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UBIQUINONE AND RELATED COMPOUNDS XX. COENZYMATIC ACTIVITY OF UBIQUINONE AND RELATED COMPOUNDS (II)

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SUMMARY

An enzyme preparation which requires the addition of Q alone for the restoration of the succinate oxidase system and is stable during a considerable period was obtained by the acetone-extraction procedure from rat-liver mitochondria. Structure–activity relationships of Q were investigated using this preparation and the *trans*-2′,3′-double bond in the isoprenoid side chain was found to be essential for maximum restoration of succinate oxidase activity.

INTRODUCTION

The mitochondrial electron transfer chain catalyzes the oxidation of succinate and NADH by molecular oxygen. These activities are lost on solvent extraction and they can be restored when the extracted mobile components such as Q and cytochrome *c* are added to the depleted particles (see ref. 1).

For the solvent extraction, acetone extraction[2] was known to be the most effective procedure until pentane extraction[3,4] was reported to give a preparation in which the succinate and NADH oxidase systems are restored by the addition of Q. By using these preparations the structure and activity relationships of Q have been widely investigated (see ref. 5).

In this paper a modified method for the preparation of a Q-depleted enzyme and studies on the structure-activity relationships using this preparation are described.

MATERIALS AND METHODS

Materials

Cytochrome *c* and NADH were purchased from Sigma, and antimycin A from Kyowa Hakko Kogyo. Q homologs and related compounds were isolated (see refs 6, 7) or synthesized[8–10], respectively, in the authors' laboratories. Rotenone was kindly

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

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supplied by Professor H. Fukami, Kyoto University. Mitochondria were prepared from the liver of Sprague–Dawley rats (body weight 150–200 g, 2 months old) as described by Hogeboom[11] as a modification of Schneider's method.

Assay and analytical methods

The succinate oxidase system (succinate:O_2 oxidoreductase)[2], succinate–cytochrome *c* reductase[12] (succinate:cytochrome *c* oxidoreductase), the NADH oxidase system (NADH:O_2 oxidoreductase)[13], NADH–$K_3Fe(CN)_6$ reductase[14] (NADH:$K_3Fe(CN)_6$ oxidoreductase, EC 1.6.99.3), NADH–2,6-dichlorophenolindophenol (DCIP) reductase[14] (NADH:DCIP oxidoreductase, EC 1.6.99.3) and NADH–cytochrome *c* reductase[14] (NADH:cytochrome *c* oxidoreductase, EC 1.6.99.3) were assayed by the procedure already described, respectively, at 25 °C. Cytochrome *c* was removed from the assay mixtures of the succinate and NADH oxidase systems unless stated otherwise. Q and related compounds were added in the form of an aqueous solution containing five times their weight of a detergent, Nikkol OP-10 (Nikko Kogyo) unless noted otherwise. Rates of oxygen consumption were recorded polarographically[15]. The difference of the succinate oxidase activities before and after the addition of a compound was expressed as relative succinate oxidase activity since a small amount of oxygen consumption was observed in a few of the acetone-extracted preparations. Protein was determined by Folin–Ciocalteu reagent[16].

Preparation of Q-depleted mitochondrial preparation

The suspension (50 mg protein per ml) (4 ml) of mitochondria in 0.25 M sucrose was added to cold acetone (−30 °C, 100 ml) with stirring. After being stirred for 10 min, the mixture was kept standing for 5 min, then the opalescent supernatant (90 ml) was siphoned out. To the residue was added cold acetone (100 ml) and the mixture was treated in a similar manner. The precipitates were treated with acetone similarly once more and the mixture was centrifuged at 6000 × *g* for 10 min. The precipitates were dried under reduced pressure at 0 °C overnight to obtain the gray powder (100 mg) of Q-depleted mitochondrial preparation. The preparation was stored at −30 °C and suspended in 0.25 M sucrose (2 ml) just before use.

Assay of Q in mitochondrial preparation

The mitochondrial preparation (100 mg) was heated in ethanol–acetone (1:1, v/v) (20 ml) at 70 °C with stirring for 30 min, and then the mixture was filtered by suction. The extraction was repeated three times in the same manner. The combined filtrates were evaporated to dryness under reduced pressure. A solution of the residue in chloroform was applied on a silicagel layer (20 cm × 20 cm, 10 g) and then developed with benzene. The part corresponding to Q-9 was taken off and extracted with three 20-ml portions of ether. The extracts were similarly purified by using hexane–ether (3:2, v/v) as the developing solvent. The purified extracts were divided into two. The first part was dissolved in an aliquot volume of ethanol for analysis by the ultraviolet method[17] and the second part was dissolved in hexane for analysis by means of gas chromatography using an electron capture detector[33].

RESULTS AND DISCUSSION

Enzyme preparation and its oxidase activities

The authors have obtained an enzyme preparation by a slightly modified version of the acetone extraction of Lester and Fleischer[2]. The washing with 0.88 M sucrose was avoided in the authors' procedure because it often led to an inability to restore activity. The acetone remaining in the preparation was almost completely removed by suction. Q was found to be rather completely removed with this procedure (Table I). The succinate and NADH oxidase activities of the preparation were mostly lost (Table II). The former was fully restored by the addition of a Q homolog such as Q-9, Q-7 and Q-2, but not restored by phylloquinone and tocopherylquinone showing a specific requirement of the Q structure. Cytochrome *c* did not affect the activity (Fig. 1), unlike the acetone-extracted preparation already described[2,18]. The preparation was rather stable and 78% of the activity remained after storage at −30 °C for a month (Table III). The addition of neutral lipid[19–22], a phospholipid[2,3,23,24] or a detergent[22] with Q is necessary for restoring the activity of a Q-depleted enzyme preparation, and this is considered to be effective for the

TABLE I

Q-9 CONTENT IN MITOCHONDRIAL PREPARATION

The analytical procedures were referred in Methods.

Treatment of mitochondria	*Content of Q-9 (nmoles/mg protein)*	
	Ultraviolet method	*Gas chromatography method*
Lyophilized	1.23	0.83
Acetone-treated	Not detected	0.01

TABLE II

ELECTRON-TRANSPORT ACTIVITIES OF RAT-LIVER MITOCHONDRIAL PREPARATION

The assay procedures were referred in Methods. All activities are terms of nmoles of substrate oxidized per min per mg protein.

Donor–acceptor pair	*Additions to basal assay mixture*		
	None	*Q-7 ($1.5 \cdot 10^{-5}$ M)*	*Cytochrome c (100 µg)*
(1) Lyophilized rat-liver mitochondria			
Succinate–O_2	50		
NADH–O_2	160		
(2) Acetone-treated rat-liver mitochondria			
Succinate–O_2	0	50	0
Succinate–cytochrome *c*	0	51	
NADH–O_2	20	20	170
NADH–$K_3Fe(CN)_6$	540		
NADH–DCIP	92		
NADH–cytochrome *c*	158		

positioning of Q in the electron-transfer chain[22]. An appropriate medium was required as well for the restoration in the authors' preparation. Q-7 showed a full restoration in an aqueous solution containing a non-ionic detergent of the ether type

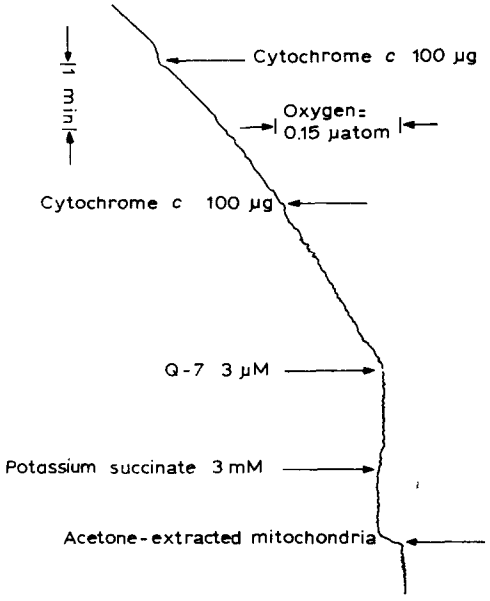


Fig. 1. Restoration of succinate oxidation of acetone-extracted rat-liver mitochondria. Rates of oxygen consumption were recorded directly by means of a vibrating platinum electrode. The final concentration of the reactants in the flasks were as follows: 10 mM Tris–HCl buffer (pH 7.4); KCl, 20 mM; extracted mitochondria, 1.6 mg/ml; potassium succinate, 3 mM; sucrose, 200 mM. Preparation of Q-7 solution as in Methods.

TABLE III

RESTORATION OF SUCCINATE OXIDASE ACTIVITY OF ACETONE-EXTRACTED RAT-LIVER MITOCHONDRIA AFTER PRESERVATION AT −30 °C

Assay details as in legend for Fig. 1.

Days preserved	*Succinate oxidase activity restored with Q-7 ($1.3 \cdot 10^{-5}$ M)*	
	natoms oxygen per min per mg protein	%
0	35.7	100
1	39.7	111
3	30.6	86
11	29.3	82
20	28.0	78
30	28.0	78

such as Nikkol OP-10. But it showed only slight activity in an aqueous solution containing a non-ionic detergent of the ester type such as Nikkol HCO-50, or in an aqueous suspension containing a phospholipid (Table IV), and did not show any activity in an ethanol solution. The succinate oxidase activity thus restored with

TABLE IV

EFFECTS OF DETERGENTS ON RESTORATION OF SUCCINATE OXIDASE ACTIVITY WITH Q-7

Assay details as in legend for Fig. 1 except the detergent used.

Detergent (trade name)	*Succinate oxidase activity restored with Q-7 (natoms oxygen per min per mg protein)*	
	Q-7 ($1.7 \cdot 10^{-6}$ M)	*Q-7 ($3.3 \cdot 10^{-6}$ M)*
(1) Phospholipid		
Yolk lecithin	−0.9	−2.5
Soybean lecithin	1.3	3.8
(2) Glyceride		
Octanoate (Neo B-M5) *	0	0
(3) Non-ionic detergent		
Polyoxyethylene fatty acid ester		
Hydrogenated ricinoleate (HCO-50) **	0.9	1.3
Hydrogenated ricinoleate (HCO-120) **	4.5	4.5
Stearate (Mys-40) **	3.2	3.2
Stearate (Mys-45) **	3.2	3.2
Polyoxyethylene sorbitan fatty acid ester		
Monolaurate (Tween-50) ***	2.8	3.5
Monolaurate (Tween-20) ***	6.4	9.6
Stearate (Myrj-45) ***	2.2	12.9
Stearate (Myrj-52S) ***	4.1	6.3
Polyoxyethylene fatty alcohol ether		
Lauryl (BL-25) **	19.1	17.5
Octylphenol (OP-10) **	14.8	17.0
Cetyl (Brij-58) ***	13.5	13.8
Oleyl (DOP) **	15.9	14.3

* Drew.
** Nikko.
*** Atlas.

a Q homolog was inhibited by antimycin A and KCN similarly to that found in intact mitochondria, and its electron-transfer pathway was considered to be identical with an intact chain.

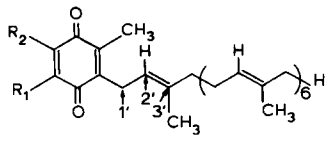
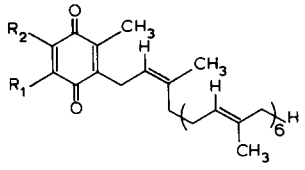
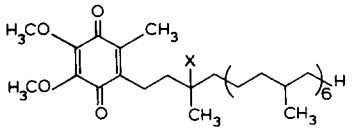
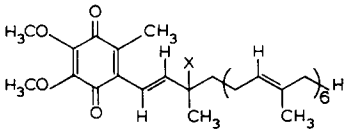
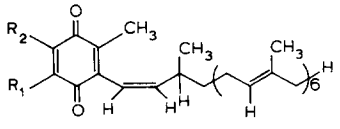
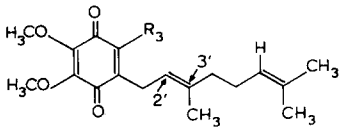
The NADH oxidase activity in our preparation could not be restored with Q, but a similar NADH oxidase activity to that of lyophilized mitochondria was observed rotenone-insensitively in the presence of cytochrome *c*, unlike the preparation of Lester and Fleischer[2]. This may be due to the damage at the NADH dehydrogenase–Q juncture in addition to the loss of Q, since the NADH dehydrogenase activity remained (Table II) and the washing prior to drying is known to affect the NADH oxidase activity[25].

Restoration of succinate oxidase system with Q and related compounds

The structure and activity relationships of Q have been made clear by many authors using various Q-depleted enzyme preparations (see ref. 5). The relationships

TABLE V

STRUCTURES OF UBIQUINONE AND RELATED COMPOUNDS

trans-Q-7 $R_1 = R_2 = H_3CO$ *trans*-Monoethoxy-Q-7 $R_1 = H_3CO$ $R_2 = H_5C_2O$ (equimolar mixture) $R_1 = H_5C_2O$ $R_2 = H_3CO$	
cis-Q-7 $R_1 = R_2 = H_3CO$ *cis*-Monoethoxy-Q-7 $R_1 = H_3CO$ $R_2 = H_5C_2O$ (equimolar mixture) $R_1 = H_5C_2O$ $R_2 = H_3CO$ *cis*-Diethoxy-Q-7 $R_1 = R_2 = H_5C_2O$	
Perhydro-Q-7 X = H 3′-Ethoxyperhydro-Q-7 $X = OC_2H_5$ 3′-Methoxyperhydro-Q-7 $X = OCH_3$	
trans-3′-Hydroxyiso-Q-7 X = OH *trans*-3′-Ethoxyiso-Q-7 $X = OC_2H_5$ *trans*-3′-Methoxyiso-Q-7 $X = OCH_3$	
cis-Iso-Q-7 $R_1 = R_2 = H_3CO$ *cis*-Monoethoxyiso-Q-7 $R_1 = H_3CO$ $R_2 = H_5C_2O$ (equimolar mixture) $R_1 = H_5C_2O$ $R_2 = H_3CO$ *cis*-Diethoxyiso-Q-7 $R_1 = R_2 = H_5C_2O$	
trans-Q-2 $R_3 = CH_3$ 2′,3′ = *trans* *cis*-Q-2 $R_3 = CH_3$ 2′,3′ = *cis* *trans*-5-Ethyl Q-2 $R_3 = C_2H_5$ 2′,3′ = *trans*	

between the activity and the configuration of the substituents at the 2′,3′-double bond of the isoprenoid side chain are, however, in confusion[26–28]. Our preparation was considered to be suitable for the assay of Q-like activity on Q related compounds. The effect of Q-related compounds having modified double bonds (Table V) on the activity was investigated by using this preparation. Since the length of the isoprenoid side chain did not affect the restoration activity as described above, Q-7 related compounds were compared with *trans*-Q-7 in the region in which the activity was proportional to the compound concentration and the following results were obtained. (a) the restoration with *cis*-Q-7 having *cis*-2′,3′-double bond was decreased by about a half (Fig. 2a); (b) *cis*-iso-Q-7 in which the 2′,3′-double bond was transferred to 1′,2′ showed further lower activity (Figs 2b, 2c); (c) perhydro-Q-7 in which all double

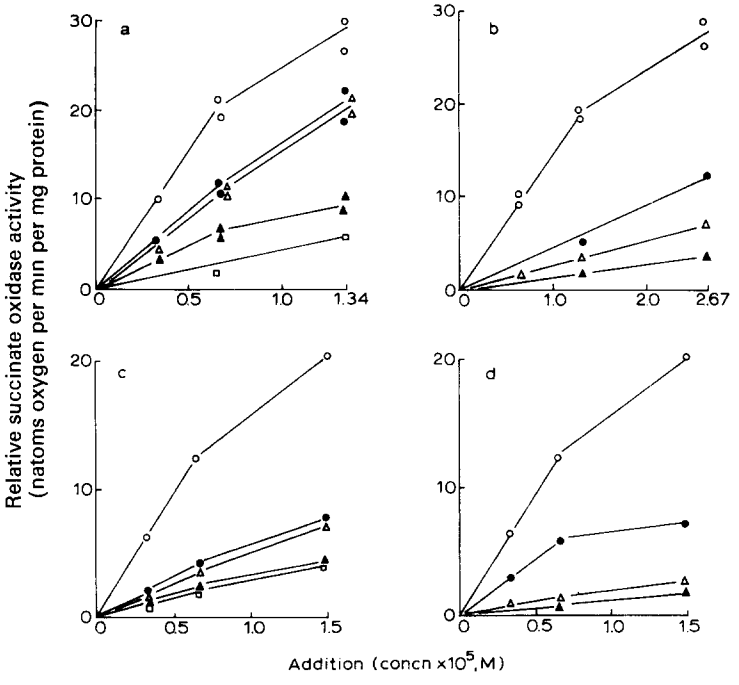


Fig. 2. Restoration of succinate oxidase with *trans*-Q-7 and related compounds. Basal components and procedure for the assay as in legend for Fig. 1. Relative succinate oxidase activity as in Methods. Compounds: (a) ○, *trans*-Q-7; ●, *trans*-monoethoxy-Q-7; △, *cis*-Q-7; ▲, *cis*-monoethoxy-Q-7; □, *cis*-diethoxy-Q-7. (b) ○, *trans*-Q-7; ●, *cis*-iso-Q-7; △, *cis*-monoethoxyiso-Q-7; ▲, *cis*-diethoxyiso-Q-7. (c) ○, *trans*-Q-7; ●, *cis*-iso-Q-7; △, *trans*-3′-methoxyiso-Q-7; ▲, *trans*-3′-ethoxyiso-Q-7; □, *trans*-3′-hydroxyiso-Q-7. (d) ○, *trans*-Q-7; ●, perhydro-Q-7; △, 3′-ethoxyperhydro-Q-7; ▲, 3′-methoxyperhydro-Q-7. Preparation of the compounds solution as in Methods.

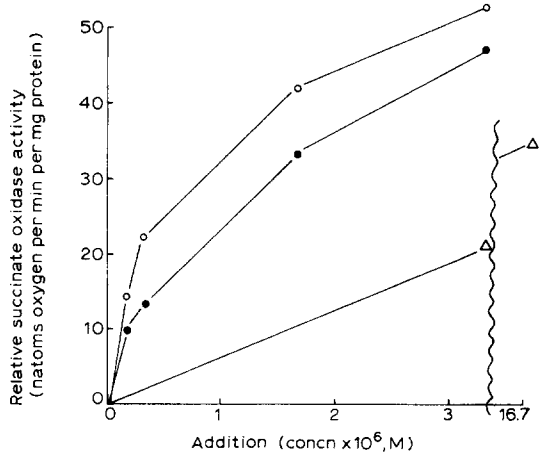


Fig. 3. Restoration of succinate oxidase with *trans*-Q-2 and related compounds. Basal components and procedure for the assay as in legend for Fig. 1. Relative succinate oxidase activity as in Methods. Compounds: ○, *trans*-Q-2; ●, *cis*-Q-2; △, *trans*-5-ethyl-Q-2. Preparation of the compounds solution as in Methods.

bonds were saturated also resulted in the decrease of the activity (see ref. 28) (Fig. 2d); (d) 3'-substituted compounds showed lower activities than those of the corresponding unsubstituted compounds (Figs 2c, 2d). As expected, the decreased activity of the *cis* configuration was observed also in the case of Q-2 (Fig. 3). From these results the 2',3'-double bond with *trans* configuration was found to give maximum activity. This is interesting since a similar physiological significance of the 2',3'-double bond configuration has been reported on phylloquinone[29-31]. As already described[2,5,32], the modification of methoxyl and ring methyl resulted in a decrease also in the present study (Figs 2a, 2b, 3).

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