Antioxidative effect of ubiquinones on mitochondrial membranes

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Peroxidation of mitochondria occurs extensively in ubiquinone-depleted membranes. Reincorporation into the membranes of either the physiological ubiquinone or a short-chain homologue protects mitochondria against peroxidation. The ability to prevent this phenomenon is more evident in mitochondria that have incorporated ubiquinone-3 and might be ascribed to an ordering structural effect on the lipid bilayer.

Mitochondria, owing to their high content of polyunsaturated fatty acids, peroxidize readily *in vitro* under certain conditions (Tappel & Zalkin, 1959).

Extensive peroxidation of mitochondria by Fe^{2+} , ascorbate or glutathione produces 'mitochondrial ghosts', which retain the size and shape of mitochondria but have apparently lost their matrix material and have undergone clumping of cristae in the intramitochondrial space (Hunter *et al.*, 1964; McKnight *et al.*, 1965; McKnight & Hunter, 1966).

The importance of α -tocopherol in preventing a possible free radical attack on membrane phospholipids has been widely stressed (Tappel, 1962). Similar antioxidative properties were first ascribed to UQ by Mellors & Tappel (1966) who found that light-induced peroxidation of mitochondrial phospholipids was effectively prevented by UQ-6. More recently, Takayanagi *et al.* (1980) showed that not only NADPH, as was previously reported by Pfeifer & McCay (1972), but also NADH, can support lipid-peroxidation reactions in heart mitochondria and suggested that this peroxidation is controlled by a potent antioxidant, ubiquinol.

On the other hand, during a study of the effect of endogenous UQ on the interaction of UQ-1 with the respiratory chain of bovine heart mitochondria (Cabrini *et al.*, 1981) we observed that pentane-extracted mitochondria, which were UQ-depleted, appeared more labile than lyophilized controls. Furthermore, mitochondria extracted in the presence of an antioxidant such as α -tocopherol were improved in stability and ability to restore oxida-

tive activity on addition of exogenous UQ-10 (Cabrini *et al.*, 1981). We have further investigated this aspect and in the present paper we report studies on the antioxidative effect of UQs in mitochondrial membranes.

Lipid peroxidation reactions catalysed by bovine heart mitochondria with NADH as an electron donor and in the presence of ADP and Fe^{3+} were studied in mitochondria extracted and reconstituted either with UQ-10 or with UQ-3.

Comparing the effect of the natural UQ with that of the short-chain homologue we suggest that the antioxidant role of UQ could be ascribed both to a reaction with lipid free radicals (Mellors & Tappel, 1966) and to a structural effect on the lipid bilayer.

Materials and methods

Preparation of mitochondrial membranes

Beef heart mitochondria were prepared by a large-scale procedure (Smith, 1967). UQ-depleted mitochondria were obtained by pentane extraction of lyophilized mitochondria (Szarkowska, 1966). Since mitochondria depleted by this method still showed residual endogenous UQ ranging from 7 to 10% an additional extraction with pentane/acetone (9:1, v/v) was used (Pasquali *et al.*, 1981).

The UQ-depleted mitochondria were reconstituted either with UQ₁₀ or UQ₃ (Esai Co., Tokyo, Japan) as described by Norling *et al.* (1974) and the amounts of incorporated UQ were determined (Kröger, 1978).

Determination of enzyme activities

NADH oxidase and succinate oxidase activities were measured with a Clark oxygen electrode (YSI

Abbreviation used: UQ, ubiquinone.

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1531) at 30°C. The reaction mixture consisted of 0.25M-sucrose, 0.01M-Tris/HCl buffer, pH 7.4, 0.8–1 mg of membrane protein and either 0.3mM-NADH or 5mM-succinate.

The redox state of UQ was determined in the steady state of respiration. Mitochondrial suspension was sampled from the oxygen-electrode vessel and thoroughly mixed with methanol/light petroleum (b.p. 40–60°C) (3:2, v/v). The extraction and determination of UQ_{ox.} and UQ_{red.} in the extracts was performed by measuring the absorbance at 280–289nm in a Sigma ZWS II dual-wavelength spectrophotometer as described by Kröger (1978). The difference absorption coefficient used was 8.8mm⁻¹·cm⁻¹.

Assay of lipid peroxidation

The formation of malondialdehyde due to the peroxidation was measured by the thiobarbituric acid method (Beuge & Aust, 1979) with the following procedures.

(a) The reaction mixture, containing 0.3–0.5mg of membrane protein, 2mM-ADP and 0.2mM-FeCl₃ in 1 ml of 50mM-Mes (4-morpholine-ethanesulphonic acid) buffer, pH 6.75, was shaken in air at 37°C for 10 min. The reaction was started by the addition of 0.5mM-NADH and stopped by the addition of 1.5 ml of 20% (v/v) acetic acid adjusted to pH 3.5 with NaOH and 0.01 ml of 3.5mM-3,5-di-*t*-butyl-4-hydroxytoluene.

(b) In a reaction mixture containing the same amount of membrane proteins in 1 ml of 50mM-Mes buffer, peroxidation was catalysed by cold freshly prepared 0.2mM-ferrous ammonium sulphate (McKnight *et al.*, 1965). After shaking in air at 37°C for 20 min, the reaction was stopped as above.

Mixtures were heated for 15 min in a boiling-water bath and, after cooling, the flocculent precipitate was removed by centrifugation at 1000g for 10 min. The absorbance of the sample was determined at 532 nm.

1,1,3,3-Tetramethoxypropane from E. Merck, Darmstadt, Germany, was used as external standard.

Other analytical procedures

Protein was assayed by a biuret method (Gornall *et al.*, 1949). Phospholipid content was calculated by measuring phosphorus content (Marinetti, 1962). UQ₁₀ and UQ₃ were stored as ethanolic solutions at -20°C, at concentrations ranging between 10 and 30mM as determined spectrophotometrically from the decrease in absorption at 275 nm observed upon the reduction of the chromophore with NaBH₄ (5 mg/ml).

Other reagents used were of analytical grade.

Results and discussion

To elucidate the antioxidative role of the physiological UQ we have compared the effect of this quinone with that of the non-physiological UQ₃. The chain length of UQ₃ is similar to that of DL- α -tocopherol, which is the biological antioxidant of choice. The content of UQ₁₀ and UQ₃ reincorporated into depleted mitochondria is shown in Table 1. It can be seen that the two quinones were incorporated at the same extent into mitochondria, whereas incorporation of UQ homologues from an aqueous medium into the membranes depends on both the length of the isoprenoid chain and the type of membrane (Degli Esposti *et al.*, 1981). The respiratory activities of the various mitochondrial preparations are shown in Table 2. Exogenous UQ₁₀ completely restore NADH oxidase and succinate oxidase activities in extracted mitochondria, whereas UQ₃-recon-

Table 1. Ubiquinone content of mitochondria after various treatments

Mitochondrial preparations were obtained as described in the Materials and methods section. For full experimental details see the text.

Mitochondria	UQ content	
	(nmol/mg of protein)	(% of control)
Lyophilized	5.50	100
UQ-depleted*	0.49	9
UQ-depleted†	0.16	3
UQ ₁₀ -reconstituted	6.29	114
UQ ₃ -reconstituted	6.04	110

* Standard pentane extraction according to Szarkowska (1966).

† Pentane extraction plus an additional pentane/acetone extraction.

Table 2. NADH and succinate oxidase activities of mitochondria after various treatments

Enzymic activities were measured polarographically. UQ₁₀ was added as an ethanolic solution to the assay medium at a final concentration of 35 μ M. Results reported are the means of three or four experiments with different mitochondrial preparations.

Mitochondria	UQ ₁₀ addition	Oxidase activity (nmol of O ₂ /min per mg of protein)	
		NADH	Succinate
Lyophilized	—	104	86
UQ-depleted	—	20	19
	+	138	98
UQ ₁₀ -reconstituted	—	158	90
	+	163	95
UQ ₃ -reconstituted	—	47	90
	+	71	88

stituted mitochondria show a decreased NADH oxidation even when supplemented by exogenous UQ₁₀. In fact, studies of the respiratory activity of mitochondria depleted of endogenous UQ showed that succinate oxidation can be completely restored by exogenous UQ with isoprenoid chain lengths ranging from 2 to 10 (Lenaz *et al.*, 1975) but NADH oxidase activity can only be fully recovered by adding long-chain homologues (Lenaz *et al.*, 1978). Moreover, it has been shown that UQ₃ competitively inhibits NADH oxidation and affects non-specifically the structure of complex I (Landi *et al.*, 1984).

When bovine heart mitochondria are incubated in the presence of ADP and FeCl₃ and with NADH as an electron donor, lipid peroxidation is observed. Takayanagi *et al.* (1980) showed that electrons are supplied to the lipid-peroxidation reactions from a component between the substrate site and the rotenone-sensitive site of complex I and that the reduction of ADP-Fe³⁺ chelate is an essential step in lipid peroxidation (Takeshige *et al.*, 1980).

Formation of malondialdehyde by the various mitochondrial preparations supported by NADH in the presence of ADP-Fe³⁺ chelate is shown in Table 3. This end product of lipid peroxidation is very high in UQ-depleted membranes, particularly when the residual UQ content was 3% of the control; on the contrary, reincorporation of UQ decreases malondialdehyde formation in the same membranes. This is more evident when the short-chain quinone is used.

It is interesting to note that pentane extraction removes not only UQ but also α -tocopherol (Norling *et al.*, 1974) and that the reincorporation

of the short-chain quinone is able to replace this outstanding biological antioxidant.

It has been claimed that only reduced UQ acts as a potent antioxidant in mitochondrial membranes (Takayanagi *et al.*, 1980). However, when the redox state of ubiquinones was determined in the steady state of respiration with NADH as substrate (Table 4) the proportion of reduced UQ₃ was far lower than that of reduced UQ₁₀, but nevertheless the antioxidative effect was higher in UQ₃-reconstituted membranes. The same effect is seen when Fe²⁺ was used for initiation of lipid peroxidation (Table 5): in UQ₃-reconstituted mitochondria less formation of malondialdehyde occurs. Moreover, when autoxidation of egg lecithin vesicles in which either UQ₁₀ or UQ₃ were incorporated was tested, the antioxidative effect of UQ was confirmed, particularly with respect to UQ₃ (L. Landi, L. Cabrini, A. M. Sechi & P. Pasquali, unpublished results).

Table 4. Redox state of ubiquinones in mitochondria in the presence of NADH

In the steady state of respiration, 0.5 ml of the suspension was sampled from the oxygen-electrode vessel and mixed with 2.5 ml of methanol/light petroleum (b.p. 40–60°C) (3:2, v/v). In these extracts active and reduced UQ were calculated according to Kröger & Klingenberg (1973).

Preparation of mitochondria	Reduced UQ (%)	Active UQ*
Lyophilized	67	83
UQ ₁₀ -reconstituted	40	51
UQ ₃ -reconstituted	8	46

* Active UQ was that part of the total UQ reducible under equilibrium conditions in the presence of 1 mM-KCN and NADH.

Table 3. Effect of ubiquinones with different isoprenoid chain length on NADH-dependent malondialdehyde formation by heart mitochondria

For full experimental details see the text. The amount of malondialdehyde formation supported by NADH was obtained by subtracting the amounts obtained without NADH.

Preparation of mitochondria	Malondialdehyde formation	
	(nmol/mg of protein)	(nmol/mg of phospholipid)
Lyophilized	1.23	5.40
UQ-depleted*	13.04	63.44
UQ-depleted†	19.18	93.30
UQ ₁₀ -reconstituted	3.19	16.09
UQ ₃ -reconstituted	1.27	6.19

* Standard pentane extraction according to Szarkowska (1966).

† Pentane extraction plus an additional pentane/acetone extraction.

Table 5. Effect of ubiquinones with different isoprenoid chain length on Fe²⁺-dependent malondialdehyde formation by heart mitochondria

Mitochondrial preparations were obtained as described in the Materials and methods section. The reaction mixtures were incubated at 37°C for 20 min.

Mitochondria	Malondialdehyde formation	
	(nmol/mg of protein)	(nmol/mg of phospholipid)
Lyophilized	0.43	2.13
UQ-depleted*	6.02	29.30
UQ ₁₀ -reconstituted	2.48	12.09
UQ ₃ -reconstituted	1.72	8.38

* Pentane extraction plus an additional pentane/acetone extraction.

From our experiments it appears that not only the reduced form of the quinones, but also the oxidized form, can behave as a powerful antioxidant.

The more efficient protection against peroxidation exerted by the short-chain quinone with respect to the physiological quinone could be ascribed to their different disposition in the membrane.

It has been shown that the polyisoprenoid chain length influences the interaction of UQ with the phospholipid bilayer (Katsikas & Quinn, 1982). The physical state of UQ₁₀ might be an equilibrium of monomers and clusters, as suggested by the relatively low partition coefficient and by the disorganization produced in the membrane (Lenaz *et al.*, 1984).

Studies with spin labels and fluorescent probes indicate that UQ₃, whose side chain length approximates to that of half the lipid bilayer, decreases membrane fluidity (Lenaz *et al.*, 1981). Moreover, calorimetric data (Katsikas & Quinn, 1982) clearly show that UQ₃ has a tendency to intercalate between the phospholipid molecules.

On the basis of these data we suggest that the ability of UQ to act as antioxidants might be ascribed not only to a reaction with lipid free radicals, as postulated by Mellors & Tappel (1966), but also to a structural effect on the lipid bilayer.

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