ENERGY-LINKED REACTIONS IN MITOCHONDRIA: A REQUIREMENT FOR UBIQUINONE AFTER PENTANE EXTRACTION

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1. Introduction

A role for ubiquinone in oxidative phosphorylation and related reactions has been suggested by many workers and many hypotheses have been advanced to explain the role of quinones in energy conservation reactions. There is little or no evidence which directly supports the hypotheses that have been proposed and the role of ubiquinone remains obscure [1, 2].

We have investigated the effect of pentane extraction on energy-linked reactions in submitochondrial particles prepared from lyophilised beef heart mitochondria. The reactions studied were the energy-linked transhydrogenase reaction (ATP-driven) and the energy-linked reduction of NAD^+ by succinate (ATP-driven). Pentane extraction by the method of Szarkowska [3] as modified by Horio et al. [4], caused complete loss of energy-linked activities in derived submitochondrial particles.

The energy-linked transhydrogenase reaction in submitochondrial particles could be restored by addition of the pentane extract or ubiquinone alone to the lyophilised extracted mitochondria. This was a specific effect of ubiquinone and it could not be achieved by addition of vitamin K_1 or by addition of phospholipids. The energy-linked reduction of NAD^+ by succinate could not be restored by ubiquinone alone but could be restored by the pentane extract which contains ubiquinone and other unidentified lipid components.

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2. Methods

The methods of preparation of mitochondria, and submitochondrial particles and the assay of protein, energy-linked transhydrogenase and the energy-linked reduction of NAD^+ by succinate have been fully described in previous communications [5, 6]. Oxidase activities with NADH and succinate as substrates were measured polarographically with an oxygen electrode.

Pentane extraction was carried out by the method of Szarkowska [3] as modified by Horio et al. [4]. Heavy beef heart mitochondria (20–30 mg/ml) suspended in 0.15 M KCl were lyophilised for 3–4 hr. The lyophilised mitochondria (250–300 mg protein) were shaken at 2–3° for 30 min 50 ml pentane followed by centrifugation at 35,000 *g*. The pellet was homogenised in 20 ml pentane and re-extracted for a further 5–10 min, followed by centrifugation at 35,000 *g*. The pellet was homogenised in pentane (5–10 ml) and the pentane removed by rotary evaporation at room temperature. The dried mitochondria were then divided into three batches:

(1) Suspended in 0.25 M sucrose – 0.01 M tris-HCl buffer (pH 7.6) and called Lyophilised-Extracted Particles.

(2) Suspended in the pentane extract (5 ml) and called Lyophilised-Extracted Particles + Pentane Extract.

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The pentane extract had been previously concentrated by evaporation and an amount of concentrated extract equivalent to a 2–3 fold excess of ubiquinone was added.

(3) Suspended in 5 ml pentane containing the appropriate amount of ubiquinone-(30) and called Lyophilised-Extracted Particles + Ubiquinone.

Batches (2) and (3) were allowed to stand for 5 min and then the pentane was removed by rotary evaporation at room temperature. The dried particles were resuspended in 0.25 M sucrose – 0.01 M tris-HCl buffer (pH 7.6).

Submitochondrial particles were prepared from all three batches by sonic oscillation [5, 6]. A control preparation of submitochondrial particles was prepared from non-lyophilised mitochondria and from lyophilised mitochondria which had not been subjected to the pentane extraction treatment. Lyophilised preparations normally yielded particles which had only 25–30% of the energy-linked activities of normal submitochondrial particle preparations. The method described above gave reproducible results showing loss of energy-linked activities on pentane extraction and restoration of energy-linked activities on reincorporation of ubiquinone or the lipid components in the pentane extract.

3. Results and discussion

The method of choice to demonstrate restoration of energy-linked activities by ubiquinone would be to extract submitochondrial particles directly after lyophilisation and to restore energy-linked activities by addition of ubiquinone directly to the assay system. This was the method used in initial studies with varying degrees of success. No restoration of energy-linked reduction of NAD^+ by succinate by addition of ubiquinone was observed and this activity was usually lost completely during the lyophilisation step. The energy-linked transhydrogenase reaction was decreased to 25–30% of the control level by the lyophilisation procedure and to about 3% by subsequent extraction with pentane. Restoration of the energy-linked transhydrogenase activity was achieved by addition of ubiquinone directly to the assay medium (table 1). These results were highly variable but on several occasions restoration of activity equivalent to 50–90% of the activity exhibited by lyophilised submitochondrial particles was observed. When active lyophilised particles were prepared then partial restoration of the energy-linked activity by ubiquinone was observed in all experiments.

Table 1
Restoration of ATP-dependent reduction of $NADP^+$ by NADH by addition of ubiquinone in beef heart submitochondrial particles.

Particles	NADPH formed (nmoles/min/mg protein)	
Normal	69	(1.39)
Lyophilised	23	(0.90)
Lyophilised-extracted	2.4	(1.00)
Lyophilised-extracted Plus ubiquinone	16.1	(1.00)

Pentane extracted submitochondrial particles were prepared as described in the Methods section. The optical density increase at 340 nm was measured in a Beckman DK 2 recording spectrophotometer fitted with a time drive attachment. *Assay system*: 2.7 ml reaction medium containing 675 μmole sucrose, 16 μmole magnesium chloride, 3 μmole potassium cyanide, 135 μmole tris-HCl buffer (pH 8.0) and 0.5–2.0 mg beef heart submitochondrial particle protein were placed in a cuvette followed by 10 μl (290 μg) yeast alcohol dehydrogenase (ADH) and 10 μl ethanol. Subsequent additions were made in the following order; 40 nmole NAD^+, 0.6 μmole $NADP^+$, 6 μmole ATP and 10 μl of an ethanolic solution of ubiquinone (130 nmole). Temperature 30°. The reference cuvette contained all the components of the reaction except the NADH regenerating system (ADH, ethanol and NAD^+). The reaction was initiated by the addition of ATP to the experimental cuvette to give a final volume of 3 ml. Ubiquinone caused a large absorbance increase which could be compensated for by the addition of an equivalent amount of ubiquinone to the reference cuvette, The figures in brackets refer to the amounts of particle protein (mg) present in the assay medium.

The effect on the transhydrogenase was not due to a non-specific absorbance increase produced by ubiquinone, because when ubiquinone was added before ATP, no restoration was obtained until ATP was added (fig. 1, b). Furthermore in both cases addition of the NADPH specific enzyme glutathione reductase plus glutathione at the end of the experiment caused a decrease in absorbance to the original level.

The restored reaction was sensitive to 2,4-DNP and oligomycin at the same concentrations as the normal reaction, demonstrating that an energy-linked system had been restored on addition of ubiquinone (fig. 1c,d).

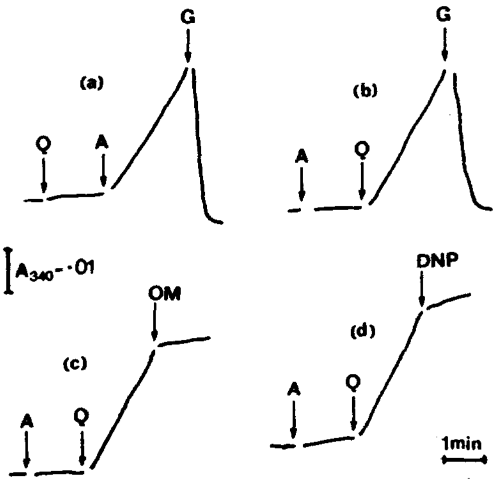

Fig. 1. Restoration of ATP-dependent reduction of $NADP^+$ by ubiquinone: effect of inhibitors and formation of NADPH. Conditions were as described in the legend to table 1, except that 1.0 mg submitochondrial particle protein was used in all assays. Q: 130 nmole ubiquinone-(45) added with thorough mixing; A: 6 μmole ATP added; G: 5 μmole glutathione plus 5 μg glutathione reductase; OM 2.5 μg oligomycin added; DNP: 2,4-dinitrophenol added to give a final concentration of 500 μM.

Table 2
Restoration of oxidase and energy-linked activities in beef heart submitochondrial particles prepared from pentane extracted beef heart mitochondria.

Particles	Succinate	NADH	ATP-driven reduction of $NADP^+$ by NADH	ATP-driven reduction of NAD^+ by succinate
Normal	140	188	72	48
Lyophilised	125	142	17.4	5.5
Lyophilised-extracted	12	14	0.9	0
Lyophilised-extracted plus ubiquinone (reincorporated)	108	86	9.0	0
Lyophilised-extracted plus pentane extract	89	98	14.1	4.5
Lyophilised-extracted plus ubiquinone (direct addition)	25	24	–	–

Submitochondrial particles were prepared from pentane extracted beef heart mitochondria as described in the Methods section. Ubiquinone-(30) (15 nmole/mg protein) and pentane extract (containing an amount of ubiquinone equal to twice that originally present in the particles) were incorporated into the mitochondria as described in the Method section. Oxidase activities were measured polarographically in a medium containing 750 μmole sucrose, 150 μmole tris-HCl buffer (pH 7.5) and 18 μmole magnesium chloride. Either 10 μmole succinate or 5 μmole NADH were added as substrate to give a final volume of 3 ml. Transhydrogenase activity was measured as described in the legend to table 1. ATP-driven reduction of NAD^+ by succinate was determined at 340 nm in a Beckman DK 2 recording spectrophotometer. Blank and experimental cuvettes contained 675 μmole sucrose, 135 μmole tris-HCl buffer (pH 8.0), 16 μmole magnesium chloride, 3 μmole potassium cyanide, 3 μmole NAD^+ and 15 μmole succinate at 30°. 0.5–2.0 mg submitochondrial particle protein were added to both cuvettes and the reaction was started by the addition of 6 μmole ATP to the experimental cuvette to give a final volume of 3 ml. The amounts of normal, lyophilised, lyophilised-extracted, lyophilised-extracted plus ubiquinone (reincorporated), lyophilised-extracted plus pentane extract and lyophilised-extracted plus ubiquinone (direct addition) were 0.75, 0.7, 0.81, 0.95, 0.82 and 0.81 mg protein respectively for the oxidase activities and 1.5, 1.4, 1.6, 1.9 and 1.6 mg protein for the energy-linked activities.

One problem with the experiments was that the concentration of ubiquinone required for restoration was in excess of that originally present in the mitochondria (in table 1, 130 nmole ubiquinone/g particle protein were added whereas the amount extracted was equivalent to 5.9 nmole/g protein).

More reproducible results demonstrating a requirement for ubiquinone in energy-linked reactions were obtained when pentane extraction and reincorporation of ubiquinone were performed on intact mitochondria prior to preparation of submitochondrial particles (see Methods). Some typical results are summarised in table 2. Restoration of the energy-linked transhydrogenase was achieved by incorporation of ubiquinone alone but restoration of the energy-linked reduction of NAD^+ by succinate required the addition of the pentane extract and was not observed on addition of ubiquinone alone. In agreement with Ernster et al. [7] NADH and succinate oxidation were both restored by ubiquinone and the restored reactions were sensitive to antimycin A and cyanide.

Restoration of the energy-linked transhydrogenase was achieved by addition of ubiquinone-(30) or ubiquinone-(50), but ubiquinone-(30) was more effective in restoring activity. This was probably a reflection of the relative rates of reincorporation of ubiquinone isoprenalogues into extracted mitochondria. Dispersal of ubiquinone in soybean phospholipids did not appear to enhance the restorative action of ubiquinone. No restoration of activity was observed on addition of vitamin K_1 or vitamin K_2-(35), nor was there any restoration of activity on addition of purified phospholipids alone.

These results suggest that restoration of the energy-linked transhydrogenase reaction is achieved specifically by ubiquinone and that it is not due to a non-specific lipid effect. However, restoration of the energy-linked reduction of NAD^+ by succinate reaction requires the presence of unidentified lipid components in the pentane extract. The extent of restoration of the transhydrogenase was dependent on the amount of ubiquinone-(30) added; and a near maximum effect was found at the concentration of ubiquinone originally present in the particles (fig. 2). This was in contrast with the direct addition of ubiquinone, which required a twenty-fold excess of the quinone for maximum restoration. The restored

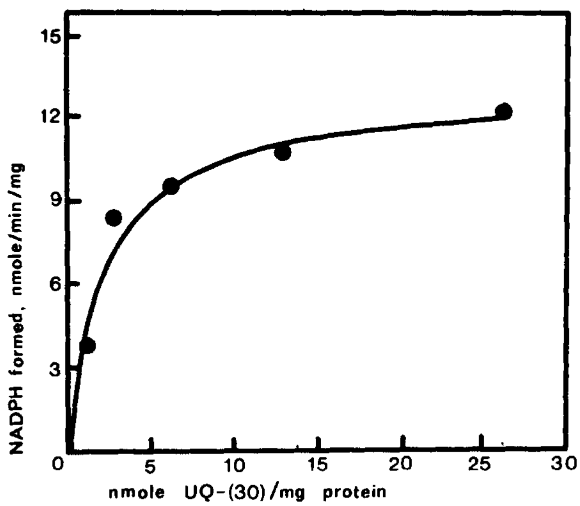

Fig. 2. Relationship between ubiquinone-(30) concentration and restoration of the ATP-driven reduction of $NADP^+$ by NADH. Conditions were as described in the legend to table 2 and 1.5 mg particle protein were used in all determinations. Ubiquinone-(30) was reincorporated into the particles at the concentrations shown.

Table 3
Effect of inhibitors on ATP-dependent reduction of $NADP^+$ by NADH in pentane extracted beef heart submitochondrial particles.

Particles	Oligomycin (2.5 μg)		2,4-DNP (240 μM)	
	Control	+Oligomycin	Control	+2,4-DNP
Normal	70	9	65	13
Lyophilised	19	4.2	17.4	4.5
Lyophilised-extracted plus ubiquinone (reincorporated)	8.5	1.0	8.7	2.3

Conditions were as described in the legend to table 2. Ubiquinone-(30) was reincorporated into the particles as described in the Methods section at a concentration of 15 nmole/mg particle protein. The amounts of normal, lyophilised and lyophilised-extracted plus ubiquinone were 1.5, 1.4 and 1.9 mg particle protein respectively. The figures in the table represent nmole NADPH formed/min/mg particle protein.

transhydrogenase reaction was sensitive to the same concentrations of inhibitors that affected the control reactions (table 3).

To summarise, the results presented in this paper point to a specific role for ubiquinone in the energy-linked transhydrogenase reaction of beef heart submitochondrial particles and a role of unidentified lipid components in the energy-linked reduction of NAD^+ by succinate. The failure of ubiquinone to restore the energy-linked reduction of NAD^+ by succinate constitutes evidence against the participation of a common quinone intermediate (e.g. a high-energy derivative of a quinone) in both energy-linked reactions.

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