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Quinone specificity of Complex I

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Abstract

This review considers the interaction of Complex I with different redox acceptors, mainly homologs and analogs of the physiological acceptor, hydrophobic Coenzyme Q. After examining the physical properties of the different quinones and their efficacy in restoring mitochondrial respiration, a survey ensues of the advantages and drawbacks of the quinones commonly used in Complex I activity determination and of their kinetic properties. The available evidence is then displayed on structure–activity relationships of various quinone compounds in terms of electron transfer activity and proton translocation, and the present knowledge is discussed in terms of the nature of multiple quinone-binding sites in the Complex. © 1998 Elsevier Science B.V.

Keywords: Complex I (NADH Coenzyme Q reductase); Coenzyme Q analog

1. Introduction

Not only is Complex I the respiratory enzyme having the most complicated structural organization, but, in addition, there is no universally accepted and reliable assay for its activity, mainly due to uncertainty on the nature of the optimal quinone acceptor to be used. Understanding the specificity of the enzyme for its quinone acceptor would not only shed light on the functional mechanism of the complex, but also contribute to standardize the enzymatic determination. Owing to the alterations of Complex I in some mitochondrial disorders [1] and to its suggested involvement in some neurodegenerative diseases [2] and in aging [3], an unambiguous definition of quinone specificity in its assay is highly desirable.

This review summarizes present knowledge on the interaction of Complex I with its redox acceptors.

2. Physical properties of Coenzyme Q homologs

Coenzyme Q (CoQ) or ubiquinone (2,3-dimethoxy-5-methyl-6-polyprenyl-1,4-benzoquinone) is the natural electron acceptor of Complex I from eukaryotic sources. The side chain in the 6 position of the benzoquinone ring ranges from 6 to 10 isoprenoid units, being CoQ_6 common in yeasts, CoQ_8 in some bacteria, and CoQ_9 and CoQ_{10} in vertebrates [4].

Natural CoQ homologs are exceedingly hydrophobic molecules [5,6] so that they were postulated to be located in the hydrophobic core of the lipid bilayer

Abbreviations: CoQ_n, Coenzyme Q homologs having *n* isoprenoid units; DB, decyl-ubiquinone; DCIP, dichlorophenolindophenol; DQ, duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone); PB, pentyl-ubiquinone; SMP, beef heart submitochondrial particles; UBQ, undecyl-ubiquinone

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[7]. Nevertheless, the precise localization and orientation of the CoQ molecule in artificial bilayers and natural membranes has been the subject of considerable research [8–14].

Linear dichroism studies in model systems [15,16] strongly support the concept that CoQ is located in the lipid bilayer in a highly dynamic state, existing as the time average of two limiting orientations, one with the benzoquinone ring in the bilayer midplane and lying parallel to the membrane surface, and the other with the ring parallel to the lipid acyl chains near the polar heads, such that the benzoquinone ring with the first couple of isoprenoid units oscillates between the two limit positions [17,18]. In a recent study [19] employing molecular dynamics simulation and energy minimization of CoQ homologs, a folded structure was achieved for long chain homologs (CoQ_6 to CoQ_{10}), where the last isoprenoid unit is in close contact with the benzoquinone ring. The geometry of the molecules thus obtained allowed to calculate theoretical diffusion coefficients according to the free volume theory [20]; the values in the range of 10^{-7} cm^2 s^{-1} are strikingly in accordance with the high diffusion coefficients of natural quinones experimentally found by fluorescence quenching of membrane probes [18,21–23]. EPR studies of the radical anions and cations of CoQ_6 and CoQ_{10}, together with molecular orbital calculations [24] also yielded a folded conformation as the one having the minimum free energy.

An aliquot of CoQ is protein-bound [25–29], but its significance, whether representing a fixed coenzymatic form of the CoQ-reactive enzymes or merely substrate binding in the active site, is not completely clarified [30]. Protein binding is required to stabilize the semiquinone form in the Q-cycle [31–33]; in Complex III, whose crystalline X-ray structure has been recently solved [34,35], only one molecule of bound ubiquinone, out of the two to three molecules predicted by functional models [32,33,36–38] has been observed [34]. In Complex I, one molecule of ubiquinone might be firmly bound [39], in analogy with Q_A of the reaction center of photosynthetic bacteria [40], whereas another molecule could be in equilibrium with the quinone pool, in analogy with Q_B.

Being natural ubiquinones extremely hydrophobic molecules, a series of homologs and analogs having shorter chains in the 6 position are used as substrates for Complex I assays [41]. These quinones have finite membrane/water partition coefficients [42], that have to be taken into account in any consideration concerning their specificity and kinetics of interaction.

Table 1
Partition coefficients of some quinones used as Complex I acceptors

Quinone	log *P* cyclohexane/water	log *P* membrane/water
CoQ_0	0.39	–
DQ	2.45	–
CoQ_1	2.65	2.9
PB	3.7	3.6
CoQ_2	5.1	4.0
DB	7.2	4.7
CoQ_3	7.7	4.8
UBQ	8.0	–

The cyclohexane/water partition coefficients of different quinones are good parameters of their hydrophobicities and are known from the literature [43,44] (Table 1). Membrane/water partition coefficients can be determined indirectly, e.g., by fluorescence quenching by the quinones of fluorescent probes inserted in the membrane [45,46]. The membrane/water partition coefficients of CoQ_1 and pentyl-ubiquinone (PB), determined by fluorescence quenching, agree with the cyclohexane/water corresponding values, but more hydrophobic quinones are underevaluated [42] because their partition from water to the membrane competes with their micellization in water [21]. The fluorescence quenching-derived coefficients are valuable because they reflect the incorporation of the quinones in the membrane under usual experimental conditions.

An additional consequence of the high hydrophobicity of ubiquinones, related to their partition coefficients, is their solubility in monomeric state [21,47]; only quinones with very short chains (as CoQ_1 or PB) are monomeric in the concentration ranges used in Complex I assays, whereas already CoQ_2 and decyl-ubiquinone (DB) form micelles at or below micromolar concentrations in the assay medium. If the micelle-to-monomer transition is rate-limiting with respect to the enzymatic kinetic steps, then any rate determination would become meaningless.

Water insolubility is particularly serious a phenomenon for oxidized quinones, as in Complex I

activity determination, since the hydroquinone forms, used in Complex III activity determination, are significantly less hydrophobic [6,48,49].

An important consideration concerning the use of ubiquinones as substrates is their reaction with the partner enzymes from within the lipid bilayer: in most cases the kinetic constants of these enzymes are calculated taking into account the total substrate concentration in the heterogeneous assay mixture, rather than the *true* substrate concentration in the membrane. This can lead to ambiguity when performing comparisons of the specificity of substrates having different partition coefficients. Some investigators have developed enzyme kinetics in two-phase systems [50–54]; a method to determine the true membrane K_m and the partition coefficients was described for ubiquinols in ubiquinol cytochrome *c* reductase by steady-state kinetic measurements at varying phospholipid fractional values in the assay medium [53]. Alternatively, knowing the partition coefficient, the true membrane concentration of the substrate and hence the true K_m and other kinetic constants can be derived [42].

3. Kinetics of the Coenzyme Q pool

The isolation of four discrete lipoprotein redox complexes from the inner mitochondrial membrane [55] and the finding that the respiratory chain could be reconstituted from the isolated complexes [56,57] led Green and Tzagoloff [57] to postulate that overall respiratory activity is the result of intercomplex electron transfer ensured by rapid diffusion of mobile components acting as cosubstrates, i.e., CoQ and cytochrome *c*. In the subsequent years the organization of the respiratory chain was enriched of an increased number of respiratory complexes [58]. A *Random Collision Model* was systematically elaborated by Hackenbrock [59], Schneider et al. [60], and Hackenbrock et al. [61], who provided convincing evidence that the respiratory chain components undergo independent lateral diffusion, so that electron transfer is a diffusion-coupled kinetic process. Hackenbrock also postulated that electron transfer is rate-limited by the diffusion of the fastest components (CoQ and cytochrome *c*) [61]; this latter proposal has been questioned by our group [18,21,23,30].

The first detailed kinetic analysis of the redox reactions involving CoQ in the respiratory chain was elaborated by Kröger and Klingenberg [62,63], who showed that steady-state respiration could be modelled as a single two-enzyme system, the first causing reduction of CoQ and the second causing oxidation of ubiquinol; the overall flux is determined by the redox state of the quinone and hence is a hyperbolic function of the rates of quinone reduction and reoxidation, according to the well known *pool equation* (cf. Refs. [64,65]. The pool behavior allows to calculate one unknown or uncertain parameter if the other two parameters are known (the three parameters being overall rate of respiration, rate of quinone reduction and rate of its reoxidation). This procedure has been useful for determining the rate of Complex I activity indirectly when its direct determination had been found unreliable for the unsuitability of the quinone acceptors [66].

If the CoQ concentration in the membrane is not saturating for the activity of reducing and oxidizing enzymes, the pool equation is modified by feeding it into the Michaelis–Menten equation for enzyme kinetics [65,67], taking into account the total CoQ concentration, $[Q_t]$, the individual maximal velocities of the enzymes reducing and reoxidizing the CoQ pool, and their dissociation constants for ubiquinone; the rate of respiration is hyperbolically related to $[Q_t]$ and maximal turnovers of electron transfer are attained only at $[Q_t]$ saturating both CoQ reduction and reoxidation.

Direct titrations of lyophilized beef heart mitochondria depleted of their CoQ complement and reconstituted with different CoQ supplementations yielded a K_m of NADH oxidation for Q_t in the range of 2–5 nmol mg^{-1} mitochondrial protein [68,69]; considering a phospholipid content of 0.5 mg mg^{-1} protein [70], this value corresponds to a $[Q_t]$ of 4–10 mM in the lipid bilayer [69,71]. This K_m value is practically important in defining the hyperbolic relation between membrane CoQ concentration and electron transfer rate [69]. Analysis of the literature [4,72,73] shows that the physiological CoQ content of several types of mitochondria is in the range of the K_m for NADH oxidation, and therefore not saturating for this activity.

In contrast to NADH oxidation, the K_m values of succinate [69] and of glycerol-3-phosphate oxidation

[74] for CoQ were found to be one order of magnitude lower; since in all cases CoQ, once reduced, is reoxidized by Complex III, and since the rates of oxidation, at least of NADH and succinate, do not differ to large extents [64], it follows that the high K_m for CoQ of aerobic NADH oxidation must be a reflection of a correspondingly high K_m of Complex I for the quinone. This lower affinity of the CoQ-binding sites in Complex I with respect to the other mitochondrial CoQ-binding enzymes may indicate some unique structural feature of this site.

A consequence of such low affinity for CoQ is the rate enhancement of NADH cytochrome *c* reductase upon CoQ_{10} incorporation by cosonication of the quinone with bovine heart submitochondrial particles (SMP): NADH oxidation, but not succinate oxidation, was almost doubled when CoQ_{10} was incorporated [75]. This finding argues against a possible artifactual decrease of quinone affinity for Complex I due to the lyophilization and solvent extraction procedures used in the reconstitution experiments.

The implications of a 'high' K_m of Complex I for CoQ are worth considering, since a deficiency of CoQ, not corrected by sufficient biosynthesis, was postulated to occur in different kinds of human pathologies [76,77].

Although the CoQ content can be raised in vitro in the inner mitochondrial membrane above the physiological level and close to kinetic saturation, in vivo experiments have failed to indicate a mitochondrial CoQ increase when the quinone was administered by perfusion [78] or dietary means [79,80]. This failure does not appear to be due to an upper limit of solubility of long-chain ubiquinones in the membrane phospholipids [75].

4. Quinone specificity in NADH oxidation

In their early extraction–reconstitution studies, Lenaz et al. [81] first reported that succinate oxidase is not very specific in the structural and steric requirements for the isoprenoid side chain, whereas NADH oxidase is rather specific, being reactivated only by CoQ homologs having long isoprenoid side chains (>6 units). Since CoQ reduced by either enzyme is reoxidized by Complex III, the specific requirements of NADH oxidation must be referred to properties in the Complex I active site. Accordingly, CoQ homologs having short isoprenoid chains (like CoQ_2 and CoQ_3) inhibit NADH oxidation competitively with long-chain homologs [42,82]. Although the inhibitory action is well documented, no rigorous study has yet excluded the possibility that inhibition is exerted by contaminating impurities of the quinones.

The difference in specificity of the isoprenoid side chain of CoQ in restoring NADH oxidase and succinate oxidase activities of CoQ-depleted mitochondria led to the formulation of the existence of two sterically different sites for CoQ in NADH CoQ reductase and succinate CoQ reductase [75,81], having different sensitivities to the lipoidal and steric nature of the isoprenoid side chain, as also indicated by the different specificity of the two enzymes for the quinones as acceptors (Section 5) and by the different sensitivity to CoQ competitive inhibitors [83].

It was subsequently found that the saturated chain analog DB was as active as CoQ_{10} in restoring NADH oxidation [75], in agreement with its high acceptor activity [42] (cf. Section 5). A practical consequence of these findings is that low or intermediate CoQ homologs are unlikely to restore respiratory chain deficient activities under pathological conditions, whilst the low analog DB would represent an interesting candidate for inserting an effective quinone in intact tissues [75]. The hydroxydecyl analog idebenone, used in clinics to correct respiratory chain deficiencies [84–86], is however a potent respiratory chain inhibitor at the level of Complex I [87], making its therapeutical use of questionable efficacy.

5. A survey of routinely used quinones as complex I acceptors

From hence on, I will define the site in Complex I where the natural CoQ from the pool is reduced as the 'physiological site', whereas any other site in the enzyme where exogenous quinones or other compounds can accept electrons will be considered 'non-physiological'.

Among the oxidants used, ferricyanide [88] accepts electrons prior to the physiological reduction site, as shown by the lack of inhibition by rotenone of NADH

ferricyanide reductase [89] and by retention of NADH ferricyanide reduction activity in the solubilized type I NADH dehydrogenases [90] lacking the hydrophobic sector of the enzyme. Other oxidants are reduced by Complex I in a rotenone-insensitive pathway, such as hexammineruthenium(III) [91] and a series of quinones and nitro-compounds [92] but their kinetic mechanism of reduction is different from that of ferricyanide. Degli Esposti et al. [93], investigating the rotenone-insensitive component of reduction by Complex I of Coenzyme Q analogs differing in the substituents in the 6 position, provided evidence for the existence of a rotenone-insensitive, nonphysiological site for the most hydrophilic quinones, in addition to two different rotenone-sensitive quinone-binding sites (cf. Section 7). Moreover the reduction of such oxidants as ferricyanide, Wurster's Blue, and 2,6-dichlorophenol-indophenol (DCIP) is not coupled to energy transduction [94].

The electron acceptors most commonly employed as substitutes of the physiological ubiquinones are CoQ homologs and analogs [41,95]. It is assumed that these compounds interact with the physiological site(s), 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 [42,53,96]. The discovery that the enzyme contains bound ubiquinone essential for its activity [97] reopens the question of the mode of interaction of exogenous quinones with the acceptor site(s) (Section 8).

Among the quinone acceptors used are the homolog series from CoQ_0 up [41,95], including less frequently long isoprenoid chain homologs in spite of their insolubility in water [89], the tetramethyl benzoquinone analog, duroquinone (DQ), and analogs having straight saturated chains such as 6-pentyl, 6-decyl and 6-undecyl ubiquinones (usually abbreviated as PB, DB, and UBQ, respectively). Among these, the most commonly used in the assay of Complex I have been CoQ_1 and DB; however, CoQ_0 [94], CoQ_2 [27,98], PB [42,83,99], and UBQ [100,101] have also been employed.

The suitability of many of these commonly used acceptors has been questioned [102]; the main reason has been the observation that NADH CoQ reductase activity, as experimentally determined, is often paradoxically found lower than NADH cytochrome *c* reductase or NADH oxidase [66,103].

The reasons for underevaluating the reduction of exogenous quinones by NADH may be summarized as follows.

(a) The water solubility of the quinones with respect to their K_m is fundamental for assessing that kinetic saturation is reached during assay (Section 2).

(b) Some quinones, as CoQ_0, are significantly reduced in a rotenone-insensitive fashion, indicating that they accept electrons from a site situated upstream from the flow into the hydrophobic moiety of the enzyme, thus sharing in part the behavior of water-soluble oxidants such as ferricyanide. Degli Esposti et al. [93], in a detailed study of the specificity of quinones differing in their side chain composition, found that compounds eliciting highly rotenone-insensitive activities were clustered in two classes having different hydrophobicities.

In beef heart mitochondria or SMP, both CoQ_1 and DB exhibited high rotenone sensitivity ($>90\%$) [42,93]; however, even in beef heart SMP [104] and in purified Complex I from bovine heart [105], a high rotenone-insensitive rate with CoQ_1 was found in absence of added phospholipids, but significant only at high quinone concentrations. Using CoQ_1, the rotenone sensitivity was about 80% in non-synaptic mitochondria from rat brain cortex [106], but only 0–30% in rat liver mitochondria [103] and in human platelets mitochondria [107]. DB reduction exhibited higher rotenone sensitivity, i.e., $>90\%$ in both brain and liver mitochondria quoted above, whereas it was 70–80% in platelet mitochondria [107]; in the latter, it was reported that UBQ reduction is over 90% inhibited by rotenone [100].

(c) The respiratory chain contains quinone reduction sites in Complex III [108–111], where exogenous quinones may accept electrons in addition to or in competition with the natural site(s) in Complex I. The use of Complex III inhibitors to avoid reduction downstream the dehydrogenase may involve additional problems, since some of the so-called center o inhibitors of Complex III [32], as myxothiazol, also inhibit Complex I [112]. The additive inhibition of quinone reduction [42] by antimycin A and mucidin [113] demonstrates that both centers i and o behave as quinone reduction sites in Complex III when NADH is the electron donor.

By far the quinone which is mostly reduced at center i is duroquinone (DQ) (ca. 50%), whereas CoQ_1 and DB are usually least affected by antimycin [42]. The preferential reduction of DQ by Complex III with respect to Complex I (or even more Complex II [108,109]) is not due to thermodynamic reasons, as the midpoint potential of the DQ/DQH_2 couple is slightly lower than that of ubiquinone/ubiquinol [114]; thus it must depend on the extent to which the exogenous quinone competes with endogenous CoQ_{10} for the reduction site in the dehydrogenase [42,115]: if the exogenous quinone competes strongly with CoQ_{10}, then it is preferentially reduced by the dehydrogenase; otherwise the electrons are transferred to Complex III by the endogenous pool and find additional reduction sites for the exogenous acceptor [42,116]. Poor acceptors from Complex I, such as DQ, would compete such that 50% of the electrons still reach Complex III through the pool.

(d) Some quinones are Complex I inhibitors. The inhibitory action of CoQ_2 and other short chain isoprenoid homologs (but not of CoQ_1), well documented in beef heart mitochondria [42,81,82], was also observed in human lymphoblast mitochondria [117]; moreover, CoQ_2 was shown to inhibit cell growth in culture [118]. As pointed out in Section 4, clinically used idebenone also inhibits Complex I [87].

6. Kinetics of quinone reduction

In bovine heart SMP only CoQ_1 and DB were found to elicit rates comparable to those calculated for reduction of endogenous CoQ_{10} using the pool equation [71], and their reduction was almost completely inhibited by rotenone and only marginally affected by Complex III center i inhibitors [42].

Using CoQ_1, as well as other acceptors, NADH CoQ reductase 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 [42]. The overall turnover number of the enzyme (two electrons from NADH to CoQ) calculated on the basis of Complex I content of SMP, approached 500 s^{-1}, close to that found for DCIP reduction [115].

The parameter k_{cat}/K_m, the minimum second-order association rate constant of an enzyme with a substrate (k_{min}) [119], is one order of magnitude higher for DB than for CoQ_1, due to the low K_m of the enzyme for the former compound [42]; however, calculation of the effective quinone concentration in the lipid phase, which is a function of the partition coefficient [53] (Section 2), shows that CoQ_1 is a better substrate than DB, with a k_{min} of 4.3×10^4 M^{-1} s^{-1}, to be compared with 1.9×10^3 M^{-1} s^{-1} for DB; the k_{min} for CoQ_1 is close to the value that can be calculated for CoQ_{10} (8.8×10^4 M^{-1} s^{-1}) (cf. Ref. [42]). Table 2 summarizes the kinetic properties of a series of quinones as Complex I acceptors.

The relatively low value of the *true* k_{cat}/K_m for CoQ_1 (which approaches k_5, the association rate constant of CoQ_1 with the enzyme) and the high activation energy of the same parameter derived from Arrhenius plots [42] suggest that NADH CoQ_1 reductase activity is not diffusion-limited. The close value

Table 2
Kinetic constants of NADH–CoQ reductase in bovine heart SMP using various acceptors[a] [42]

	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	70	65	–	1.1	–
CoQ_1	380	20.1	8.9	18.9	43
CoQ_2	112	1.3	36	86	3.1
CoQ_3	66	0.8	61	82.5	1.1
PB	360	21.0	98	17.1	3.7
DB	225	1.8	138	125	1.6
CoQ_{10}	441[b]		5[b]		88

[a]Data were obtained from titrations at variable quinone concentrations, keeping NADH concentration at 75 μM. Thus, k_{cat} are slightly lower than those obtained from secondary kinetic plots [42]. K_m(Q) and k_{min}(Q) in the lipid phase were calculated using the partition coefficient (cyclohexane/water) from Table 1.
[b]Cf. Ref. [42] for discussion.

of the k_{cat}/K_m ratio for CoQ_{10} with respect to CoQ_1 allows us to suggest that also the physiological activity with the natural acceptor is not diffusion-limited, similarly to what found for ubiquinol cytochrome *c* reductase [71].

7. Structure–activity relationships and the nature of the quinone-binding sites

Evidence that Complex I contains multiple quinone-binding sites was largely collected by studies with inhibitors (cf. Degli Esposti in this issue). Most models of proton translocation by Complex I take in account the existence of at least two quinone sites [27,39,120–123]; these postulated sites are physiological by definition, being involved in the natural mechanism of energy conservation. In addition, extensive evidence demonstrates the existence of a *non-physiological* site, capable of reducing quinones and other oxidants. This site seems different from the ferricyanide site [91,92] for its kinetic properties. The reduction of soluble quinones, including CoQ_1, by isolated Complex I [92] is less sensitive to the action of inhibitors of the CoQ-binding site, in comparison to that of SMP [88]; this may be determined by the diminished phospholipid to protein ratio [105], since phospholipids appear to increase the affinity of quinones to the rotenone-sensitive site [105]. In isolated Complex I, the rotenone-insensitive site has higher turnover and lower k_{min}, whereas the rotenone-sensitive site has lower turnover and higher k_{min} [92]. Quinones at this site are largely reduced by a one-electron mechanism, as shown by the dependence of the log k_{min} on their *one-electron* reduction potentials.

The stoichiometry of electron transfer from NADH to CoQ approaches 1 for many straight chain quinones but is approximately 0.5 with CoQ_2 or with idebenone [87], indicating that they are incompletely reduced, presumably at the expense of molecular oxygen, as demonstrated by the antimycin-insensitive, superoxide dismutase-sensitive reduction of cytochrome *c*, similar to naphthoquinones and other quinones which stimulate a rotenone-insensitive oxidation of NADH [92,124]. Since CoQ_2 reduction is more sensitive to rolliniastatin-2 and rotenone than idebenone reduction [87], it was concluded that idebenone reacts with a site upstream to the action of inhibitors, possibly iron–sulfur centers, whereas CoQ_2 interacts with a site downstream the action of inhibitors. The instability of the enzyme-bound quinone intermediates was taken as the mechanism of inhibition of the enzyme [87]. Some other more specific mechanism must exist, however, to explain why CoQ_2 *inhibits* NADH oxidation, whereas other quinones, which also presumably react with oxygen [92], *activate* electron transfer from NADH. The study by Fato et al. [42] revealed that the K_i of CoQ_2 as a competitive inhibitor was identical to its K_m as an acceptor substrate and that the inhibitory form was presumably the reduced quinone. On the other hand the analog DB, which differs from CoQ_2 only for having a decyl saturated rather than a diprenyl side chain of ten carbon atoms, was not an inhibitor at all. The difference in V_{max}, but not in K_m, between the two acceptors suggests that the isoprenoid chain of CoQ_2 is not correctly positioned at the active site for optimal electron transfer. The same type of hindrance is expected for all isoprenoid quinones having side chains between two and five units, as they all are unable to restore NADH cytochrome *c* reductase activity [81]. The inhibition by short isoprenoid chain quinones is a peculiar character of Complex I, not shared by either Complex II [125] or Complex III [49,71,126]. Any explanation of the inhibitory action of short chain isoprenoid homologs has to explain the lack of inhibition of the corresponding saturated chain analogs.

Also the different rotenone sensitivity of the direct and reverse reaction catalyzed by Complex I [127] in the active or *pulsed* state [128] was ascribed to the existence of two different sites, one for ubiquinone and the other for ubiquinol binding, having different rotenone sensitivities.

The $Arg^{340} \rightarrow His$ mutation occurring in subunit ND4 of the Complex in the ND4/11778 form of Leber's hereditary optic neuropathy is accompanied by an increased K_m for CoQ_2 and by a decreased rotenone sensitivity in platelet mitochondria [100,129]; the decreased stability of an ubisemiquinone intermediate, due to replacement of the basic arginine residue with a less basic histidine, would be responsible for the decrease of affinity for both the acceptor CoQ_2 and the inhibitor rotenone [100]. If the affinity for the natural acceptor, CoQ_{10}, is also de-

Table 3
Synopsis of some properties of quinones as electron acceptors of Complex I

Quinone	Properties	Reference	Comment[a]
Isolated Complex I			
CoQ_0	Amytal-insensitive	[142]	Nonphysiological site
	High rotenone-insensitive rate	[105]	
CoQ_1	High activity, 100% amytal- and rotenone-sensitive, K_m higher than in situ	[89,142]	Physiological site
	Only partial rotenone sensitivity, partly $1e^-$	[92]	Two sites
	Partial rotenone sensitivity, increased by PL	[105]	Two sites
CoQ_2	Low activity, amytal-sensitive	[142]	Poor acceptor at physiological site
CoQ_6	Low activity	[89]	Water insoluble
CoQ_{10}	Low activity	[4,89]	Water insoluble
PB	High rotenone sensitivity	[105]	Physiological site
DB	High rotenone sensitivity	[105]	Physiological site
BQ[b]	Rotenone-insensitive, partly $1e^-$	[92]	Nonphysiological site
2,3-dimethoxy-5,6-dimethyl-BQ	Amytal-insensitive	[142]	Nonphysiological site
2,5-dimethyl-BQ	Rotenone-insensitive, partly $1e^-$	[92]	Nonphysiological site
2-methyl-BQ	Rotenone-insensitive, partly $1e^-$	[92]	Nonphysiological site
NQ[c]	Rotenone-insensitive, partly $1e^-$	[92]	Nonphysiological site
2-methyl-NQ (menadione)	Rotenone-insensitive, partly $1e^-$	[89,92,142]	Nonphysiological site
5,8-dioxy-NQ	Rotenone-insensitive, partly $1e^-$	[92]	Nonphysiological site
5-oxy-NQ	Rotenone-insensitive, partly $1e^-$	[92]	Nonphysiological site
9,10-phenanthrene quinone	Rotenone-insensitive, partly $1e^-$	[92]	Nonphysiological site
Adriamycin	Rotenone-insensitive, partly $1e^-$	[92]	Nonphysiological site
5-hydroxy-NQ (Juglone)	High activity, piericidin-insensitive	[88]	Nonphysiological site
Beef heart SMP[d]			
CoQ_0	High rate, $>90\%$ rotenone-sensitive, high $P/2e^-$	[104]	Physiological site
	$\sim 50\%$ rotenone sensitivity, low $H^+/2e^-$	[141]	Two sites
	Low rate, 68% piericidin-sensitive	[88]	Two sites
	Low rate, low rotenone sensitivity	[42]	Two sites
	Low rate, low $\Delta\psi$ and ΔpH generation	[93,133]	Two sites
CoQ_1	High rate, $>90\%$ rotenone-sensitive, high $P/2e^-$	[104,140]	Physiological site
	High rotenone sensitivity, high $H^+/2e^-$	[141]	Physiological site
	High rate, high piericidin sensitivity	[88]	Physiological site
	High rate, high rotenone sensitivity, highest k_{min}	[42]	Physiological site
	High rate, high rotenone sensitivity, low $\Delta\psi$ and ΔpH generation	[93,133]	Dual site

CoQ_2	Low rate, $>90\%$ rotenone-sensitive, high $P/2e^-$	[104,140]	Poor acceptor at physiological site
	Low rate, high piericidin sensitivity	[88]	Poor acceptor at physiological site
	Low rate, high rotenone sensitivity, inhibitor	[42]	Poor acceptor at physiological site
	Low rate, relatively low rotenone sensitivity, inhibitor	[87,93,133]	Dual sites, O_2 reactivity
CoQ_3	Low rate, high rotenone sensitivity	[42]	Little solubility
	Low rate	[93]	
6-methyl-CoQ	High rate, high rotenone sensitivity, low ΔpH generation	[93]	Dual site
6-propyl-CoQ	High rate, high rotenone sensitivity, low $\Delta\psi$ and ΔpH generation	[93,133]	Dual site
6-propenyl-CoQ	Intermediate rate, high rotenone sensitivity, low $\Delta\psi$ and ΔpH generation	[93,133]	Dual site
6-butenyl-CoQ (2 isomers)	High rate, high rotenone sensitivity	[93]	
6-pentyl-CoQ (PB)	High rate, 95% rotenone sensitivity, high $P/2e^-$	[140]	Physiological site
	High rate, high rotenone sensitivity, low k_{min}	[43]	Physiological site
	High rate, high rotenone sensitivity, high $\Delta\psi$ and ΔpH generation	[93,133]	Dual site
	High activity	[131]	
6-hexyl-CoQ	High rate, high rotenone sensitivity, high $\Delta\psi$ and good ΔpH generation	[93,133]	Mainly physiological site
6(2′4′-hexadienyl)-CoQ	Low rate, low rotenone sensitivity, relatively low $\Delta\psi$ generation	[93]	Dual site
6(1′-methyl-2′,4′-pentadienyl) CoQ	Low rate, low rotenone sensitivity	[93]	Dual site
5-desmethyl-6-hexyl-CoQ	Activity higher than PB	[131]	
5-desmethyl-6-(1-methylpentyl) CoQ	As active as PB	[131]	
5-desmethyl-6(2-methylpentyl) CoQ	Activity higher than PB	[131]	
5-desmethyl-6-(3-methylpentyl) CoQ	Activity lower than PB	[131]	
5-desmethyl-6-(4-methylpentyl) CoQ	Activity lower than PB	[131]	
5-desmethyl-6-(1-ethylbutyl) CoQ	Activity slightly lower than PB	[131]	
6-heptyl-CoQ	Intermediate rate, high rotenone sensitivity, high $\Delta\psi$ and ΔpH generation	[93,133]	Physiological site
6-octyl-CoQ	Low rate, relatively low rotenone sensitivity, high $\Delta\psi$ generation, inhibits H^+ pumping	[93,133]	Inhibitor
6-nonyl-CoQ	Intermediate rate, high rotenone sensitivity, high $\Delta\psi$ and ΔpH generation	[93,133]	Physiological site
6-decyl-CoQ (DB)	High rate, 100% rotenone-sensitive, high $P/2e^-$	[140]	Physiological site
	High rate, high rotenone sensitivity, low k_{min}	[42]	Physiological site (but less efficient than CoQ_1)
	High rate, high rotenone sensitivity, high $\Delta\psi$ and ΔpH generation	[93,133]	Physiological site
	High rate	[131]	

Table 3 (continued)

Quinone	Properties	Reference	Comment[a]
3-ethoxy-DB	Same rate as DB	[131]	
2-ethoxy-DB	Rate lower than DB	[131]	
2,3-diethoxy-DB	Rate lower than DB	[131]	
6-undecyl-CoQ (UBQ)	High rate, high rotenone sensitivity, high $\Delta\psi$ and ΔpH generation	[93,133]	Physiological site
6-hydroxydecyl-CoQ (idebenone)	Low rate, low rotenone sensitivity, inhibits electron transfer and $\Delta\psi$	[87]	Inhibitor
BQ	Low rate, low piericidin sensitivity	[88]	Poor acceptor at nonphysiological site
2-methyl-BQ	Low rate, low piericidin sensitivity	[88]	Poor acceptor at nonphysiological site
2,3-dimethyl-BQ	Low rate	[88]	Poor acceptor
2,5-dimethyl-BQ	Low rate	[88]	Poor acceptor
2,6-dimethyl-BQ	Low rate	[88]	Poor acceptor
2,3,5-trimethyl-BQ	Low rate, high piericidin sensitivity	[88]	Poor acceptor
2,3,5,6-tetramethyl-BQ (DQ)	Low rotenone sensitivity, low $H^+/2e^-$	[141]	Nonphysiological site
	Low rate, high piericidin sensitivity	[88]	Poor acceptor
	Low rate, high rotenone sensitivity, high reduction from Complex III	[42]	Poor acceptor
2,5-dimethoxy-3,6-dimethyl-BQ	Low rate, high piericidin sensitivity	[88]	Poor acceptor
2,6-dimethoxy-3,5-dimethyl-BQ	Low rate, high piericidin sensitivity	[88]	Poor acceptor
2,3-dimethoxy-5-methyl-6-chloro-BQ	High rate, 57% piericidin-sensitive	[88]	Two sites
2,3-dimethoxy-5-methyl-6-bromo-BQ	High rate, 50% piericidin-sensitive	[88]	Two sites
2-methoxy-3-hydroxy-5-methyl-6-chloro-BQ	Very low rate, 35% piericidin-sensitive	[88]	Poor acceptor
2,3-dichloro-5-methyl-BQ	Very low activity, piericidin-insensitive	[88]	Poor acceptor
2,3-dichloro-5,6-dicyano-BQ	Low activity, piericidin-insensitive	[88]	Poor acceptor
2,5-dihydroxy-BQ	No activity	[88]	
NQ[b]	Low rate, 37% piericidin sensitivity	[88]	
1,2-NQ	High rate, piericidin-insensitive	[88]	Nonphysiological site
2-methyl-NQ (menadione)	Low rate, low rotenone sensitivity, low $P/2e^-$	[104]	Nonphysiological site
	Low rate, 73% piericidin-sensitive	[88]	Two sites
5-hydroxy-NQ	Low rate, low piericidin sensitivity	[88]	Nonphysiological site
2-hydroxy-NQ	No activity	[88]	
2-hydroxy-3-methyl-NQ	No activity	[88]	
Anthraquinone derivatives	No activity	[88]	
Doxorubicin	High rotenone-insensitive reduction and O_2 radical formation	[143]	Redox cycling
Daunorubicin	As above	[143]	
5-imino-daunorubicin	Low rotenone-insensitive reduction	[143]	

[a]'Nonphysiological' refers to interaction with Fe–S clusters in the Complex; 'physiological' refers to rotenone-sensitive site(s) without further specification; 'two sites' means both physiological and nonphysiological; 'dual site' refers to the dual nature of the rotenone-sensitive site according to Refs. [93,133].
[b]BQ = 1,4-benzoquinone.
[c]NQ = 1,4-naphthoquinone.
[d]Rat liver mitochondria in Ref. [141].

creased, this could provide an explanation why the rate of integrated electron transfer is decreased, whereas NADH CoQ reductase activity is retained [130]. An alternative explanation would be if the unstable ubisemiquinone reacted with oxygen, establishing a short-circuit of electron transfer regenerating ubiquinone [100] and preventing reoxidation of ubiquinol by the respiratory chain.

The decrease of rotenone sensitivity of Complex I activity observed in aging [106,107] might be related either to specific changes of the CoQ-binding site, as in Leber's disease [100], or, alternatively, to modified assembly of the complex due to changes in the lipid environment (cf. Ref. [105]), or also to changes in the proportion of resting and active forms of the enzyme [127].

Sakamoto et al. [131] observed that the quinone-binding site of Complex I from beef heart, contrary to that in *Escherichia coli* glucose dehydrogenase, is not stringent in its specificity of substituents in the benzoquinone ring, and concluded that the site is spacious enough to accommodate a large variety of quinone compounds; in particular, substitution of the methoxy groups at position 2 and 3 with ethoxy groups or removal of the methyl group at position 5 did not affect reductase activity in a significant way. This lack of specificity reminds the properties of the quinol binding site in Complex III [132], at difference with the high specificity of Complex II for the benzoquinone ring substituents [132]. The low specificity of Complex I for the quinone ring substituents agrees with the substantial activity of the 2-amino-derivative rhodoquinone in NADH oxidation (but not in succinate oxidation) [81].

Sakamoto et al. [131] also studied the effect of the structural changes in a six-atom side chain at position 6, and also found no specific requirement for Complex I activity. In view of these findings, the inhibition by CoQ_2 [42] and other analogs of similar size [87,93] is even more puzzling. Studying the effect of structural changes in side chains having 10 or more carbon atoms might shed light on the mechanism of inhibition.

Degli Esposti et al. [93] and Helfenbaum et al. [133] performed a detailed study of the specificity of six-substituents on for NADH CoQ reductase activity of beef heart SMP, investigating the rates of electron transfer, the generation of membrane potential, and proton translocation elicited by Complex I activity. CoQ analogs with a saturated substituent of 1 to 3 carbons have the fastest rates of electron transfer activity, and analogs with substituents of 7 and 9 carbons have the highest association rate constants, as defined by the k_{cat}/K_m ratios [93]; these ratios are not very sensitive to hydrophobicity as defined by the cyclohexane/water partition coefficients, contrary to Complex III [49]; this means that the *true* association rate constants, corrected for the partition coefficient in the membrane, actually *decrease* with increasing hydrophobicity, as also shown by Fato et al. [42] in their comparison between CoQ_1 and DB. As pointed out by Degli Esposti et al. [93], the bell-shaped dependence of k_{cat}/K_m on the hydrophobicity of the CoQ analogs resembles the bell-shaped dependence of the inhibition constants for various Complex I inhibitors [83,131,134–139], reinforcing the idea that chemically different classes of Complex I inhibitors act as CoQ antagonists [93,98].

Contrary to electron transfer, the rate of generation of membrane potential, measured by use of the probe oxonol VI, increased steadily with CoQ hydrophobicity [93]; the energetic efficiency of Complex I with respect to the entire respiratory chain was maximal for quinones having more than nine carbons.

The proton pumping activity, studied by the extent of fluorescence quenching of a pH-sensitive acridine dye [133], was also consistent with the presence of two sites of interaction of CoQ analogs with the Complex, both of which are rotenone-sensitive. One site, which is more hydrophobic and interacts with CoQ analogs having more than six carbons at the 6 position, leads to a rapid proton pumping and a stoichiometric consumption of NADH; another site preferentially reacts with CoQ analogs having less than six carbons, including CoQ_1; this site drives a slow proton pumping activity associated with NADH oxidation that is overstoichiometric to the reduced quinone acceptor, the latter finding being interpreted as redox cycling from an unstable semiquinone [133]. Hydrophilic CoQ analogs also exhibit low respiratory control ratios for their NADH CoQ reductase activity, contrary to the hydrophobic analogs. On top of these two sites is the nonphysiological interaction of the most hydrophilic quinones with iron–sulfur clusters.

In the studies quoted above [93,133] CoQ_1 was found to belong to the group interacting in part with

the nonphysiological site; this contrasts with the ability of CoQ_1 to support oxidative phosphorylation with a P:$2e^-$ ratio approaching 1 [104,140] and H^+ translocation with a $H^+/2e^-$ ratio approaching 4 [141]. The differences in the systems employed (e.g., extent of partition to the hydrophobic site due to membrane lipid concentration, cf. Ref. [105]) may be responsible for some of the differences, which are, however, far from being explained. Moreover, the role of endogenous CoQ_{10} is not well defined, and it is not clear how protein-bound quinone may interact with the exogenous acceptors under different experimental conditions. Table 3 synoptically summarizes major properties of quinones as electron acceptors from Complex I.

Table 4
Effect of endogenous ubiquinone on the kinetics of NADH–CoQ_1 reductase[a] [42]

Particles	V_{max} (μmol min^{-1} mg^{-1})	K_m (μM)
SMP	1.1	22
BHM[b]	0.35	20
BHM lyophilised	0.19	25
BHM extracted[c]	0.21	56
BHM extracted + CoQ_{10}	0.21	21
BHM extracted + CoQ_5	0.22	27
BHM extracted + CoQ_3	0.20	24

[a]Similar qualitative behavior was shared by PB [42] and DB (unpublished).
[b]Bovine heart mitochondria (frozen and thawed).
[c]No detectable CoQ present.

8. Role of endogenous ubiquinone

It was shown that endogenous CoQ_1 accepts electrons directly from Complex I in pentane-extracted mitochondria without the involvement of the CoQ pool [96]; the presence of tightly bound CoQ_{10} in Complex I [39,97] means, however, that exogenous CoQ is likely to accept electrons from a site containing the tightly bound CoQ molecules [42]. Unfortunately, it seems difficult, using extraction and reconstitution procedures, to differentiate between endogenous quinone belonging to the free pool and quinone bound to the Complex.

It was found [42] that V_{max} is the same in mitochondria before CoQ extraction and in extracted mitochondria containing no detectable CoQ: it is therefore 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 behavior is also in agreement with the strong reversible increase of K_m for exogenous quinones upon removal of endogenous CoQ_{10} [42,75,144] (Table 4). A simple interaction of the exogenous quinone directly with a protein site in place of the endogenous CoQ_{10} would exhibit competition by endogenous CoQ_{10} or by CoQ_{10} upon reconstitution; this pattern is actually exhibited by succinate CoQ reductase where the K_m for exogenous quinone is *decreased* by CoQ extraction [116,141]. The opposite behavior in the case of NADH CoQ reductase where the K_m for CoQ_1 is *increased* by CoQ extraction [42,75,116,144] may be interpreted to mean that the quinone active site is different when the endogenous CoQ_{10} is removed. This effect would 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 of 5 mM in the lipids, and a K_m for CoQ_1 of 10 mM in the lipid phase [42] and using the actual membrane concentrations of both quinones under the assay conditions (i.e., 50 mM CoQ_1 and 5 mM CoQ_{10}) it can be calculated 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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