Steady-State Kinetics of the Reduction of Coenzyme Q Analogs by Complex I (NADH:Ubiquinone Oxidoreductase) in Bovine Heart Mitochondria and Submitochondrial Particles†

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ABSTRACT: The reduction kinetics of coenzyme Q (CoQ, ubiquinone) by NADH:ubiquinone oxidoreductase (complex I, EC 1.6.99.3) was investigated in bovine heart mitochondrial membranes using water-soluble homologs and analogs of the endogenous ubiquinone acceptor CoQ_{10} [the lower homologs from CoQ_0 to CoQ_3, the 6-pentyl (PB) and 6-decyl (DB) analogs, and duroquinone]. By far the best substrates in bovine heart submitochondrial particles are CoQ_1 and PB. The kinetics of NADH−CoQ reductase was investigated in detail using CoQ_1 and PB as acceptors. The kinetic pattern follows a ping-pong mechanism; the K_m for CoQ_1 is in the range of 20 μM but is reversibly increased to 60 μM by extraction of the endogenous CoQ_{10}. The increased K_m in CoQ_{10}-depleted membranes indicates that endogenous ubiquinone not only does not exert significant product inhibition but rather is required for the appropriate structure of the acceptor site. The much lower V_{max} with CoQ_2 but not with DB as acceptor, associated with an almost identical K_m, suggests that the sites for endogenous ubiquinone bind 6-isoprenyl- and 6-alkylubiquinones with similar affinity, but the mode of electron transfer is less efficient with CoQ_2. The k_{min} (k_{cat}/K_m) for CoQ_1 is 4 orders of magnitude lower than the bimolecular collisional constant calculated from fluorescence quenching of membrane probes; moreover, the activation energy calculated from Arrhenius plots of k_{min} is much higher than that of the collisional quenching constants. These observations strongly suggest that the interaction of the exogenous quinones with the enzyme is not diffusion-controlled. Contrary to other systems, in bovine submitochondrial particles, CoQ_1 usually appears to be able to support a rate approaching that of endogenous CoQ_{10}, as shown by application of the "pool equation" [Kröger, A., & Klingenberg, M. (1973) *Eur. J. Biochem. 39,* 313−323] relating the rate of ubiquinone reduction to the rate of ubiquinol oxidation and the overall rate through the ubiquinone pool.

NADH:coenzyme Q oxidoreductase (EC 1.6.99.3), also called complex I at the time of its original isolation from bovine heart mitochondria [cf. Hatefi (1963)], catalyzes the reduction of lipid soluble coenzyme Q (ubiquinone, CoQ)[1] by water soluble NADH (Weiss et al., 1991). This enzyme represents the first step of the mitochondrial respiratory chain (Ragan, 1987) and is linked to outward translocation of protons, with a $H^+/2e^-$ stoichiometry of 4 (Weiss et al., 1991) (site I of oxidative phosphorylation).

The enzyme has been isolated and characterized in detail from bovine heart (Ragan, 1987) and *Neurospora crassa* (Leonard et al., 1987); purified complex I has a molecular mass of approximately 700 kDa and is an assembly of at least 41 different polypeptides (Fearnley & Walker, 1992). Seven hydrophobic intrinsic membrane subunits, designated as ND1−ND6 and ND4L, are encoded by the mitochondrial genome (Chomyn et al., 1986). The enzyme also has a rich collection of prosthetic groups, including one flavin mononucleotide (FMN) and at least four EPR-detectable iron−sulfur clusters (Walker, 1992), besides bound ubiquinone (Suzuki & Ozawa, 1986), of which two molecules, occupying two different sites, are present (Degli Esposti & Ghelli, 1994).

The enzyme is inhibited by several compounds, of which rotenone and piericidin A are the most commonly used (Singer, 1979); a large collection of inhibitors is available (Shimomura et al., 1989; Friedrich et al., 1994; Degli Esposti et al., 1994), among which are compounds widely used as pesticides (Hollingworth et al., 1994). Moreover, compounds previously believed to act as specific inhibitors of complex III, as myxothiazol and stigmatellin, are also potent inhibitors of NADH−CoQ reductase (Degli Esposti et al., 1993).

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[1] Abbreviations: 12-AS, 12-(9-anthroyloxy)stearic acid; BHM, bovine heart mitochondria; CoQ_n, coenzyme Q (ubiquinone) with *n* isoprenoid units; DB, decylubiquinone (2,3-dimethoxy-5-methyl-6-decylbenzoquinone); DCIP, 2,6-dichlorophenolindophenol; DQ, duroquinone (2,3,5,6-tetramethylbenzoquinone); EPR, electron paramagnetic resonance; ETP_H, phosphorylating submitochondrial particles from beef heart; k_{coll}, bimolecular collisional frequency; k_{min}, the minimal association rate constant of enzyme to substrate, corresponding to the k_{cat}/K_m ratio; PB, pentylubiquinone (2,3-dimethoxy-5-methyl-6-pentylbenzoquinone); PL, phospholipids; SMP, bovine heart submitochondrial particles obtained by ultrasonic irradiation; pyrene-PC_6 (or pyrene-PC_{10}), 1-hexadecanoyl-2-(1-pyrenylhexanoyl[or -decanoyl])-*sn*-glycero-3-phosphocholine; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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Recently, interest in complex I has increased as a consequence of the discovery that some degenerative diseases, including the so-called mitochondrial enkephalomyopathies (Morgan-Hughes et al., 1988), Parkinson's disease (Parker et al., 1989), and Huntington's disease (Parker et al., 1990), may be associated with genetic or acquired defects of this enzyme and that complex I defects may be one of the major consequences of primary somatic mutations of mitochondrial DNA in the aging process (Cooper et al., 1992). This clinical interest, on one hand, has prompted further investigations on the structure and genetics of complex I and, on the other, has focused on the requirement of reliable assays for its analysis.

The physiological electron donor of complex I is NADH, although NADPH may also serve as a substrate at low pH (Ragan, 1976) even in the purified enzyme in the absence of the NADPH:NAD^+ transhydrogenase reaction. On the other hand, the physiological acceptor is lipid soluble endogenous coenzyme Q (CoQ_{10} in beef heart), as first demonstrated in extraction−reconstitution experiments (Szarkowska, 1966).

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 employed as substrates in the assay media.

Among the acceptors used, ferricyanide [cf. Ruzicka and Crane (1970a)] accepts electrons prior to the physiological reduction site, as shown by a lack of inhibition by rotenone of NADH:ferricyanide reductase (Hatefi et al., 1962) and by retention of ferricyanide reduction activity in the solubilized type I NADH dehydrogenases (Ringler et al., 1963) that lack the hydrophobic sector of the enzyme. Moreover, the reduction of such oxidants as ferricyanide, Wurster's Blue, and 2,6-dichlorophenolindophenol (DCIP) is not coupled to energy transduction (Kotlyar & Gutman, 1992).

The electron acceptors most commonly employed as substitutes of the physiological ubiquinone are CoQ homologs and analogs (Ragan, 1976). It is assumed that these compounds interact with the physiological site, in place of the endogenous CoQ, by first partitioning from the water phase to the membrane and that exogenous quinones are reduced directly by complex I, without the mediation of the ubiquinone pool (Cabrini et al., 1981). The discovery that the enzyme contains bound ubiquinone essential for its activity (Degli Esposti & Ghelli, 1994) reopens the question of the mode of interaction of exogenous quinones with the acceptor site(s).

Among the quinone acceptors used are the homolog series from CoQ_0 to higher homologs (Singer, 1974; Ragan, 1976), including less frequently long isoprenoid chain homologs in spite of their insolubility in water (Hatefi et al., 1962), the tetramethyl benzoquinone analog, duroquinone (DQ), and analogs having straight saturated chains such as 6-pentyl- and 6-decylubiquinones (PB and DB, respectively). Among these acceptors, the most commonly used in the assay of complex I have been CoQ_1 and DB; however, CoQ_0 [e.g. Kotlyar and Gutman (1992)], CoQ_2 [e.g. Suzuki and King (1983) and Friedrich et al. (1994)], and PB (Tan et al., 1993; Ackrell et al., 1994) have also been employed.

Kinetic studies using some of these acceptors have mainly been undertaken to research the mechanism of action of different inhibitors [cf. Tan et al. (1993) and Friedrich et al. (1994)]. No extensive studies have been published on the kinetic mechanism of interaction of a quinone acceptor with the enzyme taking into account the chemical, physical, and steric properties of the exogenous acceptor used and the role of bound ubiquinone(s). The kinetic mechanism of complex I (type I NADH dehydrogenase) using either ferricyanide (one electron) or DCIP (two electrons) as acceptors was found to be a ping-pong bi-bi mechanism (Dooijewaard & Slater, 1976).

Another important, though unanswered, question is the possible presence of a diffusion-limited step in the reaction of ubiquinones with the complex. The presence of diffusion control in the activity of complex I would have important physiological implications; Hackenbrock et al. (1986) proposed a "random collision model" for the mitochondrial respiratory chain, where the respiratory complexes are randomly dispersed in the lipid bilayer and electron transfer occurs by collisional encounters between the complexes and smaller connecting molecules, such as ubiquinone between complexes I and III. In this model, the overall respiratory activity was postulated to be controlled by the lateral diffusion of ubiquinone. Previous studies in our laboratory (Fato et al., 1993; Cavazzoni et al., 1993) showed that ubiquinol−cytochrome *c* reductase is not subject to diffusion control of the interaction of exogenous ubiquinols; no such study of the interaction of complex I with exogenous ubiquinones is available however. Such a study requires the use of a substrate eliciting the highest possible turnover in order to reveal the presence of a diffusion-limited step (Brouwer & Kirsch, 1982).

The main criterion utilized to assess the suitability of quinones to be used as complex I electron acceptors has been their rotenone sensitivity, for instance to distinguish between exogenous and endogenous pathways of NADH oxidation (Ragan, 1978). Nevertheless, the issue is much more complex, and other factors have to be considered, as pointed out by Estornell et al. (1993).

In addition to water soluble quinones, cytochrome *c* and oxygen, which accept electrons at the level of complex III and complex IV, respectively, of the respiratory chain, have also been used as alternative indicators of the reduction of endogenous CoQ by complex I; in fact, according to the "pool equation" (Kröger & Klingenberg, 1973), the rate of electron flux in the respiratory chain via the CoQ pool (V_{obs}) is a function of the rate of CoQ reduction (V_{red}):

$$V_{obs} = V_{red}V_{ox}/(V_{red} + V_{ox})$$

where V_{ox} is the rate of CoQ reoxidation by complex III. According to this relation, if V_{ox} and V_{red} differ significantly, V_{obs} will approach the rate of the slower step.

In view of the fact that complex I is present at lower levels than complex III [less than one-third in beef heart mitochondria (Capaldi, 1982)] and that it may have a lower turnover (Gutman, 1985), it seems justified to postulate that V_{obs} approaches V_{red} and that the reduction of either cytochrome *c* or oxygen with NADH as substrate may be a convenient indication of complex I activity. On the other hand, this assumption would not be valid if the turnover of complex I is underevaluated, a reasonable hypothesis considering that purified preparations of the enzyme readily lose activity (Gluck et al., 1994) and in consideration of the lack of suitable assays, as suggested by Estornell et al. (1993).

One further complication of electron withdrawal downstream with respect to endogenous ubiquinone is in the demonstration that endogenous CoQ_{10} is not saturating for maximal electron transfer activity from NADH (Estornell et al., 1992); thus, the electron flow through the endogenous CoQ pool may not represent the V_{max} of the enzyme which reduces the pool, viz complex I.

For all of these reasons, we have decided to undertake a systematic examination of the steady-state kinetics of complex I, using a series of quinones as electron acceptors and attempting to evaluate their reduction site(s) in the respiratory chain; in this study, we have also considered the role of the CoQ pool with respect to the electron acceptors employed and taken into account the possible extent of diffusion control.

In consideration of the fact that upon isolation the enzyme loses activity (Ragan, 1976) and is subject to changes in the properties of its hydrophobic sector (Gluck et al., 1994), this study has been performed on the enzyme *in situ* in mitochondrial membranes.

A preliminary note on the assay conditions of NADH−CoQ reductase has been published (Estornell et al., 1993).

MATERIALS AND METHODS

Materials. Coenzyme Q homologs were kind gifts from Eisai Co., Tokyo; NADH (β-form), DB, DQ, and CoQ_0 were purchased from Sigma Co., St. Louis, MO. Asolectin (mixed soybean phospholipids), from Sigma, was purified with the method of Kagawa and Racker (1971). Horse heart cytochrome *c*, type III, was also from Sigma. Flavin mononucleotide was from Boehringer Manheim, Germany. The fluorescent probes 12-AS, pyrene, pyrene-PC_6, and pyrene-PC_{10} were purchased from Molecular Probes, Junction City, OR.

For the assay of ubiquinol−cytochrome *c* reductase, the ubiquinols (usually ubiquinol-2 and reduced DB) were formed from the corresponding quinones by the method of Rieske (1967). All quinones and quinols were conserved as ethanolic solutions in the dark, and their titer was determined spectrophotometrically from their absorbance at the appropriate wavelengths (Estornell et al., 1993); in particular, the wavelengths and extinction coefficients, respectively, were as follows: CoQ_1 and the other CoQ homologs, 275 nm and 13.7 mM^{-1} cm^{-1}; DB and PB, 278 nm and 14 mM^{-1} cm^{-1}; DQ, 270 nm and 19.2 mM^{-1} cm^{-1}; and CoQ_0, 263 nm and 13.6 mM^{-1} cm^{-1}.

Inhibitors were purchased commercially when available; rotenone, antimycin A, and myxothiazol were from Sigma. Mucidin was a kind gift from Dr. J. Subik, University of Bratislava, Slovakia.

Preparative Procedures. Beef heart mitochondria (BHM) were obtained by a large scale procedure (Smith, 1967) and submitochondrial particles (SMP) therefrom by sonic irradiation of the frozen and thawed mitochondrial preparation (Beyer, 1967) in a MSE sonifier at 30 s intervals for a total time of 5 min; the particles were essentially broken membrane fragments (Fato et al., 1993). Alternatively, when the presence of well-coupled closed particles was required, ETP_H were prepared by the method of Hansen and Smith (1964).

All preparations were kept frozen at −80 °C; SMP were used after thawing once, while BHM were frozen and thawed two to three times before use (unless otherwise specified). It was shown that under these conditions the permeability barrier for NADH was completely lost, as demonstrated by the lack of further stimulation by detergents.

CoQ-depleted mitochondria were prepared by pentane extraction after lyophilization (Szarkowska, 1966), and reconstitution was accomplished as described by Estornell et al. (1992) after the method of Norling et al. (1974).

Phospholipid vesicles were prepared from dry phospholipids by sonic irradiation as described by Fato et al. (1986).

Analytical Determinations. Protein was evaluated by the biuret method of Gornall et al. (1949) with addition of 10% sodium deoxycholate and using bovine serum albumin as standard; lipid phosphorus was assayed by the micromethod of Marinetti (1962), and the content of phospholipids was calculated by multiplication of the phosphorus content by 25.

The content of cytochromes was evaluated according to Vanneste (1966) in a JASCO 7850 UV−vis spectrophotometer using the following wavelength couples and extinction coefficients (reduced minus oxidized): cytochrome *b*, 561−575 nm and 25 mM^{-1} cm^{-1}; and cytochromes *c* and c_1, 550−540 nm and 20 mM^{-1} cm^{-1}. The content of complex III was evaluated by division of cytochrome *b* concentration by 2. The FMN content was assayed by the high-performance liquid chromatography (HPLC) method of Light et al. (1980) after water extraction at 80 °C according to Yagi (1971), using a Nova-Pak C_{18} column, 3.9 × 150 mm (Waters); the mobile phase was 20% methanol in water, containing 5 mM ammonium acetate (pH 6), at a flow rate of 0.8 mL/min, in a Waters Millennium 2010 Chromatography Manager equipped with a Waters 996 Photodiode Array Detector. Alternatively, a Perkin-Elmer 1020 Chromatography System with a fluorimetric detector LC 240 at λ_{ex} = 450 nm and λ_{em} = 530 nm was employed, with identical results. Since the FMN content overevaluates complex I content (Smith et al., 1980), the latter was routinely calculated from the NADH ferricyanide assay (see later).

The content of endogenous CoQ_{10} was analyzed by reverse phase HPLC analysis as above after methanol/light petroleum extraction as previously described (Estornell et al., 1992).

Enzyme Assays. Ubiquinol−cytochrome *c* reductase activity was measured as described elsewhere (Fato et al., 1993) and NADH−cytochrome *c* reductase according to Battino et al. (1991). Oxygen uptake linked to NADH oxidation was determined with a Clark oxygen electrode (Estabrook, 1967). Aerobic ubiquinol oxidation was determined by following the increase of absorption at 275 nm due to ubiquinol oxidation using an extinction coefficient of 12.5 cm^{-1} mM^{-1} (oxidized minus reduced).

NADH−CoQ reductase was assayed essentially as described by Yagi (1990) and modified by Degli Esposti et al. (1993), except that only 2 mM KCN was routinely added in the assay mixture to block electron flow through cytochrome oxidase; unless otherwise specified according to the design of the individual experiments, antimycin A (4.6 μM) was also included in the reaction mixture.

Determination of the kinetic constants, unless otherwise specified, was accomplished at quasi-saturating concentrations of NADH (75 μM) and varying ubiquinone concentrations. Since ubiquinones interact with the enzyme active site from within the lipid bilayer, their concentrations in the

lipid bilayer were expressed on a molar basis using the method of Fato et al. (1988) for ubiquinol interaction with complex III; this method allows for the calculation of the true K_m for the quinone [K_m(Q)] and k_{min} ($=k_{cat}/K_m$), the minimum association rate constant of complex I with its quinone substrate. The method employs steady-state kinetic measurements at varying membrane fractional volumes in the assay medium; alternatively, the concentrations of ubiquinones corresponding to the true K_m in the lipid phase were calculated using the partition coefficients previously established (Battino et al., 1986; Fato et al., 1986) or investigated as described below.

NADH−ferricyanide reductase activity was assayed at 420 minus 500 nm in a Sigma Biochem ZWS2 dual wavelength spectrophotometer, with an extinction coefficient of 1 mM^{-1} cm^{-1}, at 30°, with 150 μM NADH and variable amounts of potassium ferricyanide in the same buffer as that used for NADH−CoQ reductase activity.

The specific activity of ferricyanide reductase with 2 mM potassium ferricyanide was used to estimate the content of active complex I in the membrane by considering half of the turnover maximum [8×10^5 min^{-1} (Cremona & Kearney, 1964)], since the concentration of ferricyanide was approximately equal to the K_m [cf. Smith et al. (1980) and Degli Esposti et al. (1994)].

Biophysical Determinations. The partition and diffusion coefficients of ubiquinone analogs and homologs in phospholipid vesicles or mitochondrial membranes were simultaneously determined by fluorescence quenching of either 12-(9-anthroyl)stearic acid (12-AS), pyrene, pyrene-PC_6, or pyrene-PC_{10}, using the procedure described in detail by Fato et al. (1986) using 12-AS with corrections for two-dimensional diffusion (Cavazzoni et al., 1993), using the following excitation and emission wavelengths: 12-AS, 366 and 443 nm; and pyrene and its derivatives, 342 and 393 nm, respectively.

The fluorescence-quenching experiments were performed using both static fluorescence in a Jasco FP 777 spectrofluorometer and time-resolved fluorescence in an IBH Ltd. single photon-counting spectrofluorometer. Fluorescence lifetimes of the different probes were also determined by time-resolved fluorescence.

In the calculation of the membrane concentration of quinones, they were considered to uniformly occupy the entire volume of the membrane phospholipids, assumed to have an average density of 1 mg/μL; the mass of the protein was considered to be impermeable to quinones, and no correction was applied for any bound form of the quinone, taken to be quantitatively irrelevant. Moreover, no distinction was made between free bilayer and boundary lipids (Lenaz & Parenti Castelli, 1985) as a solvent for ubiquinones.

Partition coefficients in cyclohexane/water were evaluated from data in the literature or calculated therefrom (Ragan, 1978; Battino et al., 1986; Fato et al., 1986; Braun et al., 1986; Rich & Harper, 1990).

The critical micelle concentrations (cmc) of the various ubiquinones were calculated from the partition coefficients by applying the equation (Hill, 1974; Ragan, 1978)

$$PS = 2$$

where P is the partition coefficient (in units of mole fractions) and S is solubility in moles of quinone per mole of water. The values obtained were rearranged to obtain solubility values in moles of quinone per liter of water and reported as the logarithms thereof. Alternatively, cmc were determined spectrophotometrically as described by Battino et al. (1986).

RESULTS

Screening of NADH−CoQ Reductase Activity with Various Acceptors. NADH−CoQ reductase has been assayed at quasi-saturating concentrations of NADH and of different quinone acceptors, previously determined by rate inspection at different acceptor concentrations. Since a general screening of various quinones as acceptors was reported in a previous publication (Estornell et al., 1993), these data are not included herein. The kinetics was linear using CoQ_1, PB, and DQ, although the rate was more than double using CoQ_1 and PB compared to that using DQ, whereas DB systematically showed a nonlinear behavior irrespective of concentration, approximating first-order kinetics; nonlinear kinetics was also observed with CoQ_2 and higher homologs.

In a number of different preparations of BHM and SMP examined, the rates were consistently higher in the SMP preparations with all acceptors, with a rate ratio approaching 2. It is noteworthy that, in spite of their similar hydrophobicity, CoQ_2 was a much poorer acceptor in comparison with DB, whereas CoQ_1 and PB elicited comparable rates, in the range of 1 μmol min^{-1} mg^{-1} or higher in the SMP. Intact phosphorylating particles, ETP_H, exhibited rates in the high range but not significantly different from those of SMP.

Under the conditions of our experiments, all quinones employed, except CoQ_0, were found to accept electrons at or close to the physiological site, as shown by the almost total (>90%) inhibition by rotenone. The rotenone sensitivity using CoQ_0 was variable (30−80%) but always significantly lower than with other quinone acceptors [cf. Table 1 in Estornell et al. (1993)].

We have also measured, for comparison, the rates of reduction of potassium ferricyanide and DCIP; as it is known, these compounds do not act at or near the physiological CoQ site. Accordingly, their reduction was completely insensitive to rotenone. The rate of ferricyanide reduction was several times that of the most effective quinone acceptor (CoQ_1) (in the range of 20−25 μmol min^{-1} mg^{-1} in BHM and 40−50 μmol min^{-1} mg^{-1} in SMP).

The nonlinear behavior of the more hydrophobic homologs and analogs (DB and CoQ_2) may be the result of their low water solubility, preventing monomeric dispersion in the assay medium and hence attainment of the actual saturating concentrations; accordingly, addition of detergents or of mixed sonicated phospholipids to the assay medium enhanced the rate of DB reduction and yielded linear kinetics [cf. Table 1 in Estornell et al. (1993)].

We have determined the critical micelle concentrations from the partition coefficients (cyclohexane/water) of the quinone analogs employed in this study (Table 1); while CoQ_1 and PB were presumably monomeric in the concentration ranges employed, CoQ_2 formed micelles above 2 μM, and DB polymerized well below micromolar concentrations. The consequence is that ubiquinones at concentrations above cmc were not fully suitable as substrates of the enzyme because the micelle-to-monomer transition was rate-limiting with respect to quinone reduction.

Table 1: Partition Coefficients and Critical Micelle Concentration (cmc) of Various Quinones

homolog or analog	log *P* of cyclohexane/water[a]	log cmc		log *P* of membrane/water[d]
		from *P*[b]	direct[c]	
CoQ_0	0.39	0.04	−	−
DQ	2.45	−2.0	−	−
CoQ_1	2.65	−2.2	−	2.9
PB	3.7	−3.3	−	3.6
CoQ_2	5.1	−5.7	−4.8	4.0
DB	7.2	−7.8	−	4.7
CoQ_3	7.7	−8.3	−7.0	4.8

[a] Partition coefficient in (moles of Q per liter of cyclohexane)/(moles of Q per liter of water) taken or calculated from the literature (Braun et al., 1986; Rich & Harper, 1990). [b] Solubility $s = 2/P$ (Hill, 1974; cf. Materials and Methods) using the *P* values from column 2 and expressed in moles per liter. [c] From the wavelength shift (Fato et al., 1986; Battino et al., 1986). [d] From fluorescence quenching of either 12-AS, pyrene, or pyrene-PC_6 and PC_{10} probes, with closely overlapping values, according to Fato et al. (1986). Data expressed in (moles of Q per liter of phospholipids)/(moles of Q per liter of water).

The membrane/water partition coefficients of CoQ_1 and PB, determined by fluorescence quenching of different probes (Fato et al., 1986) agreed with the cyclohexane/water corresponding values, but more hydrophobic quinones were strongly underevaluated because their partition from water to the membrane competed with their micellization in water [cf. Fato et al. (1986)].

A slight rate increase in the presence of phospholipids was also observed using more hydrophilic quinones such as CoQ_1 [cf. Table 1 in Estornell et al. (1993)], for which no solubility problem exists up to millimolar concentration (Ragan, 1978); in this case, it is likely that phospholipids act by removing some lipophilic inhibitor, presumably free fatty acids, as their effect was mimicked by bovine serum albumin addition in the assay medium.

Quinone Homologs as Inhibitors of Complex I. As reported in previous publications [e.g. Landi et al. (1984)], short-chain ubiquinone homologs, besides being electron acceptors, also inhibit complex I activity. The kinetic features of CoQ_2 in comparison with those of DB, which has the same number of carbon atoms, are displayed in Figure 1. As an acceptor of NADH−CoQ reductase (Figure 1A), CoQ_2 showed an affinity comparable with that of DB, with K_m in the range of 1 μM, but a much lower V_{max}.

On the other hand, CoQ_2, but not DB, progressively decreased the rate of electron transfer from NADH to CoQ_1 to values corresponding to those obtained with CoQ_2 alone as acceptor. This may be interpreted to indicate that, under the conditions employed, CoQ_2 behaves as a competitive poorer substrate in comparison to CoQ_1. However, particularly at low concentrations (<4 μM), CoQ_2 behaved as a pure competitive inhibitor with respect to CoQ_1 (Figure 1B), with a K_i of 1.2 μM. The inhibitory effect was quantitatively the same using reduced CoQ_2 (Figure 1C). Moreover, NADH−cytochrome *c* reductase and NADH oxidase were inhibited by CoQ_2 but not by DB (Figure 1D), indicating that the former is also a competitive inhibitor of endogenous CoQ_{10}.

Qualitatively similar results were obtained using either CoQ_2 or CoQ_3 [for CoQ_3, cf. Landi et al. (1984)], although the lower solubility of the latter compound made the use of this homolog less reproducible than that of CoQ_2. It is worth noting that the partition coefficient of CoQ_3 is very close to that of DB, which is not an inhibitor (see above).

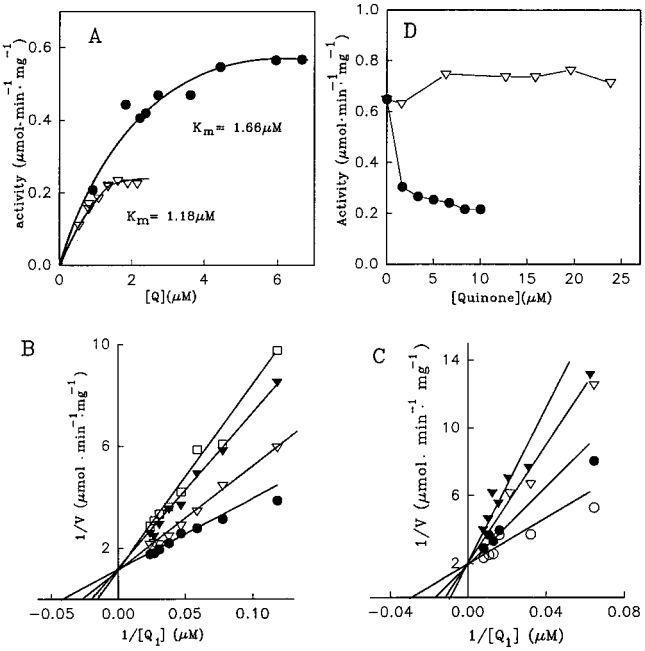


FIGURE 1: Inhibitory effect of CoQ_2 on NADH oxidation in SMP. (A) NADH−CoQ_2 reductase (▽) and NADH−DB reductase (●) were assayed using different quinone concentrations, as described in Materials and Methods. (B) Effect of CoQ_2 on NADH−CoQ_1 reductase: (●) control, (▽) 0.8 μM CoQ_2, (▼) 1.5 μM CoQ_2, and (□) 3.0 μM CoQ_2. (C) Effect of reduced CoQ_2 on NADH−CoQ_1 reductase: (○) control, (●) 0.8 μM reduced CoQ_2, (▽) 1.6 μM reduced CoQ_2, and (▼) 2.4 μM reduced CoQ_2. (D) Effect of CoQ_2 (●) and DB (▽) on aerobic NADH oxidation.

Effect of Complex III Inhibitors. We have investigated whether and to what extent sites in complex III are involved in CoQ reduction, using specific inhibitors of complex III. We have previously shown (Degli Esposti et al., 1993) that center o [for notation, cf. Berry and Trumpower (1985)] inhibitors also inhibit complex I, although to different extents depending on which inhibitor is used. When the redox interaction of cytochrome *b* with exogenous quinones was completely blocked, NADH−CoQ_1 reductase was strongly inhibited by further additions of myxothiazol, while mucidin has little effect. On the other hand, antimycin A had little effect on complex I activity.

We have therefore chosen to use antimycin A as diagnostic of center i and mucidin to block electron transfer at center o. The combination of antimycin and mucidin represents the so-called "double kill", by which no electron transfer to the *b* cytochromes is possible (Berry & Trumpower, 1985).

Table 2 shows the effect of the inhibitors on NADH oxidation with a series of quinone acceptors; in these experiments, complex III inhibitors were present only as indicated in the headings. The results are not identical when mitochondria or submitochondrial particles are used, particularly using DB, but show a general trend of partial inhibition with either antimycin or mucidin and further inhibition by addition of both inhibitors. The extent of inhibition was somewhat different with different acceptors, presumably reflecting their relative affinities for complex I and complex III. Considering one individual acceptor (e.g. duroquinone, cf. lines 4 and 5 in column 2 in the table),

Table 2: NADH−CoQ Oxidoreductase Activity in Beef Heart Mitochondria (BHM) and Submitochondrial Particles (SMP) in the Presence of Inhibitors of the bc_1 Complex[a]

		residual activity %			
acceptor (μM)	relative rate	4.6 μM antimycin A	1.5 μM mucidin	antimycin A + mucidin	1.5 μM myxothiazol
BHM					
CoQ_0 (100)	0.4	71 ± 15	77 ± 11	45 ± 12	92
CoQ_1 (50)	1	85 ± 3	85 ± 9	58 ± 11	20
CoQ_2 (30)	0.2	95 ± 18	83 ± 6	69 ± 7	65
DQ (100)	0.3	51 ± 19	76 ± 21	62 ± 21	10
DB (40)	0.5	70 ± 32	72 ± 30	48 ± 21	55
SMP					
CoQ_0 (100)	0.3	79 ± 11	77 ± 16	63 ± 12	45
CoQ_1 (50)	1	83 ± 10	86 ± 10	63 ± 6	30
CoQ_2 (30)	0.3	64 ± 14	85 ± 19	67 ± 14	67
DQ (100)	0.5	80 ± 10	77 ± 19	45 ± 6	36
DB (40)	0.6	100 ± 9	91 ± 12	94 ± 8	57

[a] Data are means ± the standard deviation of three to five different preparations, except in the last column (myxothiazol) where the data given for comparison are the means of two determinations only [cf. Degli Esposti et al. (1993) and illustrations therein].

lowering electron transfer through complex I by rotenone enhanced antimycin inhibition.

The rate of electron transfer in the presence of both antimycin and mucidin may be taken as a measure of the real complex I activity with a given quinone acceptor; the rate in the presence of both inhibitors was usually lower than in the presence of antimycin alone, indicating that both center i and center o are involved in an appreciable way in quinone reduction.

Table 2 also reports for comparison the effect of myxothiazol, confirming its inhibitory effect on complex I; an apparent exception was CoQ_0 reduction in BHM (but the reaction was partly rotenone-insensitive) and CoQ_2 reduction in both BHM and SMP (but CoQ_2 is also a complex I inhibitor; cf. above).

Saturation Kinetics of NADH−CoQ$_1$ Reductase. For reasons outlined above, the steady-state kinetics of NADH−CoQ reductase was investigated more systematically using CoQ_1 as electron acceptor.

Double reciprocal plots of NADH−CoQ_1 reductase using CoQ_1 as the variable substrate at fixed concentrations of NADH (Figure 2) yielded secondary plots which are shown in the inset of the figure for a SMP preparation.

The parallel straight lines of the primary plots conform to a ping-pong mechanism (Cleland, 1970). The absolute constants derived from these plots for either BHM or SMP are reported in Table 3.

The concentration of complex I, determined by the ferricyanide assay (cf. Materials and Methods), was in good agreement with the values of Degli Esposti et al. (1994) and considerably lower than the values obtained by FMN determination, in accordance with the finding of Smith et al. (1980); using the concentrations obtained by the former method, the turnover number of NADH−CoQ_1 reductase was 469 s^{-1} in BHM and 498 s^{-1} in SMP.

The second-order rate constants of enzyme reduction by NADH and its oxidation by CoQ_1 ($k_{min} = k_{cat}/K_m$) in SMP were 5.4×10^7 and 2.4×10^7 M^{-1} s^{-1}, respectively. The kinetic pattern of the enzymatic reaction and the significance of the kinetic constants are exhibited in Scheme 1.

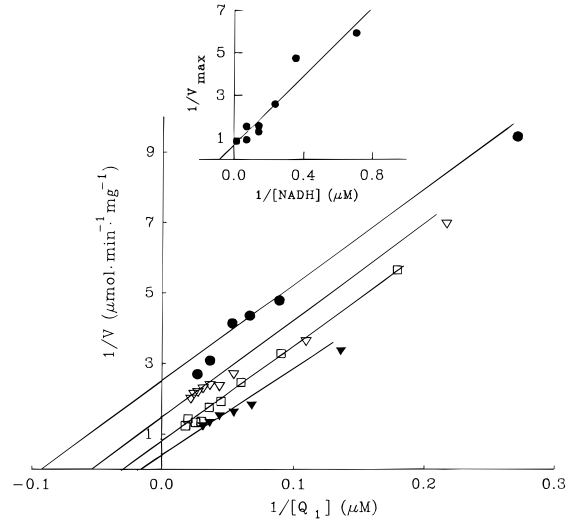


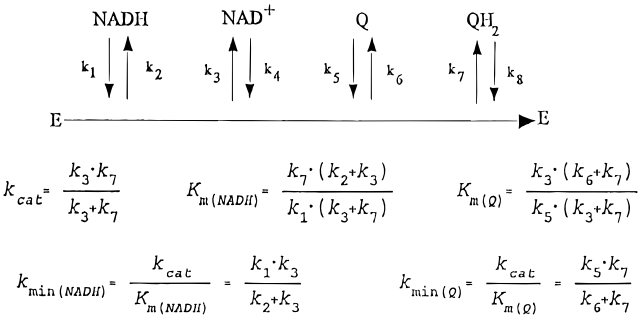
FIGURE 2: Titrations of NADH−CoQ reductase in SMP using CoQ_1 as the variable substrate at the following fixed NADH concentrations: (●) 4.27 μM, (▽) 7.13 μM, (□) 14.19 μM, and (▼) 75 μM. The inset shows the replot of the intercepts obtained from the above titration.

Table 3: Kinetic Constants of NADH−CoQ_1 Reductase in BHM and SMP Therefrom[a]

	BHM	SMP
complex I content (pmol mg^{-1})	27 ± 10	52 ± 9
V_{max} (μmol min^{-1} mg^{-1})	0.76	1.56
k_{cat} (s^{-1})	469	498
K_m (NADH) (M)	8.7×10^{-6}	9.2×10^{-6}
k_{min} (NADH) (M^{-1} s^{-1})	5.4×10^7	4.8×10^7
K_m(Q) (M, in water)	1.9×10^{-5}	2.1×10^{-5}
K_m(Q) (M, in lipids)	1.1×10^{-2}	1.2×10^{-2}
k_{min}(Q) (M^{-1} s^{-1}, in water)	2.5×10^7	2.4×10^7
k_{min}(Q) (M^{-1} s^{-1}, in lipids)	4.3×10^4	4.1×10^4

[a] The values were obtained from secondary plots like those in Figure 2, inserting all data points from three different experiments in the same plots. The correlation coefficient was 0.98 for both BHM and SMP. The k_{cat} values were obtained by division of the V_{max} by the concentration of complex I, calculated by the ferricyanide assay (see Materials and Methods). The true K_m for CoQ_1 in the lipid phase [and the corresponding k_{min}(Q)] was obtained by calculation of the quinone concentration in the lipid phase, Q_m, by the following equation: $Q_m = PQ_{tot}/[P\alpha + (1 - \alpha)]$, where α is the relative volume of the lipid phase with respect to the total assay medium and P is the partition coefficient of CoQ_1 in molar units (from Table 1).

Scheme 1



The true K_m for CoQ_1 in the membrane phase was obtained by using membrane concentrations determined from the partition coefficient of the quinone between the phospholipid and aqueous phase or, alternatively, by titration of the activity with CoQ_1 as acceptor at progressively increased concentrations of phospholipids in the assay medium, according to

Table 4: Kinetic Constants of NADH−CoQ Reductase in Bovine Heart SMP Using Various Acceptors[a]

	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	K_m(Q) in water (μM)	K_m(Q) in lipids (mM)	k_{min}(Q) in water (μM^{-1} s^{-1})	k_{min}(Q) in lipids (mM^{-1} s^{-1})
CoQ_0	0.18	70	65	0.16	1.1	437
CoQ_1	0.98 ± 0.4	380	20.1 ± 5.4	8.9	18.9	43
CoQ_2	0.29	112	1.3	36 (11.5)	86	3.1 (9.7)
CoQ_3	0.17	66	0.8	61 (28)	82.5	1.1 (2.3)
PB	0.93	360	21.0	98	17.1	3.7
DB	0.58 ± 0.15	225	1.8 ± 0.8	138 (120)	125	1.6 (1.9)
CoQ_{10}		441[b]		5[b]		88

[a] Data were obtained from titrations at variable quinone concentrations, keeping NADH concentration at 75 μM. Data for CoQ_1 and DB are means ± the standard deviation of six different titrations, and all other data are means of two titrations. K_m(Q) and k_{min}(Q) in the lipid phase were calculated using the partition coefficient (cyclohexane/water) from Table 1 as explained in the footnote of Table 3. The values in parentheses were calculated using the apparent partition coefficients in the phospholipids obtained from fluorescence-quenching experiments, also taken from Table 1. The content of complex I was 43 pmol mg^{-1} in this SMP preparation. [b] Taken as the maximal value of the turnover number found with CoQ_1 as acceptor (cf. Table 3, and see below and Table 6 for discussion of CoQ_{10} reduction rate according to the pool equation) (Kröger & Klingenberg, 1973). [c] Average value of K_m for CoQ_{10} (expressed as concentration in the lipid phase) of NADH−cytochrome *c* reductase (Estornell et al., 1992), assuming this value to express the K_m of NADH−CoQ reductase, since this activity has lower affinity for CoQ than ubiquinol−cytochrome *c* reductase [cf. also Fato et al. (1993) for discussion thereupon].

the method developed in this laboratory for ubiquinol−cytochrome *c* reductase (Fato et al., 1988).

Plotting the apparent K_m values obtained at different phospholipid concentrations against the phospholipid relative volume with respect to the water phase yielded both the "true" K_m expressed as concentration in the lipid phase and the partition coefficient of CoQ_1. These true K_m values, also shown in Table 3, are in the millimolar range.

Throughout this study, we have usually investigated steady-state enzyme kinetics using CoQ_1 (or other acceptors) as variable substrates at a fixed concentration of 75 μM NADH, which is practically saturating for activity. Using CoQ_1, the apparent K_m was in fair agreement with the values derived from the absolute plots. Table 4 lists the kinetic features concerning the quinone analogs and homologs investigated in this study.

It can be observed that CoQ_1, among all quinones tested, elicits the highest k_{cat}. The K_m values, expressed as concentrations in the water phase, were in general progressively lower with increasing quinone hydrophobicity. However, when they were expressed as concentrations in the lipid phase, all the K_m values for the isoprenoid homologs were similar, especially when the concentrations in the lipid phase were calculated from the effective partition coefficients in the membrane, obtained from fluorescence quenching, and not from the theoretical cyclohexane/water partition coefficients (cf. Table 1); the former, in fact, takes into account the effective membrane concentrations, lower than expected from theory, due to partial micellization of the hydrophobic quinones in water.

The K_m value for CoQ_0, calculated in the lipid phase, was exceptionally low, whereas the K_m values for the alkyl analogs, PB and DB, were 1 order of magnitude higher than those of the isoprenoid homologs.

Accordingly, the k_{min} (k_{cat}/K_m) for CoQ_1, when expressed as lipid concentration, was 1 order of magnitude higher (>4 × 10^4 M^{-1} s^{-1}) than for all other quinones tested, if we exclude CoQ_0, whose k_{min} was 1 order of magnitude higher, due to its exceedingly low K_m. Nevertheless, interpretation of the results with CoQ_0 is questionable, since they were obtained from the low rotenone-sensitive component of the reaction rate (ca. 30%).

An Arrhenius plot of the enzyme activity was linear if care was taken that the concentrations of both substrates were kept quasi-saturating. We had previously shown that a nonlinear Arrhenius plot of ubiquinol−cytochrome *c* reductase was the artifactual result of the strong temperature dependence of the K_m for cytochrome *c* (Fato et al., 1993). On the contrary, in complex I activity, the temperature dependence of K_m for CoQ_1 was very small.

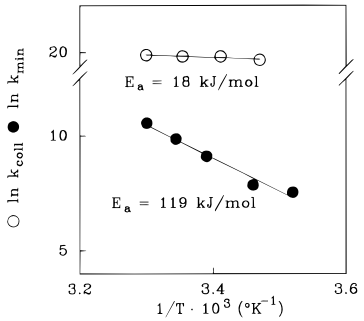


FIGURE 3: Arrhenius plot of k_{min} of NADH−CoQ_1 reductase in beef heart SMP calculated for CoQ_1 concentration in the lipid phase and of the collisional quenching constant (k_{coll}) of CoQ_1 in PL vesicles using as the probe pyrene-PC_6 according to Fato et al. (1986).

The Arrhenius plot of the ratio k_{cat}/K_m for CoQ_1, which approaches the k_{min}, the bimolecular constant of enzyme interaction with the quinone, was linear and yielded an activation energy of 83 kJ mol^{-1}; this value increased to 119 kJ mol^{-1} when E_a was calculated for CoQ_1 concentration in the lipid phase (Figure 3). The corresponding E_a for CoQ diffusion, obtained from Arrhenius plots of the bimolecular collision constants of CoQ_1 in the membrane, exploited by fluorescence quenching by the quinone of the membrane probe pyrene, was 18 kJ mol^{-1}.

Effect of Endogenous Ubiquinone. We have examined the rates of electron transfer to different quinones in lyophilized, pentane-extracted mitochondria and in mitochondria reconstituted with CoQ_{10} after pentane extraction. The extent of extraction was such that the residual CoQ_{10} contents ranged from less than 10 to 40 pmol/mg of protein and were at times low enough to be undetectable.

The enzyme activity was affected differently by CoQ removal, depending upon the nature of the quinone acceptor used. While the rate of electron transfer was reduced maximally by CoQ_{10} removal when DQ was the acceptor (residual rate between 17 and 36% in four different preparations), smaller changes occurred when either CoQ_1 or DB

Table 5: Effect of CoQ Depletion and Reconstitution on the Kinetic Constants of NADH−CoQ_1 and NADH−PB Reductase of Lyophilized BHM[a]

	CoQ_1		PB	
particles	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)
experimental set 1				
lyophilized	0.35 ± 0.23	28.2 ± 14.0(4)	0.37 ± 0.12	25.1 ± 2.3(3)
extracted	0.52 ± 0.19	90.4 ± 15.2(3)	0.51 ± 0.22	107.9 ± 30.8(3)
experimental set 2				
lyophilized (4)	0.19 ± 0.05	24.6 ± 7.23		
extracted (4)	0.21 ± 0.11	55.5 ± 26.06		
reconstituted (CoQ_{10}) (5)	0.21 ± 0.09	21.2 ± 11.26		
reconstituted (CoQ_5)	0.22	26.9		
reconstituted (CoQ_3)	0.20	23.7		

[a] Numbers in parentheses are the number of preparations.

was used (63 and 83%, respectively), indicating that reduction of DQ requires the presence of the CoQ pool and confirming that it largely occurs through complex III (see above).

Determination of the saturation kinetics of NADH−CoQ_1 reductase in pentane-extracted and CoQ-reconstituted mitochondria (Table 5) showed little effect of the endogenous quinone on V_{max}, confirming that the endogenous CoQ pool is not required for electron transfer to the exogenous acceptor. The lyophilization procedure had some effect on the properties of the enzyme, since the V_{max} was significantly decreased with respect to intact mitochondrial membranes. On the other hand, the K_m of the enzyme for either CoQ_1 or PB was significantly increased by CoQ extraction; reconstitution with CoQ_{10} or other homologs restored the original K_m values.

Cytochrome c and Oxygen as Acceptors. Since the maximal rate of CoQ reduction by NADH through complex I in our SMP preparations was ca. 1200 nmol (corresponding to 2400 electron equivalents) min^{-1} mg^{-1}, whereas the maximal rate of ubiquinol oxidation by cytochrome *c* through complex III in the same preparation was ca. 3000 nmol min^{-1} mg^{-1}, the rate of NADH−cytochrome *c* reductase calculated according to the pool equation (Kröger & Klingenberg, 1973) was expected to be slightly over 50% of that of NADH−CoQ reductase (assuming the same V_{ox}/V_{red} ratio occurs at the level of the endogenous CoQ_{10} pool). The same considerations applied for overall aerobic electron transfer in the respiratory chain.

When we compared the activities of NADH−CoQ_1 reductase and NADH oxidase in the same preparations, we observed that the integrated activity was very close to that expected from the application of the pool equation (Table 6), using ubiquinol oxidase as V_{ox}, indicating that V_{red} was correctly evaluated by the NADH−CoQ_1 reductase assay. This once more confirmed that CoQ_1 is a good substrate analog of the endogenous CoQ_{10}.

Table 6: Enzymatic Activities of SMP and Calculation of NADH Oxidation from the Pool Equation[a]

	preparation 1		preparation 2	
	experimental	calculated[b]	experimental	calculated[b]
NADH−CoQ_1	1.19	1.31 (110%)	1.08	1.29 (119%)
ubiquinol 2-O_2	2.25		1.05	
NADH−O_2	0.83	1.01 (122%)	0.58	0.53 (91%)

[a] Each experimental figure is the mean of three different determinations. [b] Calculated according to the pool equation $V_{obs} = V_{ox}V_{red}/(V_{ox} + V_{red})$ taking as unknown either V_{red} (NADH−CoQ_1) or V_{obs} (NADH-O_2). Numbers in parentheses are percentages of calculated with respect to experimental.

DISCUSSION

Suitability of Various Quinones as Acceptors. The kinetic data generated in this study were largely obtained employing either CoQ_1 or PB as the electron acceptor; in fact, these compounds elicited rates comparable to those calculated for reduction of endogenous CoQ_{10} using the pool equation (Kröger & Klingenberg, 1973), and their reduction was almost completely inhibited by rotenone and only marginally affected by complex III center i inhibitors. It was therefore concluded that the bulk of electrons reduces either CoQ_1 or PB at the same rate as CoQ_{10} and reduction occurs at or near the physiological CoQ_{10} site in complex I (cf. discussion below). However, this optimal behavior was not shared by other quinone acceptors, as is summarized below.

(a) CoQ_0 was significantly reduced in a rotenone-insensitive fashion, indicating that it accepts electrons at a site upstream from the flow in the hydrophobic moiety of the enzyme, therefore sharing in part the behavior of water soluble reductants such as ferricyanide.

(b) Duroquinone (DQ) was largely reduced by the cytochrome bc_1 complex, as shown by the high extent of inhibition by antimycin and mucidin. This behavior, already shown for succinate oxidation (Von Jagow & Bohrer, 1975; Chen et al., 1986) and also for NADH oxidation (Zhu & Beattie, 1988; Beattie et al., 1992), indicates that DQ has a low affinity for the CoQ sites in the dehydrogenases.

(c) The saturated-chain analog DB is too hydrophobic to be kept monomeric in aqueous solution even at concentrations much below K_m; for this reason, it could not be reduced with linear kinetics unless phospholipids were added to the reaction medium [cf. Estornell et al. (1993)], indicating that micellar DB does not monomerize at rates comparable to those of its reduction by complex I [cf. Fato et al. (1986) for discussion].

The phospholipid requirement was of particular inconvenience in the kinetic studies we intended to accomplish and prevented the use of DB in our subsequent investigation. In fact, phospholipid addition to the assay medium increased the apparent K_m of complex I for the quinone analog as a result of its partition in the lipids. This K_m increase with phospholipid concentration is common to any hydrophobic substrate and was previously used to calculate the true K_m values of mitochondrial ubiquinol−cytochrome *c* reductase

for the ubiquinol substrates, expressed as substrate concentration in the lipid phase (Fato et al., 1988); however, if phospholipids also change V_{max}, as is the case for NADH−CoQ reductase using DB as acceptor, the interpretation of the data becomes impossible. This drawback was not encountered when the corresponding hydroquinone, DBH_2, was used as an electron donor to the cytochrome bc_1 complex (Degli Esposti & Lenaz, 1991); the water solubility of oxidized quinones is lower than that of the corresponding quinols (Battino et al., 1986; Rich, 1990), so that the problem is much more relevant to complex I activity than to complex III determinations.

(d) CoQ_2 and other short-chain homologs (but not CoQ_1), besides sharing with DB their low water solubility, are also complex I inhibitors (Lenaz et al., 1975; Landi et al., 1984), making their use unsuitable for kinetic studies.

It is important to point out that the optimal behavior of CoQ_1 and PB in the assay of NADH−CoQ reductase, verified in bovine heart mitochondrial membranes, is not shared by all biological systems investigated. For example, in rat liver mitochondria or in platelet-derived mitochondrial membranes, CoQ_1 reduction has a strong rotenone-insensitive component (Lenaz et al., 1995) so that DB is a more suitable acceptor. Even in beef heart submitochondrial particles (Schatz & Racker, 1966) and in purified complex I from bovine heart (Ragan, 1978), a relatively high rotenone-insensitive rate with CoQ_1 was found in the absence of added phospholipids, but it was significant only at high quinone concentrations. In our study, the rotenone-insensitive rate was not significant in beef heart mitochondrial membranes.

Short-Chain CoQ Homologs as Inhibitors of Complex I. A study of the organic structural specificity of CoQ homologs in NADH and succinate oxidation (Lenaz et al., 1968) demonstrated that only CoQ homologs containing more than five isoprenoid units are fully active in restoration of NADH oxidase in CoQ_{10}-depleted mitochondria from bovine heart, while all homologs are active in succinate oxidase; this selectivity for NADH oxidation was not present in mitochondria from *Saccharomyces cerevisiae* (Lenaz et al., 1971). It was subsequently found that short-chain homologs inhibit NADH oxidation competitively with CoQ_{10} (Lenaz et al., 1975) and that this inhibition is partly associated with the rotenone-binding site (Landi et al., 1984).

Results of the present study have revealed that CoQ_2, but not DB, inhibits complex I activity, as shown by the competitive inhibition of CoQ_1 reduction in the NADH−CoQ_1 assay and by the hyperbolic decrease of NADH oxidation rate (Figure 1D). The K_i for CoQ_2 as an inhibitor (Figure 1B) was identical to its K_m as an acceptor substrate (Table 4). On the other hand, DB, which is not an inhibitor, has a K_m for complex I comparable with that of CoQ_2 (Table 4), suggesting that the binding affinities of the two quinones to the complex are almost identical. The inhibitory effect of CoQ_2 was not related to its hydrophobicity, since CoQ_2 is actually less hydrophobic than DB (Table 1). Since reduced CoQ_2 is as inhibitory as the oxidized form (Figure 1C), and considering that CoQ_2 is an electron acceptor, though poor, from the complex, it is reasonable to suggest that the inhibitor is the reduced form of this homolog.

The effect of a chemical difference between a diprenyl and a decyl side chain in the 6-position of the benzoquinone on V_{max}, but not on K_m, suggests that the isoprenoid chain of CoQ_2 is not correctly positioned at the active site for optimal electron transfer.

These observations suggest that exogenous quinones replace the endogenous CoQ_{10} in one or both of its binding sites and participate in the electron transfer reactions inherent to the endogenous quinone; nevertheless, bound CoQ_2 is unable to elicit the same rates of electron transfer of either the physiological CoQ_{10} or DB. Presence of a trans double bond and/or of branching in a ten-carbon side chain at the 6-position of the benzoquinone ring is sufficient to dramatically alter the mode of electron transfer in the complex.

On the other hand, CoQ_1 and PB, which correspond to CoQ_2 and DB, respectively, with respect to the five-carbon side chain, are comparable in eliciting the highest V_{max} (Table 4), indicating that the inhibitory action requires a chain with at least two isoprenoid units. The previous finding that only ubiquinones having six or more isoprenoid units are able to restore NADH oxidation in pentane-extracted mitochondria (Lenaz et al., 1968, 1971) also indicates that the inhibitory action of isoprenylquinones on complex I ranges between two and five isoprenoid units.

Electron Withdrawal by Quinones at the Level of Complex III. The respiratory chain contains quinone reduction sites downstream from the dehydrogenases, discovered as early as 1970 in NADH oxidation (Ruzicka & Crane, 1970b); in particular, Mitchell's Q-cycle (Mitchell, 1975) predicts a physiological reduction site for CoQ in complex III at the so-called center i. It is therefore expected that exogenous quinones are also able to receive electrons from center i in addition to the acceptor site(s) in complex I; moreover, center o in the cytochrome bc_1 complex is also a potential interaction site by virtue of reversal of the enzyme reduction by ubiquinol [cf. Berry and Trumpower (1985)].

The location of quinone interaction sites in the respiratory chain is assessed by using specific inhibitors whose binding localization is known (Zhu & Beattie, 1988). Thus, the validity of the interpretations derived from inhibitor effects on electron transfer pathways is dependent upon knowledge and specificity of the inhibitor binding site.

The reduction of water soluble quinones at center i and also at center o was suggested by a number of experiments (Zhu & Beattie, 1988; Beattie et al., 1992) using antimycin A and/or myxothiazol to block electron flow within complex III pathways. The finding that myxothiazol is a significant inhibitor of complex I (Degli Esposti et al., 1993), confirmed in this study, reopens the interpretation of those results. The observation that mucidin (Rieske, 1980), which does not significantly inhibit complex I (Degli Esposti et al., 1993), partly inhibits DQ reduction supports the involvement of center o. The combined use of antimycin A and mucidin represents the optimal means of obtaining the double kill, whereby electrons are prevented from reaching complex III via complex I, in studies where the electron donor is NADH (Berry & Trumpower, 1985). The additive inhibition of quinone reduction by antimycin A alone and by antimycin A in combination with mucidin demonstrates that both centers i and o behave as quinone reduction sites in complex III.

The preferential reduction of DQ by complex III with respect to complex I [or complex II, cf. Von Jagow and Bohrer (1975) and Chen et al. (1986)] is not due to thermodynamic reasons, as the midpoint potential of the DQ/DQH_2 couple is slightly lower than that of ubiquinone/

ubiquinol (Boveris et al., 1972); thus, it must reflect a different steric requirement of the two sites.

The involvement of complex III in quinone reduction may depend on the extent to which the exogenous quinone competes with endogenous CoQ_{10} for the reduction site in the dehydrogenase. If the exogenous quinone competes strongly with CoQ_{10}, then it would be preferentially reduced by the dehydrogenase; otherwise, the electrons would be transferred to complex III by the endogenous pool and find additional reduction sites for the exogenous acceptor. DQ is a poor acceptor for complex I, and endogenous CoQ_{10} would compete such that 50% of the electrons would still reach complex III through the pool. Our interpretation is that reduced CoQ_{10} is oxidized by center o through the physiological pathway in the absence of mucidin and directly by center i in the presence of mucidin, enabling center i to act as a transhydrogenase between reduced CoQ_{10} and oxidized DQ [cf. Weiss (1987)].

Kinetics of NADH−CoQ Reductase. In agreement with the previous study of Dooijewaard and Slater (1976), who used ferricyanide and DCIP as acceptors, the NADH−CoQ reductase reaction catalyzed by complex I using CoQ homologs as acceptors follows a ping-pong kinetic mechanism, whereby the enzyme is first reduced by aqueous NADH with release of NAD^+ and then reoxidized by ubiquinone with release of ubiquinol in the lipid phase (cf. Scheme 1).

The overall turnover number of the enzyme (two electrons from NADH to CoQ), calculated on the basis of complex I content, was comparable with that of 500 s^{-1} calculated for DCIP reduction (Dooijewaard & Slater, 1976). When calculated for one-electron transfer, it was also closely comparable to that of complex III, which was about 700 s^{-1} under optimal conditions for one-electron transfer from ubiquinol to ferricytochrome *c* (Degli Esposti & Lenaz, 1991), but usually lower (average 370 s^{-1}) in mitochondrial membranes (Fato et al., 1993).

Since complex I content in bovine heart mitochondria is much less than that of complex III (Capaldi, 1982; Fato et al., 1993; Degli Esposti et al., 1994; this study), the specific activities are such that complex I is usually only slightly rate-limiting [cf. Gutman (1985)]. This is reflected by the pool equation (Kröger & Klingenberg, 1973), showing that the overall rate of electron transfer is approximately half the rate of either ubiquinol oxidation or ubiquinone reduction (Table 6). The close correspondence between the experimental rate of ubiquinone reduction and that calculated from the pool equation indicates that, in bovine heart SMP, the complex I assay is highly reliable if CoQ_1 is employed as acceptor [cf. Estornell et al. (1993)].

This is not necessarily true for other types of mitochondria, where all acceptors employed, including CoQ_1, elicit complex I activities much lower than those predicted by the pool equation and, in addition, may have a high degree of rotenone-insensitive activity (Lenaz et al., 1995).

Absence of Diffusion Control. The possible presence of a diffusion-limited step in the bimolecular reaction of CoQ with the enzyme *in situ* is amenable to experimental investigation using CoQ_1 as acceptor, since the mode of interaction of this quinone and its reduction turnover are comparable with those of the physiological CoQ_{10} (see above).

The parameter k_{cat}/K_m, the minimum second-order rate constant of the reaction of an enzyme with a substrate, reflects the rate constants of all processes up to, and including, the first irreversible step of the reaction (Hardy & Kirsch, 1984). It is expected, in the case of a diffusion-controlled enzymatic reaction, that substrate analogs yielding lower turnovers and lower association rate constants must result in less strongly diffusion-limited rates (Brouwer & Kirsch, 1982; Bazelyansky et al., 1986).

The present study has shown that the k_{cat} of NADH−CoQ reductase is maximal using CoQ_1 (or PB) and is approached by that of DB under its optimal conditions of use; however, the K_m is 1 order of magnitude lower for DB, making k_{min} (k_{cat}/K_m) 1 order of magnitude higher (ca. 10^8 M^{-1} s^{-1} for DB vs 10^7 M^{-1} s^{-1} for CoQ_1). This finding could lead to the conclusion that DB is the preferred substrate. However, if we calculate the kinetic constants taking into consideration the quinone concentration in the lipid phase, which is a function of the partition coefficient between water and lipids (Fato et al., 1988), CoQ_1 is shown to be a better substrate than DB, with a k_{min} of up to 5.5×10^4 M^{-1} s^{-1}, calculated for quinone dissolved in the lipid phase. The latter observation is valid also for PB, since this analog has both K_m and V_{max} on the order of those for CoQ_1 but is considerably more hydrophobic than CoQ_1 (Table 1). Thus, the apparent k_{min} values are quite high when calculated using the quinone concentration in the assay medium and approach values suggesting a diffusion-limited regime; they are, however, considerably lower when calculated as true constants using membrane concentrations.

The corresponding collisional frequency of quinone with complex I can be calculated using the Smoluchowski relation (Smoluchowski, 1917)

$$k_{coll} = 4\pi RDN/1000$$

where R is the encounter distance of the two molecules, taken as the sum of the radii of the two interacting molecules or sites, D is the sum of the diffusion coefficients in cm^2 s^{-1}, and N is Avogadro's number.

Assuming an encounter radius of 8 Å (Fato et al., 1986, 1993) and a diffusion coefficient of 4×10^{-7} cm^2 s^{-1} for CoQ_1 in the membrane (Fato et al., 1993) and assuming complex I to be immobile compared with the diffusion time of ubiquinone (Gupte et al., 1984), the k_{coll}, calculated from the Smoluchowski relation, is 2.5×10^8 M^{-1} s^{-1}.

The collisional frequency of ubiquinone with complex I in the membrane is therefore 4 orders of magnitude greater than the minimum bimolecular association rate constant, making it rather unlikely that the reaction is diffusion-controlled. This conclusion is further supported by the very high activation energy of k_{min} for CoQ_1 (119 kJ mol^{-1}, compared with 18 kJ mol^{-1} for diffusion expressed as the collisional frequency of quinone measured by quenching of pyrene; cf. Figure 3).

The steep temperature dependence of k_{min} results from the very low temperature coefficient of the true K_m for CoQ_1; the constancy of the K_m for quinone, within the temperature range considered, results in an activation energy for k_{cat} very similar to that for k_{min}. The high activation energy of complex I activity agrees with previous studies (Heron et al., 1979; Poore & Ragan, 1982) and suggests that a chemical

step having a high activation energy is rate-limiting in the enzyme mechanism.

This conclusion reached using exogenous CoQ_1 can be extended to endogenous CoQ_{10}, since the rate of CoQ_1 reduction is equal to that rate of reduction of the endogenous CoQ pool (this study) and the diffusion coefficient of CoQ_{10} is almost the same as that of CoQ_1 (Fato et al., 1986). Assuming a K_m for CoQ_{10} of NADH−CoQ reductase of 5 mM in the lipid phase (Estornell et al., 1992; cf. also footnote in Table 4), the k_{min} with CoQ_{10} of NADH−CoQ reductase would still be less than 10^5 M^{-1} s^{-1}.

It is therefore concluded that NADH−CoQ reductase is not subjected to diffusion control; since the same conclusion was previously reached for ubiquinol−cytochrome *c* reductase (Fato et al., 1993), it is strongly suggested, though not proven, that the operation of the respiratory chain is not under a regime of diffusion control, consistent with our previous interpretation (Lenaz, 1988) and contrary to the conclusions reached by Chazotte and Hackenbrock (1988).

Effect of Endogenous Ubiquinone. It has been demonstrated that exogenous CoQ_1 accepts electrons directly from complex I in pentane-extracted mitochondria without the involvement of the CoQ pool (Cabrini et al., 1981); the presence of tightly bound CoQ_{10} in complex I (Degli Esposti & Ghelli, 1994; Singer & Ramsay, 1994) means, however, that exogenous CoQ is likely to accept electrons from a site containing the tightly bound CoQ molecules.

Using a variety of quinones as acceptors, the rates of reduction by NADH were differentially affected by removal of the CoQ pool. The rate of electron transfer was decreased most significantly when using those acceptors whose reduction is inhibited by center i inhibitors (vz. DQ), confirming the observation that endogenous CoQ_{10} of the pool efficiently competes with them for electron withdrawal from complex I.

No evidence has been found in this study that endogenous CoQ_{10} is required for electron transfer to the exogenous CoQ acceptor, although the presence of residual amounts of CoQ_{10} in the enzyme might be responsible for such activity. Since, however, V_{max} is routinely the same in lyophilized mitochondria before CoQ extraction and in extracted mitochondria lacking any detectable CoQ (less than 10 pmol/mg of protein; therefore substoichiometric with complex I), it is most likely that exogenous CoQ molecules substitute for the endogenous CoQ_{10} in the binding site(s) and interact with additional exogenous molecules in place of the CoQ_{10} pool.

This complex behavior is also in agreement with the strong reversible increase of K_m for exogenous quinones upon removal of endogenous CoQ_{10}. A simple interaction of the exogenous quinone directly with a protein site would exhibit competitive inhibition by endogenous CoQ_{10} or by CoQ_{10} upon reconstitution; this pattern is actually exhibited by succinate−CoQ_1 reductase where the K_m for CoQ_1 is decreased by CoQ extraction (Lenaz et al., 1994). The opposite behavior in the case of NADH−CoQ reductase may be interpreted to mean that the quinone active site is different when the endogenous CoQ_{10} is removed. This effect may make it difficult to observe a possible competition.

The fact that CoQ_1 is poorly reduced at center i of the bc_1 complex is direct proof that endogenous CoQ_{10} is not an efficient competitive acceptor at the concentrations at which the exogenous quinone is added. Assuming a K_m for CoQ_{10} of complex I approaching that found by Estornell et al. (1992) for CoQ_{10} in NADH−cytochrome *c* reductase, i.e. 5 mM in the lipids, and a K_m for CoQ_1 of 10 mM in the lipid phase (this study) and using the actual membrane concentrations of the two quinones calculated under standard assay conditions (50 mM CoQ_1 and 5 mM CoQ_{10}), we calculate that over 70% of the electrons are channeled to reduce exogenous CoQ_1. The slight competition of CoQ_{10} with respect to CoQ_1, however, is overwhelmed by the change of nature of the binding site when CoQ_{10} is present, so that the K_m for the exogenous acceptor is actually decreased.

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