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Direct Interaction between Mitochondrial Succinate-Ubiquinone and Ubiquinol-Cytochrome *c* Oxidoreductases Probed by Sensitivity to Quinone-Related Inhibitors

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The electron-transfer activities of bovine heart mitochondrial complexes I, II, and III, but not complex IV, were simultaneously inhibited by 2-alkyl-4,6-dinitrophenols to a different extent. The extent of inhibition of NADH and succinate oxidase activities by dinitrophenols was compared with that of individual complex activities using submitochondrial particles. The extent of inhibition of succinate oxidase activity by 1-methylpropyl and 1-methylbutyl derivatives was much larger than that of NADH oxidase activity. This large inhibition of succinate oxidase activity seemed not to be explainable by the extent of inhibition of individual complex activities (*i.e.*, complexes II and III activities), based upon the homogeneous ubiquinone pool model. On the other hand, other dinitrophenols (*n*-propyl, 1-methylpentyl, 1-methylhexyl, and *tert*-butyl derivatives) very similar to the above compounds did not elicit such anomalous inhibitory action, indicating that the action of 1-methylpropyl and 1-methylbutyl derivatives is highly specific to their structure. The anomalous inhibition by these two compounds was also observed with the isolated succinate-cytochrome *c* oxidoreductase, in which there is no ubiquinone pool behavior [Rich, P.R. (1984) *Biochim. Biophys. Acta* 768, 53-79]. However, when the succinate-cytochrome *c* reductase of which the activity had been partially restored by adding phospholipid and exogenous quinone to the phospholipid- and ubiquinone-depleted succinate-cytochrome *c* reductase was assayed, the anomalous inhibitory action of interest was undetectable. These results indicated that electron-transfer between complexes II and III, which is mediated not only by free-form, but also by protein-bound ubiquinone, occurs in the mitochondrial membrane. The fact that the anomalous inhibition of succinate oxidase activity of submitochondrial particles was sensitive to changes in the external osmotic pressure which affected the total area of the particle supports this notion.

Key words: electron-transfer inhibitor, mitochondrial electron-transfer, succinate-ubiquinone oxidoreductase, ubiquinol-cytochrome *c* oxidoreductase, ubiquinone-pool.

The electron-transfer system of the mitochondrial inner membrane consists of four multisubunit complexes: NADH-ubiquinone oxidoreductase (complex I), succinate-ubiquinone oxidoreductase (complex II), ubiquinol-cytochrome *c* oxidoreductase (complex III), and cytochrome *c* oxidase (complex IV). Ubiquinone is considered to act as a mobile electron carrier between complexes I, II, and III. In support of this view, Kröger and Klingenberg (*1*) demonstrated that virtually all the mitochondrial ubiquinone reacts in a homogeneous fashion during electron transfer. The homogeneous ubiquinone pool model accounted for the sigmoidal inhibition of mitochondrial respiration by antimycin A (*1*). Studies in Hackenbrock's laboratory (*2-4*) have supported the random collision model of mitochondrial electron transport, in which the respiratory chain complexes as well as ubiquinone are free to diffuse laterally and independently of each other in the plane of the mitochondrial membrane.

On the other hand, several approaches have provided evidence for the direct interaction of the complexes. For example, Beattie and colleagues (*5-7*) have proposed an interaction between complexes II and III based upon the antimycin-sensitivity of the exogenous quinone reduction by succinate in submitochondrial particles and isolated succinate-cytochrome *c* oxidoreductase from yeast and beef heart mitochondria. Gwak *et al.* (*8*) have suggested a specific interaction between complexes II and III based upon differential scanning calorimetry and electron paramagnetic resonance (EPR) studies with mammalian electron-transfer complexes embedded in phospholipid vesicles. Using the Q_i inhibitor-resistant mouse cytochrome *b* mutant, Howell (*9*) proposed that the mutation in cytochrome *b* protein affects complex II assembly or stability. Furthermore, the identification of ubiquinone-binding proteins in mitochondrial electron-transfer complexes (*10-*

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Abbreviations: complex I, NADH-ubiquinone oxidoreductase; complex II, succinate-ubiquinone oxidoreductase; complex III, ubiquinol-cytochrome *c* oxidoreductase; complex IV, cytochrome *c* oxidase; DCIP, dichloroindophenol; DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone; DBH_2, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinol; I_{50}, molar concentration in the reaction medium needed to halve the full activity; SF6847, 3,5-di-*tert*-butyl-4-hydroxybenzylidene malononitrile.

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13) suggests that the active species of ubiquinone during electron transfer is a ubiquinone-protein complex and not the free-form ubiquinone. Thus, whether these complexes are present individually or as macromolecular assemblies (supercomplexes) in the membrane is still a matter of controversy.

The 2-alkyl-4,6-dinitrophenols are well known protonophore-type uncouplers (14) and they also inhibit mitochondrial electron-transfer (15). Although the inhibition pattern varies depending upon the different electron-transfer complexes and quinol analogue substrates used, they inhibit complexes I, II, and III activities, but not that of complex IV (15, 16). In addition, some dinitrophenols are fairly potent inhibitors of the Q_B site of photosystem II (15, 17) and of *bo*-type ubiquinol oxidase of *Escherichia coli* (18). It is therefore possible that some 2-alkyl-4,6-dinitrophenols serve as universal inhibitors of electron-transfer complexes in which quinone plays a functional role. On the other hand, a molecular orbital calculation study (15) showed that the conformation of the alkyl substituent at the 2-position, which may correspond to the isoprenoid side chain of ubiquinone, is the significant factor for the inhibitory action, irrespective of substitution patterns at the 4- and 6-positions (19).

In preliminary investigations with bovine heart submitochondrial particles, we found that when the inhibition of succinate oxidase activity is assayed, some dinitrophenols seem to elicit greater inhibition of succinate oxidase activity than would be expected from their inhibitory potencies toward individual complex activities. By contrast, this anomalous inhibition was not observed when the inhibition of NADH oxidase activity was assayed. Considering the proposed direct interaction between complexes II and III, the anomalous inhibitory action of some dinitrophenols would be associated with the electron-transfer process between the two complexes, which is not mediated through the ubiquinone pool. To examine this possibility, the inhibitor sensitivity of succinate and NADH oxidase activities of bovine heart submitochondrial particles was compared with that of individual complex activities using a series of 2-alkyl-4,6-dinitrophenols. Furthermore, their inhibitory action with the isolated succinate-cytochrome *c* oxidoreductase, in which ubiquinone pool behavior is lost (20, 21), was compared with that with submitochondrial particles.

EXPERIMENTAL PROCEDURES

Materials—Rotenone, antimycin A and cytochrome *c* (horse heart) were purchased from Sigma. The 2-alkyl-4,6-dinitrophenols, DB, and DBH_2 were as described (15). Other reagents were of the purest grade commercially available.

Methods—Submitochondrial particles were prepared from beef heart mitochondria by the method of Matsuno-Yagi and Hatefi (22) and stored in a buffer containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4) at −78°C. The averaged respiratory control ratio (*RCR*) was 1.8 with succinate as the substrate. The orientation of the submitochondrial particles was checked by the method of Degli Esposti and Lenaz (23) and they were orientated usually 90-95% inside-out. The protein concentration was determined by the biuret method (24) using bovine serum albumin as the standard.

The NADH-DB oxidoreductase activity of submitochondrial particles was measured spectrophotometorically at 30°C by the decrease in the absorbance due to NADH at 340 nm ($\varepsilon = 6.22\ mM^{-1} \cdot cm^{-1}$) using a stirred cuvette in a Shimadzu UV3000 spectrometer. The reaction medium, in a final volume of 2.5 ml, contained 50 mM phosphate (pH 7.4), 0.25 M sucrose, 2.0 mM potassium cyanide, 1.0 mM $MgCl_2$, 0.1 μM antimycin A, 0.1 μM SF6847, 50 μM NADH, and 60 μM DB. The final mitochondrial protein concentration was 15 μg/ml.

Succinate-DB oxidoreductase activity was coupled to the reduction of DCIP ($\varepsilon = 21\ mM^{-1} \cdot cm^{-1}$), and the rate was followed spectrophotometorically at 600 nm at 30°C. The reaction medium contained 50 mM phosphate (pH 7.4), 0.25 M sucrose, 2.0 mM potassium cyanide, 1.0 mM $MgCl_2$, 1 μM rotenone, 0.1 μM antimycin A, 0.1 μM SF6847, 50 μM DCIP, 20 mM succinate, and 60 μM DB. The final mitochondrial protein concentration was 15 μg/ml. Submitochondrial particles (20 mg/ml) were preincubated at 30°C with 1 mM malonate for 10 min to fully activate the succinate dehydrogenase (25). The amount of malonate carried over into the assay was negligible.

DBH_2-cytochrome *c* oxidoreductase activity was measured at 30°C as the rate of cytochrome *c* reduction with the wavelength pair 550-540 nm ($\varepsilon = 19\ mM^{-1} \cdot cm^{-1}$). The particles were treated with sodium deoxycholate (0.2 mg/mg protein) before dilution with reaction medium (23), consisting of a mixture of 50 mM phosphate (pH 7.4), 0.25 M sucrose, 2.0 mM potassium cyanide, 1.0 mM $MgCl_2$, 1 μM rotenone, 10 μM DBH_2, and 40 μM cytochrome *c*. The final mitochondrial protein concentration was 15 μg/ml.

Succinate oxidase activity, after activation as described above, was assayed with a Clark-type oxygen electrode at 30°C in medium consisting of 50 mM phosphate (pH 7.4), 0.25 M sucrose, 1 μM rotenone, 1.0 μM SF6847, and 20 mM succinate. NADH oxidase activity was also measured polarographically at 30°C. The reaction medium contained 50 mM phosphate (pH 7.4), 0.25 M sucrose, 1.0 μM SF6847, and 1.0 mM NADH. The final mitochondrial protein concentration was 0.3 mg/ml in the assay of succinate and NADH oxidases activities.

The succinate-cytochrome *c* oxidoreductase was prepared from submitochondrial particles according to the method of Yu and Yu (26). The purified enzyme was dissolved in 50 mM phosphate buffer (pH 7.4) containing 0.25 M sucrose and stored at −78°C. The determinations of cytochrome c_1 (27), cytochrome *b* (28), and ubiquinone (29) were carried out by the reported methods. The phospholipid- and ubiquinone-depleted preparation of succinate-cytochrome *c* oxidoreductase was prepared from ordinary succinate-cytochrome *c* oxidoreductase by means of repeated ammonium sulfate fractionation (30). The restoration of succinate-cytochrome *c* oxidoreductase activity was carried out according to the reported method (30), except that exogenous quinone (DB) was added prior to the phospholipid (asolection), as described in "RESULTS."

Succinate-DB oxidoreductase activity of the isolated enzyme was determined at 30°C in the reaction medium containing 0.1 M phosphate (pH 7.4), 0.3 mM EDTA, 0.1 μM antimycin A, 60 μM DB, and 50 μM DCIP, final protein concentration being 3 μg/ml. The reaction was started by adding 20 mM succinate. DBH_2-cytochrome *c* oxidore-

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ductase activity was measured in medium containing 0.1 M phosphate (pH 7.4), 0.3 mM EDTA, and 40 μM cytochrome *c*, the final protein concentration being 3 μg/ml. The reaction was started by the addition of 10 μM DBH_2. Succinate-cytochrome *c* oxidoreductase activity was similarly measured, except that the reaction was started by adding 20 mM succinate.

For all assays, the test compounds were dissolved in ethanol and added 2 min before the reaction was started. The final ethanol concentrations were less than 0.5% (v/v).

RESULTS

The homogeneous pool model of Kröger and Klingenberg (*1*) suggests that quinone acts as a diffusible carrier of reducing equivalents between dehydrogenases and complex III. Under steady-state conditions, the reaction rate is given by

$$V_{obs} = V_{red} \cdot V_{ox}/(V_{red} + V_{ox})$$

where V_{obs} is the rate of succinate- and NADH-cytochrome *c* oxidoreductase activity, V_{red} and V_{ox} being the rates of ubiquinone reduction and oxidation, respectively.

The compounds tested in this study simultaneously inhibited the activities of complexes I, II, and III activities, but not that of complex IV, to a different extent, as shown below. If the rates of individual complex activities, V_I (complex I), V_{II} (complex II), and V_{III} (complex III), are reduced to V'_I, V'_{II}, and V'_{III}, respectively, in the presence of an inhibitor at a given concentration, the rates of NADH ($V'_{I\text{-}III}$) and succinate ($V'_{II\text{-}III}$) oxidases activities are expressed by Eqs. 1 and 2, respectively.

$$V'_{I\text{-}III} = V'_I \cdot V'_{III}/(V'_I + V'_{III}) \quad (1)$$

$$V'_{II\text{-}III} = V'_{II} \cdot V'_{III}/(V'_{II} + V'_{III}) \quad (2)$$

The ratio of NADH oxidase to succinate oxidase activities is then given by Eq. 3.

$$\frac{V'_{I\text{-}III}}{V'_{II\text{-}III}} = \frac{V'_I V'_{II} + V'_I V'_{III}}{V'_I V'_{II} + V'_{II} V'_{III}} \quad (3)$$

Assuming $V'_I/V'_{II} = n$, Eq. 3 is rewritten:

$$\frac{V'_{I\text{-}III}}{V'_{II\text{-}III}} = \frac{n(V'_{II} + V'_{III})}{nV'_{II} + V'_{III}} \quad (4)$$

When $V'_{III} \gg V'_{II}$, $V'_{I\text{-}III}/V'_{II\text{-}III}$ ratio is equal to n. This is an example where the inhibition of complex III activity is negligibly small compared to that of complex II. When $V'_{II} \gg V'_{III}$, the $V'_{I\text{-}III}/V'_{II\text{-}III}$ ratio is unity. This holds where the compound of interest specifically inhibits complex III.

Other than the above, $V'_{I\text{-}III}/V'_{II\text{-}III}$ ratio varies depending upon both V'_{II}/V'_{III} ratio and n. Under any given conditions, however, the $V'_{I\text{-}III}/V'_{II\text{-}III}$ ratio should be,

$1 > n$: $1 > V'_{I\text{-}III}/V'_{II\text{-}III} > n$

$n = 1$: $V'_{I\text{-}III}/V'_{II\text{-}III} = 1$

$n > 1$: $1 < V'_{I\text{-}III}/V'_{II\text{-}III} < n$

It is therefore concluded that when complexes I, II, and III activities are inhibited simultaneously, the $V'_{I\text{-}III}/V'_{II\text{-}III}$

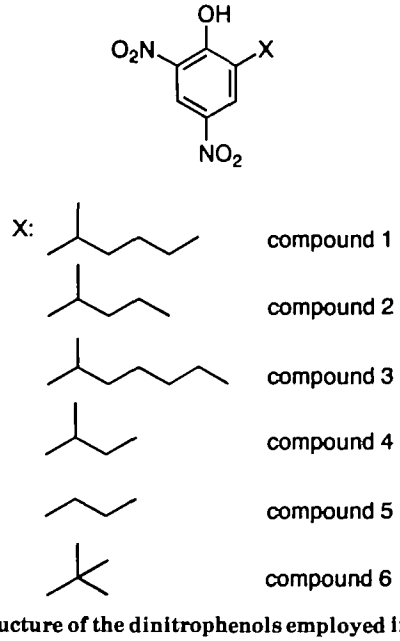

Fig. 1. **Structure of the dinitrophenols employed in this study.**

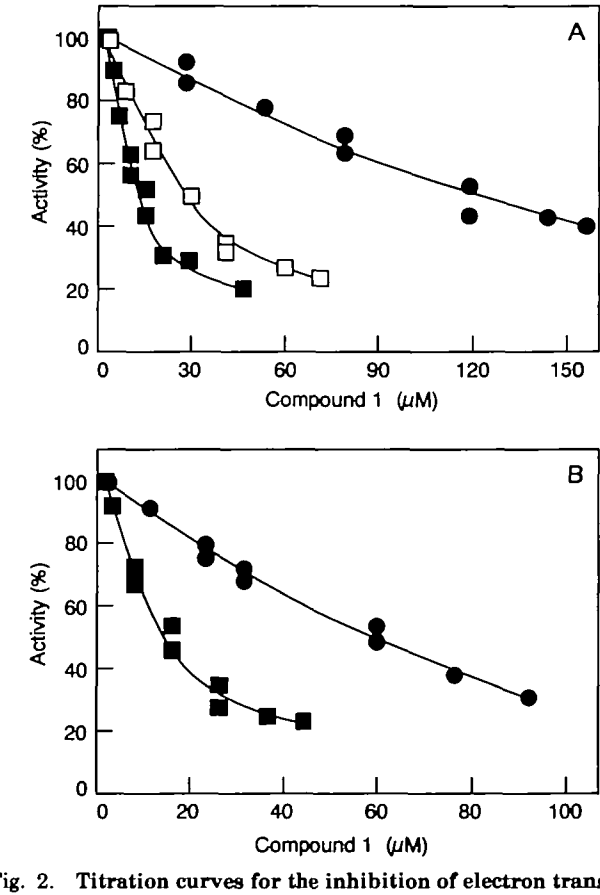

Fig. 2. **Titration curves for the inhibition of electron transfer by compound 1.** A: The inhibition of NADH-DB (●), succinate-DB (■), and DBH_2-cytochrome *c* oxidoreductases (□) by compound 1. The averaged 100% activities of NADH-DB, succinate-DB, and DBH_2-cytochrome *c* oxidoreductases were 0.79 μmol NADH, 0.32 μmol DCIP, and 4.5 μmol cytochrome *c* reduced per min per mg protein, respectively. B: The inhibition of NADH (●) and succinate (■) oxidases activities by compound 1. The averaged 100% activities of NADH and succinate oxidases activities are 330 and 155 nmol O_2/min per mg protein, respectively. For details, see "*Methods*."

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ratio should be comparable to, or less than the V'_I/V'_{II} ratio regardless of V'_{III}. This relationship is visualized in Fig. 5 of Ref. *31*, where variations in respiratory activity were determined when the donor and acceptor activities are simultaneously varied by specific inhibitors. Looking at this from the perspective of "inhibitor sensitivity," a difference in the extent of inhibition of individual complex activities (*i.e.*, complexes I and II activities) is not enhanced when NADH and succinate oxidase activities are assayed through the ubiquinone pool, since the extent of inhibition of complex III activity (acceptor activity) is identical regardless of the respiration substrates at a definite concentration of the inhibitor.

Inhibition of Individual Complex Activities and NADH and Succinate Oxidases Activities by Dinitrophenols—The titrations for the inhibition of NADH-DB oxidoreductase (complex I), succinate-DB reductase (complex II), and DBH_2-cytochrome *c* oxidoreductase (complex III) activities of submitochondrial particles by compounds 1 and 2 are shown in Figs. 2A and 3A, respectively. The extent of inhibition of these complexes activities decreased in the order of complexes II, III, and I. These compounds did not attain the complete inhibition of individual complex activities. In addition, since the dinitrophenols studied here do not inhibit complex IV activity (*15, 16*), the complex IV inhibition can be neglected in the following experiments.

The titrations for the inhibition of NADH and succinate oxidases activities of submitochondrial particles by compounds 1 and 2 are shown in Figs. 2B and 3B, respectively. Since the concentration of mitochondrial protein was higher in this oxygraphic experiment than that used in the spectroscopic experiment described in Figs. 2A and 3A, the molar concentration of the inhibitor in bulk aqueous phase can not be directly compared. To examine whether the extent of inhibition of NADH and succinate oxidases activities is explainable by that of individual complex activities based on the homogeneous pool model, we tried to compare the difference in inhibitor sensitivity between NADH oxidase and succinate oxidase activities with that between complex I and complex II activities. As an index of the inhibitor sensitivity for each activity, the molar concentration required to halve the control activity (I_{50}) was used. The difference in the inhibitor sensitivity between complexes I and II activities and also between NADH and succinate oxidase activities was compared as the ratio of I_{50} values for convenience. Since the absolute turnover values are different between complexes I and II, the same degree of inhibition of each activity does not result in the same degree of reduction of the ubiquinone pool (*31*). Therefore the ratio of I_{50} values does not directly correspond to the ratio of the reaction rates described above.

As shown in Table I, the I_{50} ratio of NADH oxidase to succinate oxidase activities for compound 1 was less than that of complex I to complex II activities (3.4 *vs.* 8.5), indicating that the extent of inhibition of NADH and succinate oxidases activities by compound 1 can be accounted for by that of individual complex activities based on the homogeneous pool model. By contrast, compound 2 inhibited succinate oxidase activity more effectively than it did NADH oxidase activity. The difference in the extent of inhibition of individual complex activities should be not enhanced when NADH and succinate oxidase activities are assayed, but this was not so (95.8 *vs.* 11.2). Thus, this anomalous inhibition seemed not to be explainable by a simple combination of the partial inhibition of individual

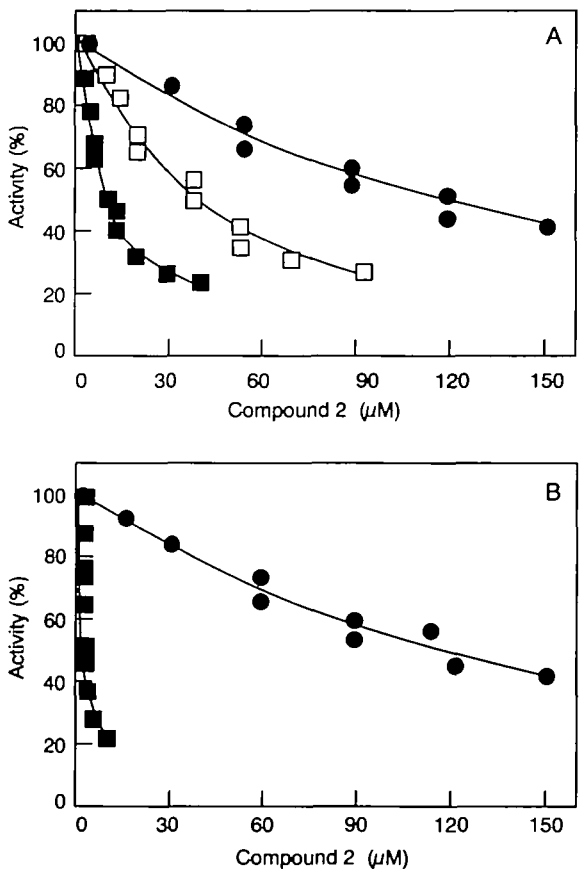

Fig. 3. **Titration curve for the inhibition of electron-transfer by compound 2.** A: The inhibition of NADH-DB (●), succinate-DB (■), and DBH_2-cytochrome *c* oxidoreductases (□) activities by compound 2. B: The inhibition of NADH (●) and succinate (■) oxidases activities by compound 2.

TABLE I. **Inhibitory potency of dinitrophenols with submitochondrial particles.** The I_{50} value is the molar concentration required to halve the control enzyme activity. I_{50}(I), I_{50} value for complex I activity; I_{50}(II), I_{50} value for complex II activity; I_{50}(I)/I_{50}(II), the ratio of I_{50} (I) to I_{50}(II); I_{50}(N), I_{50} value for NADH oxidase activity; I_{50}(S), I_{50} value for succinate oxidase activity; I_{50}(N)/I_{50}(S), the ratio of I_{50}(N) to I_{50} (S). The results are means of at least two independent measurements.

Compounds	I_{50}(I) (μM)	I_{50}(II) (μM)	I_{50}(III) (μM)	I_{50}(I)/I_{50}(II)	I_{50}(N) (μM)	I_{50}(S) (μM)	I_{50}(N) I_{50}(S)
1	128	15	29	8.5	58	17	3.4
2	123	11	36	11.2	115	1.2	95.8
3	8.0	4.3	4.0	1.9	15	8.0	1.9
4	251	133	110	1.9	135	11	12.3
5	631	380	145	1.7	405	253	1.6
6	182	307	19	0.6	25	35	0.7

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complex activities. Considering that compounds 1 and 2 are structurally similar, it is likely that the anomalous inhibition of succinate oxidase activity by compound 2 is highly specific to its structure, in other words, some mode of action of the inhibitor is responsible for the inhibition of interest.

To confirm the structural specificity of dinitrophenols, we also examined the inhibition using other dinitrophenol derivatives (compounds 3, 4, 5, and 6) that are similar to compounds 1 and 2. For compounds 3, 5, and 6, the I_{50} ratio of NADH oxidase to succinate oxidase activities was comparable to that of complex I to complex II activities (Table I). This indicated that the inhibition of NADH and succinate oxidases activity by these compounds is explainable by a combination of the inhibition of individual complex activities. In contrast, the extent of inhibition of succinate oxidase activity by compound 4 was much greater than that of NADH oxidase activity, and seemed not to be accounted for by the effects on individual complex activities (12.3 *vs.* 1.9). In particular, comparing the inhibition of succinate oxidase activity by compounds 2 and 3, the anomalously potent inhibition by compound 2 was apparent, because compound 3 was a more potent inhibitor than compound 2 when individual complex activities were assayed separately. It is therefore concluded that the anomalous inhibition of succinate oxidase activity is highly specific to the inhibitor structure, and that the mode of action of the inhibitor is responsible for this type of inhibition. The branching structure at the α-position (1-position) and the main chain length of 3 or 4 carbons are the structural requirements for the 2-alkyl substituent to bring about the anomalous inhibition.

Osmotic Sensitivity of Inhibition of Succinate Oxidase Activity by Dinitrophenols—The primary effect of an increase in the external osmotic pressure is a decrease in the volume of mitochondria, leading to a decrease in the total area of the particle (*32*). If the random distribution of electron-transfer complexes and ubiquinone in the plane of the mitochondrial membrane is considered, a decrease in the area of the membrane results in a decrease in the distance between the complexes (or reaction sites) (*32*). If there is a direct interaction between the complexes, the mode of the interaction would be somewhat affected by changes in external osmotic pressure. To determine whether the anomalous inhibitory action of compounds 2 and 4 is associated with a specific interaction between complexes II and III, the osmotic sensitivity of the inhibition of succinate oxidase activity, as well as NADH oxidase activity was studied.

The concentration of sucrose in the reaction medium was set as 0.25, 1.0, or 1.5 M. Table II shows the osmotic sensitivity of the inhibition of succinate and NADH oxidase activities by dinitrophenols. The inhibitory potency of compounds 2 and 4 with respect to succinate oxidase activity decreased with an increase in external osmotic pressure. Contrary to this, the inhibition of NADH oxidase activity was not sensitive to changes in osmotic pressure. On the other hand, the inhibitory potency of compounds 1 and 6 towards succinate and NADH oxidase activities was free from the effects of changes in osmotic pressure. These results suggested that the inhibition of succinate oxidase activity by compounds 2 and 4 is associated with direct interaction between complexes II and III, as discussed later.

Inhibition of the Isolated Succinate-Cytochrome c Oxidoreductase by Dinitrophenols—To examine the possibility that the anomalous inhibitory action of compounds 2 and 4 is the result of a direct interaction between complexes II and III (or to the electron-transfer process involving protein-bound quinone), the inhibitor titrations were carried out with the isolated succinate-cytochrome *c* oxidoreductase. In the assay system using the isolated succinate-cytochrome *c* oxidoreductase, there is no ubiquinone pool behavior (*20, 21, 33*). The enzyme preparations had a succinate-cytochrome *c* oxidoreductase activity of 4-5 μmol cytochrome *c* reduced per min per milligram of protein, and contained 0.7-0.8 mol of ubiquinone per mol of cytochrome c_1, being comparable to those reported (*26, 30*).

Using the isolated succinate-cytochrome *c* oxidoreductase, inhibition of succinate-DB, DBH_2-cytochrome *c*, and succinate-cytochrome *c* oxidoreductases activities by compounds 1, 2, 3, and 4 was examined. The inhibitory potency, in terms of I_{50} value, for each of the three activities and the relative extent of inhibition of individual complex activity at the concentration where succinate-cytochrome *c* reductase activity was inhibited by 50% are listed in Table III. The I_{50} values of compound 2 for succinate-DB and DBH_2-cytochrome *c* oxidoreductases activities were similar to those of compound 1, whereas the inhibitory potency of compound 2 for succinate-cytochrome *c* oxidoreductase activity was much larger than that of compound 1. It is notable that while compound 3 was a more potent inhibitor of individual complex activities than compound 2, the latter more potently inhibited succinate-cytochrome *c* oxidoreductase activity than the former. A similar tendency of variation in inhibitory potency was observed for compounds 1 and 4. These results also indicated that compounds 2 and 4 bring about more potent inhibition of succinate-cytochrome *c* oxidoreductase than that predicted from the effects on the individual complex activity. Incidentally, the relative extent of the inhibition of individual complex activities in the presence of compounds 2 or 4 at concentrations causing 50% inhibition of succinate-cytochrome *c* oxidoreductase activity was less than 10%. Considering that there is no ubiquinone pool behavior with the isolated succinate-cytochrome *c* oxidoreductase, it is very likely that the anomalous inhibitory action can be attributed to electron-transfer involving the protein-bound ubiquinone.

Inhibition of the Restored Succinate-Cytochrome c Oxidoreductase by Dinitrophenols—To further explore the

TABLE II. **Osmotic sensitivity of inhibitory potency (I_{50}) of dinitrophenols.** Submitochondrial particles were prepared in a buffer containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.4). The sucrose concentration in the reaction medium was set as 0.25, 1.0, or 1.5 M. The averaged I_{50} value from at least two determinations is listed.

Compound	I_{50} value (μM)					
	Succinate oxidase activity			NADH oxidase activity		
	[sucrose]			[sucrose]		
	0.25	1.0	1.5 (M)	0.25	1.0	1.5 (M)
1	17	20	19	58	52	55
2	1.2	2.0	3.1	115	113	110
4	11	23	38	135	131	140
6	35	40	37	25	22	27

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TABLE III. **Inhibitory potency of dinitrophenols with the isolated succinate-cytochrome *c* reductase.** The I_{50} value is the molar concentration required to halve the control enzyme activity. Complex II, succinate-DB oxidoreductase activity; complex III, DBH_2-cytochrome *c* oxidoreductase activity; complex II-III, succinate-cytochrome *c* oxidoreductase activity. The relative extent of inhibition of complexes II and III activities at the concentration where succinate-cytochrome *c* oxidoreductase activity was inhibited by 50% is listed. Results are means of at least two independent measurements.

Compounds	I_{50} (μM)			Relative inhibition (%)	
	Complex II	Complex III	Complex II-III	Complex II	Complex III
1	23	90	5.2	35	10
2	21	95	0.85	5	—[a]
3	8.0	18	1.8	33	15
4	43	—[b]	2.5	8	—[a]

[a]Inhibition was negligible. [b]At least up to 150 μM, 50% inhibition was not observed.

TABLE IV. **Inhibitory potency of dinitrophenols with the partially restored succinate-cytochrome *c* oxidoreductase.** The I_{50} value is the molar concentration required to halve the control enzyme activity. Complex II, succinate-DB oxidoreductase activity; complex III, DBH_2-cytochrome *c* oxidoreductase activity; complex II-III, succinate-cytochrome *c* oxidoreductase activity. The relative extent of inhibition of complexes II and III activities at the concentration where succinate-cytochrome *c* oxidoreductase activity was inhibited by 50% is listed. Results are means of at least two independent measurements.

Compounds	I_{50} (μM)			Relative inhibition (%)	
	Complex II	Complex III	Complex II-III	Complex II	Complex III
1	10	25	6.4	33	25
2	3.6	21	3.1	42	18
3	3.5	9.0	3.0	45	20
4	19	42	13	41	15

role of protein-bound ubiquinone, we examined the inhibitory action of dinitrophenols using succinate-cytochrome *c* oxidoreductase of which the activity had been partially restored by the addition of phospholipid (asolectin) and exogenous quinone (DB) to the phospholipid- and ubiquinone-depleted succinate-cytochrome *c* oxidoreductase. By employing the technique of repeated ammonium sulfate-cholate precipitation (*30*), we obtained a phospholipid- and ubiquinone-depleted preparation of succinate-cytochrome *c* oxidoreductase. After five cycles of precipitation, the preparations contained 0.15-0.2 mol of ubiquinone per mol of cytochrome c_1 and they had no succinate-cytochrome *c* oxidoreductase activity. This result was in agreement with the observation that succinate-cytochrome *c* oxidoreductase activity is drastically reduced even by the partial removal of phospholipid and ubiquinone (*30*). The maximal restoration of the enzyme activity requires the addition of quinone prior to the addition of phospholipid and an incubation period of more than 1 h (*30*). However, to see whether the electron transfer involving tightly protein-bound ubiquinone is associated with the anomalous inhibition of interest, we required the enzyme preparations in which the "binding" manner of quinone differs from that in the undepleted enzyme; in other words, the enzyme in which quinone does not occupy the proper site to reform a functionally active unit. Therefore, we prepared a partially restored enzyme by adding phospholipid (0.3 mg asolectin/mg protein) *prior to* DB (20 nmol/ mg protein) and incubating for just 3 min. The enzyme prepared by this procedure recovered about only 55% activity of the undepleted enzyme. It was further confirmed that even if the incubation time was prolonged, full activity was not restored by changing the sequence of the components addition, as reported (*30*).

The same set of inhibitory potencies as those shown in Table III was determined with the partially restored succinate-cytochrome *c* reductase, and these are listed in Table IV. The discrepancy in the I_{50} values between the normal and restored enzymes may be due to a difference in the "binding" manner of quinone. Comparing the inhibition of individual complex activities and succinate-cytochrome *c* reductase activity between compounds 1, 2, 3, and 4, it is apparent that the extent of inhibition of succinate-cytochrome *c* oxidoreductase activity by compound 2 or 4 is not necessarily larger than that expected from the effects on the individual complex activities. It is therefore concluded that the anomalous inhibitory action of compounds 2 and 4 is closely associated with the electron-transfer process involving protein-bound ubiquinone.

DISCUSSION

Ubiquinone acts as the electronic connector between primary dehydrogenases (*i.e.*, complexes I and II) and complex III. Although many electron-transfer systems follow the homogenous ubiquinone pool theory, there are reports of systems which do not precisely fit this model (*20, 21, 33*). Two extreme types of molecular organization of electron-transfer components in the membrane have been posited, being termed "liquid-state" and "solid-state" (*20*). In a liquid-state system like the mitochondrial membrane, all components diffuse freely, and thus ubiquinone pool behavior is applicable. In a solid-state system like the isolated succinate-cytochrome *c* reductase studied here, the required ubiquinone is associated with complex II-III units. Under these conditions, there is no ubiquinone pool behavior. Compounds 2 and 4 elicited potent inhibition of succinate oxidase activity with submitochondrial particles, which could not be explained based on the homogenous ubiquinone pool model alone. This anomalous inhibition was also observed with the isolated succinate-cytochrome *c* oxidoreductase, indicating that it is closely associated with the electron-transfer process involving protein-bound ubiquinone. The fact that the anomalous inhibition was not observed when the partially restored succinate-cytochrome *c* oxidoreductase was assayed supports this notion. These results indicated that the electron-transfer process between complexes II and III, which is mediated by protein-bound ubiquinone, occurs to some extent with mitochondrial membranes.

Several investigators (*5-9, 13*) have proposed that both the free movement of quinone molecules and direct collision involving protein-bound ubiquinone play a role in electron transfer between ubiquinone-reducing and ubiquinol-oxidating enzymes. Based upon inhibitor sensitivity, this study demonstrated support for the notion that both free and protein-bound ubiquinone are involved in the electron transfer between complexes II and III, and that the anomalous inhibition of succinate oxidase activity by compounds 2 and 4 is attributed primarily to disturbance of

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the electron transfer involving the protein-bound ubiquinone (or direct collision involving protein-bound ubiquinone). The idea that ubiquinone functions as a mobile pool and that it binds to protein as a prosthetic group would be compatible if bound quinone is capable of equilibrating with free quinone (*34*).

Although there have been numerous reports suggesting a direct physical interaction between complexes II and III as mentioned above, it may be interesting to discuss the roles of the supposed interaction in the inter or intracomplex electron-transfer reactions. In this context, recent molecular biological study by Bruel *et al.* (*36*), in which the function of subunit 8 of mitochondrial complex III in *Saccharomyces cerevisiae* was investigated by generating site-directed mutants, might be suggestive. They showed that all of the mutations in subunit 8 inhibit complex II activity by as much as 40–60% without significant effect on complex III activity. These observations indicated not only that subunit 8 interacts with complex II, but also that subunit 8 facilitates, but is not essential for, electron transfer from succinate to ubiquinone. Considering the proposed notion that activation of complex II involves a conformational change in the enzyme which alters the electronic environment around iron-sulfur clusters (*25*), subunit 8 might regulate the conformational change involved in the activation of complex II. Thus it would be possible that redox components functionally regulate each other through direct physical interaction, although this notion needs to be verified by further experimental evidence.

It is important to explain mechanistically how compounds 2 and 4 inhibit electron-transfer mediated by protein-bound ubiquinone. The first step to solve this question might be to elucidate which of the two redox reactions (namely, succinate-bound ubiquinone or bound ubiquinol-cytochrome *c* oxidoreductase reactions) shows the more markedly enhanced inhibition when succinate-cytochrome *c* oxidoreductase activity is assayed. Although the two enzyme reactions can be assayed in isolation using exogenous ubiquinone or ubiquinol, individual assay of the two intrinsic reactions involving endogenous bound ubiquinone or ubiquinol is experimentally impractical. Taking into account the fact that the anomalous inhibition of interest was detectable only when succinate-cytochrome *c* oxidoreductase activity mediated by protein-bound ubiquinone was assayed, this experimental limitation hampers the elucidation of the inhibitory mechanism at present.

In this study, we measured individual complex activities using exogenous ubiquinone (DB) or ubiquinol (DBH_2), while endogenous ubiquinone (or ubiquinol) served as a diffusible mediator when NADH and succinate oxidase activities were determined. Therefore the possibility that the individual complex activities measured using exogenous ubiquinone do not strictly correspond to those occurring in the native mitochondrial membrane cannot be ruled out. However, even if this is so, the experimental conditions under which individual complex activities and succinate and NADH oxidase activities were determined were identical for all compounds studied. In other words, all compounds are equally subject to the effects of a discrepancy between exogenous and endogenous ubiquinone (or ubiquinol) on individual complexes. Therefore the anomalous inhibitory action of compounds 2 and 4 is not due to the assay procedures.

The primary effect of an increase in external osmotic pressure is a decrease in the volume of submitochondrial particles, leading to a decrease in their total area (*35*). Essentially, three mitochondrial parameters are mainly influenced by external osmotic pressure: (i) lateral diffusion, (ii) the mean distance between the complexes, and (iii) the catalytic velocity of the individual electron-transfer complexes (*32*). This notion is based upon a random distribution model of redox components in the plane of the membrane. The three respiratory complexes of interest may react differently and simultaneously to variations in osmotic pressure, making it difficult to interpret the effect of osmotic pressure on the above three parameters. However, if a direct interaction involving bound-ubiquinone between the complexes is indeed present, the mode of interaction would be at least somewhat affected by changes in the external osmotic pressure, and consequently a phenomenon which is closely associated with the interaction would be affected. It is noteworthy that the osmotic sensitivity of the inhibition was determined *only* when the inhibition of succinate oxidase activity, but not NADH oxidase activity, by the inhibitors which elicited the anomalous inhibition was assayed. These observations indicate that changes in the lateral diffusion and/or the velocity of the individual enzyme reaction may not affect the apparent profile of osmotic sensitivity of the inhibitor titration. It is therefore reasonable to conclude that the anomalous inhibitory action of these compounds reflects a direct interaction between complexes II and III.

The mode of inhibition by compounds 2 and 4 remains to be elucidated, whereas structural specificity of the inhibitors required for the inhibition is apparent, indicating that the manner of inhibitor binding is closely related to the anomalous inhibition. In other words, even if there is electron transfer involving protein-bound ubiquinone (or a specific interaction between complexes II and III), it may not necessarily be detectable by inhibitor titration of the respiration rate. The conformation and length of the alkyl substituent at the 2-position of dinitrophenol, corresponding probably to the isoprenoid side chain of ubiquinone (*15*), might be the factor governing the action of this series of inhibitors. The main chain length of three or four carbons with an α-branch is the key factor in bringing about the anomalous inhibition of interest. Saitoh *et al.* (*15*) have studied the effect of the α-branch structure of 2-substituents in detail. This structure makes the main chain extend almost perpendicularly to the benzene ring plane, resulting in a configuration similar to the portion of the isoprenoid side chain next to the natural ubiquinone ring. In this study, structural variations were limited to the substituent at the 2-position of 4,6-dinitrophenol. To elucidate mechanism of the inhibitory action and also the structural characteristics of the inhibitor required to elicit this kind of unique inhibition, further studies of derivatives with systematically modified structures are needed.

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