc1 complex that differ from those induced in its absence. Antimycin A has induced certain conformational changes of cyt. bc1 complex.11,12 Although we can not predict the extent of conformational change induced by antimycin A binding at
the concentrations studied here, it may be very slight, but not negligible. Such slight conformational change probably makes cyt. bcl complex more susceptible to dissipation of the transmembrane electrical potential by SF6847, resulting in conformational change which differed from that induced in the absence of antimycin A.

In this study, we show that the binding affinity of even “simple” respiration inhibitors like compound A was sufficiently affected by the conformational state of cyt. bcl complex. This study provokes our interest in investigating the interaction between electron transport inhibitors and their binding niches on intact energy-transducing biomembranes, since these binding niches can be predicted to involve fairly dynamic conformational changes according to the energy state of the biomembrane.

References