

The specificity of mitochondrial complex I for ubiquinones

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We report the first detailed study on the ubiquinone (coenzyme Q; abbreviated to Q) analogue specificity of mitochondrial complex I, NADH:Q reductase, in intact submitochondrial particles. The enzymic function of complex I has been investigated using a series of analogues of Q as electron acceptor substrates for both electron transport activity and the associated generation of membrane potential. Q analogues with a saturated substituent of one to three carbons at position 6 of the 2,3-dimethoxy-5-methyl-1,4-benzoquinone ring have the fastest rates of electron transport activity, and analogues with a substituent of seven to nine carbon atoms have the highest values of association constant derived from NADH:Q reductase activity. The rate of NADH:Q reductase activity is potently but incompletely inhibited by

rotenone, and the residual rotenone-insensitive rate is stimulated by Q analogues in different ways depending on the hydrophobicity of their substituent. Membrane potential measurements have been undertaken to evaluate the energetic efficiency of complex I with various Q analogues. Only hydrophobic analogues such as nonyl-Q or undecyl-Q show an efficiency of membrane potential generation equivalent to that of endogenous Q. The less hydrophobic analogues as well as the isoprenoid analogue Q-2 are more efficient as substrates for the redox activity of complex I than for membrane potential generation. Thus the hydrophilic Q analogues act also as electron sinks and interact incompletely with the physiological Q site in complex I that pumps protons and generates membrane potential.

INTRODUCTION

Complex I catalyses electron transport from intramitochondrial NADH to the ubiquinone (coenzyme Q; abbreviated to Q) contained within the mitochondrial inner membrane and is the most intricate respiratory enzyme complex present [1–3]. Although complex I is still commonly referred to as NADH dehydrogenase [2], its most characteristic enzymic activity is rotenone-sensitive NADH:Q reductase (EC 1.6.99.3). In complex I, electron transport to Q is coupled to proton pumping which generates membrane potential [1–7], but there is little consensus of opinion on how this coupling occurs [1–3,8–10], even if there seems to be a general agreement on the stoichiometry of two protons per electron transported [1,2,6,7,11–13].

It is clear that the heart of the enzymic mechanism of complex I lies in its reaction with Q [1,2,8,9,13,14]. The site of this reaction is traditionally called the ‘rotenone site’, since rotenone is the classical potent inhibitor of the complex which blocks interaction with Q and proton pumping [1,2,14,15]. However, inhibition by rotenone is incomplete [4,6,16,17]. An increasingly large number of inhibitors seem to act at the rotenone site in mammalian complex I; conversely, the wide variation in the chemical structures of the inhibitors is suggestive of multiple interaction sites in the enzyme complex [2,18–21]. These inhibitory compounds basically act as Q antagonists [2,8,14,18–20,22], which suggests that Q could also react with complex I at different sites. This idea has been sustained by binding studies with rotenone and piericidin A [2,15,16] and by the combined use of Q analogues and inhibitors [18,20,21,23–25].

The concept of multiple binding sites for Q within the intricate structure of complex I seems to be logical because of its complicated sequence of proton and electron transport reactions

and also its functional analogies with the photosynthetic Q-reacting systems [13,26]. To date, however, the experimental evidence supporting this concept has been scant [1,2,18,25,27–30], and there has been no detailed study on the efficiency of exogenous Q analogues in stimulating the energy-conserving function of complex I.

This study presents the first detailed evaluation of the specificity of complex I for Q analogues in coupled mitochondrial particles, and focuses on the energetic function of the complex. The results indicate that mitochondrial complex I possesses multiple quinone-reacting sites with different specificities for the redox and energy-conserving functions of the complex.

MATERIALS AND METHODS

Synthesis and sources of Q analogues

Various analogues of 2,3-dimethoxy-5-methyl-1,4-benzoquinone (Q-0) (I) were synthesized using two different methods depending upon whether a saturated or unsaturated substituent was to be added at position 6 of the ring. The method of Wan et al. [31] was used to prepare the alkyl-Q analogues III, VI, VIII, XI, XII and XIII. The unsaturated Q analogues, IV, V, VII, IX and X, were synthesized by the method of Naruta [32] from Q-0 and allylated tributyltins. The allyltin reagents were prepared by sonication from commercial 3-chloro-1-propene, (*E*)-1-chloro-2-butene and 1-chloro-3-methyl-2-butene, and from (*E,E*)-2,4-hexadien-1-ol as described [33]. Q analogues were purified by flash chromatography and characterized by ¹H (Table 1) and ¹³C NMR, and infrared, ultraviolet/visible (Table 1) and mass spectroscopy. The spectroscopic properties of the new compounds (V, X and XII) were consistent with their expected structures. Only ana-

Abbreviations used: ETP_{IT}, electron transfer submitochondrial particles; P, partition coefficient; Q, ubiquinone (coenzyme Q).

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Table 1 Spectral, physico-chemical and catalytic properties of Q analogues

The maximal velocities of the NADH:Q reductase activity were extrapolated from quinone titrations with 0.15 mM NADH and 0.02–0.04 mg/ml submitochondrial particles, which were treated with both antimycin and mucidin. R is the substituent at position 6 of the benzoquinone ring.

| No. | -R | λ_{\max} UV (nm) | λ_{\max} VIS (nm) | $^1\text{H NMR}$ (CDCl ₃) | Log P (cyclohexane) (water) | NADH:Q reductase activity V_{\max} ($\mu\text{mol min}^{-1} \text{mg}^{-1}$) |
|------------|---|--------------------------|---------------------------|--|-----------------------------|--|
| I | -H Coenzyme Q-0 (Sigma) | 265 | 401 | 2.04, d, 3H, 1.6Hz, Ar-CH ₃ ; 4.00, 4.03, 2s, 2x3H, CH ₃ O; 6.45, q, 1H, 1.6Hz, H-6 | 0.4 | 0.46 |
| II | -CH ₃ methyl-Q (aurantiocladin) (Apin) | 275 | 417 | | 0.7 | 1.64 |
| III | -C ₃ H ₇ propyl-Q | 279 | 411 | 0.96, t, 3H, 7.3Hz, H-3'; 1.45, m, 2H, H-2'; 2.02, s, 3H, Ar-CH ₃ ; 2.45, t, 2H, 7.8 Hz, H-1'; 4.00, 2s, 2x3H, CH ₃ O | 2.3 | 1.31 |
| IV | -CH ₂ CH=CH ₂ 2'-propenyl-Q | 275 | 413 | 2.03, s, 3H, Ar-CH ₃ ; 3.24, d, 2H, 6.3Hz, H-1'; 4.00, 4.01, 2s, 2x3H, CH ₃ O; 5.00-5.05, 5.05-5.10, 2m, 2x1H, H-3'; 5.65-5.85, m, 1H, H-2' | 1.3 | 0.59 |
| V A V B | -CH ₂ CH=CHCH ₃ (E) (60%) -CH(CH ₃)CH=CH ₂ (40%) A: 2'-butenyl-Q B: 1'-methyl-2'-propenyl-Q | 275 | 411 | A: 1.63, dd, 3H, 1.2, 6.1Hz, H-4'; 3.16, d, 2H, 6.1Hz, H-1'; 5.26-5.56, m, 2H, H-2', 3'; B: 1.32, d, 3H, 7.1Hz, CH ₃ CH; 3.80, m, 1H, H-1'; 5.00-5.06, 5.06-5.11, 2m, 2x1H, H-3'; 5.96-6.13, m, 1H, H-2'. A & B: 2.02, 2.05, 2s, 2x3H, Ar-CH ₃ ; 3.99, 4.00, 2s, 2x6H, CH ₃ O | -2.0 | 0.85 |
| VI | -C ₅ H ₁₁ pentyl-Q | 279 | 412 | 0.89, t, 3H, 6.4Hz, H-5'; 1.23-1.47, m, 6H, H-2'-4'; 2.01, s, 3H, Ar-CH ₃ ; 2.45, t, 2H, 7.4Hz, H-1'; 3.99, 4.00, 2s, 2x3H, CH ₃ O | 3.6 | 0.90 |
| VII | -CH ₂ CH=C(CH ₃) ₂ Coenzyme Q-1 (Eisai) | 276 | 416 | 1.68, d, 3H, 1.2Hz & 1.75, s, 3H, C(CH ₃) ₂ ; 2.02, s, 3H, Ar-CH ₃ ; 3.18, d, 2H, 7.1Hz, H-1'; 3.98, 4.00, 2s, 2x3H, CH ₃ O; 4.94, m, 1H, 1.4, 7.1Hz, H-2' | 2.6 | 0.89 |
| VIII | -C ₆ H ₁₃ hexyl-Q | 279 | 410 | 0.89, t, 3H, 6.4Hz, H-6'; 1.23-1.35, m, 8H, H-2'-5'; 2.02, s, 3H, Ar-CH ₃ ; 2.45, t, 2H, 7.3Hz, H-1'; 3.99, 4.00, 2s, 2x3H, CH ₃ O | 4.3 | 1.17 |
| IX | -CH ₂ CH=CHCH=CHCH ₃ (E,E) 2',4'-hexadienyl-Q | 273 | shoulder | 1.72, d, 6.2Hz, 3H, H-6'; 2.03, s, 3H, Ar-CH ₃ ; 3.23, d, 2H, 6.8Hz, H-1'; 3.99, 4.00, 2s, 2x3H, CH ₃ O; 5.40-5.62, 1H, 5.62-5.70, 1H, 5.90-6.11, 2H, 3m, H-2'-5' | 3.2 | ≤0.20 |
| X | -CH(CH ₃)CH=CHCH=CH ₂ (E) 1'-methyl-2',4'-pentadienyl-Q | 275 | shoulder | 1.34, d, 3H, 7.1Hz, CH ₃ CH; 2.05, s, 3H, Ar-CH ₃ ; 3.75-3.95, m, 1H, H-1'; 3.98, 4.00, 2s, 2x3H, CH ₃ O; 4.99-5.20, m, 2H, H-5'; 5.88-6.40, m, 3H, H-2'-4' | 3.0 | 0.31 |
| XI | -C ₇ H ₁₅ heptyl-Q | 279 | 413 | 0.88, t, 3H, 6.5Hz, H-7'; 1.20-1.50, m, 10H, H-2'-6'; 2.02, s, 3H, Ar-CH ₃ ; 2.45, t, 2H, 7.2Hz, H-1'; 3.99, 4.00, 2s, 2x3H, CH ₃ O | 5.0 | 0.59 |
| XII | -C ₈ H ₁₇ octyl-Q | 279 | 411 | 0.88, t, 3H, 6.4Hz, H-8'; 1.20-1.47, m, 12H, H-2'-7'; 2.01, s, 3H, Ar-CH ₃ ; 2.45, t, 2H, 7.1Hz, H-1'; 3.99, 2s, 2x3H, CH ₃ O | 5.7 | 0.44 |
| XIII | -C ₉ H ₁₉ nonyl-Q | 279 | 410 | 0.88, t, 3H, 6.7Hz, H-9'; 1.18-1.50, m, 14H, H-2'-8'; 2.01, s, 3H, Ar-CH ₃ ; 2.45, t, 2H, 7.1Hz, H-1'; 3.99, 4.00, 2s, 2x3H, CH ₃ O | 6.5 | 0.52 |
| XIV | -C ₁₀ H ₂₁ decyl-Q (decylubiquinone) (Sigma) | 279 | 405 | 0.88, t, 3H, 6.7Hz, H-10'; 1.20-1.43, m, 16H, H-2'-9'; 2.01, s, 3H, Ar-CH ₃ ; 2.45, t, 2H, 7.3Hz, H-1'; 3.99, 2s, 2x3H, CH ₃ O | 7.3 | 0.71 |
| XV | -[CH ₂ CH=C(CH ₃)CH ₂] ₂ -H (E) Coenzyme Q-2 (Eisai) | 277 | (420) shoulder | | 5.1 | 0.18 |
| XVI | -C ₁₁ H ₂₃ undecyl-Q (E. Berry, gift) | 279 | 405 | | 8.0 | 0.76 |
| XVII | -[CH ₂ CH=C(CH ₃)CH ₂] ₃ -H (E,E) Coenzyme Q-3 (Eisai) | 275 | shoulder | | 7.7 | 0.29 |

logue V could not be separated into the two isomers A and B (Table 1) and was therefore used as a mixture.

The isoprenoid Q analogues Q-1 (VII), Q-2 (XV) and Q-3 (XVII) were generously provided by Eisai Co., Tokyo, Japan, and the decyl-analogue (XIV) was purchased from Sigma, St. Louis, MO, U.S.A. The undecyl-analogue (XVI) was a gift from Dr. E. Berry, University of California, Berkeley, CA, U.S.A. The methyl-analogue (II) was purchased from Apin Ltd., Glamorgan, U.K. Other chemicals were from Aldrich or Sigma.

Characterization of reagents

Quinones were stored in ethanolic solutions in the dark at

-20 °C. An average absorption coefficient of 14.5 mM⁻¹·cm⁻¹ at 275–280 nm was utilized for determining the concentration of all Q analogues except for Q-0, for which an absorption coefficient of 13.8 mM⁻¹·cm⁻¹ was used at 265 nm [17]. Piericidin A was generously donated by Professor F. L. Crane, Purdue University, West Lafayette, IN, U.S.A., and rolliniastatin-1 and -2 were kindly provided by Professor D. Cortes, University of Valencia, Spain. Mucidin was a gift from Dr. J. Subik, University of Bratislava, Slovakia. Methoxyacrylatestilbene was kindly given by Professor R. A. Gennis, University of Illinois, Urbana-Campaign, IL, U.S.A. The concentrations of all inhibitors were determined spectrophotometrically in absolute ethanol using the reported absorption coefficients (cf. [20] and references therein).

The partition coefficients in cyclohexane/water of the Q analogues were evaluated using the offset parameters reported by Braun et al. [34] or were taken from the literature [35].

Preparations and enzymic assays

Submitochondrial particles from beef heart were prepared using the procedure of Hansen and Smith [36] for electron transfer particles (ETP_H). The particles were stored at -70°C at a protein concentration of 30–60 mg/ml, measured by the biuret assay as previously described [20]. The NADH:Q reductase activity of frozen–thawed ETP_H was assayed at 30°C in 50 mM potassium phosphate, 1 mM EDTA, pH 7.6, containing 2–5 mM KCN and 0.12–0.15 mM NADH [20]. The particles were diluted to a protein concentration of about 3 mg/ml, pre-activated with 0.1 mM NADH and treated with antimycin A and mucidin or methoxyacrylatestilbene [20,22,37]. Enzymic rates were usually expressed in units of $\mu\text{mol}/\text{min}$ per mg of protein. Treatment of the particles with rotenone was carried out essentially as described previously [20] with 1–2 nmol of inhibitor per mg of protein.

Measurements of membrane potential

The electric membrane potential generated by the mitochondrial particles during NADH oxidation was measured by following the absorbance changes of oxonol VI at 630–601 nm [13,38–41]. The ETP_H were first diluted to 6–12 mg/ml and treated with 2 nmol of oligomycin/mg of protein to block proton leak through the ATP synthase complex, and with 1 nmol of antimycin plus 3 nmol of methoxyacrylatestilbene to block complex III [13,20]. The particles were then adjusted to a final protein concentration of 0.10–0.15 mg/ml in the assay medium, consisting of 0.125 M sucrose, 0.05 M Tricine/OH, 0.04 M KCl and 2.5 mM MgCl_2 , pH 8, containing $1\ \mu\text{M}$ nigericin and 2.5–3.0 μM oxonol-VI. The treatment of particles with complex III inhibitors was omitted in the experiments designed to measure the membrane potential generated by NADH oxidase, i.e. when NADH oxidation proceeded through the entire respiratory chain. The reaction was usually started by the addition of 0.1 mM NADH after the particles were equilibrated with the Q analogues, and the absorbance changes were measured at $22\text{--}25^{\circ}\text{C}$ in a dual-wavelength spectrophotometer equipped with a rapid mixing system.

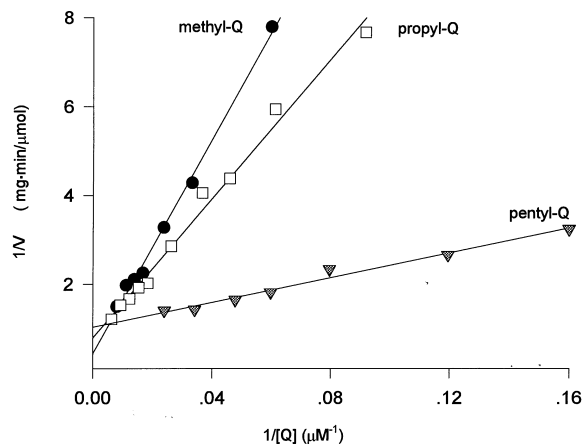
The ratio between the initial velocity of the signal elicited by the potential-sensitive dye and the initial velocity of NADH oxidation (the flow ratio) reflects the number of charges separated across the membrane per electron transported by complex I [6]. This flow ratio has also been used to evaluate the effect of the exogenous Q analogues on the membrane potential generated by the entire respiratory chain in submitochondrial particles and their relative energetic efficiency.

RESULTS

Nomenclature of Q analogues

Herein, the synthetic Q analogues we have used are identified by the chemical name of the substituent at position 6 of the ring followed by -Q or -analogue, and also by the serial Roman numerals given in Table 1. Other nomenclatures have been avoided to eliminate confusion in the identification of various analogues. For the isoprenoid analogues such as Q-1 (VII), we have adopted the abbreviations recommended by IUPAC-IUB [42] (Table 1).

A



B

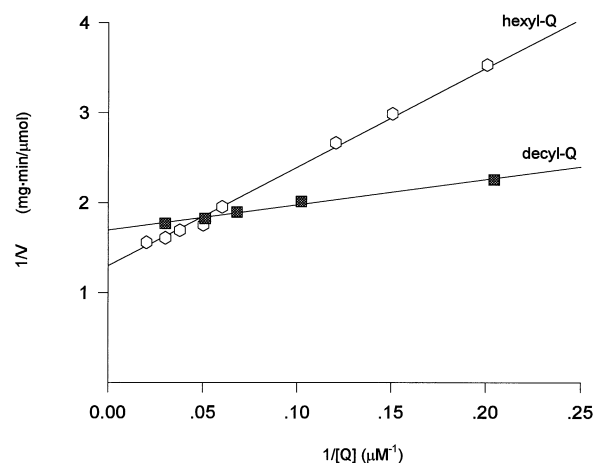


Figure 1 Double-reciprocal plots of NADH:Q reductase activity with different Q analogues

The experimental conditions were the same as those described in Table 1, with particles at 0.02–0.04 mg/ml. The initial rates (V) were corrected for the portion insensitive to both rotenone and rollinastatin-1, as described previously [20].

Specificity of NADH:Q reductase for Q analogues

The activity of NADH:Q reductase in mammalian mitochondria shows a characteristic specificity for quinone acceptor substrates: the analogues with the shortest saturated substituents, namely methyl-Q (II) and propyl-Q (III), have the highest maximal rates (Figure 1 and Table 1). For analogues I–VII, the maximal rates of NADH:Q reductase measured without treating the particles with inhibitors of the myxothiazol site of complex III are higher than those reported in Table 1. For instance, treatment of particles with mucidin decreased the maximal rate with methyl-Q (II) by 30% (from 2.3 to 1.6 units). However, the maximal rates with analogues having more than five carbons in the substituent are negligibly influenced by inhibitors such as mucidin (results not shown and [20,37]).

The interaction of complex I with Q substrates and Q-antagonist inhibitors is greatly altered after its isolation from the membrane [2], contrary to complex III which generally retains similar properties when isolated as it has within mitochondrial

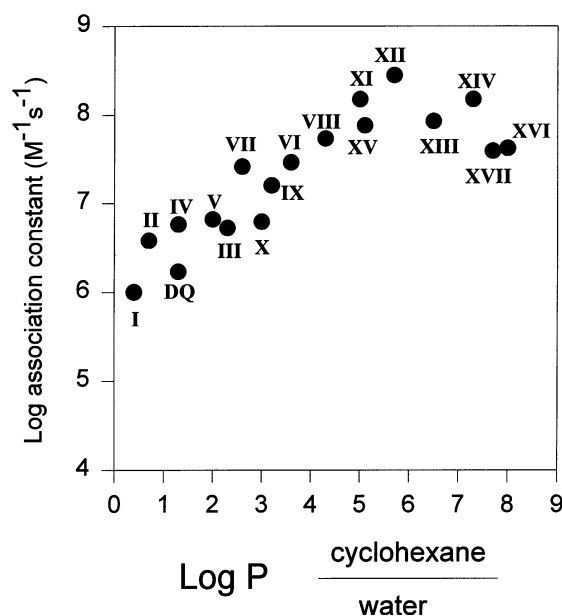


Figure 2 Hydrophobicity dependence of the association constants of Q analogues

The Q analogues are identified by Roman numerals as listed in Table 1. Minimal association constants of NADH:Q reductase were obtained as the ratio between the maximal rates (Table 1) and the K_m values (cf. [35]).

membranes [35]. Unfortunately, detailed data on the Q specificity of complex II and complex III are available mostly from studies carried out with isolated enzyme complexes [35,43], thereby preventing a direct comparison with the present data on complex I in submitochondrial particles. We observe, however, that the Q specificity of complex I within the particles seems to be more comparable with that of isolated complex III than with that of isolated complex II in terms of the flexibility and stereochemistry of the substituent at position 6 of the ring. This observation is in accordance with the data of Tan et al. [29] obtained with nitrophenol inhibitors acting as Q antagonists in submitochondrial particles.

In other Q-reacting membrane systems, the hydrophobicity of the Q analogues plays a fundamental part in their reactivity [34,35,43]. For example, in isolated complex III the minimal association constants of the Q analogues with the enzyme, which can be evaluated as the ratio of the maximal rate and the K_m ($K_{1\text{min}}$), increase by approximately one order of magnitude per order of magnitude increase in the partition coefficient (P) in cyclohexane/water (in the range of log P values from 0 to 4; cf. [35]). By contrast, complex I activity in submitochondrial particles shows that all the Q analogues which have partition coefficients in the range of log P values from 0 to 3 display similar association constants (Figure 2). Taken together, the association constants for complex I activity show a bell-shaped dependence on the hydrophobicity of the Q analogues (Figure 2) which resembles the bell-shaped dependence of the inhibition constants for various complex I inhibitors: capsaicin analogues [44], pteridin analogues [45], 4-alkylacridones [46], 2-alkyl-4,6-dinitrophenols [29], *N*-alkyl-4-aminoquinazolines [47] and 4-alkylphenylpyridine compounds related to the neurotoxic 1-methyl-4-phenylpyridinium [21,48]. The resemblance of the hydrophobicity relationship is striking between the Q analogues and the 4-alkylacridones, which both show an optimum for a

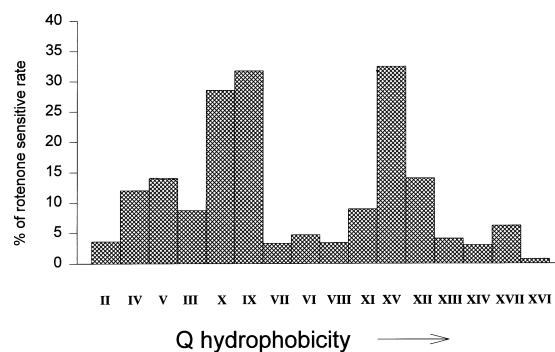


Figure 3 Rotenone-insensitive rates of NADH oxidation with various Q analogues

The rate of NADH oxidation was measured with either untreated or rotenone-treated mitochondrial particles under the conditions described in Figure 1, with a concentration of 20 μM for each of the Q analogues, which are identified by Roman numerals and shown in increasing order of hydrophobicity (see Table 1). The data represent the rotenone-insensitive rates expressed as a percentage of the rotenone-sensitive rates.

substituent with eight carbons (Figure 2 and [46]). These similarities strengthen the idea that chemically different classes of complex I inhibitors could act as Q antagonists [18,19].

Q analogues and the rotenone sensitivity of complex I

Although natural compounds such as pteridins and annonaceous acetogenins are the most potent inhibitors of mammalian NADH:Q reductase [20,47,49], commercially available rotenone is still the most commonly used inhibitor of complex I [2,49]. Rotenone does not fully inhibit the NADH:Q reductase activity [4,6,16,17,37,50] and leaves a 'leak' of electrons which generates free radicals including superoxide [11,51,52]. This electron leak in complex I is similar to the electron leak in antimycin-treated complex III [51,53,54].

The sensitivity of NADH:Q reductase to saturating concentrations of rotenone depends on the structure and the hydrophobicity of the quinones. Q analogues show a direct proportionality between the rate of NADH:Q reductase activity and the extent of rotenone sensitivity, which generally is lower for Q analogues with double bonds in the substituent than for Q analogues with a saturated substituent (Figure 3). Q-1 seems to be an exception to this generalization because of its high rotenone sensitivity, which is the highest among analogues with unsaturated substituents (Figure 3). Moreover, the sensitivity of complex I to rotenone shows a multiphasic dependence on Q hydrophobicity: there are two groups, one of hydrophilic analogues (V-IX) and another of more hydrophobic analogues, including Q-2, which display low rotenone sensitivity, whereas the most hydrophobic analogues exhibit the highest sensitivity to rotenone (Figure 3). Additionally, the rotenone-insensitive rate stimulated by Q analogues having five or more carbons in the substituent is further inhibited by other complex I inhibitors, notably rolliniastatin-1 (results not shown; cf. [13]). This complicated pattern of rotenone sensitivity has not been observed before (e.g. [17]).

Q analogues and membrane potential generated by NADH oxidation

The interpretation of the results of enzyme specificity for Q analogues is complicated by the vexing question of how the

Table 2 Energetic efficiency (flow ratio) of some Q analogues in the oxidation of NADH by submitochondrial particles

ETP_H (final protein concentration 0.15 mg/ml) were assayed for the generation of membrane potential with 2.7 μ M oxonol-VI on addition of 0.1 mM NADH either in the absence of complex III inhibitors (NADH oxidase activity through the entire respiratory chain) or in the presence of antimycin and mucidin to inhibit complex III (NADH:Q reductase activity of complex I). Q analogues were all added to the assay mixture at a concentration of 40 μ M before the addition of NADH. The parallel measurements of the rate of NADH oxidation were carried out under the same conditions (except for the omission of the oxonol dye) at 350–500 nm. Reference values for these redox activities were 0.42 unit for NADH oxidase and 0.45 unit for NADH:Q reductase with undecyl-Q. The flow ratios were calculated by dividing the initial rates of the oxonol absorbance changes [representing the initial velocity of membrane potential; expressed in milli-absorbance units (milliA)] by the initial rates of NADH oxidation.

| Q analogue | Flow ratio (milliA/ μ M) | | |
|------------------------|------------------------------|--------------|-------------------------------------|
| | NADH:Q reductase | NADH oxidase | NADH:Q reductase/NADH oxidase ratio |
| None (endogenous Q-10) | — | 193 | — |
| Undecyl-Q | 109 | 210 | 0.52 |
| Decyl-Q | 83 | 197 | 0.42 |
| Nonyl-Q | 107 | 215 | 0.50 |
| Q-2 | 53 | 160 | 0.33 |
| Q-7 | 52 | 160 | 0.32 |
| Methyl-Q | 47 | 127 | 0.36 |

redox activity of exogenous Q compounds relates to the physiological function of natural Q. In fact, exogenous Q analogues are redox-active compounds which may aspecifically react with various redox cofactors of the respiratory chain and thus function also as redox sinks or pro-oxidants. This is a recurrent problem in studies with Q-interacting enzymes and it has been addressed previously by the approach of solvent extraction and re-incorporation of Q analogues [23,55,56]. Solvent extraction, however, not only damages complex I [2] but also disrupts the integrity of the mitochondrial membrane and thus prevents the evaluation of the energy-conserving functions.

In contrast to the electron transport activity, which can be artificially stimulated by exogenous quinones, the function of membrane potential can only be generated when exogenous Q analogues react with the physiological site(s) in the membrane where endogenous Q-10 promotes proton translocation coupled to electron transport [4,6]. Therefore we have employed the measurement of membrane potential generated by NADH oxidation in the presence of Q analogues to obtain an effective means of assessing the interaction of these quinones with complex I and the respiratory chain.

In order to properly evaluate the energetic interaction of various Q analogues with the respiratory chain, we have taken as a reference the flow ratio of the membrane potential generated by the NADH oxidase activity in submitochondrial particles. This latter activity involves electron transport from complex I via endogenous Q-10, complex III and complex IV up to O₂, and therefore generates membrane potential by the cumulative charge separation produced by the vectorial proton translocation of the three respiratory complexes. Complexes III and IV each produce one charge separation per electron transported, whereas it is now generally agreed that complex I alone produces a separation of two charges (two vectorial protons) per electron transported [1,2,6,7,11–13]. Consequently, the flow ratio of membrane potential generated by complex I reducing endogenous Q should be approximately half that generated by the NADH oxidase activity.

From the above considerations it follows that if exogenous Q analogues interact efficiently with the physiological site of proton pumping in complex I, the flow ratio of the membrane potential generated by the NADH:Q reductase activity should also approximate to half that generated by NADH oxidase activity measured in their presence. This is indeed found with nonyl-Q, decyl-Q and undecyl-Q (Table 2). These Q analogues stimulate

both the rate of membrane potential generation and the rate of NADH oxidase activity to a similar extent (Table 2). Other Q analogues, especially the least hydrophobic of the present series and also Q-2, show a flow ratio which is lower than those of decyl-Q or undecyl-Q (Table 2 and results not shown). The reduced forms of these analogues, however, produce a flow ratio which is almost identical to that produced by the reduced form of decyl-Q in the membrane potential generated by the quinol oxidase activity, namely complex III to oxygen (results not shown). Therefore the low energetic efficiency of NADH oxidase in the presence of the hydrophilic Q analogues and Q-2 is due to a decreased capacity of NADH:Q reductase for generating membrane potential. In other words, these Q analogues function more as electron sinks than as physiological energy-stimulating substrates of complex I.

Q specificity of energy production by complex I

The parallel measurements of membrane potential and redox activity (Table 2) suggest that only some hydrophobic Q analogues can react with the proton pumping site of complex I as efficiently as endogenous Q-10. In order to provide a quantitative estimation of the relative energetic efficiencies of various Q analogues, we have evaluated the time course of the oxonol signals of membrane potential generated by the NADH:Q reductase activity (Figure 4A). The most hydrophobic Q analogues such as undecyl-Q show a sharp transient signal in the optical changes of oxonol VI, whereas less hydrophobic Q analogues show a broader signal with a slow decay (Figure 4A). Parallel experiments have indicated that the initial rates of the optical changes are linearly correlated with the ratio of the height of the maximum signal and the half-time of its decay. This ratio is thus used in Figure 4B to quantify the time course of the oxonol signals obtained in the presence of various Q analogues. Analogues with five or more carbons in the saturated side chain have a comparable efficiency, whereas those analogues containing less than five carbons in their substituent clearly show a lower efficiency.

In the experiments of Table 2 and Figure 4 the Q analogues were used at a fixed concentration which is largely not saturating for the redox activity with the less hydrophobic analogues (cf. Figure 1). However, the possibility that the low energetic

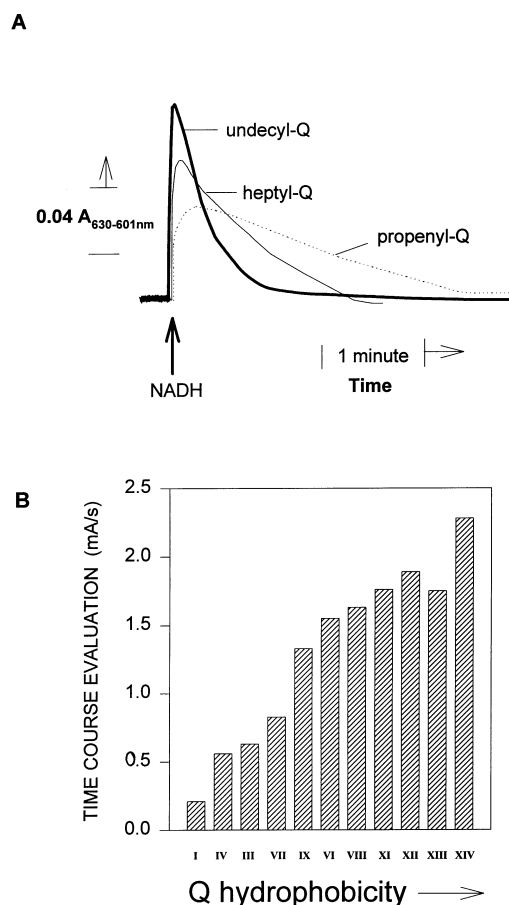


Figure 4 Time course of membrane potential generation with various Q analogues

The submitochondrial particles (0.14 mg/ml protein) were treated with complex III inhibitors and assayed for membrane potential generation with $3 \mu\text{M}$ oxonol-VI as described in the Materials and methods section. (A) Time course of the optical changes of oxonol-VI with three different Q analogues. (B) Relative efficiency of membrane potential generation by various Q analogues ($40 \mu\text{M}$) shown in order of increasing hydrophobicity (cf. Figure 3). mA represents milli-absorbance units. The ratio between the maximal extent of the optical signal of oxonol (cf. A) and the half-time of its decay was used to quantify the relative efficiency of the various Q analogues in generating the membrane potential (see text).

efficiency of these analogues could arise from their incomplete saturation of the catalytic activity of complex I is excluded by the data in Figure 5. The titrations of the initial velocity of membrane potential generation show a saturation around $30\text{--}40 \mu\text{M}$ for Q analogues having largely different hydrophobicities such as undecyl-Q and propenyl-Q. The maximal initial velocities, however, are roughly proportional to the hydrophobicity of the Q analogues, reflecting the progressive increase in the energetic efficiency of NADH:Q reductase with increasing hydrophobicity of the Q substrates (Table 2 and Figure 4). Because the K_m values for NADH:Q reductase of both propenyl-Q and methyl-Q are higher than $40 \mu\text{M}$ (Figure 1 and Table 1), the data in Figure 5 also indicate that these hydrophilic Q analogues saturate the proton pumping function well before they saturate the redox activity of complex I. On the contrary, hydrophobic Q analogues such as decyl-Q have similar saturation kinetics for both the electron transport and membrane potential activities of complex I (results not shown; cf. Figures 1 and 5).

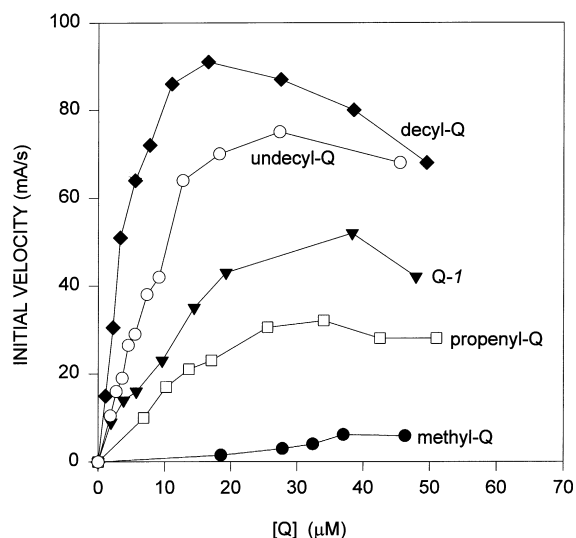


Figure 5 Q titrations of the initial velocity of membrane potential generation

Submitochondrial particles (0.15 mg/ml) were treated with complex III inhibitors and assayed as in the experiments of Table 2. Titrations with Q-2 under the same conditions yielded maximal rates of about 20 milli-absorbance units (mA)/s with saturation at a concentration of approx. $6 \mu\text{M}$ (data not shown for the sake of clarity). In these experiments the reaction was started by the addition of Q analogues.

DISCUSSION

This study significantly increases our understanding of the mechanism of Q reduction by mitochondrial complex I, a major conclusion being that Q analogues interact with complex I at different sites. A first indication supporting this conclusion emerges from the evaluation of the specificity of NADH:Q reductase for Q analogues, which is multiphasic and shows a less pronounced dependence of the association constants upon the hydrophobicity of the quinone substrates than for other membrane enzymes reacting with Q, for instance complex III (Figure 2; cf. [35]). A second indication is provided by the multiphasic pattern of rotenone sensitivity as a function of Q hydrophobicity (Figure 3). However, the most compelling evidence suggesting that complex I has different Q reaction sites which are accessible to exogenous Q analogues derives from our novel study of membrane potential generation by complex I (Table 2; Figures 4 and 5). In particular, the measurements of the flow ratio of membrane potential generation (Table 2) provide for the first time a solid basis for evaluating the energetic capacity, and consequently the physiological competence, of exogenous Q analogues reacting with complex I in coupled submitochondrial particles. Hydrophobic Q analogues such as undecyl-Q yield a stoichiometry of approximately two charges translocated per electron transported from NADH (Table 2), in agreement with the current consensus that in complex I two protons are translocated per electron transported to Q [1,6,7,13]. The energetic efficiency of Q analogues gradually decreases on decreasing the hydrophobicity of their substituent (Table 2 and Figure 4B). Furthermore, the most hydrophilic Q analogues exhibit an energetic efficiency as low as that of Q-2 (Table 2), suggesting that their membrane interaction with complex I is incomplete or non-physiological.

To rationalize our results, we propose that complex I can reduce Q analogues at two sites, each having a different structural specificity for the substituent at position 6 of the quinone ring

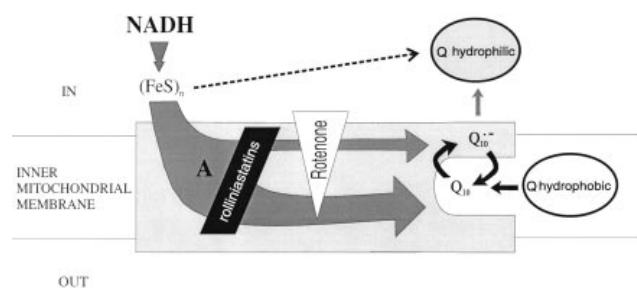


Figure 6 Scheme for the interactions of Q analogues with complex I

The scheme illustrates our interpretations of the present results according to the concepts of the dual Q-gated pump model for the mechanism of complex I [13]. Electrons flow from NADH through the iron–sulfur clusters (FeS_n) to site A in the membrane, which is proposed to contain a tightly bound semiquinone radical [13,26]. From site A, electrons flow to Q analogues along a series of steps which are postulated to be sequentially inhibited by rollinastatins and rotenone [13]. Endogenous Q (shown as Q_{10} in the figure) interacts with complex I at the hydrophobic pocket within the transmembrane part of the enzyme and forms a stabilized semiquinone intermediate ($\text{Q}_{10}^{\bullet-}$). Unlike endogenous Q and its hydrophobic analogues, hydrophilic Q analogues do not efficiently penetrate the hydrophobic pocket in complex I or form stable semiquinone intermediates. Instead, they interact with the hydrophilic site and essentially get reduced via the endogenous semiquinone intermediates. The residual rotenone-insensitive oxidation of NADH in the presence of hydrophilic Q analogues is mainly due to pro-oxidant interactions with the iron–sulfur cofactors of complex I (broken line). Such pro-oxidant interactions are less pronounced for those analogues which efficiently penetrate the hydrophobic site.

(Figure 6). One site binds most specifically Q analogues having a substituent of less than six carbons; consequently, we define this site as the hydrophilic site. We propose that at this site the hydrophilic quinones receive electrons primarily through the protein-bound semiquinone that is formed from endogenous Q during the catalysis of complex I [9,10,57–59]. The fact that various Q analogues differing by three orders of magnitude in their hydrophobicity have similar association constants for NADH:Q reductase activity (Figure 2) suggests that a process internal to complex I could be rate-determining in the electron transport to all these quinones. We believe that this internal step is associated with the formation of a stable ubisemiquinone in complex I, an idea which is further supported by the evidence that: (i) the extraction of endogenous Q abolishes the NADH oxidation sensitive to piericidin which is stimulated by duroquinone, a hydrophilic Q analogue [23]; and (ii) the time course of NADH oxidation initiated by the very hydrophilic Q-0 has a pronounced lag which reflects the formation of a reduced intermediate during NADH oxidation, most likely ubisemiquinone [10,59]. Other hydrophilic analogues such as methyl-Q and propyl-Q also show a lag in NADH oxidation; by contrast, the hydrophobic analogues usually do not show this lag (results not shown; cf. [37]).

We propose that the physiological site of the interaction of Q with complex I is distinct from the hydrophilic site and is embedded within the transmembrane domain of the enzyme complex. Here endogenous Q coming from the lipid membrane binds to the complex and, after full reduction, dissociates to return free in the lipids. Exogenous Q analogues can reach this hydrophobic site where endogenous Q reacts, but only if they are sufficiently hydrophobic to effectively partition into the lipid membrane under the conditions of the assay, as in other membrane complexes interacting with Q (cf. [35]). Under the conditions of our NADH:Q reductase assay, Q analogues need to have at least six carbons in their substituent to penetrate efficiently into the hydrophobic site of complex I (Figure 4B).

Ubisemiquinone intermediates are likely to play a crucial role in the mechanism of proton pumping by complex I [10,13,58]. We propose that exogenous Q analogues can also form stable semiquinone intermediates once bound to the hydrophobic pocket where endogenous Q reacts with complex I. Consequently, hydrophobic Q analogues will directly stimulate the proton pumping activity associated with NADH oxidation, whereas hydrophilic Q analogues will stimulate the same activity only indirectly through endogenous ubisemiquinone. The latter interaction at the hydrophilic site will be less efficient energetically because it will reoxidize the endogenous semiquinone intermediates instead of generating additional stable semiquinones. In line with our interpretation, the hydrophilic analogues such as Q-0 and duroquinone stimulate little proton pumping in mitochondria, while they apparently function as effective acceptor substrates for the redox activity of complex I [4,6]. This redox activity essentially reflects a redox sink interaction at the hydrophilic site, as indicated by the present results of membrane potential generation in submitochondrial particles (Figures 4 and 5; Table 2). We believe that the low energetic efficiency of Q-2 (Table 2) is also related to semiquinone instability, which in turn may derive from an incomplete reduction of the analogue at the hydrophobic site (M. Degli Esposti, unpublished work). This interpretation would also explain the inhibitory properties of Q-2 in complex I activity [55], but requires further experimentation to properly assess it.

To conclude, our detailed evaluation of the specificity of complex I for Q substrates has important implications for clinical and biomedical studies reporting complex I deficiencies in human patients. These enzymic defects are described in several mitochondrial and neurodegenerative diseases, e.g. Parkinson's disease [2]. They are most frequently ascertained by measurements of the NADH:Q reductase activity with Q-1 (for reviews see [1,2,60,61]). Although traditionally Q-1 is considered to be an ideal substrate for complex I [17,27,35], our results show that this Q analogue has a low efficiency in stimulating the energetic function of complex I (Figures 4 and 5). Decyl-Q or undecyl-Q should be preferred to Q-1 in biomedical research because their enzymic reactions with complex I closely match those of the endogenous ubiquinone. In the context of the biomedical aspects of complex I research, we are currently extending the present studies on Q specificity by approaches at both the cellular and the tissue level.

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