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Electron transport inhibition of the cytochrome bc_1 complex of rat-liver mitochondria by phenolic uncouplers

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The respiration inhibition of rat-liver mitochondria by a series of substituted phenolic uncouplers was studied. The inhibitory effects were classified into three types, I–III, depending on the pattern of the changes in inhibitory potency observed when the potent uncoupler SF6847 was simultaneously applied. The extent of inhibition by type I phenols did not change as the transmembrane potential was dissipated by SF6847, but the extent of inhibition by type II and III phenols was decreased and increased, respectively. With the addition of another potent uncoupler, fluazinam, the uncoupling activity of which disappears with time, the inhibitory potency of type II phenols was decreased, but increased reversibly with the disappearance of the uncoupling effect of fluazinam. However, the inhibitory potency of type III phenols increased by fluazinam was not reduced. The inhibitory site of the phenols studied here was the cytochrome bc_1 complex. This complex undergoes conformational changes when the transmembrane potential changes. The findings suggested that inhibition by substituted phenolic uncouplers depends partially on conformational changes of the cytochrome bc_1 complex that accompany variations in the transmembrane potential.

Introduction

Phenol-type inhibitors of the photosynthetic electron transport system have been widely investigated with isolated thylakoid membrane [1–4]. There is less information available on the phenol-type respiration inhibitors of mammal mitochondria [5]. Some phenolic uncouplers such as 2,4,6-trichlorophenol and 2-*s*-butyl-4,6-dinitrophenol (Dinoseb) inhibit the respiratory chain of rat-liver mitochondria [6]. The inhibition is highly specific to their structures. Compounds with similar structures, including diortho halogenated phenols such as ioxynyl (2,6-diiodo-4-cyanophenol) and 2-alkyl-4,6-dinitrophenols, are powerful inhibitors of Photosystem II (the Q_B site) [1,7]. The structural similarities in electron-transport inhibitors between inner mitochondrial and thylakoid membranes are not unreasonable, if the similarity in the redox carriers and the peptide subunit involved in quinone binding is considered between cytochrome bc_1 complex and Photosystem II [8–10].

Compounds that simultaneously uncouple and inhibit the photosynthetic electron-transport chain of the thylakoid membrane have been termed 'inhibitory uncouplers' by Moreland [11]. The uncoupling effect of phenolic inhibitory uncouplers is usually observed at concentrations higher than that required for inhibition [1,4,12]. 2,4,6-Trichlorophenol and Dinoseb, however, have these two effects on rat-liver mitochondria in almost the same concentration range [6]. The observed uncoupling activity is, in fact, somewhat distorted by the inhibition. Looking at this from the other way around, the inhibition of inhibitory uncouplers is hidden by the uncoupling effect. Therefore, the inhibitory effects of phenol-type inhibitory uncouplers have sometimes been overlooked in studies of the uncoupling activity of phenols with rat-liver mitochondria.

In this study, we examined the uncoupling and inhibitory activities of various substituted phenols on the respiration chain in rat-liver mitochondria. The phenolic inhibitors were classified into three types depending on the pattern of variations in the inhibition accompanied by a steep reduction in the transmembrane potential caused by the potent uncoupler SF6847. The inhibi-

Abbreviations: SF6847, 3,5-di(*t*-butyl)-4-hydroxybenzylidenemalononitrile; fluazinam, 3-chloro-*N*-(3-chloro-2,6-dinitro-4-trifluoromethyl phenyl)-5-trifluoromethyl-2-pyridinamine.

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tion by phenols was suggested to depend partially on the conformational changes in their binding niche in the cytochrome bc_1 complex.

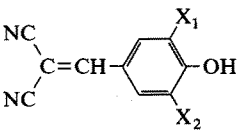
Materials and Methods

Materials. *N,N,N′,N′*-Tetramethylphenylenediamine (TMPD), dichlorophenolindophenol (DCPIP) and cytochrome *c* were purchased from Sigma. Fluazinam and SF6847 were the same samples as those used previously [6,13]. The substituted phenols used here are listed in Table I. Compounds 1–5 are the same as those studied before [14]. Compounds 17 and 18 were purchased from Lancaster Synthesis (U.K.). Other reagents were of the purest grade commercially available.

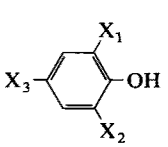
Synthesis. Compounds 6–15 in Table I were synthesized as shown in Fig. 1. The reaction conditions of steps A, B and C were as described by Smith [15], Tarbell et al. [16] and Bastus [17], respectively. Compound 7 was synthesized via steps B and C. Compounds 8–12 were derived from the corresponding 3,5-disubstituted-4-hydroxybenzaldehydes, which are commercially available, via step C. 2,6-Disubstituted phenols from which compounds 6, 13 and 14 were prepared through steps A and C were purchased from Nacalai Tesque (Kyoto). 2-*s*-Butyl-6-chlorophenol (for compound 15) was prepared with sulfuryl chloride from 2-*s*-butylphenol. Compound 16 was synthesized from 2,6-dichlorophenol by the usual nitration method [18]. Compound 19 was prepared from 3,5-diiodo-4-hydroxybenzaldehyde with ethyl cyanoacetate. The structures of the newly synthesized compounds were confirmed by elementary analysis and spectra.

TABLE I

Chemical structures and type of inhibitory pattern of phenols



No.	mp (°C)	X_1	X_2	Type	V_R
1	161–163	Me	Me	II	70
2	139–140	Et	Et	II*	83
3	153–155	*i*-Pr	*i*-Pr	no	103
4	107	*s*-Bu	*s*-Bu	no	102
5	143–145	*t*-Bu	*t*-Bu	no	100
6	141–143	F	F	III*	68
7	174	Cl	Cl	III	45
8	167–169	Br	Br	III	42
9	202–204	I	I	III	32
10	159–161	MeO	MeO	II	44
11	190–192	MeO	I	I	85
12	168–170	MeO	Br	I	67
13	141	Me	Cl	I	69
14	226–228	Me	*t*-Bu	I	87
15	133	*s*-Bu	Cl	no	97



No.	mp (°C)	X_1	X_2	X_3	Type	V_R
16	118–121	Cl	Cl	NO_2	I	66
17	149–151	I	I	NO_2	III *	41
18	200–202	I	I	CN	I	44
19	189–190	I	I	$CH{=}C(CN)CO_2Et$	I	69

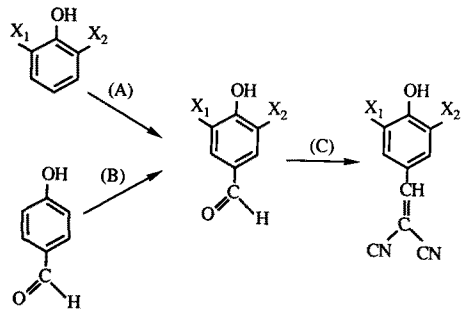


Fig. 1. Scheme of synthesis. (A) A mixture of 2,6-disubstituted phenol, hexamethylenetetramine and trifluoroacetic acid was heated at 90 °C for 12 h. (B) A mixture of 4-hydroxybenzaldehyde and sulfuryl chloride was heated at 80 °C for 1 h. (C) A mixture of substituted benzaldehyde and malononitrile in 70% aqueous ethanol was left at room temperature overnight.

Methods. Mitochondria were isolated from the liver of adult male Wistar rats in a medium containing 250 mM sucrose and 2 mM Tris-HCl (pH 7.4) as described by Myers and Slater [19]. The amount of mitochondrial protein was measured by the method of Bradford [20] 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 $MgCl_2$, 1 mM EDTA and 2.5 μM rotenone in 2.5 mM potassium phosphate buffer (pH 7.4) and the total volume was 2.5 ml. In each of the mitochondrial preparations, the respiration control ratio was stable at 4.5 or more. The maximum respiration rate, V_{max}, induced by each test compound was normalized with use of the V_{max} value of SF6847 as the reference and was expressed by the relative value V_R [6] as follows:

$$V_R = \left\{ \frac{V_{max} \text{ of test compound} - \text{rate(state 4)}}{V_{max} \text{ of SF6847} - \text{rate(state 4)}} \right\} \times 100$$

The activity of succinate-coenzyme Q reductase (complex II) was measured by monitoring of the reduction of DCPIP at the wavelength of 600 nm with Shimadzu UV-3000 spectrophotometer [21]. The incubation medium was a mixture of 20 mM succinate, 0.18 mM phenazine methosulfate, 1 mM EDTA, 2.5 μM

rotenone, 0.1 μM antimycin A, 0.3 mM KCN and 0.05 mM DCPIP in 50 mM potassium phosphate buffer (pH 7.4). The activity of cytochrome bc_1 complex (complex III) was estimated by subtracting the complex II activity from the sum of complex II and III activities which was determined from the reduction of cytchrome *c* at the wavelengths of 550 and 540 nm [22]. The incubation medium was a mixture of 20 mM succinate, 0.5 mg cytochrome *c* per ml of medium, 2.5 μM rotenone and 0.3 mM potassium cyanide in 50 mM potassium phosphate buffer (pH 7.4). The activity of cytochrome *c* oxidase (complex IV) was measured with a Clark-type oxygen electrode with use of TMPD (0.6 mM) plus sodium ascorbate (5 mM) as the artificial electron donor [23]. The reaction medium was a mixture of 5 mM sodium ascorbate, 0.6 mM TMPD, 0.2 M sucrose, 2 mM $MgCl_2$ and 1 mM EDTA in 2.5 mM potassium phosphate buffer (pH 7.4). During the measurement of the activities of complexes II and III, the mitochondrial membrane may break partially because of the osmotic difference between the medium in which the mitochondria were isolated and that in which the activity was measured. The final concentration of mitochondrial protein in each of the reaction media was 0.7 mg/ml and the final volume of the reaction medium was 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 except that succinate was not included. The concentration of TPP^+ in the incubation medium was monitored continuously with use of a TPP^+-sensitive membrane electrode prepared as described by Kamo et al. [24]. The initial concentration of TPP^+ in the medium was 10 μM. This concentration was found in preliminary experiments to have no effect on mitochondrial respiration. Because only the changes with time of the transmembrane potential was needed, correction for the amount of TPP^+ bound to the mitochondrial membrane was not made.

Results

The maximum respiration rate, V_{max}, induced by SF6847 in rat-liver mitochondria is decreased in the presence of a respiratory inhibitor [6]. Here, we first examined whether substituted phenols inhibit the respiration so that the V_{max} caused by SF6847 (35 nM) is reduced. This concentration of SF6847 elicits the maximum respiration rate with rat-liver mitochondria in preliminary experiments. Phenols without showing the respiratory inhibition, i.e., the V_R value of which is close to 100, did not affect the V_{max} of SF6847 in the range of concentrations studied here (Fig. 2A). Phenols

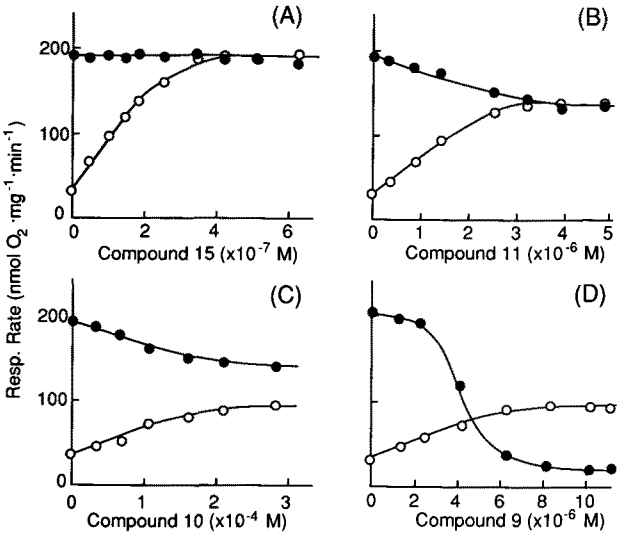


Fig. 2. Respiration rate induced by compounds 15 (A), 11 (B), 10 (C) and 9 (D) in the presence (●) or absence (○) of 35 nM SF6847.

exhibiting an inhibitory effect with the V_R value significantly lower than 100 lowered the V_{max} of SF6847 to various degrees (Fig. 2B–D). The inhibitory patterns of phenols were classified into three types depending on how the V_{max} value of each phenol changed when SF6847 (35 nM) was added. Phenols for which the V_{max} value was not changed by the addition of SF6847 were called type I compounds (Fig. 2B). The maximum respiration rates of compound 11 before and after the addition of SF6847 were also identical, being lowered gradually with increasing the concentration higher than those shown in Fig. 2B. Phenols for which the V_{max} value was increased by the addition of SF6847 were called type II compounds (Fig. 2C). Although the maximum respiration rate of compound 10 alone decreased

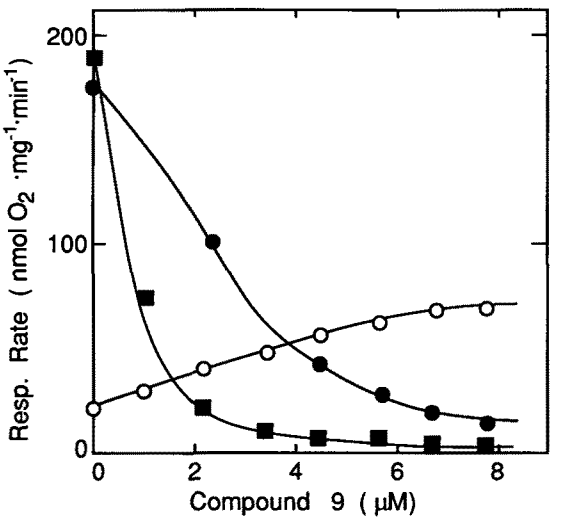


Fig. 3. Effects of various concentrations of compound 9 on the maximum respiration rate induced by 2 μM 2-*t*-butyl-4,6-dinitrophenol (■) or 30 nM valinomycin (●). Open circles show the respiration rate with compound 9 only.

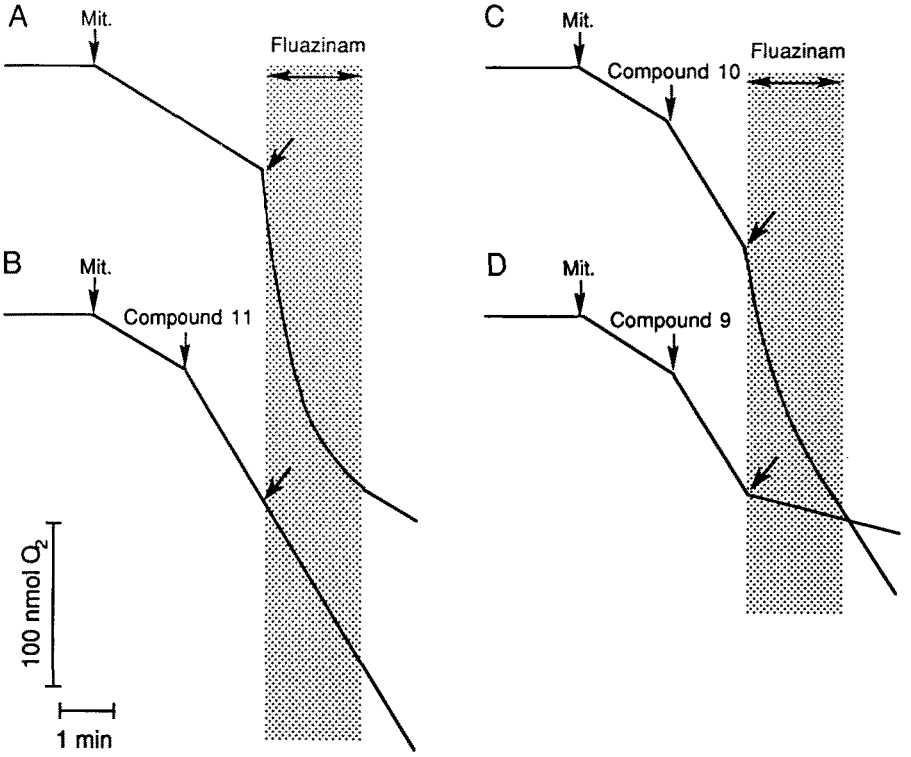


Fig. 4. Effects of fluazinam (20 nM) on the maximum respiration rates induced by compound 11 (B, 4.2 μM), 10 (C, 0.34 mM) and 9 (D, 6.6 μM). The trace A shows the effect of fluazinam alone (20 nM) on the respiration rate. The uncoupling activity of fluazinam disappears at the end of the period shown by the stippling.

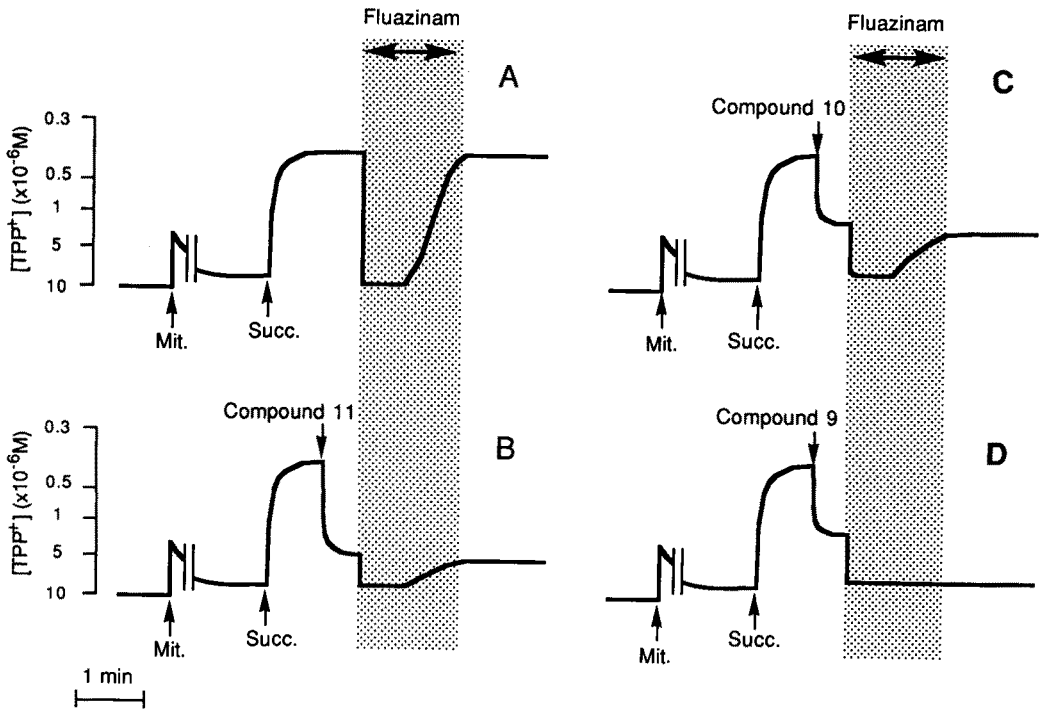


Fig. 5 Effects of fluazinam (20 nM) on the transmembrane potential decreased by compound 11 (B, 4.2 μM), 10 (C; 0.34 mM) and 9 (D, 6.6 μM). The trace A shows the effect of fluazinam alone (20 nM) on the membrane potential. The upward change in the TPP^+ concentration reflects the uptake of TPP^+ from the incubation medium into the mitochondrial matrix (i.e., an increase in the transmembrane electrical potential). The uncoupling activity of fluazinam disappears at the end of the period shown by the stippling.

very gradually above 0.4 mM, the addition of SF6847 still enhanced the maximum rate of compound 10 of at least up to 0.8 mM. Type III compounds were those for which the V_{max} value was significantly decreased by the addition of SF6847 (Fig. 2D). The inhibitory patterns and the V_R values are listed in Table I. In Table I, compounds 2, 6 and 17 are marked with asterisks because their V_{max} values were either increased or decreased by SF6847 slightly but significantly.

These inhibitory patterns were also examined with the use of the powerful uncoupling reagents 2-*t*-butyl-4,6-dinitrophenol (2 μM) and the potassium ionophore valinomycin (30 nM). The V_{max} values of these uncouplers were almost equivalent to the V_{max} of SF6847. The inhibitory pattern with compound 9, a type III compound, is shown in Fig. 3. Like the effect of SF6847 shown in Fig. 2D, the V_{max} value of compound 9 was significantly decreased by the addition of 2-*t*-butyl-4,6-dinitrophenol or valinomycin. The V_{max} value of Type I and II compounds also showed responses similar to those caused by SF6847 (data not shown).

We examined the effect of a potent uncoupler, fluazinam (20 nM), on the V_{max} value of these three classes of phenols. The uncoupling activity of fluazinam rapidly disappears with the metabolic transformation of this compound by the glutathione conjugation mechanism in rat-liver mitochondrial matrix [13]. Changes in the transmembrane potential were monitored with the TPP^+ electrode. The results are shown in Figs. 4 and 5. The uncoupling activity of fluazinam disappears with time, so the mitochondrial energy state is changed continuously from the complete uncoupling to the conditions in state 4 (Figs. 4A and 5A).

The V_{max} value of compound 11 (type I) did not change after the addition of fluazinam (Fig. 4B), as was also found with the addition of SF6847 (Fig. 2B). The residual transmembrane potential after the addition of compound 11 was rapidly dissipated by the uncoupling action of fluazinam (Fig. 5B). With the metabolic transformation of fluazinam, the transmembrane potential returned again to its level before the addition of fluazinam. The V_{max} value of compound 10 (type II) was increased by the addition of fluazinam (Fig. 4C). The enhanced respiration rate was gradually reduced to the initial V_{max} value with the gradual disappearance of the effects of fluazinam. The effect on the transmembrane potential of compound 10 was similar (Fig. 5C); the membrane potential, once dissipated, completely recovered with time to the level before the addition of fluazinam. The V_{max} value of compound 9 (type III) was significantly decreased by the addition of fluazinam (Fig. 4D), as it was when SF6847 had been added (Fig. 2D). However, the reduced V_{max} value of compound 9 did not return to the initial value even after the disappearance of the uncoupling activity of fluazinam. The residual transmembrane potential after the addition of compound 9 was rapidly dissipated by the addition of fluazinam. In contrast to the findings for type I and II compounds, the transmembrane potential did not recover even after the disappearance of the uncoupling activity of fluazinam.

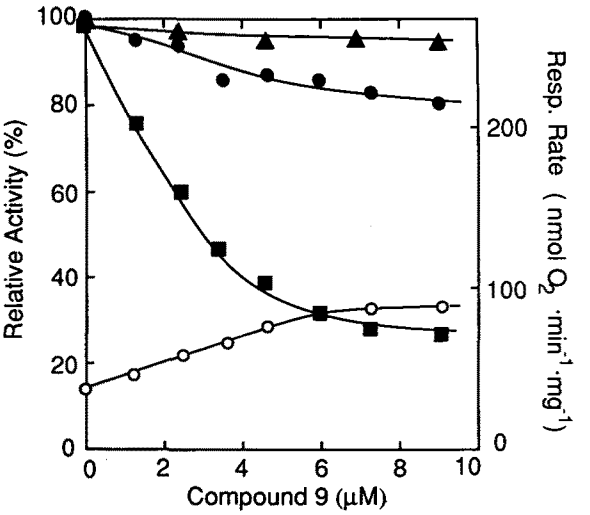


Fig. 6. Effects of compound 9 on the electron transport activities of complexes II (●), III (■) and IV (▲). Open circles show the respiration rate induced by compound 9 in the same concentration range.

The inhibitory effects of compound 9 (type III) on the activity of complexes II, III and IV are shown in Fig. 6, with the titration curve for the respiration rate. Compound 9 strongly inhibited the activity of complex III (cytochrome bc_1 complex). The inhibition of complex II and IV activities was small in the concentration range studied. The inhibition by compounds 11 (type I) and 10 (type II) of complex II and IV were almost negligible at the concentration corresponding to that used for the respiration measurement. Inhibition by these compounds of the activity of complex III was less than that caused by compound 9, but it was highly significant, with 10–15% inhibition at 4.2 μM for compound 11 and 15–20% inhibition at 0.34 mM for compound 10. Thus, regardless of the patterns of the inhibitory effect, the inhibitory site of phenols seemed to be complex III.

Discussion

The inhibition of respiration in rat-liver mitochondria by a series of phenolic uncouplers was not uniform. The effects were classified as type I, II and III depending on how the strength of inhibition changed when the potent uncoupler SF6847 was added. The type I pattern of inhibition can be explained by the consideration of two opposite effects acting on the respiration assemblies: the acceleration of the respiration rate by uncoupling and the inhibitory effect on respiration. Under the conditions in the mitochondria in which type

I compounds induce the maximum respiration rate, the acceleration of respiration by type I compounds is balanced by their inhibitory effect. In this situation, the respiration rate is governed completely by the extent of the inhibition. Therefore, the later addition of such uncouplers as SF6847 can no longer accelerate the respiration rate. Type II and III patterns of inhibition, however, cannot be explained by consideration of accelerative and inhibitory effects only, because their V_{max} values were either decreased or enhanced by the addition of SF6847.

One common effect on mitochondria by such uncoupling reagents as SF6847, 2-*t*-butyl-4,6-dinitrophenol and valinomycin is the reduction of the transmembrane potential. The steep change in the transmembrane potential may cause such a conformation change in the binding site as that responsible for the inhibitory activities of type II and III compounds. The cytochrome bc_1 complex has been found in experiments to be the component of the respiration assembly in which conformation changes take place owing to changes in the mitochondrial energy state [25–27]. In this study, the binding site of phenols was identified to be the cytochrome bc_1 complex. This finding suggests that the difference in interaction between phenols and their binding niche is essential in differentiating type II and III inhibitory patterns from the type I pattern. It also suggests that the conformation change in the binding niche in the cytochrome bc_1 complex participates in type II and III inhibition but not in type I inhibition. The binding affinity of type III compounds with their binding niche could be enhanced by a conformation change in the cytochrome bc_1 complex, increasing the inhibition. The affinity of type II compounds to their binding niche could be decreased, resulting in decreased inhibition. That of type I compounds would be neither increased nor decreased.

Results shown in Fig. 4 support this working hypothesis about type II and III inhibition patterns. After the addition of fluazinam, the binding affinity of type II compounds, which had decreased, returned to the initial level, because the cytochrome bc_1 complex returned to its original conformation with the disappearance of the uncoupling activity of fluazinam. On the other hand, the binding affinity of type III compounds, which had increased, did not return to the initial level even after the disappearance of the uncoupling activity of fluazinam. The interaction between type III compounds and their binding niche was so strong that type III compounds did not lose their high binding affinity when the conformation changed. In contrast to type II and III, the mode of binding of type I compounds to the binding niche was only slightly dependent on its conformation state, if at all.

The inhibition by type III compounds of cytochrome bc_1 complex activity was greater than that of type I and II compounds (Fig. 6). This finding is consistent with our hypothesis. The cytochrome bc_1 complex could take a conformational state similar to that occurring when a low transmembrane potential is induced by SF6847, as the mitochondrial membrane under the conditions we used for the measurement of activity may be leaky because of the osmotic difference mentioned in the experimental section.

In Fig. 2, we showed the three inhibitory patterns of phenols using, as examples, three compounds that give the extreme response to the dissipation of the transmembrane potential. With other compounds, these patterns were not always readily distinguishable. A continuous spectrum of patterns was observed. For example, the pattern for compound 2 was type II but close to type I and the patterns for compounds 6 and 17 were type III but close to type I.

Of the compounds used here, type III compounds seemed to require halogen atoms at both ortho positions and also para substituents with suitable steric bulk and electron-withdrawing ability. It is indistinct whether both ortho substituents have to be identical halogen atoms only from the present study. The structural requirements for type II compounds seemed to be similar to those for type III compounds, but small alkyl or alkoxy groups may be needed as the ortho substituents. Other derivatives without these structural features gave a type I inhibitory pattern. Phenols where two ortho positions were substituted with bulky alkyl groups such as *i*-Pr (compound 3), *s*-Bu (compound 4) and *t*-Bu (compound 5; SF6847 itself) had no inhibitory effect on the respiratory chain in the range of concentrations studied here. To draw conclusions about the structural factors that give rise to different inhibitory patterns, further studies with derivatives having structures systematically modified are needed, as is the physicochemical rationalization of various effects of substituents located on each of the positions on the benzene ring.

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