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On the Mechanism of Inhibition of NADH Oxidase by Ubiquinone-3

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Abstract

The combined effects of rotenone and ubiquinone-3 on the kinetics of NADH dehydrogenase and NADH oxidase have been investigated. The two inhibitors do not show additivity; on the other hand, ubiquinone-3, when preincubated with the enzyme, partially removes rotenone sensitivity. The inhibition of NADH oxidase by ubiquinone-3 is the result of at least two combined effects: the competition of the less active ubiquinone-3 with endogenous ubiquinone-10 in the acceptor site of the dehydrogenase, and a nonspecific action on the structure of complex I. The latter effect is perhaps mediated by a physical change of the phospholipid bilayer similar to that observed with agents such as butanol, perturbing lipid–protein interactions in the membrane.

Key Words: NADH oxidase; NADH dehydrogenase; NADH-ubiquinone reductase; ubiquinone homologs; rotenone; submitochondrial particles.

Introduction

Although ubiquinone (UQ) was discovered more than 20 years ago (Morton, 1961) and recognized as a component required for mitochondrial electron transfer (Green and Wharton, 1963; Szarkowska, 1966; Ernster *et al.*, 1969), it is still questionable whether this component is a mobile electron carrier (Kröger and Klingenberg, 1973) or a prosthetic group of one or more redox proteins (Yu *et al.*, 1978).

We have previously observed (Lenaz *et al.*, 1968) that NADH oxidation in pentane-extracted mitochondria is restored by the ubiquinone homologs having long isoprenoid side chains, while the homologs having short isoprenoid

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chains are considerably less active. Such difference in effectiveness is not observed in succinate oxidation. We have also observed that UQ_3 inhibits NADH oxidation but not succinate oxidation; the inhibition is competitive in nature, since high levels of long-chain quinones could restore the activity inhibited by ubiquinone-3 (Lenaz *et al.*, 1975). Since short-chain ubiquinones can be reduced by NADH dehydrogenase (Lenaz *et al.*, 1978), and short-chain ubiquinols can be oxidized by the bc_1 complex, the specificity of NADH oxidase for long-chain homologs has been ascribed to the fact that only the latter, once reduced by complex I, can be reoxidized by the bc_1 complex in a concerted pathway (Lenaz *et al.*, 1978; Lenaz *et al.*, 1977a). This finding suggests a functional compartmentation of endogenous UQ in the membrane.

Short-chain ubiquinones, used by us and by other investigators (Degli Esposti and Lenaz, 1982; Hatefi and Rieske, 1967; Ragan, 1978; Boveris *et al.*, 1972; Lawford and Garland, 1973; Schatz and Racker, 1966) as electron donors or acceptors in partial reactions of the respiratory chain, directly interact with their redox sites in the respiratory complexes and not with endogenous ubiquinone (Cabrini *et al.*, 1981; Zhu *et al.*, 1982). This finding demonstrates that exogenous ubiquinones share the same behavior of endogenous ubiquinone-10, and their redox pathways appear to be physiological, although Ruzicka and Crane (1970) have shown multiple sites for ubiquinone reduction in NADH dehydrogenase.

Gutman (1980) suggested that the inhibitory effect of UQ_3 on NADH oxidase reported by Lenaz *et al.* (1975) is not exerted at the quinone-reducing site in complex I, but at the same binding site of piericidin, the most potent inhibitor known of NADH oxidation in mammalian mitochondria and in submitochondrial particles (Hall *et al.*, 1966). The suggestion was supported by means of structural similarities between the two molecules.

In order to clarify this problem we report recent studies undertaken on the kinetics of NADH dehydrogenase and NADH oxidase utilizing rotenone as inhibitor of these activities. It is well known that although the structure of rotenone bears no similarity to that of piericidin, these two inhibitors are specifically bound at the same site and block NADH oxidation at the same point, i.e., at the NADH dehydrogenase–ubiquinone junction (Horgan *et al.*, 1968).

The results of this investigation show that the inhibitory effect of UQ_3 on NADH oxidase is not as simple as it was initially postulated, representing the combination of a competitive inhibition with endogenous UQ_{10} at the UQ-reducing site of NADH dehydrogenase and of a nonspecific effect on membrane structure. Although we have found that UQ homologs can enter the rotenone site preventing its inhibitory effect, no evidence is obtained that UQ_3 binding to the above site is inhibitory to NADH oxidation.

Materials and Methods

From beef heart mitochondria isolated according to the large-scale procedure of Smith (1967), submitochondrial particles (SMP) were obtained by ultrasonic irradiation (Beyer, 1967). NADH-UQ reductase was assayed spectrophotometrically by the method of Schatz and Racker (1966) following the decrease in absorption of NADH at 340 nm. The reaction mixture contained, in a total volume of 3 ml, 0.23 M sucrose, 33 mM K-phosphate buffer, pH 7, 160 μM NADH, SMP (0.36 mg protein), and 1 μg antimycin A. The reaction was started by adding various amounts of either UQ_1 or UQ_3 in ethanolic solution. When rotenone was present, the inhibitor was preincubated for 10 min in the presence of the particles. NADH oxidase activity was measured either spectrophotometrically or polarographically at 30°C in 0.25 M sucrose, 0.01 M Tris-Cl buffer, pH 7.4. When determinations were made with a Clark oxygen electrode, NADH concentration was 1 mM and particle protein 0.75 mg. In the spectrophotometric assay, NADH and particle protein concentrations were the same as in the NADH-UQ reductase assay. The experiments to determine the K_m for NADH were carried out with a Cary model 15 spectrophotometer equipped with a rapid-mixing apparatus and recording the initial rates of oxidation of NADH at 340 nm (full scale 0.1 A); accurately measured amounts of NADH (15–60 nmol) were added to start the reaction.

The ubiquinone homologs used in these studies were generously donated by Eisai Co., Tokyo, Japan; stock solutions (10–30 mM) of the compounds in absolute ethanol were kept at −20°C.

The concentrations of the various homologs were determined by measuring the absorption decrease at 275 nm upon addition of KBH_4 and using an absorption coefficient of 12.25 mM^{-1} cm^{-1} (Kröger and Klingenberg, 1966). Protein was determined by the biuret method (Gornall *et al.*, 1949). All the biochemicals as well as the inhibitors used were obtained from Sigma Chemical Co. (USA) in the purest available form. The inhibitors were dissolved in absolute ethanol and kept at −20°C.

Results

NADH-UQ Reductase

In a previous work we demonstrated that both UQ_1 and UQ_3 can function as electron acceptors in NADH dehydrogenase activity (Lenaz *et al.*, 1978); it can be seen in Fig. 1 that although NADH-UQ_3 reductase shows typical saturation kinetics, this activity appears considerably lower in comparison

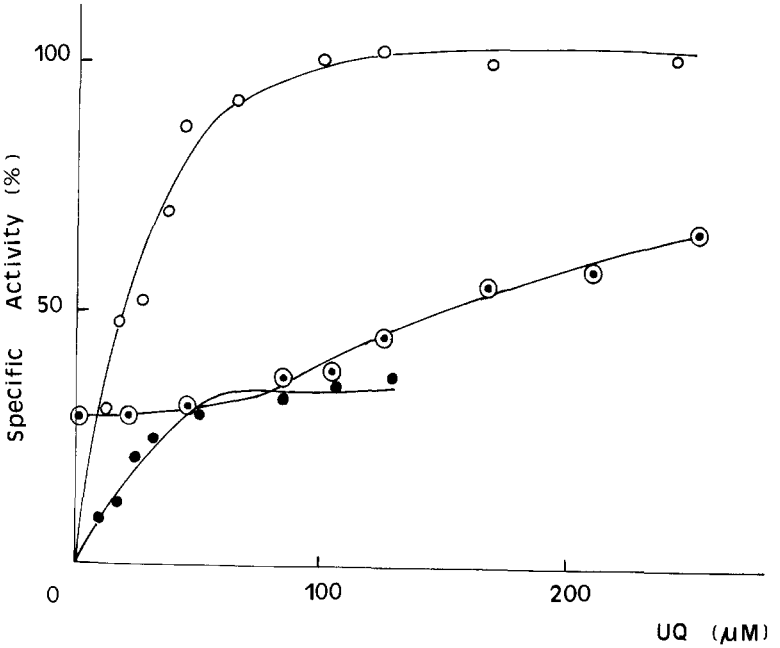

Fig. 1. NADH dehydrogenase activity with either UQ_1 or UQ_3 as electron acceptors. (○) NADH-UQ_1 reductase (maximal rate 308 nmol of NADH oxidized/min · mg protein); (●) NADH-UQ_3 reductase (maximal rate 109 nmol of NADH oxidized/min · mg protein); (⊙) NADH-UQ_1 reductase in the presence of 100 μM UQ_3.

with NADH-UQ_1 reductase; moreover, the NADH-UQ_1 activity is inhibited by UQ_3. Since high concentrations of UQ_1 can partially remove the inhibition, this appears to be competitive (Fig. 1), even if kinetic parameters are difficult to evaluate since UQ_3 acts at the same time as an inhibitor and as an electron acceptor.

Combined studies with UQ_3 and rotenone on NADH dehydrogenase have been devised in attempt to clarify the UQ_3 inhibition site. It can be seen in Fig. 2 that the two inhibitors do not show additivity in their effects; in fact, rotenone inhibition of NADH-UQ_1 and NADH-UQ_3 reductase activities is superimposable (K_i apparent = 37 nmol/mg protein). The whole NADH oxidase activity also shows a similar inhibition pattern (cf. below).

This means that UQ_3 does not interact significantly with the inhibitor binding site under our experimental conditions. On the other hand, it is noteworthy that when NADH-UQ_1 reductase is assayed with the amount of mitochondrial protein lowered to that supporting the same rate found for NADH-UQ_3 reductase, lower concentrations of rotenone are required for inhibition, resulting in an identical K_i. Results which are partially in contrast with the above findings have been observed when UQ_3 is preincubated with

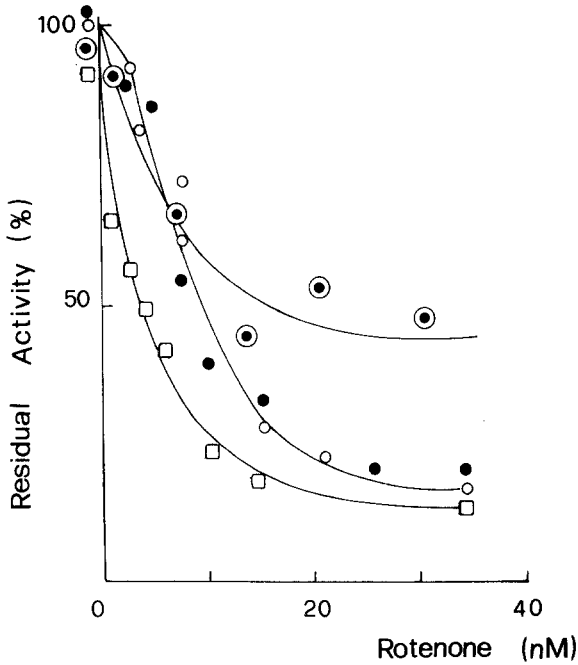

Fig. 2. Inhibition of NADH-UQ reductase activity by rotenone. (□) NADH-UQ_1 reductase (0.12 mg SMP protein); (○) NADH-UQ_1 reductase (0.36 mg SMP protein; absolute rate with no rotenone: 360 nmol NADH oxidized/min · mg protein); (●) NADH-UQ_3 reductase (0.36 mg SMP protein; absolute rate with no rotenone: 152 nmol NADH oxidized/min · mg protein); (⊙) NADH-UQ_3 reductase (UQ_3 preincubated with the particles prior to addition of NADH). UQ concentration was 100 μM in each assay.

the particles prior to the addition of NADH (Fig. 2). In such a case, a partial loss of rotenone sensitivity is observed, but there is no additivity of inhibition at any rotenone concentration.

NADH-Oxidase

As already shown (Lenaz *et al.*, 1975), NADH oxidase activity is inhibited by UQ_3, and this inhibition is partially relieved by addition of a long-chain ubiquinone (Fig. 3); since inhibition appears to be competitive, it is not likely that UQ_7 simply enables a bypass of the UQ_3 inhibition site. On the other hand, UQ_1 does not inhibit NADH oxidase activity (Table I) although it receives electrons from NADH dehydrogenase, as shown by the high NADH-UQ_1 reductase activity. We have then investigated the combined effects of rotenone and UQ_3 on NADH oxidase. The effect of preincubation with UQ_3

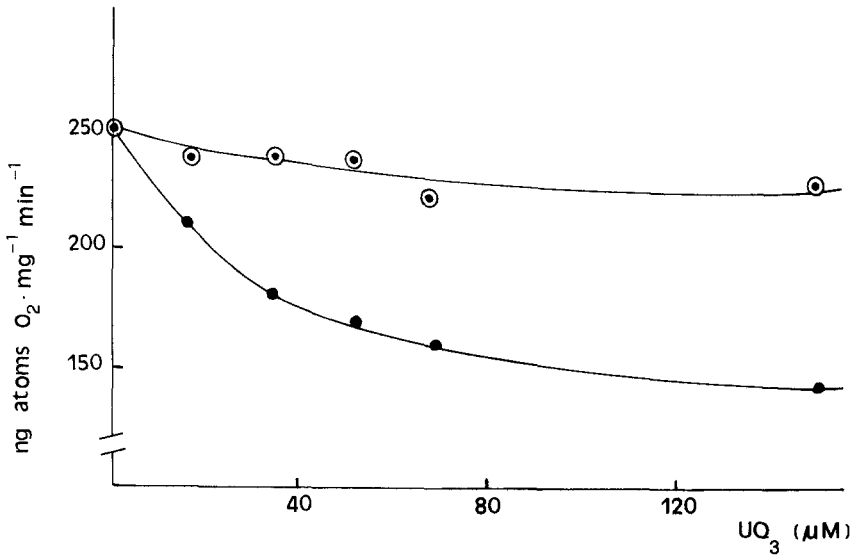

Fig. 3. Effect of a long-chain ubiquinone (UQ_7) on NADH oxidase activity inhibited by UQ_3. (●) NADH oxidase in the presence of increasing amounts of UQ_3; (⊙) NADH oxidase in the presence of increasing amounts of UQ_3 and 60 μM UQ_7.

on the inhibition of NADH oxidase by rotenone, shown in Fig. 4, points out that at low rotenone concentrations the inhibitory effect is similar both in the presence and in the absence of UQ_3, while at high concentrations the UQ_3-preincubated SMP become partially rotenone-insensitive.

These results could be interpreted, according to Gutman (1980), as a displacement of rotenone from the inhibitory site by ubiquinone-3; in such a case, however, the bound quinone would not be able to block electron transfer through the occupation of the rotenone site. In fact, the results of the experiments (shown in Fig. 5) set to ascertain mutual competition or lack of mutual competition between fully competitive and fully noncompetitive

Table I. Effect of UQ_1 and UQ_3 on NADH Oxidation and O_2 Consumption in SMP[a]

Addition	NADH oxidation (nmol · min^{-1} mg^{-1} protein)		O_2 consumption (ng-atoms O_2 · min^{-1} mg^{-1} protein)	
		+ Antimycin		+ Antimycin
—	243	0	287	12
UQ_1	215	186	220	37
UQ_3	127	102	144	45

[a]NADH oxidation was assayed spectrophotometrically following the decrease in NADH absorption at 340 nm. O_2 consumption was determined polarographically. For reaction mixtures, see Materials and Methods. Antimycin, when present, was 1 μg. Final volume was 3 ml, temperature 30°C. UQ_1 and UQ_3 were added as ethanolic solutions to achieve a final concentration of 83 and 80 μM, respectively. The results reported are the means of three or four determinations.

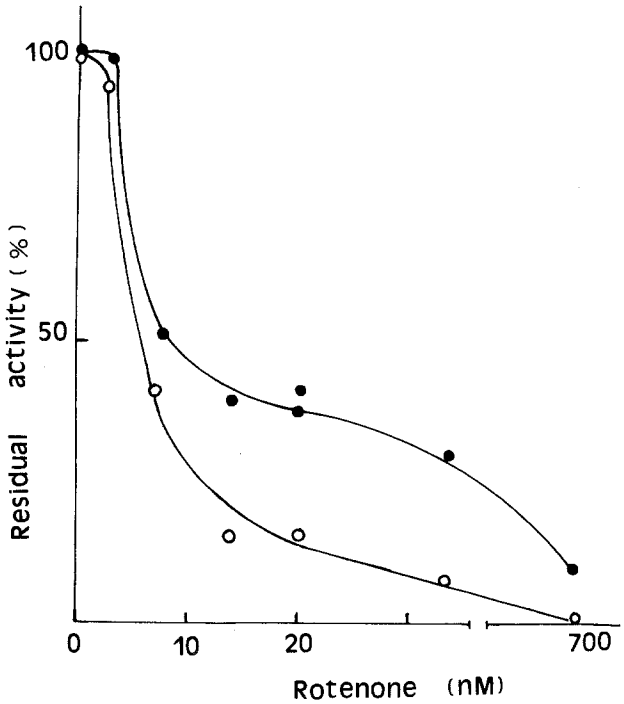

Fig. 4. Effect of preincubation with UQ_3 on the inhibition of NADH oxidase activity by rotenone. (O) NADH oxidase activity (absolute rate with no rotenone: 379 nmol NADH oxidized/min · mg protein); (●) NADH oxidase activity in the presence of 100 μM UQ_3 (absolute rate with no rotenone: 237 nmol NADH oxidized/min · mg protein).

inhibitors according to Semenza and Balthazar (1974) do not fit any known inhibition pattern.

Moreover, the preincubation of SMP with a long-chain UQ homolog like UQ_7, which is well known not to inhibit the oxidation of NADH, also makes the particles partially rotenone-insensitive (Fig. 6). For the above reasons, we conclude that the UQ_3 effect of releasing rotenone inhibition of NADH oxidase is due to ubiquinone binding to the rotenone site. Such binding, however, does not result in inhibition of electron transfer. The inhibition must therefore be nonspecific and might be related to a structural modification of complex I which is affected by agents modifying the membrane lipid environment (Singer and Gutman, 1971).

We have investigated the kinetics of NADH oxidase in the presence of UQ_3. From Fig. 7A it can be seen that in SMP preincubated with this short-chain quinone the apparent K_m for NADH decreases from 38 to 4 μM. The same effect on the K_m (Fig. 7B) is shown in SMP preincubated with

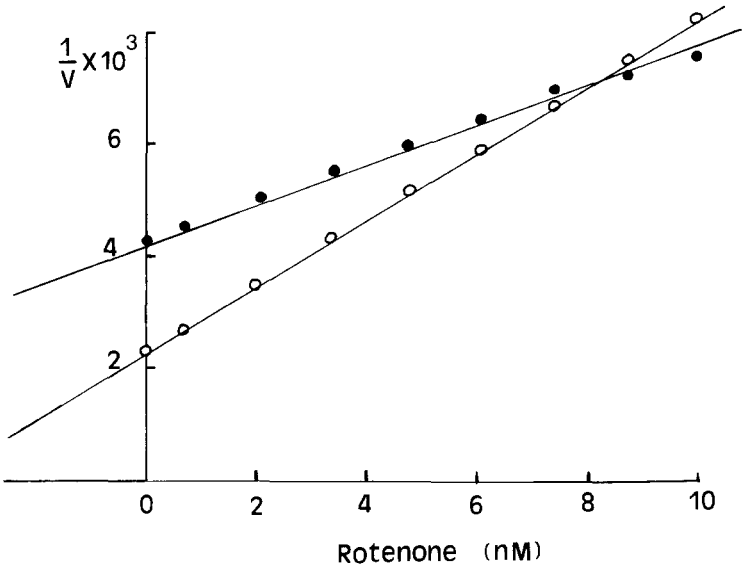

Fig. 5. Modified Dixon plot (Semenza and Balthazar, 1974) of the inhibition of NADH oxidase by rotenone (○), and of the same activity in the presence of UQ_3 (15 μM) (●).

n-butanol, a solvent which, by affecting the lipid phase, can disrupt the normal protein–lipid interactions and modify the protein conformation in the membrane (Lenaz *et al.*, 1977b).

Discussion

The interaction of mitochondrial NADH-UQ reductase (complex I) with ubiquinone is particularly intriguing; the reduction of endogenous UQ by NADH is inhibited by rotenone and piericidin A, apparently at the same site, which is not the site of exit of electrons from the complex (Gutman, 1980). However, a rotenone-insensitive UQ_1 reduction has been shown also to occur (Schatz and Racker, 1966; Machinist and Singer, 1965), indicating that there are two sites for ubiquinone-1 reduction on the enzyme.

The NADH-UQ reductase activity is complicated by the additional feature that the rotenone-sensitive activity is a sum of two reactions, one with high K_m for ubiquinone-1 and high V_{max}, and the other with low K_m and low V_{max} (Ragan, 1978), confirming the suggestion (Singer and Gutman, 1971) that the quinone is reduced at two nonidentical sites which differ in their K_m and V_{max}. Moreover, both piericidin and rotenone are able to bind the enzyme at two different sites (Gutman *et al.*, 1970) but do not show cooperative interaction between the sites; one "site" could, however, be the result of

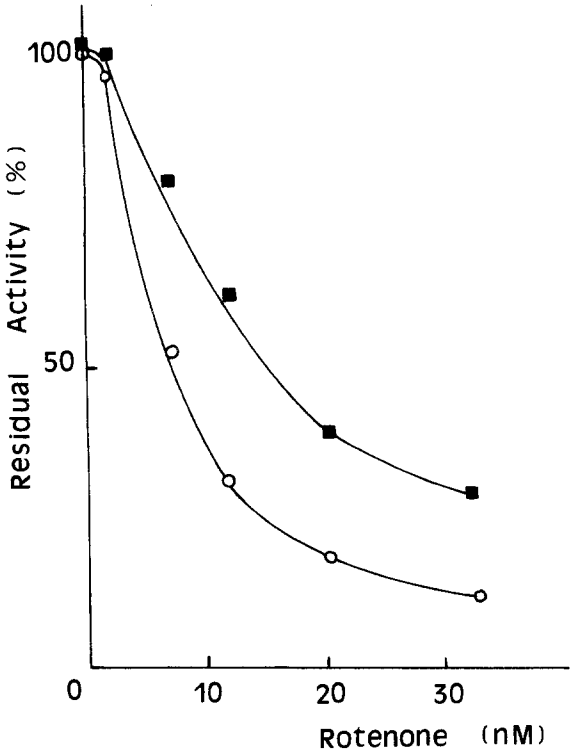

Fig. 6. Effect of preincubation with UQ_7 on the inhibition of NADH oxidase activity by rotenone. NADH oxidase activity (O) (absolute rate with no rotenone: 514 nmol NADH oxidized); NADH oxidase activity in the presence of 100 μM UQ_7 (□) (absolute rate with no rotenone: 555 nmol NADH oxidized/min · mg protein).

nonspecific binding (Horgan *et al.*, 1968; Singer, 1979). Gutman *et al.* (1971) have shown that inhibition by piericidin A is competitive with respect to UQ_{10}, indicating that ubiquinone reacts with the piericidin site in addition to the quinone-reducing site. This suggestion was used by Gutman (1980) to explain the inhibitory effect of UQ_3 on NADH oxidase previously reported by us (Lenaz *et al.*, 1975).

This study, set up to add further experimental evidence on this controversial issue in such a complex enzyme, has revealed that the inhibition of NADH oxidation by UQ_3 bears a more complex pattern than previously indicated both by Gutman (1980) and by ourselves (Lenaz *et al.*, 1977a). We have confirmed our previous indications of a competitive effect of UQ_3 with endogenous ubiquinone in overall NADH oxidase activity; UQ_3 also appears competitive with UQ_1 in the NADH-UQ_1 reductase assay, showing that the

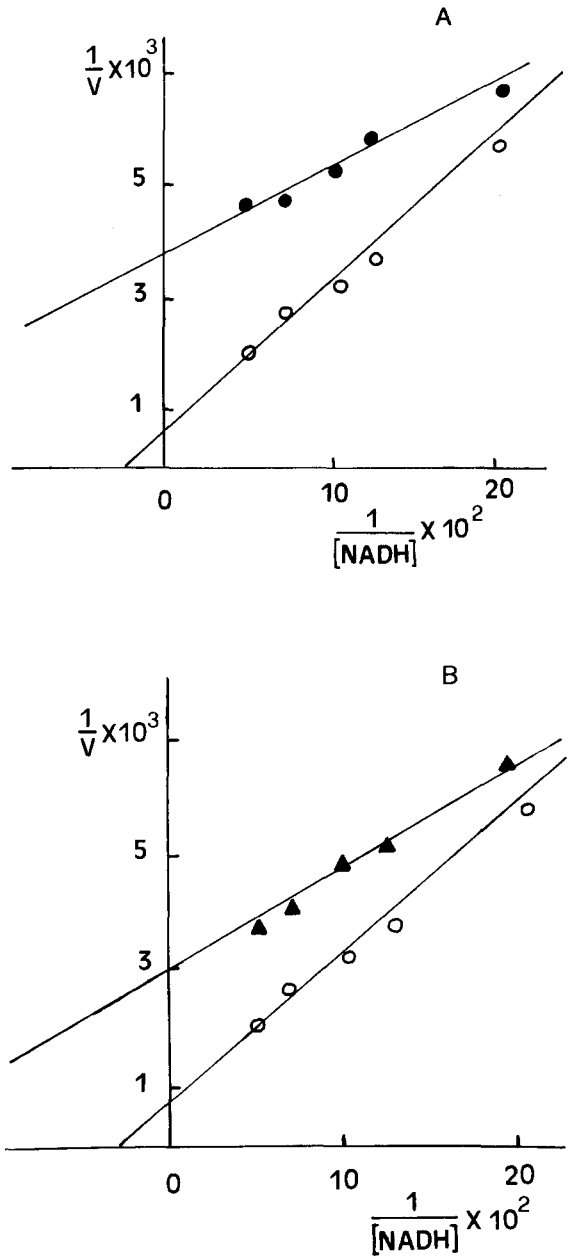

Fig. 7. (A) Double reciprocal plot of NADH oxidase activity in SMP (O) and in SMP preincubated with UQ_3 (15 μM) (●); (B) double reciprocal plot of NADH oxidase activity in SMP (O) and in SMP preincubated with *n*-butanol (55 mM) (▲).

reduction of UQ_3 by the quinone-reducing site in complex I is less effective than that of either endogenous ubiquinone or an exogenous relatively hydrophilic quinone like UQ_1. Such effect could be related to the peculiar chemical structure of UQ_3, the length of which in its extended form approximates that of a half bilayer, thus forcing it to a less flexible position in the membrane in comparison with other UQ homologs (cf. Lenaz *et al.*, 1981). Further experiments on the combined effects of rotenone and UQ_3 have revealed that the two inhibitors do not show additivity, suggesting that UQ_3 does not inhibit by interacting with the rotenone binding sites; on the other hand, significant interaction of UQ_3 with the rotenone binding sites was indicated by the relief of rotenone inhibition in particles preincubated with UQ_3. Since the effect was not additivity but relief of inhibition, and since the effect was shared by other UQ homologs (UQ_7) which do not inhibit respiration, we conclude that UQ_3, as well as UQ_7 and endogenous ubiquinone (Gutman *et al.*, 1971), can compete with rotenone (or piericidin) at the inhibitor site, but the occupation by UQ of the rotenone site does not lead to inhibition of electron transfer. We can also conclude that UQ_3 acts by displacing rotenone and not *via* bypassing of the rotenone site sustaining the rotenone-insensitive pathway, since this bypass is shown maximally with hydrophilic quinones (Ragan *et al.*, 1978) and this effect is shared by the very hydrophobic UQ_7.

The possibility exists that UQ occupation of the rotenone site is an essential prerequisite for coupled electron transfer through the complex; such prerequisite would not hold for organisms such as yeasts, which lack the rotenone sensitivity (Lenaz *et al.*, 1971). Tightly bound ubisemiquinone molecules have been described in complex I (King *et al.*, 1978), and the stability of one of these ubisemiquinone forms is decreased by rotenone (King *et al.*, 1978).

The atypical kinetic pattern of UQ_3 inhibition, which does not fit any classical inhibition type in a modified Dixon plot (Semenza and Balthazar, 1974), could be explained by an additional effect of this quinone on the structure of complex I. It is well known that complex I is very sensitive to lipophilic solvents. On the other hand, it has been previously shown that UQ_3 shares several features with organic solvents in its effect on mitochondrial ATPase (Degli Esposti *et al.*, 1981) and electron transfer (Bertoli *et al.*, 1978); such effects could be related to the interaction of the quinone with the phospholipid bilayer (Lenaz *et al.*, 1982).

We have shown in this work that UQ_3, as well as butanol, uncompetitively inhibits NADH oxidation; uncompetitive inhibition of several membrane-bound enzymes has been described in this laboratory as well as in others by lipid removal or by agents affecting lipid–protein interactions (Parenti-Castelli *et al.*, 1979; Vessey and Zakim, 1971; Yu *et al.*, 1973; Hegyvary,

1973) and suggested to result from an impaired formation of the transition state of the enzyme–substrate complex. In conclusion, the inhibition of NADH oxidation by UQ_3 is the result of at least two combined effects: (a) displacement of endogenous UQ_{10} by the less active (and perhaps less mobile) UQ_3, and (b) nonspecific effect of UQ_3 on the structure of complex I, perhaps mediated by a physical change of the lipids.

The inhibition pattern is complicated by the fact that UQ_3 is also an electron acceptor from the complex and that it can occupy the rotenone site as well as the endogenous ubiquinone.

Figure 8 attempts to explain in a schematic diagram the results of this investigation. Electron transfer from complex I to complex III may be optimal with long-chain ubiquinones or even with relatively hydrophilic UQ_1 which, once reduced by complex I, would be reoxidized by complex III through the aqueous phase. On the contrary, UQ_3 would be rather inefficient for the reasons explained above.

Further studies of the intramembrane reduction and different reoxidation of UQ homologs by rapid techniques are necessary to clarify completely this point.

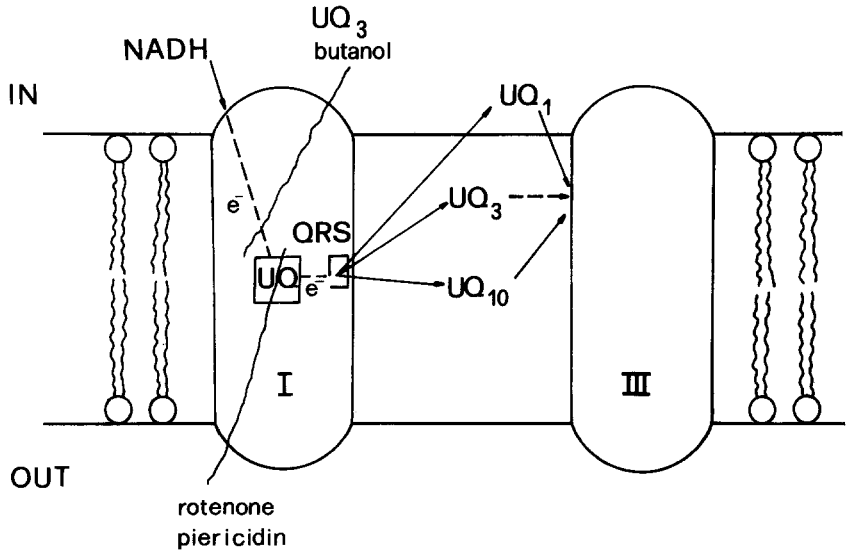

Fig. 8. Schematic diagram of electron transfer between complexes I and III mediated by different ubiquinones. Ubiquinone-3 is depicted to interact with the ubiquinone-reducing site (QRS) as well as the other homologs, while being more slowly reoxidized by complex III; rotenone and piericidin inhibit activity by competitively binding to an ubiquinone site different from QRS, which can be occupied by UQ-3 without inhibition; finally UQ-3 is shown to structurally affect the complex in a nonspecific way related to the effect of organic solvents.

Acknowledgments

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