Ubiquinone reduction pattern in pigeon heart mitochondria

Identification of three distinct ubiquinone pools

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1. Intact pigeon heart mitochondria showed 10–30% ubiquinone reduction in the absence of substrates. This reduction could not be ascribed to endogenous substrates, as judged by lack of effect of inhibitors and uncouplers and by the very low endogenous respiratory rate. 2. Addition of NADH in the presence of antimycin caused further reduction of about 10% ubiquinone, apparently coupled to the rotenone- and antimycin-sensitive exo-NADH oxidase system [Rasmussen (1969) *FEBS Lett.* 2, 157–162]. 3. Citric acid cycle substrates reduced most of the remaining ubiquinone in the presence of antimycin; 15–20% of the total ubiquinone content was still in the oxidized form under the most reducing conditions. 4. Three pools of ubiquinone therefore appeared to be present in heart mitochondria: a metabolically inactive pool consisting of reduced as well as oxidized ubiquinone, a pool coupled to oxidation of added (cytoplasmic) NADH, and the well-known pool coupled to citric acid cycle oxidations. 5. Ferricyanide selectively oxidized the ubiquinol reduced by added NADH, indicating that this pool is situated on the outer surface of the mitochondrial inner membrane. 6. Ubiquinone reduction levels were determined with a new method, which is described in detail.

Ubiquinone is generally considered as a constituent of the respiratory chain in mitochondria. This is based on (a) the redox properties in connection with oxidative phosphorylation (Redfearn & Pumphrey, 1960; Kröger & Klingenberg, 1966); (b) kinetic studies with uncoupled submitochondrial particles (Kröger & Klingenberg, 1973a); (c) incorporation studies with depleted membrane fragments (e.g. Norling *et al.*, 1974; Szarkowska, 1966) and with yeast mutants lacking ubiquinone (Brown & Beattie, 1977).

Correlation between ubiquinone redox level and respiratory rate showed that most of the ubiquinone in submitochondrial particles was kinetically homogeneous (Kröger & Klingenberg, 1973a). Some ubiquinone remained, however, always oxidized. A similar inactive pool of UQ_{ox.} was observed in intact mitochondria (Redfearn & Pumphrey, 1960; Kröger & Klingenberg, 1966).

Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine; UQ, ubiquinone; UQ_{ox.}, oxidized UQ; UQ_{red.}, reduced UQ; UQ_{tot.}, total UQ.

The possible existence within the same membrane of different pools is of particular relevance to those models of the respiratory chain that involve transverse diffusion of ubiquinone through the membrane (Mitchell, 1975; Kröger, 1976) [for reviews, see Gutman (1980); Trumpower (1981); Rich (1984)]. Compartmentation of ubiquinone in another membrane, the cytoplasmic membrane of *Pseudomonas aeruginosa*, has been suggested by Matsushita *et al.* (1980).

The present paper demonstrates the existence of three different ubiquinone pools in intact pigeon heart mitochondria. One pool, consisting of UQ_{ox.} as well as UQ_{red.}, is apparently metabolically inactive. Another pool is reduced by succinate and internal NADH and coupled to oxidative phosphorylation. The third pool exhibits the characteristics of being associated with the so-called exo-NADH oxidase system, which effects the oxidation of added NADH observed in muscle mitochondria (Rasmussen, 1969). A new ubiquinone analysis was used in this study [preliminary publication: Jørgensen & Rasmussen (1982)].

Experimental

Materials

Antimycin, FCCP and rotenone (Sigma) were used as ethanolic solutions. Glutamic, malic, pyruvic, and succinic acids (Sigma) were used neutralized. Ascorbic acid, KBH_4 , KCN and $\text{K}_3\text{Fe}(\text{CN})_6$ (ferricyanide) were from Merck. ADP, ATP, and disodium NADH (grade I) were from Boehringer Mannheim. Very dilute solutions of NADH were made up in incubation medium (pH 7.35).

Tap water was filtered through activated carbon and distilled once in glass and twice in quartz equipment.

Celite 535 (e.g. Merck) was purified by a procedure adapted from those reviewed by Bush (1961). The material (approx. 200 g) was heated at 800°C for 1 h, cooled passively, boiled in concentrated HCl (1 litre) for 1 h, washed with water (10 litres) on a filter, boiled in water for 1 h, washed with water (5 litres) on a filter, and finally dried at 120°C overnight. Silica gel for t.l.c. (60 HR, Merck) was mixed with an equal mass of purified celite. The mixture was washed on a filter with chloroform, ethanol, and chloroform in that order. It was dried at 120°C overnight.

The chloroform used in the analysis of ubiquinone was usually spectroscopic quality (Uvasol, Merck), although analytical grades from the same company could be used without problems. No special purification of absolute ethanol was required, but batches exhibiting large absorbance in u.v. light were not used.

Preparations

Pigeon heart mitochondria were prepared as described by Rasmussen (1969) and Rasmussen *et al.* (1982). The preparation medium was 225 mM-mannitol/75 mM-sucrose/1 mM-Tris/50 μM -EDTA, pH 7.35.

The rate of endogenous respiration of the preparation was very low, $0.17 \pm 0.04 \mu\text{mol}$ of O_2/min per g of protein (s.d., three preparations), indicating a low content of endogenous substrates (Rasmussen & Rasmussen, 1985). The rate of endogenous respiration could, however, be further diminished (typically by one-half) by incubating a suspension of the final pellet in medium at pH 5.18 and 0°C for 15 min (about 15 mg of protein in 40 ml). After neutralization to pH 7.35, the suspension was centrifuged at the same ratings as those used in the last centrifugation of the preparation procedure [7 min 40 s (effective time) at 4°C and 5800 g (r_{av} , 8.1 cm)]. This pellet was suspended in preparation medium to a protein concentration of 5–10 mg/ml. The procedure did not alter

the oxidative phosphorylation capability of the mitochondria.

Experiments

The incubation medium was 225 mM-mannitol/75 mM-sucrose/20 mM-Tris/0.5 mM-EDTA/10 mM-phosphate, pH 7.35. Reaction mixtures consisted of 750 μl of reaction medium and 150 μl of mitochondrial suspension with substrates and inhibitors added as described. Each determination of ubiquinone reduction level used one reaction mixture, and the results reported stem from single determinations, unless indicated. Ethanolic solutions were added in volumes of 2 μl . This amount of ethanol was without effect on the ubiquinone redox level.

Fast changes in the ubiquinone redox level were measured by parallel incubations in a special apparatus. It consisted of an open reaction vessel with a magnetic stirrer, and two gas-tight microsyringes, one for addition of substrate and one for addition of quenching agent. The microsyringes (Hamilton; Micromesure B.V., The Hague, The Netherlands) were driven by air cylinders at rates corresponding to a linear velocity at the outlets of about 30 m/s. An adjustable electronic timer controlled the time interval between the two additions. The alkali-catalysed hydrolysis of 2,4-dinitrophenyl acetate (Barman & Gutfreund, 1964) was used to evaluate the performance of the instrument. The mixing time was about 30 ms. A very similar instrument was described by Eccleston *et al.* (1980).

NADH oxidation was measured either fluorimetrically or by means of a Clarke-type oxygen electrode. Protein was determined by a method based on staining with Amido Schwartz (Schaffner & Weissmann, 1973) with bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.) as standard.

Analysis of ubiquinone

Method. The mitochondrial redox level of ubiquinone is usually determined by chemical analysis of oxidized ubiquinone [for reviews, see Crane & Dilley (1963), Redfearn (1967), Crane & Barr (1971), Kröger (1978)]. The method involves liquid–liquid extractions, evaporation of the combined extracts, resolubilization of the residue, and measurement of the decrease of absorbance following borohydride addition. The total amount of ubiquinone is determined in a separate extract to which an oxidizing agent has been added.

In the present method, the liquid–liquid extractions were replaced by a column extraction, which required less work and which, at least in our hands, produced far fewer outlying determinations of the redox level. The spectrophotometric procedure

was furthermore modified to make possible determination of total amount of ubiquinone in all samples.

HClO_4 (200 μl , 2M) was added to the mitochondrial reaction mixture (900 μl). The resulting suspension, cooled in an ice bath, was transferred quantitatively to 3g of cold celite, which after careful mixing was packed in a cold column (Fig. 1). The column was eluted in the cold with chloroform under N_2 pressure. The first 3 ml of eluate, containing all the ubiquinone, was collected in about 5 min and then evaporated at 35°C under a stream of N_2 . The residue was dissolved in 500 μl of absolute ethanol. Solution was speeded up by briefly heating the closed vessel with hot tap water (about 50°C).

A portion of the ethanolic solution (400 μl) was

transferred to a stoppered 1 cm cuvette and A_{272} and $A_{296.5}$ were measured initially, after complete reduction (3–5 min), and after complete reoxidation (1 min). Reduction was effected by addition of 1 μl of a solution of 50 mg of KBH_4 in 9.5 ml water + 0.5 ml of 1M-KOH in ethanol. The solution of KOH in ethanol had been centrifuged to remove precipitated carbonate. Reoxidation was effected by addition of 0.5 μl of 1M-HCl followed by 1.2 μl of 1M-KOH in ethanol (centrifuged solution). Reduction was best checked by $A_{296.5}$ and reoxidation by A_{272} because of the complicated nature of the reactions (cf. Kalsi & Wrigglesworth, 1981). The concentration of ubiquinone was calculated from the changes of A_{272} – $A_{296.5}$ and the molar absorption coefficient given below.

Absorbances were measured with a Zeiss PMQ-II spectrophotometer. The output voltage (linear in transmittance) was amplified and measured with a digital voltmeter interfaced to a microcomputer, which calculated the absorbance value. The spectrophotometer was operated with a halfwidth less than 0.8 nm and the precision of the absorbance measurements was 0.0002 (s.d., $n = 10$, $A = 0.0$ or 0.5). Cuvettes were washed with ethanol, water, and ethanol.

Comments. The efficiency of the extraction was checked in two ways. Freezing and thawing the mitochondria increased the amount of ubiquinone eluted from the column by light petroleum (b.p. 40–60°C) from 80% to 95% of the amount eluted by chloroform, whereas the amount eluted by chloroform was unaffected by the treatment. Furthermore, the column extraction and the liquid–liquid extraction yielded identical results with respect to UQ_{tot} . Thus, we believe all ubiquinone was extracted by the procedure.

The activated silica gel at the outlet of the column (Fig. 1) adsorbed compounds (e.g. antimycin) that caused interference in the spectrophotometric measurement. The elution pattern and recovery of ubiquinone were not affected by this silica gel layer when chloroform was used as eluant.

The crucial point of the extraction procedure was preservation of the redox state of ubiquinone. In the HClO_4 suspension, ubiquinone was contained in the precipitate, probably in close contact with oxidizing enzymes that were not completely denatured. But reoxidation also appeared to be favoured by u.v. light, and most manipulations were performed behind curtains. No other acid deproteinizing agent prevented reoxidation better than HClO_4 . The rate of reoxidation was lowered about 3-fold by cooling from room temperature to 0°C. It was highest in the most reduced samples. At $[\text{UQ}_{\text{red}}]/[\text{UQ}_{\text{tot}}] = 0.9$, the rate of decline of this ratio amounted to 0.06/h at 0°C.

Fig. 1. Glass column for extraction of ubiquinone.

Reoxidation might also occur on the column. Commercial celite, even purified grades, caused complete reoxidation of $UQ_{red.}$. The critical components in the celite were probably iron salts. The purification procedure described was most effective. It was, however, developed for another purpose and the need for every step was not checked.

Once extracted, $UQ_{red.}$ appeared more stable to reoxidation. The residues after evaporation could be stored overnight under N_2 in the refrigerator without change in the redox level. The extraction procedure could also be interrupted, at least for some hours, by storing the mixture of deproteinized suspension and celite at $-70^\circ C$.

The error due to reoxidation of $UQ_{red.}$ was judged from extrapolation of the reoxidation progress curve and comparisons with analyses carried out by the liquid-liquid extraction method. With the column extraction method, the total decline of $[UQ_{red.}]/[UQ_{tot.}]$ probably never exceeded 0.02.

Dual-wavelength measurements were used as they were less affected by changes in turbidity. Ethanolic solutions of KOH contain much less carbonate than aqueous solutions and therefore did not lead to precipitations when added to the ethanolic reaction mixture in the cuvette. Reduction of $UQ_{ox.}$ was probably complete under the conditions used, since no further reduction was observed on addition of more borohydride. The borohydride was stabilized by KOH and the solution could be pipetted without bubbling. Kept in an ice bath, this solution remained equally active for at least 5 h. Reoxidation with high yield required removal of the excess of borohydride. HCl catalysed the destruction very effectively, but also caused a slow increase of $A_{272}-A_{296.5}$. The treatment with acid was therefore limited to less than 30 s. The amount of KOH used to obtain reoxidation was critical. Smaller amounts (e.g. $0.8 \mu l$) caused only slow reoxidation, whereas larger amounts (e.g. $1.6 \mu l$) caused a slow decrease of A_{272} after a fast reoxidation. The volume of KOH was best measured with a device for delivery of variable volumes of solution protected from exposure to the atmosphere, e.g. an Agla micrometer syringe (Burroughs Wellcome, London, U.K.) The yield of the reoxidation measured with reduced pure ubiquinone was $98.2 \pm 1.4\%$ (S.D., $n = 5$).

Some discrepancies exist between the spectral data of ubiquinone in the literature, and the matter was reinvestigated. Commercial ubiquinone (Sigma) was recrystallized twice from ethanol and dried over $CaCl_2$. The standard deviations given refer to measurements of five different ethanolic solutions of this material. The maximal difference between molar absorption coefficients of $UQ_{ox.}$ and

$UQ_{red.}$ was $12430 \pm 90 M^{-1} \cdot cm^{-1}$ at 272 nm and the minimal difference was $-1130 \pm 130 M^{-1} \cdot cm^{-1}$ at 296.5 nm (mean $\pm$ S.D.). The isosbestic points were at 239, 292.5, and 303 nm. Maximal absorbance of $UQ_{ox.}$ was at 275 nm where the difference in molar absorption coefficient of $UQ_{ox.}$ and $UQ_{red.}$ was $12160 \pm 100 M^{-1} \cdot cm^{-1}$. This value agrees well with the value ($12250 M^{-1} \cdot cm^{-1}$) given by F. W. Hemming (see Redfearn, 1967).

The S.D. on determination of amount of $UQ_{tot.}$ (14.7 nmol) and $[UQ_{ox.}]/[UQ_{tot.}]$ (0.84) of mitochondrial reaction mixtures were 0.12 nmol and 0.013 ($n = 10$), respectively.

Results

Metabolically inactive ubiquinone

Ubiquinone was partially reduced in pigeon heart mitochondria incubated aerobically in the absence of added substrates (Table 1, column A). Under these conditions, the degree of reduction was insensitive to rotenone, inhibiting the NADH:ubiquinone oxidoreductase, and to antimycin and cyanide, inhibiting the respiratory chain between $UQ_{red.}$ and O_2 . ADP or the uncoupler FCCP did not affect the degree of reduction either. These findings, together with the very low rate of endogenous respiration (about 1/1000 of the succinate+glutamate State-4 rate), exclude that endogenous substrates were responsible for this background reduction. The $UQ_{red.}$ present in untreated mitochondria was therefore most likely metabolically inactive.

Table 1. Ubiquinone reduction levels in pigeon heart mitochondria incubated under various conditions

Mitochondria (1.33 mg of protein; 13.89 nmol of $UQ_{tot.}$) were incubated at $22^\circ C$ with substrates and inhibitors as indicated, without (column A) or with (column B) a further addition of NADH. The concentrations were: 7.7 mM-succinate, 7.7 mM-glutamate, 0.20 mM-NADH, 10 mM-KCN, $8.7 \mu M$ -antimycin and $2.2 \mu M$ -rotenone. Inhibitors were added prior to substrates. Incubation times were 3 min with succinate+glutamate and 15 s with NADH. Total incubation time was 8 min in all experiments.

Addition	$UQ_{red.}$ as % of $UQ_{tot.}$		
	A (-NADH)	B (+NADH)	C (B-A)
None	20.5	25.2	4.7
Rotenone	18.5	17.7	-0.8
Antimycin	19.0	30.2	11.2
KCN	22.9	33.8	10.9
Rotenone, KCN	21.1	23.0	1.9
Succinate + glutamate	77.4	81.3	3.9
KCN, succinate + glutamate	81.7	83.0	1.3

Some UQ_{ox} also appeared to be metabolically inactive because complete reduction was never observed, not even in cyanide- or antimycin-inhibited mitochondria supplied with malate + pyruvate + NADH or succinate + glutamate + NADH in great excess (Tables 1 and 4). Lack of complete ubiquinone reduction under similar reducing conditions was also observed with rat heart mitochondria (Kröger & Klingenberg, 1966).

Effect of added NADH

Incubation with NADH increased the degree of ubiquinone reduction (Table 1, column C). This effect was abolished by rotenone and enlarged by antimycin or cyanide. The increase in UQ_{red} caused by addition of NADH to antimycin- or cyanide-inhibited mitochondria in the absence of other substrates was 9.7 ± 3.3% of UQ_{tot} (mean ± s.d. for 27 experiments, 19 preparations). This was the maximal amount of ubiquinone reducible by added NADH. Prereduction of UQ with succinate + glutamate in the presence of antimycin or cyanide diminished the effect of added NADH, a point to be discussed below.

Ferricyanide is a commonly used reagent for determining sidedness of membrane constituents (Klingenberg, 1979), because it does not penetrate the membrane. As seen from Table 2, the amount of UQ_{red} reacting with ferricyanide was equal to the amount of UQ_{red} formed by added NADH and independent of UQ reduction by malate + pyruvate. The metabolically inactive UQ_{red} was appar-

ently not oxidized by ferricyanide. Some precautions were necessary when measuring the effect of ferricyanide on the ubiquinone reduction level; excess ferricyanide was removed with ascorbate before quench of the reactions as all UQ_{red} otherwise would be oxidized. The results in Table 2 indicate that the redox-active groups of some of the ubiquinone molecules are exposed to the outer surface of the mitochondria and likely electron acceptors in the oxidation of added NADH. The UQ reducible by added NADH will therefore be referred to as 'exo-UQ' in the following.

The reduction pattern of ubiquinone in subfractions of mitochondria is shown in Table 3. Most UQ was distributed evenly on a protein basis. A similar distribution was previously found for the activities of succinate oxidation and of reduction of internal NAD⁺ by succinate (Rasmussen, 1969). Amount of exo-UQ and activity of oxidation of added NADH were, however, uniquely distributed, and the ratio between these quantities was fairly constant. This supports the notion that exo-UQ is connected to the system that oxidizes added NADH.

The reaction between added NADH and ubiquinone with inhibited oxidation was fast, reaching the final level in less than 1 s (Fig. 2). The UQ reduction obtained with NADH in excess to UQ_{tot} was constant for at least 6 min. An amount of NADH, equal to only 22% of UQ_{tot}, caused the

Table 2. Effect of ferricyanide on ubiquinone reduction level

Mitochondria (1.37 mg of protein; 11.50 nmol of UQ_{tot}) were incubated at 22°C with 11 μM-antimycin and 10 mM-cyanide, after which the following additions were made in succession: substrate, as indicated (0.22 mM-NADH, 8.3 mM-malate, 8.3 mM-pyruvate), 2.2 μM-rotenone, 0.56 mM-K₃Fe(CN)₆ (in column B), and 8.9 mM-ascorbate. The total time periods from addition to quench were: malate + pyruvate, 3 min; NADH, 1 min; rotenone, 45 s; ferricyanide, 30 s; ascorbate, 15 s. No ferricyanide was added in the experiments of column A, and no rotenone in the experiments reported in parentheses.

Substrate	UQ _{red} as % of UQ _{tot}		
	A (- ferri- cyanide)	B (+ ferri- cyanide)	C (A - B)
None	12.0 (13.9)	15.4	- 3.4
NADH	31.6 (30.5)	16.7	14.9
Malate + pyruvate, NADH	86.1	68.2	17.9

Table 3. Ubiquinone reduction in subfractions of pigeon heart mitochondria

Mitochondria were prepared as usual and then fractionated by differential centrifugation at 328 g, 433 g, 555 g, 754 g (7.17 min), 1361 g and 5939 g (7.42 min); *r*_{av}, 8.3 cm; temperature 4°C. Each fraction was suspended in 500 μl of preparation medium. The protein mass concentration in fractions 1-6 was 2.32, 0.940, 0.693, 0.528, 0.487, and 0.427 g/l, respectively. Symbols used: A, UQ_{red} present in absence of substrate; B, exo-UQ, i.e. increase in UQ_{red} after incubation with 0.23 mM-NADH for 15 s in the presence of 10 mM-KCN; C, UQ remaining oxidized after the above-mentioned incubation with NADH; *f*, ratio between rate of oxidation of added NADH and amount of exo-UQ (column B).

Fraction no.	UQ amount per protein mass (μmol/g)			<i>f</i> (s ⁻¹)
	A	B	C	
1	1.86	0.785	6.84	1.41
2	1.93	0.767	7.12	1.36
3	1.83	0.929	7.28	1.72
4	1.80	1.19	6.98	1.17
5	1.36	1.62	6.66	0.87
6	1.38	1.73	6.27	1.28

Fig. 2. Reduction of ubiquinone by added NADH in antimycin-inhibited mitochondria

Mitochondria (1.46 mg of protein; 17.42 nmol of $UQ_{tot.}$) were incubated with $8.4 \mu M$ -antimycin, and $0.2 mM$ -NADH was added at time zero. The reactions were quenched at the time indicated. The slope of the straight line is the rate of NADH-oxidation with $0.2 mM$ -NADH in the absence of antimycin.

same UQ reduction as did the 70-fold larger amount used in Fig. 2. The equilibrium therefore seemed fully displaced in favour of NAD^+ and $UQ_{red.}$, but with only a part of $UQ_{tot.}$ participating.

The initial rate of ubiquinone reduction (per protein mass) in Fig. 2 was $8.8 \mu mol \cdot s^{-1} \cdot g^{-1}$ and the uninhibited steady-state rate of NADH oxidation was $4.2 \mu mol \cdot s^{-1} \cdot g^{-1}$ or approx. one-half of the initial reduction rate. This ratio was almost the same as the ratio between $UQ_{red.}$ produced by added NADH in the absence and in the presence of antimycin (Table 1, column C). The kinetic equations by Kröger & Klingenberg (1973a) predict this equality, if *exo*-UQ is assumed to be the only UQ coupled to oxidation of added NADH.

The role of *exo*-UQ in the oxidation of added NADH was further substantiated by simultaneous measurements of ubiquinone reduction level and NADH concentration (Fig. 3). The NADH concentration used in the experiment was too small to allow steady-state to be established, but some reduction of ubiquinone occurred initially and reoxidation to the previous reduction level was synchronous with the disappearance of NADH. The behaviour seen in Fig. 3 is in accordance with *exo*-UQ as being either a constituent of this oxidase system or in rapid equilibrium with it.

The results presented in this section strongly suggest that the ubiquinone of heart mitochondria is distributed between several pools, one of which is involved in the rotenone-, antimycin- and cyanide-sensitive oxidation of added NADH. This *exo*-UQ pool is apparently situated on the outer surface of the inner membrane, isolated from the

Fig. 3. Changes in ubiquinone reduction level during oxidation of NADH

NADH ($10.2 \mu M$) was added to mitochondria (1.95 mg of protein; 17.06 nmol of $UQ_{tot.}$) in the absence of inhibitors. The oxidation of NADH was followed fluorimetrically until quench at the time indicated. $\circ$, NADH; $\bullet$, $UQ_{red.}$

Table 4. Effect of citric acid cycle substrates on the apparent *exo*-UQ pool size

Mitochondria (2.48 mg of protein; 20.3 nmol of $UQ_{tot.}$) were incubated at $23^\circ C$ with $11.3 \mu M$ -antimycin followed by $8.3 mM$ -malate + $8.3 mM$ -pyruvate or $6.8 mM$ -succinate + $7.1 mM$ -glutamate as indicated, and without (column A) or with (column B) a subsequent addition of $0.22 mM$ -NADH. Incubation times were 4 min with citric acid cycle substrates and 30 s with NADH.

Addition	$UQ_{red.}$ as % of $UQ_{tot.}$		
	A (-NADH)	B (+NADH)	C (B - A)
Antimycin	20.4	35.6	15.2
Antimycin, malate + pyruvate	70.9	80.0	9.1
Antimycin, succinate + glutamate	79.2	81.6	2.4

other ubiquinone. It is distributed among subfractions of mitochondria in a characteristic manner, and it performs kinetically like a constituent of the *exo*-NADH oxidase system.

Effect of citric acid cycle substrates

Citric acid cycle substrates caused the well-known reduction of ubiquinone (Tables 1, 4 and 5). After reduction of *exo*-UQ with NADH in the presence of antimycin, the same final reduction level was obtained with malate + pyruvate and with succinate + glutamate (Table 4, column B). The reduction was terminated in about 1 min with succinate + glutamate (Fig. 4, squares) and in less than 15 s with malate + pyruvate as substrates (results not shown). This difference in rate of

reduction was probably due to the rather slow activation of succinate dehydrogenase. After activation, however, succinate dehydrogenase was more active than NADH dehydrogenase as judged by a larger UQ reduction (see Tables 1 and 5) and a higher State-4 rate of respiration with succinate+glutamate than with malate+pyruvate.

Fig. 4 shows the progress curves for reduction of ubiquinone with succinate+glutamate in the presence of antimycin and in either the absence or presence of added NADH. Despite the difference in initial level due to reduced exo-UQ, the degree of reduction reached a common final level. The lower curve was also obtained in experiments where NADH was added after succinate+glutamate. The inserted difference curve therefore represents the apparent size of the exo-UQ pool as a function of time after addition of the substrate couple. A likely explanation of the results is that succinate+glutamate reduced exo-UQ_{ox.} slowly in addition to the fast reduction of the rest of the metabolically active UQ_{ox.}

The leakage of electrons to the exo-UQ pool was slower from malate+pyruvate, i.e. from internal NADH, than from succinate+glutamate (Table 4, column C). This difference excludes that the leak was caused by direct interaction between UQ pools. The leak rate was more sensitive to a decrease in temperature from 23 to 11°C than was the rate of the fast UQ reduction with either substrate couple. It is therefore possible that the leakage was affected by mobilities in the membrane.

Under conditions of steady-state respiration, the leak rate was negligible compared with the turnover of the oxidases and therefore of no physiological significance. The previous observation (Rasmussen, 1969) that the rate of respiration of succinate+glutamate and the rate of oxidation of added NADH are additive further substantiates this.

Ubiquinone showed shifts in reduction level on State 4-3-4 transitions and on uncoupling (Table 5) similar to those in rat heart mitochondria (Klingenberg & Kröger, 1967). The exo-UQ pool showed no such transitions.

Discussion

Current models of the mitochondrial inner membrane allow three different localizations of the ubiquinone ring structure; in the middle of the lipid bilayer or on one of the surfaces (e.g. Crane, 1977). Lateral diffusion is expected to be fast (Schneider *et al.*, 1982) and ubiquinone is probably evenly distributed over the membrane area. Hauska (1977) assumed fast transverse diffusion of ubiquinone in liposomes on the basis of its ability to mediate reduction of trapped ferricyanide by added dithionite. In intact mitochondria, however, transverse diffusion appears to be slow, since ferricyanide reacts very slowly with the antimycin-inhibited respiratory chain (Grinius *et al.*, 1971; Rasmussen & Rasmussen, 1985). Thus, the liposomal membrane is probably too simple to serve as

Fig. 4. Effect of added NADH on the reduction of ubiquinone by succinate+glutamate in the presence of antimycin

Mitochondria (1.49mg of protein; 11.9nmol of UQ_{tot.}) were incubated with 9.6μM-antimycin and with (■) or without (▲) 0.22mM-NADH added at time -15s. At time zero, succinate+glutamate (7.7+7.7mM) were added and the reactions were quenched at the time indicated. The difference curve (●) is shown in the inset.

Table 5. Effect of ADP and uncoupler on the steady-state ubiquinone reduction level with malate+pyruvate as substrates

Mitochondria (0.89mg of protein; 6.0nmol of UQ_{tot.}) were incubated at 23°C as indicated; 8.3mM-malate and 8.3mM-pyruvate were present for 2min prior to quench or to a further addition of 0.44mM-ADP or 9.6μM-FCCP. In the last experiment, 11.3μM-antimycin was added prior to substrates. The ubiquinone reduction level was measured 1min (State 3) and 2min (State 4) after addition of ADP. These incubation times were chosen on the basis of fluorimetric measurements of the State 4-3-4 transition of endogenous nicotinamide coenzymes.

Additions	UQ _{red.} as % of UQ _{tot.}
None	16.2
FCCP	15.6
Malate+pyruvate	36.7
Malate+pyruvate, ADP (State 3)	21.7
Malate+pyruvate, ±ADP (State 4)	32.5
Malate+pyruvate, FCCP	12.9
Antimycin, malate+pyruvate	76.3

a proper model for ubiquinone behaviour in the mitochondrial membrane.

The results presented in the present paper suggest the existence of three ubiquinone pools in heart mitochondria: one pool is metabolically inactive, another pool participates in the oxidation of succinate and internally generated NADH through the respiratory chain, and a third pool, called the exo-UQ pool, is detected by its reduction by added NADH.

Exo-UQ is most likely associated with the system that oxidizes added NADH, either as a component of the system or in rapid equilibrium with it. This system, named the exo-NADH oxidase system, is inhibited by the normal respiratory chain inhibitors (Rasmussen, 1969). Further studies (Rasmussen & Rasmussen, 1985) have shown that its activity is high in muscle mitochondria and not due to preparation artefacts. The exo-NADH oxidase system reduced all cytochrome anaerobically and direct interaction between the system and the normal respiratory chain was demonstrated at the level of cytochrome oxidase (Rasmussen & Rasmussen, 1985). Thus, the exo-NADH oxidase system is present in all mitochondria and not confined to a damaged or permeable subpopulation thereof. A significant permeability of all mitochondria to NADH is also ruled out, because oxidation of added NADH was not coupled to phosphorylation of ADP, whereas oxidation of citric acid cycle substrates was. Several observations strongly indicate that the exo-NADH oxidase system is freely accessible to added NADH and other compounds as if it is located at the outer side of the mitochondrial inner membrane. The ability of ferricyanide to oxidize exo-UQ fits well with this notion and with the notion of exo-UQ as associated with the system.

The exo-NADH oxidase system and the normal respiratory chain converge at the level of cytochrome oxidase, as mentioned above. This may account for the complete reduction of cytochromes by added NADH in State 5. The ability of added NADH to reduce ubiquinone in State 5 was not tested for technical reasons. Added NADH did not reduce all metabolically active ubiquinone in cyanide-inhibited mitochondria (Table 1) and the reduction of cytochromes was also incomplete under this condition. Most likely, cyanide affected the interaction between the two systems at the level of cytochrome oxidase.

In the presence of antimycin, added NADH reduced only exo-UQ. The *b*-cytochromes showed a similar functional compartmentation (Rasmussen & Rasmussen, 1985). It may thus be inferred that exo-UQ and a part of the *b*-cytochromes (approximately equal amounts) are components of the exo-NADH oxidase system, and that this

system is virtually isolated from the normal respiratory chain at the substrate side of the antimycin block. The slow leakage from the normal respiratory chain to the exo-NADH oxidase system, detected in the presence of antimycin, is without any physiological significance. The leakage is not due to reaction between ubiquinone pools, as discussed in the Results section. It may occur at the level of Complex I and II and may be the result of slow rotational motions of these complexes, occasionally rendering the UQ reaction sites accessible to exo-UQ. Leakage from the exo-NADH oxidase system to the normal respiratory chain was not detected, perhaps because the leakage was even slower than the turnover of the inhibited normal respiratory chain. This turnover was consistently observed to be higher than the turnover of the inhibited exo-NADH oxidase system.

All cytochrome in the preparation is reduced anaerobically by substrates, indicating that few mitochondria are metabolically inactive (Rasmussen *et al.*, 1982; Rasmussen & Rasmussen, 1985). The metabolically inactive ubiquinone is therefore most likely present in all mitochondria, where it may be confined to parts of the membrane that are devoid of catalysts. It may here exert structural or protective effects, as suggested by Kobayashi *et al.* (1980) and Landi *et al.* (1984). Freezing and thawing of the mitochondria cause oxidation of inactive UQ_{red}. (cf. Kröger, 1978). This effect may be explained by membrane rearrangements bringing inactive ubiquinone and catalysts in contact.

A good 50% of the ubiquinone participates in the oxidation of citric acid cycle substrates. Kröger & Klingenberg (1973*a,b*) studied the kinetic behaviour of this ubiquinone in submitochondrial particles and showed that it is a homogeneous pool. Their kinetic model can account for the well-known sigmoid inhibition curve with antimycin provided that the UQ_{red} oxidizing activity exceeds the UQ_{ox} reducing activity. This prerequisite is not fulfilled in our preparation, as indicated by the high steady state reduction of ubiquinone during State-4 respiration of succinate + glutamate. But the preparation does exhibit the sigmoid antimycin inhibition curve (Rasmussen, 1969), and additional or different factors must therefore be involved in the phenomenon.

The ubiquinone reaction site of succinate: ubiquinone oxidoreductase probably binds a ubisemiquinone pair. Studies of this bound pair suggest an intramembranal location of the ubiquinone that participates in the oxidation of citric acid cycle substrates (Salerno *et al.*, 1977). Supposing preparation artefacts can be excluded, this location is also suggested by the observation that ferricyanide is unable to oxidize substrate-

reduced ubiquinone in inverted submitochondrial particles (Grinius *et al.*, 1971; Crane, 1977). The redox reactions of ubiquinone involve protons, and ubiquinone may be a mediator of vectorial proton transport as proposed by Mitchell (1975). If ubiquinone is located in the middle of the lipid bilayer and transverse diffusion is slow, some proton channels must be associated with the reaction sites. These channels may be located so that protons needed at the site of UQ_{ox} reduction are taken up from the matrix side, whereas protons set free at the site of UQ_{red} oxidation are released to the cytoplasmic side. In this way the vectorial proton transport may be driven by the affinities of the redox reactions without the need for transverse diffusion of ubiquinone.

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