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Assay conditions for the mitochondrial NADH:coenzyme Q oxidoreductase

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The assay of Complex I activity requires the use of artificial acceptors, such as short-chain coenzyme Q homologs and analogs, because the physiological quinones, such as CoQ_{10}, are too insoluble in water to be added as substrates to the assay media. The medical interest raised in the last years on the pathological changes of Complex I activity has focussed on the requirement of easy reliable assays for its analysis. We have undertaken a systematic examination of the assay conditions of Complex I in mitochondrial membranes, using a series of quinones as electron acceptors, particularly the coenzyme Q homologs CoQ_0, CoQ_1 and CoQ_2, and the analogs duroquinone and decylubiquinone. Our findings have pointed out that the most suitable electron acceptor for the NADH:CoQ reductase assay is the homolog CoQ_1. The analog DB, commercially available, although yielding a high activity, nevertheless causes some problems for the standardization of the assay conditions.

NADH:CoQ oxidoreductase; Coenzyme Q homolog and analog; Beef heart mitochondria

1. INTRODUCTION

NADH:coenzyme Q oxidoreductase (EC 1.6.99.3) or Complex I of the mitochondrial respiratory chain catalyzes the reduction of lipid-soluble coenzyme Q (ubiquinone, CoQ) by water-soluble NADH. Purified Complex I has a molecular mass of approximately 700 kDa and is now recognized to be an assembly of at least 41 different polypeptides; seven hydrophobic intrinsic membrane subunits are encoded by the mitochondrial genome [1]. Recently the interest in Complex I has increased also on biomedical grounds, as it was discovered that some degenerative diseases may be associated with genetic or acquired defects of this enzyme, and that Complex I defects may be one main result of primary somatic mutations of mitochondrial DNA in the ageing process [2,3]. This medical interest on the one hand has prompted further investigations on the structure and genetics of Complex I, and on the other has focussed on the requirement of easy reliable assays for its analysis for clinical chemistry purposes.

The physiological electron donor to Complex I is NADH and the acceptor is lipid-soluble endogenous coenzyme Q (CoQ_{10} in most mammalian mitochondria).

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Abbreviations: CoQ_n, ubiquinone or coenzyme Q (n = 0–10 refer to the number of isoprenoid units in the lateral chain); DQ, duroquinone; DB, 6-decylubiquinone; PB, 6-pentylubiquinone; BHM, beef heart mitochondria; SMP, submitochondrial particles; NADH, nicotinamide adenine dinucleotide, reduced form; PL, phospholipid vesicles. *Enzyme:* NADH:coenzyme Q oxidoreductase EC 1.6.99.3 (Complex I).

The assay of Complex I activity requires the use of artificial acceptors, because the physiological quinones, such as CoQ_{10}, are too insoluble in water to be added as substrates to the assay media. The most commonly used acceptors are short-chain CoQ homologs (from CoQ_0 up) or analogs as tetramethylbenzoquinone (duroquinone, DQ) and analogs having straight saturated chains, such as 6-pentyl and 6-decyl ubiquinones (PB and DB, respectively) [4–7]. Some of these acceptors have been used in assays concerned with mitochondrial pathologies, although in most cases the activity of Complex I has been measured indirectly from NADH oxidation by cytochrome *c* or oxygen. Nevertheless the suitability of some quinone acceptors in the assay of the Complex I activity is not clear. The water solubility of some acceptors is low and consequently it is difficult to reach kinetic saturation during the assay; some ubiquinone homologs behave as Complex I inhibitors [8] and the respiratory chain contains quinone reduction sites downstream of the dehydrogenases [9,10]. The location of the quinone interaction sites is routinely assessed by using specific inhibitors of Complex I (as rotenone and piericidin A) and Complex III (antimycin A for center *i* and myxothiazol, mucidin, stigmatellin and undecylhydroxydioxobenzothiazole (UHDBT) for center *o*) [11]. Nevertheless it was recently found in our laboratory [12] that center *o* inhibitors, maximally myxothiazol and stigmatellin, also inhibit Complex I, while piericidin A also inhibits center *o* in Complex III.

For these reasons we have decided to undertake a systematic examination of the assay conditions of Complex I in situ in mitochondrial membranes, using a series of quinones as electron acceptors, particularly CoQ_0,

CoQ_1, CoQ_2, DQ and DB (Fig. 1), and attempting to evaluate their reduction site(s) in the presence of the inhibitors antimycin A (center *i* of the bc_1 complex) and mucidin (center *o*).

2. MATERIALS AND METHODS

Isoprenoid CoQ homologs (CoQ_1 and CoQ_2) and CoQ_0 were kind gifts from Eisai Co. (Tokyo, Japan). NADH β-form, mixed phospholipids from soybean (L-α phosphatidylcholine), decylubiquinone (DB) and duroquinone (DQ) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The inhibitors antimycin A and rotenone were from Sigma Chemical Co. Mucidin was a kind gift from Dr. J. Subik, University of Bratislava, Slovakia. All quinones and inhibitors were conserved as ethanolic solutions at −20°C in the dark. Quinone concentration was determined spectrophotometrically: CoQ_0, λ 263 nm, ε 13.6 $mM^{-1} \cdot cm^{-1}$; CoQ_1 and CoQ_2, λ 275 nm, ε 13.7 $mM^{-1} \cdot cm^{-1}$; DB, λ 278 nm, ε 14.0 $mM^{-1} \cdot cm^{-1}$; DQ, λ 270 nm, ε 19.2 $mM^{-1} \cdot cm^{-1}$. The inhibitor concentration was determined as previously described [13,14].

Beef heart mitochondria were obtained by a large-scale procedure [15]. Submitochondrial particles (SMP) were prepared by sonic irradiation of the frozen and thawed mitochondrial preparation [16] and suspended in 0.33 mM sucrose, 25 mM Tris-HCl, 0.5 mM histidine buffer, pH 8.0. All preparations were kept frozen at −80°C, and frozen and thawed 2–3 times before use. It was shown that under these conditions the permeability barrier for NADH is completely lost, as demonstrated by lack of further stimulation by detergents [17]. Protein was evaluated as in [18].

NADH:CoQ reductase activity was assayed in 50 mM KCl, 10 mM Tris-HCl, 1 mM EDTA, 2 mM KCN buffer, pH 7.4. Samples were diluted to 10–15 μg/ml for SMP and 20–30 μg/ml for BHM in assay medium and incubated for 5 min without inhibitors or with either rotenone (100–200 nmol/mg protein), antimycin A (150–250 nmol/mg), mucidin (50–75 nmol/mg) and a combination of mucidin and antimycin A. Sonicated mixed phospholipids (PL) were added, when tested, at different concentrations (between 0.05 and 0.50 mg/ml) in the assay medium. NADH was added at 75 μM and acceptors at adequate concentration for maximal activity (indicated in the tables). Time-course oxidation of NADH was followed in a Sigma-Biochem double-wavelength spectrophotometer equipped with a rapid mixing apparatus (Cuv-O-Stir of Hellma) at λ 340 minus 380 nm (ε 5.5 $mM^{-1} \cdot cm^{-1}$).

3. RESULTS

NADH:CoQ reductase activity has been assayed at quasi-saturating concentrations of NADH and of quinone acceptors, previously determined by rate inspection at different acceptor concentrations. Fig. 2 shows traces of NADH oxidation using CoQ_1, DB and DQ in bovine heart submitocondrial particles (SMP). A similar behavior has been observed in beef heart mitochondria (BHM). The kinetics are linear for CoQ_1 and DQ, although the rate is more than double with CoQ_1, whereas DB systematically shows a non-linear behavior and CoQ_2 (not shown) exhibits non-linear kinetics, like DB, albeit with lower activity. Table I summarizes the NADH:CoQ oxidoreductase activity observed in BHM and SMP. The rates are higher in the SMP preparations. In spite of their similar hydrophobicity, CoQ_2 is a poorer acceptor in comparison to DB. Under the conditions of our experiments with all quinone acceptors used, except CoQ_0, the flow of electrons is physiologically channeled through Complex I, as shown by the almost total (> 90%) inhibition by rotenone (Table I). The non-linear behavior of the more hydrophobic compounds (DB, CoQ_2) may be the result of their low solubility preventing monomeric dispersion and hence attainment of the real saturating concentrations; accordingly, addition of mixed sonicated phospholipids (PL) to the assay medium greatly enhances the rate of DB and CoQ_2 reduction and yields linear kinetics (Table I). Nevertheless, phospholipids also stimulate activity with the other acceptors studied. The amount of phospholipids added is critical for each quinone acceptor. Particularly, DB showed a marked dependence on the phospholipid concentration, with an optimal PL concentration for the maximal activity, as shown in Fig. 3, while the other acceptors reach a plateau (e.g. CoQ_2).

We have previously confirmed that antimycin A and mucidin have little effect on Complex I activity [12]. Therefore we have chosen to use antimycin A as a diagnostic of center *i* inhibition and mucidin to block electron transfer to center *o*. Myxothiazol was discarded because it has been confirmed to behave as an inhibitor

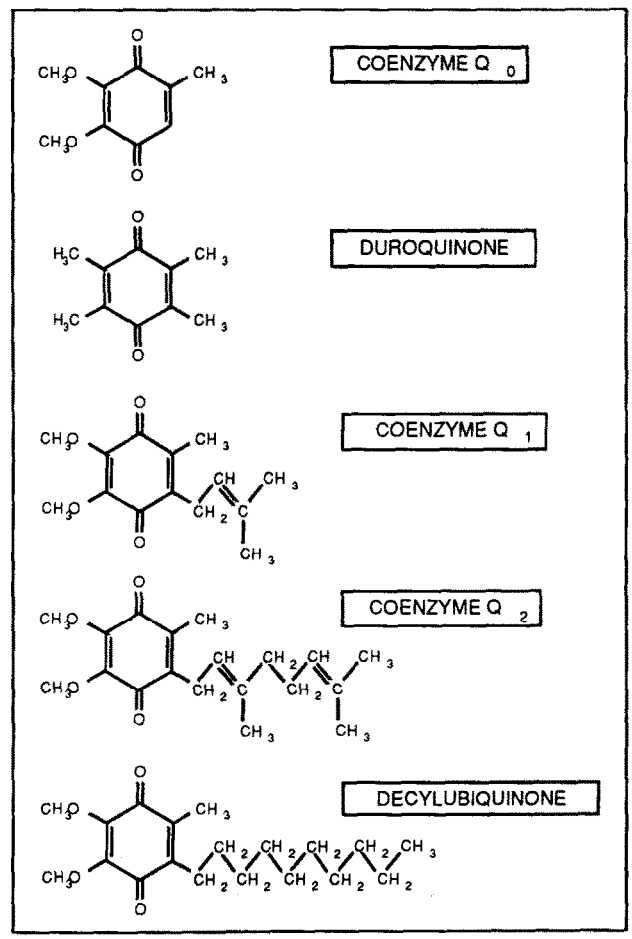

Fig. 1. Chemical structure of the Coenzyme Q homologs CoQ_0, CoQ_1 and CoQ_2 and the analogs duroquinone and decylubiquinone used as electron acceptors in the NADH:CoQ oxidoreductase assays.

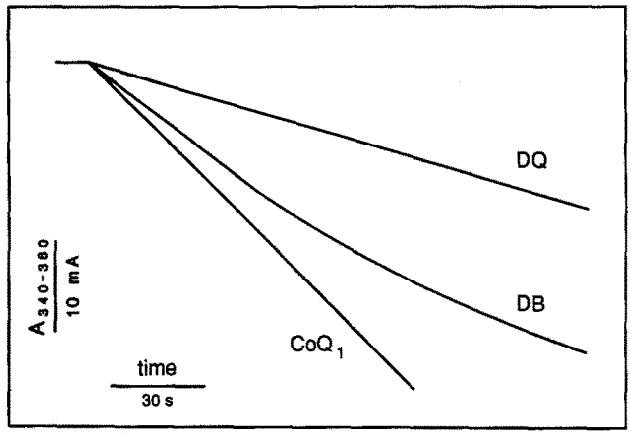

Fig. 2. Time-course oxidation of NADH by the electron acceptors duroquinone (DQ), decylubiquinone (DB) and Coenzyme Q_1 in bovine heart submitochondrial particles.

of Complex I, as previously pointed out [12]. The combination of antimycin A and mucidin represents the so-called 'double kill' of the bc_1 Complex, by which no electron transfer is possible any more to the *b* cytochromes [11]. Table II shows the effects of these inhibitors on NADH oxidation by the quinone acceptors assayed in BHM. The rate of electron transfer in the presence of both antimycin A and mucidin may be taken as a measure of the real Complex I activity with a given quinone acceptor. The results indicate a high extent of electron flow to Complex III when CoQ_0 and DQ are used, whereas this extent is smaller with the other acceptors. Since the rates with CoQ_0 and DQ are basically low, they appear to be poor acceptors for Complex I activity; this is particularly true for CoQ_0, that also has lower sensitivity to rotenone, a specific inhibitor of Complex I.

Table I

NADH:CoQ oxidoreductase activity in beef heart mitochondria (BHM) and submitochondrial particles (SMP) with the different acceptors and in presence of phospholipids (PL)

Sample	Acceptor	Activity (nmol · min^{-1} · mg^{-1})		Rotenone sensitivity (% inhibition)	Comments
		Control	+PL		
BHM	CoQ_0	275	371	72	Linear
	CoQ_1	652	878	96	Linear
	CoQ_2	177	320	91	Linear with PL
	DQ	216	302	93	Linear
	DB	399	446	99	Linear with PL
SMP	CoQ_0	463	634	77	Linear
	CoQ_1	965	1286	95	Linear
	CoQ_2	292	583	91	Linear with PL
	DQ	313	487	96	Linear
	DB	727	1282	99	Linear with PL

Assays were performed at the following acceptor concentrations: 100 μM CoQ_0, 50 μM CoQ_1, 30 μM CoQ_2, 100 μM DQ and 40 μM DB. Protein was 20–30 μg/ml for BHM and 10–15 μg/ml for SMP. Activity in the presence of phospholipids (PL) represents the maximal stimulation rate obtained as follows: 0.10 mg/ml PL for DB, 0.25–0.50 mg/ml for the others in the assay medium. Rotenone was added at 2.5 μM.

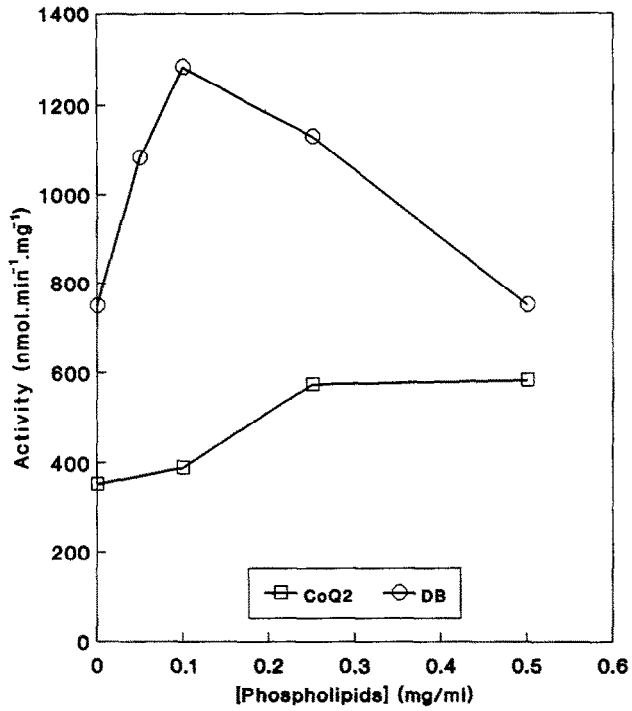

Fig. 3. Effect of phospholipid amount in the assay medium on the NADH:CoQ oxidoreductase activity in submitochondrial particles using Coenzyme Q_2 and decylubiquinone (DB) as electron acceptors.

4. DISCUSSION

Our study shows, for the first time, a screening of the suitability of the most commonly used quinone electron acceptors in the assay of NADH:coenzyme Q oxidoreductase. It is generally assumed that these ubiquinone homologs and analogs interact with the physiological site in place of endogenous CoQ, as derived from the rotenone sensitivity. Nevertheless we have shown that the issue is much more complex. The water solubility of the quinones is the fundamental factor for assessing whether kinetic saturation is reached during assay, a prerequisite for enzymatic determinations. In this sense, the most lipophilic quinones, such as CoQ_2 and DB, showed non-linear kinetics. Addition of phospholipids to the assay medium notably improved the linearity of the reaction, as well as the activity ratio. Nevertheless NADH oxidation by DB was highly dependent on the amount of phospholipids, reaching a maximum at an optimal amount and decaying beyond this, while CoQ_2 reached a plateau of maximum activity.

Some of the quinones tested, such as DQ and CoQ_2, showed a lower rate of NADH oxidation. This fact can be inconvenient because activity changes (e.g. in pa-

thological studies) might not be revealed if they occur at a step which is not rate-limiting in the enzymatic reaction. Moreover, CoQ_2 behaves as an inhibitor of Complex I, as has been pointed out previously [8]. In fact, NADH oxidation rates were the lowest when this homolog was used. This inhibitory effect could explain the differences observed when compared with its analog DB, which is more hydrophobic [19] and consequently might show lower activity.

On the other hand, some of the quinones used as electron acceptors are reduced before and beyond the physiological site in the Complex I. NADH oxidation by CoQ_0 showed a marked insensitivity to rotenone and it was greatly affected by the antimycin A–mucidin combination, whereas DQ reduction also showed a great dependence on the bc_1 Complex. The other quinones showed a lower dependence on this reduction site. Studies on the location of the reduction sites for quinones have been done, mainly concerning the succinate:CoQ oxidoreductase activity (e.g. [10]). Only a few studies have been carried out on Complex I activity and most of them have been done by using myxothiazol as inhibitor of the center *o* of the bc_1 Complex [20,21]. The finding that myxothiazol is a significant inhibitor of Complex I too [12] raises doubts about some of the interpretations of those results.

The major points of our work could be summarized as follows. CoQ_1 and DB are the best electron acceptors for the study of the NADH:CoQ oxidoreductase activity. DB has the advantage that it yields a high real Complex I activity and is easy to obtain commercially; nevertheless the non-linear kinetics with DB is a problem. It can be avoided with the addition of phospholipids to the assay medium, but it is necessary to standardize their amount for optimal assay. By contrast, CoQ_1 has been revealed as the best acceptor, yielding the highest rate of Complex I activity, linear kinetics and relative independence of the phospholipid concentration. On the other hand CoQ_1 shows a very good linear correlation between the rate of Complex I activity and the amount of protein used for the assay in a wide range of concentrations from 2 to 15 μg/ml of the total mitochondrial protein (Fig. 4) with a detection limit of ca. 1 nmol NADH oxidized per ml per min. Also, the analytical reproducibility using CoQ_1 as electron acceptor is quite good, giving an average value of 0.925 ± 0.13 ($n = 8$). Nevertheless its application for chemical chemistry purposes is difficult because it is not commercially available (at least at the moment). The other quinones studied have disadvantages and these must be avoided.

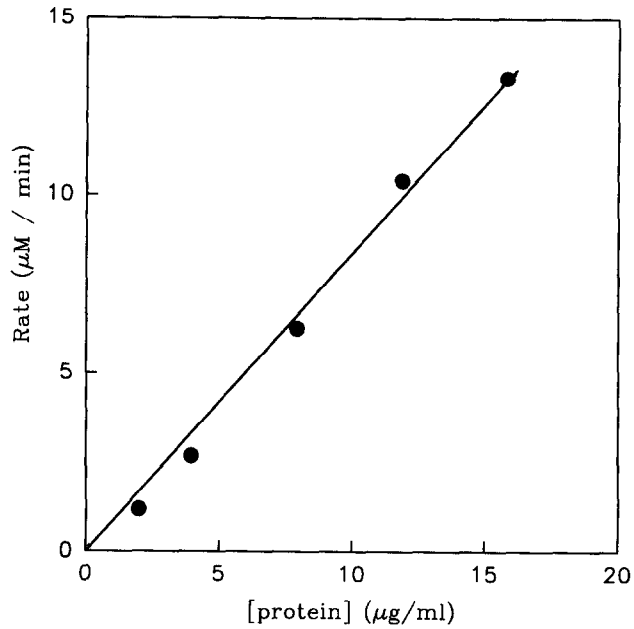

Fig. 4. Effect of protein concentration on the rate of NADH:CoQ_1 reductase activity in submitochondrial particles.

Table II

NADH:CoQ oxidoreductase activity in beef heart mitochondria (BHM) in presence of the inhibitors of bc_1 Complex

Sample	Acceptor	Residual activity (%)		
		Antimycin A	Mucidin	Both
BHM	CoQ_0	64	78	47
	CoQ_1	80	95	70
	CoQ_2	82	78	73
	DQ	55	70	40
	DB	75	97	77

Samples were incubated for 5 min at the following inhibitor concentrations: 4.6 μM antimycin A and 1.5 μM mucidin.

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