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Effect of Ubiquinone Extraction on Ubiquinol-1 Oxidase Activity in Beef Heart Mitochondria

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Abstract

Extraction of endogenous ubiquinone with different methods does not influence ubiquinol oxidase activity in lyophilized mitochondria in terms of K_M, although a decrease of V_{max} is sometimes observed. Experiments with submitochondrial particles from a UQ-deficient mutant of *S. cerevisiae* confirm the results with UQ-depleted mitochondria and support the idea that endogenous ubiquinone is not required for the oxidation of exogenous ubiquinols by complex III.

Key Words: Ubiquinone; ubiquinols; ubiquinone-depleted mitochondria; ubiquinol oxidase; ubiquinone-deficient mutant.

Introduction

In mitochondria there is a large pool of functional ubiquinone between the primary dehydrogenases and the cytochrome portion of the electron-transport chain (Kröger and Klingenberg, 1970).

The location has been established by the use of inhibitors that prevent either quinone oxidation or reduction. Extraction of quinone from the membrane has also been shown to prevent the reduction by NADH or succinate of almost all the cytochromes (Crane, 1977).

In many investigations on the role of ubiquinone in the respiratory chain, exogenous homologs of ubiquinone have been used as electron acceptors (Hatefi and Rieske, 1967; Ziegler and Rieske, 1961; Ragan and Racker, 1973) and electron donors (Hare and Crane, 1971; Lang *et al.*, 1974; Papa, 1976; Leung and Hinkle, 1975); the interpretation of the above studies, however, is complicated by the presence of natural endogenous ubiquinone in

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the mitochondrial membrane. It appears that both short-chain and long prenyl-chain analogs can be oxidized, but it is not clear if it is through endogenous quinone or by direct interaction with the dehydrogenases (Brodie and Gutnick, 1972; Ernster *et al.*, 1969; Kröger and Klingenberg, 1973). Such evaluation appears of importance in order to interpret previous findings in our laboratory showing that the rate of oxidation of exogenous ubiquinols appears to depend on the sidedness of the mitochondial membrane (Lenaz *et al.*, 1978). Clearly, any interpretation on the sidedness of the ubiquinol oxidation site depends on the knowledge whether exogenous ubiquinols interact directly with the oxidation site.

In an attempt to elucidate this problem, we have removed, by extraction with different solvents, natural endogenous ubiquinone from beef heart mitochondria and investigated the rate of oxidation of reduced short chain quinones by oxygen. We have also investigated the ubiquinol oxidase activity in an ubiquinone-deficient mutant of *S. cerevisiae*.

Materials and Methods

Materials

UQ-1 (ubiquinone-1), UQ-3, and UQ-10 were generous gifts from Dr. Gloor and Dr. Leuenberg, Hoffmann-La Roche, Basel. All the biochemicals were obtained from Sigma Chemical Co. (U.S.A.); *n*-pentane was obtained from Merck Chemical Co. (Germany) and dehydrated with anhydrous sodium sulfate just before use.

Mitochondrial Preparations

Beef heart mitochondria prepared after the method of Smith (1967) were suspended in 0.15 M KCl and lyophilized for more than 12 h. The extraction of lyophilized mitochondria was carried out four times for 5 min with *n*-pentane according to the method of Szarkowska (1966). Finally *n*-pentane was removed from the extracted residue in a rotary evaporator under reduced pressure.

As the small amount of UQ remaining in pentane-extracted mitochondria could still interact with exogenous ubiquinones, in order to achieve an unqualified interpretation of our previous data (Lenaz *et al.*, 1978) and of any other result reporting the use of exogenous quinones as redox substrates, we have thought it of importance to attempt various methods to further deplete membranes of UQ. The methods employed were the following: (a) prolongation of the pentane extraction for 15 min; b) extraction of mitochondria four times with *n*-pentane and then once with *n*-pentane + 10% acetone (Norling *et al.*, 1974).

The ubiquinone-depleted mitochondria together with the pentane extracts were transferred into a rotary evaporator and the pentane gradually removed under a slightly reduced pressure in order to reincorporate the endogenous ubiquinone into the membranes. These are the "reconstituted" mitochondria.

Ubiquinone-Deficient Mutant

An ubiquinone-deficient mutant of *Saccharomyces cerevisiae* strain E 3-24 has been used for this experiment. (Tzagoloff *et al.*, 1975). Cells were inoculated from a slant into 50 ml of 2% galactose medium containing 0.5% yeast extract and the basic salts of Wickerham (1946). After overnight growth at 30°C on a rotary shaker, the entire culture was used as inoculum for 1 liter of fresh 2% galactose medium. The cells were harvested at the end of the logarithmic phase. The mitochondria were isolated using a French Pressure cell essentially according to the Kovac method (Kovac *et al.*, 1968). Submitochondrial particles were obtained by sonication (Beyer, 1967).

Ubiquinone determination showed that the mutant is completely devoid of ubiquinone.

UQ Concentration

The concentration of UQ in the various preparations of mitochondria was determined by methanol–light petroleum extraction (Kröger and Klingenberg, 1966), followed by a thin-layer chromatography on silica gel plates (Merck, Darmstad, TLC Plates Silica gel 60, with fluorescence indicator, precoated, layer thickness 0.5 mm), using benzene:chloroform (1:1) as developing solvent (Wagner *et al.*, 1962). An appropriate UQ-10 standard was used in order to locate the spots, which were scraped off the plate with the adsorbent, extracted with absolute ethanol, and assayed spectrophotometrically in a Cary 15 double beam spectrophotometer.

The absorbance of ubiquinone was tested at 275 nm before and after reduction by BH_4^-, and the amount was computed by using an extinction coefficient of 12.25 mM^{-1} cm^{-1}. The quinones used were checked for purity by thin-layer chromatography on the silica gel plates. In this case acetone/paraffin-saturated water (9:1) was used as developing solvent and the silica gel plates were impregnated with paraffin before use (Wagner *et al.*, 1962).

Enzymatic Assay

Ubiquinol oxidase activity was evaluated polarographically with an YSI 5331 oxygen electrode (Lenaz *et al.*, 1978). The reaction mixture contained: 200 mM sucrose, 300 mM KCl, 8.3 mM Hepes (pH 6.8), 5 mM malonate, 10 μM cytochrome *c*, rotenone (0.5 μg/mg protein), and mitochondria (0.750–

0.830 mg protein) in a total volume of 3 ml. The reaction was initiated by addition of the particles after equilibration of the solution containing the ubiquinols added in ethanolic solution in a concentration ranging from 15 to 110 μM.

All assays were accomplished at 30°C. In all assays ubiquinol oxidation was found to be antimycin-sensitive.

The reduced quinones were prepared from the corresponding oxidized forms after the method of Rieske (1967).

Mitochondrial protein was determined by the biuret method (Gornall *et al.*, 1949).

Results

UQ-Depleted Mitochondria

The rates of oxidation of ubiquinol-1 in "reconstituted" mitochondria and in UQ-depleted mitochondria are reported as double reciprocal plots in Fig. 1.

Pentane extraction of lyophilized mitochondria did not affect the oxidation rate; in fact both K_M and V_{max} of the oxidation activity did not show any

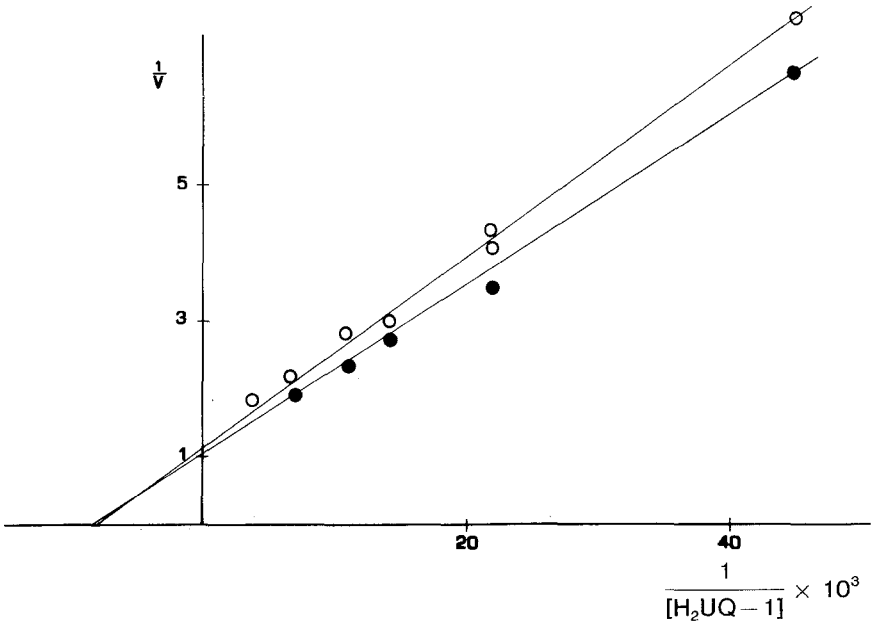

Fig. 1. Double reciprocal plot of ubiquinol-1 oxidase activity in UQ-depleted mitochondria (○) and in "reconstituted mitochondria" (●).

Table I. Content of Ubiquinone-10 in Mitochondria after Different Extraction Methods[a]

Mitochondria	UQ-10 content, nmol/mg protein
Control	4.8
"Reconstituted mitochondria"	4.0
Ubiquinone depleted[b]	0.37
Ubiquinone depleted[c]	0.33
Ubiquinone depleted[d]	0.1

[a]Mitochondria were extracted according to Kröger and Klingenberg (1966). The extracts were chromatographed on silica gel plates using the system benzene:chloroform (1:1) (Wagner *et al.*, 1962). The amount of UQ-10 in the spots was computed spectrophotometrically.
[b]Standard pentane extraction according to Szarkowska (1966).
[c]Protracted pentane extraction.
[d]Extraction with pentane plus an additional pentane–acetone extraction (see Materials and Methods).

significant change. Only in a minority of preparations did we find a decreased V_{max} in depleted mitochondria but without concomitant change of K_M.

After the standard pentane extraction the depleted mitochondria still showed residual endogenous UQ ranging from 10 to 7%. In order to test whether this small amount of UQ remaining in pentane-extracted mitochondria would be sufficient for maximal electron transport from exogenous electron donors (H_2UQ-1) to the complex III, we attempted to further deplete mitochondria of ubiquinone, using either longer extraction times with pentane or an extraction with pentane + 10% acetone.

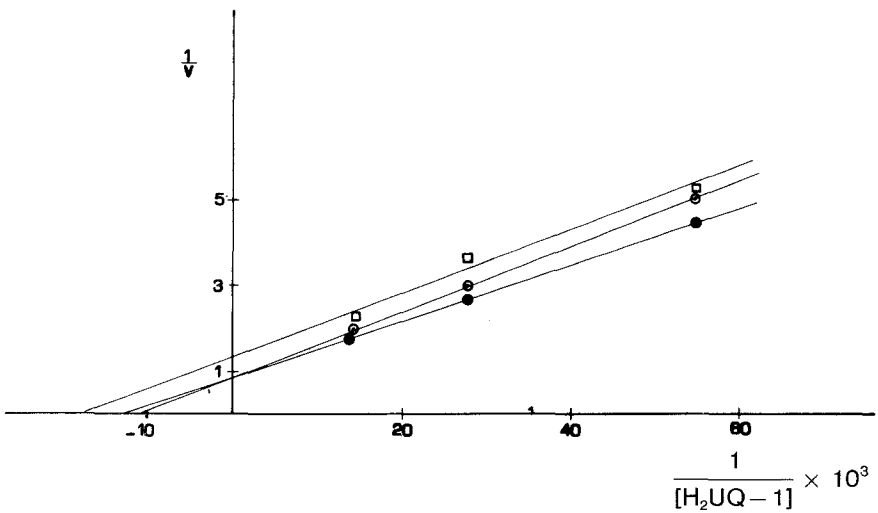

Fig. 2. Double reciprocal plot of ubiquinol-1 oxidase activity in mitochondria extracted by different procedures. ⊙, protracted extraction; □, extraction with pentane plus 10% acetone; ●, "reconstituted mitochondria." For explanations, see Materials and Methods.

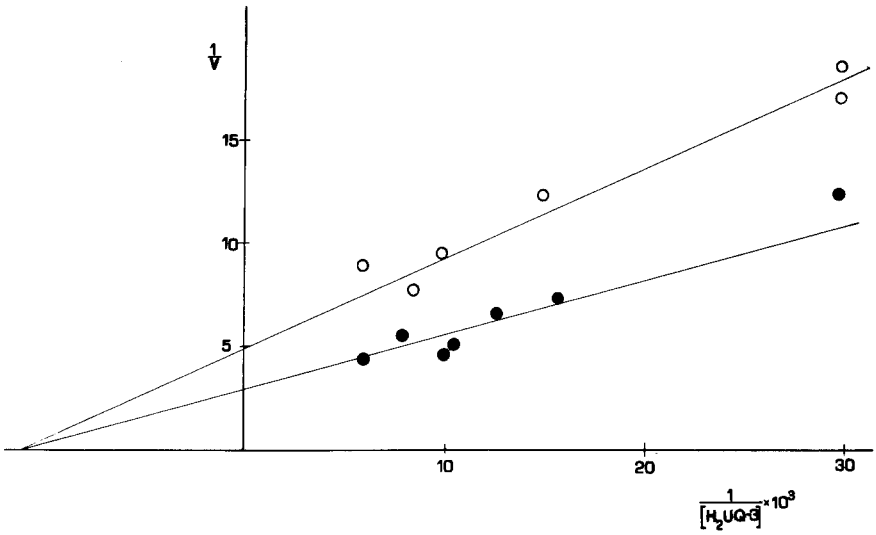

Fig. 3. Double reciprocal plot of ubiquinol-3 oxidase activity in UQ-depleted mitochondria (○) and in "reconstituted mitochondria" (●).

The residual amount of endogenous UQ in these different preparations is reported in Table I.

Figure 2 shows that ubiquinol-1 oxidase was not appreciably affected by any extraction method attempted.

Experiments with ubiquinol-3 as electron donor showed that UQ-depleted mitochondria have a slightly decreased V_{max}, although no change in K_M was apparent (Fig. 3). It should be noted that the rates of ubiquinol-3 oxidation were much lower in comparison with ubiquinol-1.

UQ-Deficient Mutant

Table II shows the rates of ubiquinol-1 oxidation by submitochondrial particles prepared by the UQ-deficient mutant of *S. cerevisiae*. The oxidation

Table II. Ubiquinol-1 Oxidase Activity in Submitochondrial Particles of UQ-Deficient Mutant

Experiment	Ubiquinol-1, μM	Treatment	Ubiquinol-1 oxidase activity, ng-atoms $O_2 \cdot min^{-1} \cdot mg^{-1}$
1	54	—	75
	90	—	122
2	90	—	128
	90	Preincubation with 17 μM UQ-3	140

of ubiquinol-1 proceeded at similar rate both in the absence and in the presence of UQ-3.

Discussion

Ubiquinol dehydrogenase activity has been studied by adding ubiquinols to intact mitochondrial membranes (Kröger and Klingenberg, 1970). The quinols with short prenyl chains, such as H_2UQ-1, H_2UQ-2, and H_2UQ-3, appear to reach easily the dehydrogenase site, while the interpretation with long prenyl-chain homologs, which give oxidation rates of the same order as lower quinols, is difficult owing to their highly hydrophobic nature leading to precipitation and aggregation during the assay. In this type of assay it has been found that the use of detergents may improve the activities (Ragan and Racker, 1973).

Since the hydrophobic nature of the long-chain quinones makes it difficult for them to penetrate into the membrane, greatly affecting the reproducibility of enzymatic assays, we have used only short-chain homologs in our experiments. Moreover the removal of endogenous quinone by solvent extraction allows a more direct approach to specificity of quinol oxidation and offers the possibility of clarifying whether exogenous quinols can be oxidized through endogenous ubiquinone or by direct interaction with the dehydrogenase. We have therefore investigated ubiquinol-1 oxidase activity in mitochondria depleted of UQ-10 after various solvent extractions, and in "reconstituted" mitochondria.

A comparison of the data of this enzymic activity in "reconstituted" mitochondria, used as controls, with pentane-extracted mitochondria showed no significant differences both in K_M and V_{max}.

In no preparation was endogenous ubiquinone required for interaction of the dehydrogenases with exogenous H_2UQ-1. It is therefore likely that H_2UQ-1 interacts directly with complex III without prior equilibration of the redox power with endogenous ubiquinone. Also extraction of lyophilized particles with pentane + 10% acetone, which results in a more effective removal of natural ubiquinone, did not change the rate of H_2UQ-1 oxidation.

Since no requirement for endogenous UQ is evident with oxidation of ubiquinol-1, other rate-limiting steps, such as transfer of exogenous ubiquinol from the aqueous phase to the lipid phase or transfer of electrons from cytochrome c_1 to oxygen, do not affect the validity of our conclusion, except in the absolute values of V_{max} and K_M found.

The results with UQ-depleted mitochondria were confirmed by the studies with submitochondrial particles from the UQ-deficient mutant of *S. cerevisiae*. Ubiquinol-1 oxidation is not affected by preincubation with UQ-3; this quinone homolog was previously (Lenaz *et al.*, 1971) found to best

support NADH and succinate oxidation in mitochondria of *S. cerevisiae* depleted of endogenous ubiquinone.

The results of this investigation convincingly show that endogenous ubiquinone is not required for oxidation of exogenous ubiquinol-1, although this conclusion does not rule out a possible involvement of endogenous UQ when it is present in the membrane. We feel, however, that the results support the idea that exogenous ubiquinol-1 interacts directly with the UQ oxidation site also when endogenous ubiquinone is present.

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