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Review

Mobility and function of Coenzyme Q (ubiquinone) in the mitochondrial respiratory chain

Giorgio Lenaz *, Maria Luisa Genova

Dipartimento di Biochimica "G. Moruzzi", Università di Bologna, Via Irnerio 48, 40126 Bologna, Italy

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ABSTRACT

The kinetic analysis by Kröger and Klingenberg on electron transfer in the Coenzyme Q region led to the conclusion that the quinone behaves kinetically as a homogeneous pool freely diffusing in the lipid bilayer, thus setting the basis for the widely accepted random diffusion model of electron transfer. The recent description of supramolecular complexes of the respiratory chain enzymes, in particular Complex I–III supercomplexes, has reopened the problem of electron transfer in the Coenzyme Q region. Flux control analysis has revealed that Complexes I and III indeed function as a single unit indicating substrate channelling by Coenzyme Q in transferring electrons from Complex I to Complex III. In this review we analyse in detail the reasons that suggested Coenzyme Q pool behaviour; although electron transfer between Complexes I and III indeed appears to be effected by substrate channelling, the Coenzyme Q pool is in equilibrium with bound quinone and is required to fill the site(s) within the supercomplex. In addition, the pool equation of Kröger and Klingenberg still describes in the most adequate way the electron transfer from Complex II and other Coenzyme Q-reducing enzymes to Complex III, besides the energy-dependent reverse electron transfer from Complex II to Complex I.

1. Introduction

In 1973, Achim Kröger and Martin Klingenberg published two fundamental papers [1,2] in which they described the kinetics of electron transfer through Coenzyme Q (CoQ, ubiquinone) for the first time in a quantitative way; they showed that steady-state respiration in submitochondrial particles from beef heart could be modelled as a simple two-enzyme system, the first causing reduction of ubiquinone and the second causing oxidation of ubiquinol, where the quinone behaves kinetically as a homogeneous pool freely diffusing in the lipid bilayer of the inner mitochondrial membrane between the dehydrogenases and the bc_1 complex. This demonstration confirmed the model of David Green [3] who anticipated, for the organization of the inner mitochondrial membrane, the more general fluid mosaic model published a few years later by Singer and Nicolson [4]. According to this view, all the integral proteins of the mitochondrial inner membrane, including the respiratory complexes and the ATP synthase, are randomly dispersed in the fluid phospholipid bilayer; within the respiratory chain, electron transfer between bulky complexes would be assured by mobility of the smaller connecting redox components, i.e. CoQ between Complex I/II and Complex III, and cytochrome *c* (cyt. c) between Complex III and Complex IV. Subsequently, the fluid mosaic model was theorized by Charles Hackenbrock [5] as applied to the respiratory chain in the random collision model of electron transfer. The random collision model was proposed on the basis of direct investigation by freeze-fracture electron microscopy of the random distribution of the intramembrane particles in the inner membrane and of demonstration of the mobility of mitochondrial components by fluorescence recovery after photobleaching, yielding lateral diffusion coefficients in the range between 10^{-9} and 10^{-10} cm^2/s for mitochondrial membrane complexes [5] and higher than 10^{-9} cm^2/s for CoQ [5,6].

More recently, a breakthrough occurred when new evidence was provided of multi-complex units in yeast and mammalian mitochondria introducing a mild one-step separation protocol for the isolation of membrane protein complexes, namely Blue Native Poly-Acrylamide Gel Electrophoresis (BN-PAGE). In particular, in bovine heart mitochondria [7,8], Complexes I–III interactions were apparent from the presence of about 17% of total Complex I in the form of a I_1III_2 supercomplex that was found further assembled into two major supercomplexes (respirasomes) comprising different copy numbers of Complex IV ($I_1III_2IV_1$ and $I_1III_2IV_2$ contain 54% and 9% of total Complex I, respectively). The presence of respiratory supercomplexes has been validated in a great number of reports concerning their detection in different organisms and tissues.

The most obvious function of supramolecular association of respiratory complexes has been considered in providing contact surfaces for substrate (CoQ and cyt. *c*) channelling [9,10], i.e. direct transfer of electrons from the donor Complex I (in the case of CoQ) to

* Corresponding author. Tel.: +39 051 2091229; fax: +39 051 209 1217.
E-mail address: giorgio.lenaz@unibo.it (G. Lenaz).

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the acceptor Complex III: it is evident that the existence of such an organization must be reconciled with experimental evidence of a pool function of CoQ (and cytochrome *c*) and of diffusion-coupled collisional interactions at the basis of inter-complex electron transfer [1,2,5,6,11–14].

In this report, we analyse available evidence related to super-complex organization with respect to the pool function of CoQ as a diffusible intermediate between the complexes donating electrons to CoQ (Complex I or II) and Complex III that receives electrons from reduced CoQ. Analogous considerations for cytochrome *c* between Complex III and Complex IV are out of the scope of this review.

2. Properties of Coenzyme Q

CoQ exists in three redox states, fully oxidized (ubiquinone), semiquinone radical (ubisemiquinone), and fully reduced (ubiquinol): nevertheless, the existence of different possible levels of protonation increases the possible redox forms of the quinone ring [15]. Due to its extreme hydrophobicity, natural CoQ can be present in three physical states only: forming micellar aggregates, dissolved in lipid bilayers, and bound to proteins. The former state is very important working with CoQ in cell-free systems [16], however in the living cell CoQ should be distributed among the other two states.

How much CoQ is protein-bound? If we consider bound CoQ as stoichiometric with one site in the complexes that have been shown to contain bound CoQ (I, II, III) [17–19], in beef heart mitochondria we come up to no more than 0.35 nmol/mg protein; this would increase to ca. 0.5 nmol assuming more than one site to be fully occupied in Complex I and Complex III. Since the total CoQ content is higher than 3 nmol/mg [20,21], we must assume that most CoQ (>84%) is free in the bilayer. A direct study [22] of the amount of CoQ bound to mitochondrial proteins in five different mammalian species has shown values between 10 and 32% of total CoQ to be protein-bound.

Currently, no common architecture can be applied to CoQ binding sites in general: several distinct types of CoQ-sites associated with respiratory electron transfer complexes can be specified with only a few very general analogous features.

The quinone/quinol head group binds into the hydrophobic site primarily by interaction of appropriately placed hydrogen bonds with the carbonyl/hydroxyl moieties, and with the ring flanked by aromatic and aliphatic residues. Further interactions with the quinone side-chain occur to give steric limitations to possible antagonists. Amino acid networks that provide a proton-conducting hydrophilic channel through the protein radically affect the properties of the bound quinone molecule that, otherwise, could cycle only between the oxidized and semiquinone forms without being protonated to quinol. The presence of a highly conserved hydrogen-bonding histidine residue, forming a triad of close contact residues with the fourth amino acid upstream and the third downstream of the His, has been proposed as a characteristic sequence motif associated with CoQ-binding sites in the reaction centre of Rhodobacter spheroides [23]. Sequences strongly resembling such a triad (aliphatic-$[X]_3$-H-$[X]_{2/3}$-[L/T/S]) can be identified in the Q(i) site of mitochondrial Complex III [23], whereas no obvious similarities were noticed to regions of the Q(o) site that is responsible for oxidation of quinol in Complex III. Moreover, analysis of sequence databases indicated that a histidine-containing element, reminiscent of the triad sequence, may be present in the mitochondrial alternative quinol oxidase and in the ND4 and ND5 subunits of Complex I [23]. Indeed, in the case of the NADH–CoQ oxidoreductase, the number of binding sites for ubiquinone is still an unsolved controversial subject; further comments about the postulated existence of multiple CoQ-binding sites in Complex I are reported in the section "Function of the Coenzyme Q pool" of the present paper [24–28].

It has been assumed for long time that the shape of the CoQ molecule is linear, with some possibility of rotation allowed for the long isoprenoid tail. Bending of the molecule is required in a model proposed by us [12], on the basis of previous evidence and of theoretical considerations; this model was confirmed by linear dichroism studies [29] of the location of CoQ_{10} in the hydrophobic mid-plane of the lipid bilayer, with the polar head oscillating about the third isoprene unit between the mid-plane (wholly linear shape) and the polar heads of the phospholipids (maximal bending of 90°). The model allows for movement of the redox centre of CoQ, that is required for interaction with other redox centres in the mitochondrial complexes.

Contrary to these predictions, a study of molecular dynamics computer simulation of CoQ homologues in the vacuum, starting from different initial configurations, has shown that the conformation with lowest energy level is a folded one, where the polar head is in tight contact with the last isoprenoid unit of the hydrophobic tail [30]. Within the series of homologues, the cut-off for the folded conformation is four isoprenoid units. The folded conformation was found for both oxidized and reduced quinones, however only small energy differences were found between oxidized and reduced ubiquinones in the folded conformation.

Although the molecular modelling has been performed in the vacuum, we have a reason to believe that the folded conformations also apply to the CoQ homologues in natural membranes; this idea is supported by the experimental demonstration by magnetic resonance techniques that ubisemiquinones are folded in organic solvents [31].

There are important implications of a folded structure. First, the similar size of short and long homologues would explain the similar high rates of lateral diffusion found in our laboratory for all quinone homologues [16,30]. In addition, protein binding during electron transfer may require unfolding, contributing to the high activation energy and low collision efficiency observed for electron transfer (e.g. [16,32]).

A variety of techniques have been employed to measure the lateral diffusion in artificial lipid bilayers and in natural membranes, yielding a broad range of values for the diffusion coefficients. The methods are based either on measuring the frequencies of encounter between probe molecules, or on the generation of a spatial gradient of labelled molecules in the membrane followed by measurement of the rate of probe redistribution. The collision-dependent methods measure lateral diffusion to distances of several nm and include excimer formation [33] or fluorescence quenching [34], whereas the methods based on the redistribution of probe molecules measure diffusion on a μm scale for which the most versatile technique is fluorescence recovery after photobleaching (FRAP). Hackenbrock et al. [5] and Rajarathnam et al. [35] exploiting the FRAP technique with fluorescent labelled ubiquinone analogues calculated diffusion coefficients in mitochondrial membranes in the range of 10^{-9} cm^2/s. On the other hand, exploiting collisional fluorescence quenching of membrane-partitioned anthroyl-stearate fluorophores by oxidized CoQ homologues, Fato et al. [16] calculated diffusion coefficients $>10^{-6}$ cm^2/s in both liposomes and mitochondrial membranes, using calculations derived from Lakowicz and Hogen [36] to account for partition and effective membrane concentration of the quencher and the Smoluchowski relation for calculating the diffusion coefficient from the second-order rate (quenching) constant. The presence of hydrophilic fluorophores bound to the CoQ molecules in the FRAP experiments throws some doubts on the excessively low values for diffusion found using that technique (cf. [12]).

3. Structural demonstration of supercomplexes

The electrophoretic evidence by both BN-PAGE and colourless native PAGE (CN-PAGE) [37] that the respiratory chain complexes are associated to form specific stoichiometric supramolecular units in all organisms and tissues is overwhelming ([7–9,38–50], cf. also [51,52]). In spite of that, we still know little about their molecular structure and their physiological role in the respiratory chain.

A well defined structure was observed for all supercomplexes that were investigated, supporting the idea of highly ordered associations of the respiratory supercomplexes and discarding most doubts on artificial interactions. Moreover, from the limited data available to date, it appears that such interactions may be species- or kingdom-specific [53].

Three-dimensional models of the I_1III_2 supercomplex isolated from plant [54,55] and mammalian mitochondria were generated by comparison of the 2D projection map of the supercomplex, as revealed by Electron Microscopy analysis (EM) and single particle image processing, with known EM and x-ray structures of Complex I and Complex III. In *Arabidopsis* [54], the specific orientation observed for the two respiratory chain complexes indicates an interaction within the plane of the membrane whereas the matrix-exposed protein domains are in one another's vicinity but probably do not (strongly) interact.

Positions and orientations of all the individual complexes were determined more in detail in a bovine supercomplex consisting of Complex I, dimeric Complex III and Complex IV ($I_1III_2IV_1$) [56]. According to this model, Complex III and IV are both associated with the membrane arm of Complex I and are also in contact with each other; the concave face of Complex IV, which is the dimer interface in the x-ray structure, is the contact surface with the rest of the supercomplex. This is in contrast to the proposed interaction in yeast [57] where the Complex IV monomer is specifically attached by its convex side to Complex III_2.

The presence of supramolecular assemblies formed by respiratory enzymes that catalyse CoQ-dependent redox reactions poses the question whether a putative hydrophobic area exists, in the supercomplexes, where CoQ molecule(s) might be restricted in order to facilitate their binding and direct switching from one redox partner to the other. To our knowledge, no clear demonstration is available of the architecture of such a site in supercomplexes and indirect indications can be only evinced from the 3D-models showing a close proximity of the CoQ-binding surfaces in the individual protein components of the supercomplex [54–56]. In particular, very recently, Bultema et al. [58] have studied the higher level conformation of respiratory mega-complexes of potato mitochondria that appear as row-like structures composed by building blocks, which are repeating $I_2III_2IV_2$ units. The structural features of those respiratory strings would allow to propose a model where the CoQ-binding sites in Complex III are deeply buried in the megacomplex and, apparently, are only accessible from the inside of a small area that is boundary-limited by Complex I.

On the basis of the structural information gained from the 3D map [56], also the binding site for cytochrome *c* in Complex III is facing the corresponding binding site in Complex IV; thus the model proposed by Schäfer et al. supports the notion of a more efficient electron transfer through the supercomplex due to the short diffusion distances of substrates. Interestingly, analysis of the characteristic features of the III_2IV_2 supercomplex in *Saccharomyces cerevisiae* revealed additional EM densities on the intermembrane-space-exposed side of Complex III in the supercomplex which could be interpreted as bound cytochrome *c* molecules [57].

Despite mounting evidence based on electrophoretic and EM results, demonstration of mitochondrial respiratory supercomplexes by genetic or biochemical means is significantly lacking [59–61] and also the functional characterization of the supercomplexes is still poor. However, it is worth mentioning that several pieces of evidence for electron-transfer supercomplexes of integral membrane proteins have been produced for the components of the photosynthetic and respiratory chains in bacteria. In particular, the study by Joliot et al. [62] provides indication on the kinetic and structural properties of a supercomplex in *Rhodobacter sphaeroides* which includes a dimer of reaction centres, cytochrome *c* and the bc_1 complex; the same authors also demonstrate that a local pool of quinone molecules is trapped in the supercomplex in the case of the wild type but not in the case of a $PufX^-$ bacterial strain. In addition, relevant information is available for the stability and functional significance of supercomplexes as observed by mutational analysis in bacteria [39,63].

4. Functional evidence for CoQ channelling: flux control analysis of the respiratory complexes

The only kinetic demonstration contrasting the model of Kröger and Klingenberg [1,2] of a pool function of CoQ as a mobile intermediate between dehydrogenases and Complex III was obtained in *S. cerevisiae* mitochondria; Boumans et al. [64] showed that the antimycin inhibition curve of succinate oxidase is linear, showing that ubiquinone does not exhibit pool behaviour, and implying that the respiratory chain in yeast is one functional and physical unit. This study anticipated the exploitation of metabolic flux control analysis to detect substrate channelling in the respiratory chain.

Kinetic testing using metabolic flux control analysis is a powerful source of information on the supra-molecular organization of enzyme complexes [65,66]. Metabolic Control Analysis predicts that if a metabolic pathway is composed of distinct enzymes freely diffusible in a dynamic organization, the extent to which each enzyme is rate-controlling may be different and the sum of all the flux control coefficients for the different enzymes should be equal to unity.

The flux control coefficient (C_i) of a step in a metabolic pathway is defined as the fractional change in the global flux through the pathway that is induced by a fractional change in the enzyme under consideration; it can be expressed in mathematical terms [65] as the ratio between the change over the metabolic flux rate $(dJ/dI)_{I\to 0}$ and the corresponding infinitesimally small change of enzyme activity $(dv_i/dI)_{I\to 0}$ induced by a specific inhibitor.

On the other hand, in a supercomplex, the metabolic pathway would behave as a single enzyme unit, and inhibition of any one of the enzyme components would elicit the same flux control. In particular, in a system in which the respiratory chain is totally dissociated from other components of the OXPHOS apparatus (i.e. ATP synthase, membrane potential, and carriers), such as open non-phosphorylating submitochondrial particles, the existence of a supercomplex would elicit a flux control coefficient near unity at any of the respiratory complexes, and the sum of all coefficients would be above 1 [67].

An alternative way for visualization of the extent of control exerted by a step on the total flux is by building threshold plots, i.e. plots of residual integrated activity (NADH oxidase or succinate oxidase) versus extent of inhibition of the single step (enzyme complex) [68]. A linear plot indicates strong control by the individual step over the total flux, while a biphasic plot indicates that there is a large reserve capacity of that individual step before the total flux is affected.

The use of flux control coefficients for the assessment of pool behaviour, although based on different theoretical grounds, has a practical exploitation very similar to that of inhibitor titrations: in fact, the presence of a lag in the inhibition of the integrated activity, as an indication that the inhibited step is not rate-limiting, is common to both types of analysis.

We addressed the problem in mammalian and in plant mitochondria. The flux control coefficients of the respiratory complexes (I, II, III, IV) were investigated in bovine heart mitochondria and submitochondrial particles devoid of substrate permeability barriers using specific inhibitors of each complex [10] (Table 1). Both Complexes I and III were found to be highly rate-controlling over NADH oxidation, a strong kinetic evidence suggesting the existence of functionally relevant association between the two complexes. On the contrary, Complex IV appeared to be randomly distributed; it is possible, however, that if any stable interaction with Complex IV exists in mammalian mitochondria, it escaped detection most likely due to a pronounced abundance of molecules in non-assembled form. Moreover, Complex II is fully rate-limiting for succinate oxidation, clearly indicating the absence of substrate channelling between Complex II

Table 1
Flux control coefficients (C_i) of the respiratory complexes in frozen/thawed mitochondria from bovine heart (BHM) and potato tuber (POM)

Step	BHM		POM	
	NADH oxidase activity	Succinate oxidase activity	NADH oxidase activity	Succinate oxidase activity
Complex I	1.06	n.a.	0.89	n.a.
Complex II	n.a.	0.88	n.a.	0.38
Complex III	0.90	0.34	1.11	1.05
Complex IV	0.26	0.20	1.15	0.94

Data were calculated using the whole set of values obtained from repeated assays for each experimental condition. Owing to that kind of mathematical analysis, the flux control coefficients shown in the table could not be expressed in the form of mean values with standard errors. (Reprinted from [58], Copyright 2008, with permission from Elsevier.)
n.a.: not applicable.

and Complex III. This latter finding contrasts the results of Boumans et al. [64] in yeast of a linear inhibition of succinate oxidation by antimycin.

In permeabilized mitochondria from freshly harvested potato tubers, where no activity of the so called alternative oxidase, AOX, is present at the level of ubiquinone [69], inhibitor titration experiments on the rotenone-sensitive and rotenone-insensitive respiration indicate that Complexes III and IV are involved in the formation of a supercomplex assembly comprising Complex I. On the other hand, the alternative dehydrogenases, as well as the molecules of Complex II, are considered to be independent structures within the inner mitochondrial membrane [70].

The electrophoretic demonstration of the existence of respiratory supercomplexes requires use of digitonin that alters the membrane and destroys the permeability barriers; on the other hand, our initial demonstration by flux control analysis that a Complex I–III aggregate exists in respiring submitochondrial particles was done under non-phosphorylating conditions in a system lacking possible rate-limiting steps external to the respiratory chain per se (NAD-linked dehydrogenases, substrate carriers, membrane potential, ATP synthase, adenine nucleotide carrier). Flux control analysis in intact mitochondria under phosphorylating or uncoupled conditions usually exhibits low flux control for respiratory complexes (cf. [71–75]) and distributed together with other components, as the adenine nucleotide carrier, the ATP synthase, and presumably the substrate carriers and the NAD-linked dehydrogenases. Thus, the presence of several rate-limiting factors would make the detection of supercomplexes quite difficult.

Nevertheless, we have obtained recent evidence of the existence of a Complex I–III association by flux control analysis in liver mitochondria from old rats under phosphorylating conditions (State 3) [70]; the reason for being able to exploit flux control analysis in such mitochondria was that, in older rats, respiration becomes rate-limiting over other accessory activities and allows to detect a Complex I–III association as described above. The threshold plots of rotenone inhibition of Complex I and of mucidin inhibition of Complex III are both linear, indicating the existence of a functional supercomplex. No information about the presence of supramolecular assemblies can be inferred based on the plots obtained in State 4 (controlled respiration) because it is known that in this condition the proton leak across the inner membrane becomes the rate-limiting step; therefore, a very low metabolic control over respiration is exerted by the enzyme complexes regardless of their assembly status.

5. Pool behaviour of CoQ

The first proposal that CoQ functions as a mobile electron carrier was given by Green [3] on the basis of the isolation of discrete lipoprotein complexes of the respiratory chain, of which the quinone was a substrate in excess concentration over the prosthetic groups in the complexes. The proposal was subsequently supported by the kinetic analysis of Kröger and Klingenberg [1]; they showed that steady-state respiration in submitochondrial particles from beef heart could be modelled as a simple two-enzyme system, the first causing reduction of ubiquinone and the second causing oxidation of ubiquinol. If diffusion of the quinone and quinol species is much faster than the chemical reactions of CoQ reduction and oxidation, the quinone behaves kinetically as a homogeneous pool. According to this assumption, during steady-state electron transfer, the overall flux observed (V_{obs}) will be determined by the redox state of the quinone and proportional to either reduced Q (Q_{red}) or oxidized Q (Q_{ox}) concentration:

$$V_{obs} = V_{ox}(Q_{red} / Q_t) = V_{red}(Q_{ox} / Q_t) \quad (1)$$

where V_{ox} is the rate of ubiquinol ($CoQH_2$) oxidation, V_{red} is the rate of CoQ reduction and Q_t is total CoQ concentration (reduced plus oxidized).

Manipulation of Eq. (1) leads to the pool equation

$$V_{obs} = (V_{red} \cdot V_{ox}) / (V_{red} + V_{ox}) \quad (2)$$

The hyperbolic relation of electron flux on the rate of either CoQ reduction (V_{red}) or $CoQH_2$ oxidation (V_{ox}) was confirmed in a variety of systems [11–13,76]. The concept of a mobile, laterally diffusing pool of CoQ molecules linking dehydrogenases and bc_1 complexes is the natural consequence of these kinetic observations.

Further evidence was provided by the characteristic effect of changing V_{red} or V_{ox} on inhibitor titration curves [2]. According to this concept, pool behaviour is characterized by a convex hyperbolic relationship between the integrated oxidation rate and the inhibitor concentration, whereas a linear relationship is expected by a stoichiometric association between the two enzymes.

A large body of experimental evidence concerning succinate oxidation has validated the pool equation in a variety of mitochondrial systems, if we except the previously quoted study in yeast by Boumans et al. [64]; on the other hand, fewer data are available for NADH oxidation [1,11,21].

CoQ-pool behaviour was shown only in reconstituted systems [14,77–79] or in mitochondria where the relations of the inner membrane complexes were altered by swelling [80], freeze-thawing cycles [81], or sonication [1]; it might be argued that in intact mitochondria inner–outer membrane contacts and the quasi-solid organization of the matrix [82,83] may keep the integral proteins in a clustered immobilized arrangement. A study dealing with presence of diffusible intermediates in the respiratory chain in intact phosphorylating mitochondria was accomplished by Stoner [84] who showed that in state 3 (i.e. synthesizing ATP), inhibition of Complex III with myxothiazol makes succinate oxidase less sensitive to the Complex II inhibitor 3′-hexylcarboxin. This finding is in accordance with the existence of a freely diffusible intermediate between the two steps, as theorized by Baum [85] on the basis of double inhibitor titration experiments. In fact, in the presence of a diffusing intermediate between two steps, inhibition of one single step increases its metabolic control and makes the other step less rate-controlling. Unfortunately, this study did not include similar titrations for search of a diffusible intermediate between Complexes I and III.

As a conclusion we may state with some certainty that, in beef heart mitochondria, succinate oxidation exhibits pool behaviour, indicating the presence of CoQ as a diffusible intermediate between Complex II and Complex III; on the other hand, the same statement for NADH oxidation is supported by less clear-cut evidence.

6. Saturation kinetics of Coenzyme Q

If the CoQ concentration is not saturating for the activity of the reducing and oxidizing enzymes, the Eq. (1) is modified [13] by feeding it in the Michaelis–Menten equation for enzyme kinetics,

taking into account the total CoQ concentration [Q_t], the individual V_{max} of the dehydrogenase and Complex III and their dissociation constants for CoQ. V_{obs} is hyperbolically related to [Q_t] and maximal turnovers of electron transfer are attained only at [Q_t] saturating both V_{red} and V_{ox} [6].

$$V_{obs} = \frac{[(V_{mr} \cdot V_{mo}) / (V_{mr} + V_{mo})] \cdot Q_t}{[(V_{mr} \cdot K_{so} + V_{mo} \cdot K_{sr}) / (V_{mr} + V_{mo})] + Q_t} \quad (3)$$

where V_{mr} and V_{mo} are the maximal velocities of CoQ reduction and ubiquinol oxidation, respectively, and K_{sr} and K_{so} are the dissociation constants for the dehydrogenase and for Complex III, respectively. An additional complication in the saturation kinetics of CoQ may be in the fact that multiple quinone-binding sites may exist in the competent enzymes (e.g. site o or P and site i or N in Complex III) [17] and that oxidized and reduced forms of CoQ may bind to the same binding sites with different affinities and mutually compete with one another.

The factor in square parentheses at the denominator of Eq. (3) is the apparent K_m for Q_t of the integrated activity (NADH oxidation or succinate oxidation). The "K_m" in the composite system is a poised function of V_{max} and dissociation constants for CoQ of the complexes involved; this "K_m" can be therefore varying with rate changes of the complexes linked by the CoQ-pool, but is anyway an important parameter, in that it is operationally described as the Q_t concentration yielding half-maximal velocity of integrated electron transfer V_{obs} [6].

The relation between electron transfer rate and CoQ concentration was seen for NADH oxidation in reconstituted systems and in phospholipid-enriched mitochondria [80,81]; in spite of the calorimetric and spin label evidence of Gwak et al. [86] suggesting association of Complexes II and III, it seems that succinate oxidation obeys pool behaviour, as it is dependent on concentration of the CoQ-pool [79,80].

The substrate-like nature of CoQ is best shown by the fact that it exhibits saturation kinetics when the natural CoQ_{10} is titrated in integrated respiration (i.e. NADH– and succinate–cytochrome *c* reductase) [6,13,79]. Direct titrations of CoQ-depleted mitochondria reconstituted with different CoQ supplements yielded a "K_m" of NADH oxidation for Q_t in the range of 2–5 nmol/mg mitochondrial protein [79], corresponding to a Q_t concentration of 4–10 mM in the lipid bilayer. A puzzling observation is that the K_m for CoQ_{10} of NADH–cytochrome *c* reductase is much higher than that of succinate–cytochrome *c* reductase [79]. On the contrary, Norling et al. [77] had found similar values for the two systems.

In particular, the results by Estornell et al. [79] show that K_m for CoQ_{10} of succinate–cytochrome *c* reductase is of the same order of magnitude of the concentration of respiratory enzymes, thus suggesting a possible interpretation in favour of a stoichiometric association of Complex II with Complex III. However, such an association was not experimentally found in the Blue Native PAGE investigations [8] nor in metabolic flux control analysis [10], but was suggested by biophysical investigations [86].

Analysis of the literature shows that the physiological CoQ content of several types of mitochondria [87] is in the range of the K_m for NADH oxidation, and therefore not saturating for this activity.

7. Deviations from pool behaviour

Deviations from pool behaviour of CoQ were also described in the past, raising doubts on its universal validity [13].

Important deviations from pool behaviour were reported in beef heart submitochondrial particles oxidizing both NADH and succinate at the same time [88], in such a case, the pool equation would be modified as described by Gutman [11]:

$$V_{obs(N+S)} = (V_{ox} \cdot \Sigma V_{red}) / (V_{ox} + \Sigma V_{red}) \quad (4)$$

where $V_{obs(N+S)}$ is the total observed rate of electron transfer in presence of both NADH and succinate and ΣV_{red} is the sum of the reduction rates $V_{red(N)}$ and $V_{red(S)}$ with NADH and succinate respectively. Actual measurement of the fluxes [88] yielded values of $V_{red(N)}$ much smaller than expected from Eq. (4), indicating that the two systems do not interact as closely as expected for a single homogeneous CoQ pool. According to Gutman [11], a source of non-homogeneity is the different redox state of the quinone molecules in the vicinity of different reduction sites, that is maintained by the finite diffusion rate of the quinone, producing a mosaic structure rather than a homogeneous pool. Nevertheless, the CoQ lateral diffusion rate as measured by fluorescence quenching of membrane probes [16] is rather high and presumably not rate-limiting for electron transfer [12], thus the non-homogeneity of the ubiquinone pool with respect to succinate and NADH oxidation may better be interpreted in terms of compartmentation of CoQ in the I–III supercomplex in contrast with the free pool used for connecting Complexes II and III.

Gutman [11] also investigated the properties of the NADH and succinate oxidation in submitochondrial particles in relation to the rates of energy-dependent reverse electron transfer from succinate to NAD^+ and of forward electron transfer from NADH to fumarate; the rates were changed by inhibition of Complex II by TTFA and of Complex I by mersalyl; succinate oxidase and NADH oxidase were assumed to represent the activities of Complex II and Complex I, respectively, since the dehydrogenases were considered rate-limiting for the whole respiration. Since reduction of NAD^+ by CoQ is the rate-limiting step in reverse electron transfer, TTFA inhibition will not affect the rate-limiting step and hence we expect a hyperbolic interrelation between the residual velocities of reverse electron transfer and succinate oxidase: indeed a hyperbolic relation identical to the theoretical curve calculated from the pool equation (Eq. (2)) was experimentally found between the rate of NAD^+ reduction by succinate and of succinate oxidase. On the other hand, the hyperbolic relation between NADH–fumarate reductase and NADH oxidase considerably deviated from the theoretical curve and was not in accordance with the pool equation. Fig. 1 shows a re-elaboration of Figs. 3 and 4 in Gutman [11] in which residual activity of reverse electron transfer is plotted as a function of succinate oxidase (i.e. Complex II) and that of NADH–fumarate reductase as a function of NADH oxidase (i.e. Complex I). We redrew the plot to resemble threshold plots exploited for flux control analysis where percent inhibition of Complex I or II is the independent variable in the abscissa. Gutman concluded that "the electron flux from succinate dehydrogenase to oxygen (forward electron transfer towards Complex III) or to NADH dehydrogenase (reverse electron transfer) employs the same carrier and is controlled by the same reaction" whereas "the electron transfer from NADH to oxygen does not share the same pathway through which electrons flow in the NADH–fumarate reductase". In other words, Complex I and Complex II are linked by a different pathway with respect to Complex I and Complex III: that is in line with what is found in the supercomplex organization, as described previously.

In their original paper on the CoQ pool function, Kröger and Klingenberg [1] already noticed that 10–20% of CoQ in submitochondrial particles is not reduced by any substrate. In a recent publication, Benard et al. [89] exploited flux control analysis in State 3 (phosphorylating) conditions with succinate as substrate and measured the reduction levels of CoQ and cytochrome *c*. Their results were interpreted to describe the existence of three different pools of CoQ (and cytochrome *c*): one utilized during steady-state respiration, another mobilizable, i.e. a reserve that is used in case of a perturbation to maintain the energy fluxes at normal values (e.g. as a consequence of inhibition of the respiratory complexes or in case of mitochondrial diseases), and a third one which is not mobilizable and is unable to participate in succinate-dependent respiration. The size of the reserve and of the non utilizable pool is, respectively, ca. 8% and 79% of the

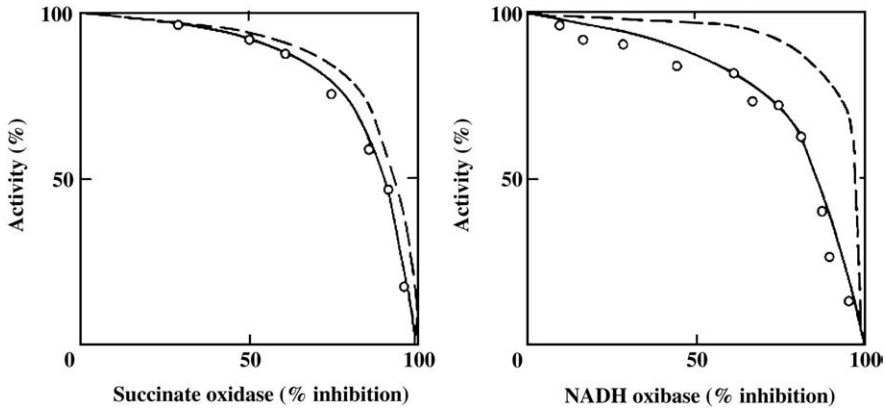

Fig. 1. Electron fluxes between Complex I and Complex II. Interrelation between (left) the residual reverse electron transfer and succinoxidase inhibition, as measured in the presence of varying concentrations of thenoyltrifluoroacetone and (right) residual NADH–fumarate reductase activity vs. NADH oxidase inhibition, as modulated by mersalyl titration. Results are expressed as percentage with respect to the rates measured in uninhibited preparation. Theoretically predicted hyperbolic curves based on pool equation (Eq. (2)) are shown as dashed lines. (Adapted from [11].)

total amount of CoQ in muscle mitochondria and, correspondingly, 23% and 21% in liver. These results are compatible with CoQ compartmentation, although similar results with NADH oxidation were not provided. It is interesting that different tissues widely differ in their contents and stoichiometries of respiratory components (cf. also [90] and Table 1 in [51]), suggesting that each tissue may be specific in terms of supercomplex organization. These observations and the detection of the different nature and extent of the rate-limiting steps of respiration by metabolic flux control analysis in different tissues allow an explanation of the tissue-specific effects of mitochondrial diseases [89,91].

Other deviations from pool behaviour occur at high membrane viscosity [92] and at low ubiquinone concentration [14], when the diffusion of the few quinone molecules over large distances may be hampered; under these conditions one dehydrogenase unit may reduce only one or few Complex III molecules within the distance scanned by diffusion, so that the system may approach solid-state behaviour.

In the already cited study [64] in *S. cerevisiae* mitochondria under physiological conditions, it was shown that neither ubiquinone nor cytochrome *c* exhibits pool behaviour, as determined from inhibitor titration experiments. The inevitable implication is that the respiratory chain in yeast is one functional and physical unit, all respiratory complexes having a control coefficient of one on respiration with either NADH or succinate. On the other hand, addition of high phosphate or trichloroacetate, acting as chaotropic agents, restores pool behaviour for both electron carriers. The authors concluded that the respiratory chain of yeast is organized as a supra-molecular unit, but ascribed their findings to a special feature of the respiratory chain in yeast at difference with higher eukaryotes where pool behaviour is normally found. It is puzzling, however, that the original study of Cruciat et al. in yeast [38] did not find Complex II as part of a supercomplex because its subunits did not co-fractionate with the III–IV supercomplex. No other studies dealing with Complex II in relation to supercomplexes in *S. cerevisiae* are available. It is however of interest that, in their recent publication, Acín-Peréz et al. [93] found minute amounts of Complex II bound in a functional supercomplex in mouse liver mitochondria.

8. Critical appraisal of the Coenzyme Q pool behaviour

The physiological implications of the specific interaction between Complex I and Complex III to form a supercomplex are not yet fully understood. It was speculated that they include enhancing of electron flow by direct channelling of ubiquinone. We will now examine this question in more detail.

The key question concerns the compatibility/incompatibility of the stoichiometric channelling of CoQ between Complex I and Complex III with the existence of pool behaviour by the bulk of CoQ molecules free in the bilayer. First of all, the problem appears to be confined to the interaction between Complex I and Complex III, since no clear demonstration exists that Complex II is part of a supercomplex. In fact, Complex II kinetically follows pool behaviour in mitochondrial membranes [1,2,11], in reconstituted systems [79] and in the double inhibitor titration experiments in intact mitochondria [84], in complete accordance with the lack of supercomplexes found by both BN-PAGE [8] and flux control analysis [10]. Nevertheless, the isolation of discrete units having succinate–cytochrome *c* reductase activity [94,95], the biophysical studies by Gwak et al. [86], the antimycin titration experiments in yeast [64], and the isolation of supercomplexes containing Complex II, though in scant amount [93], appear in strong contrast with this indication. The only possible explanation so far is in the existence of very loose but specific contacts between Complex II and Complex III, not giving rise to any kind of channelling even in phosphorylating mitochondria [84]; these contacts are lost even in frozen mitochondria and in submitochondrial particles (SMP), as appears by BN-PAGE [8], but may be strongly non-physiologically enhanced by some treatments used for mitochondrial respiratory chain fractionation.

We can summarize the available evidence against channelling in three major points: (a) electron transfer in the CoQ region obeys pool behaviour according to the Kröger–Klingenberg equation; (b) electron transfer in the CoQ region follows saturation kinetics with respect to CoQ; (c) the integral proteins of the inner membrane are randomly distributed in the bilayer and phospholipid dilution of the mitochondrial membrane proteins slows down electron transfer. The problem has been addressed in part in a previous publication [51], here we analyse it in further detail.

Actually, it may be argued that in presence of preferential associations between Complexes I and III the pool equation would be still validated if the rate of association/dissociation of the complexes from the supercomplexes were faster than the rate of electron transfer between complexes and CoQ molecules in the pool. An alternative possibility would be that CoQ reduced by one enzyme within a supercomplex has anyway to dissociate in the pool in order to meet any other supercomplex, or the same one in a different site, for being oxidized. However, the former assumption is against the stable nature of supercomplexes as appears by BN-PAGE, since they are not easily dissociated by digitonin or detergents; moreover both assumptions contrast the demonstration of CoQ channelling by flux control analysis. Thus, we have to analyse the three questions asked above to

explain in detail the reasons why the CoQ channelling was overlooked in the past.

a) Electron transfer in the CoQ region obeys pool behaviour according to the Kröger–Klingenberg equation.

Survey of the literature does not provide as many examples of pool behaviour of CoQ concerning NADH oxidation as those concerning succinate oxidation. Particularly, studies of the quantitative adherence to CoQ pool behaviour for NADH oxidation are usually not validated by direct measurement of V_{red} (NADH–CoQ reductase), that is calculated indirectly from NADH oxidase [11,14,96].

Furthermore, we have to consider that the maximal rate of Complex I activity in mitochondria is considerably slower than that of Complex III, due mainly to lower content [20] rather than different turnover [21,32]. For this reason, application of the pool equation (Eq. (2)) to a system where $V_{red} \ll V_{ox}$ would yield V_{obs} approaching V_{red}; thus for example, for NADH–CoQ reductase activity of 0.5 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ and ubiquinol$_{(2)}$–cytochrome *c* reductase activity of 2.5 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$, the calculated rate of NADH–cytochrome *c* reductase would be 0.42 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ i.e. 84% of V_{red}. Experimental values so close to V_{red} were interpreted in the past as adherence to pool behaviour whereas they are also compatible with CoQ channelling.

In fact, in a supercomplex containing stoichiometric amounts of Complex I and of dimeric Complex III, we expect that the total rate for the integrated activity (V_{obs}) would be determined by that of Complex I (V_{red}); whereas the existence of pool behaviour between the same units involved should elicit a rate V_{obs} considerably lower, according to the pool equation (Eq. (2)) yielding $V_{obs} = V_{red}/2$, as in the case of $V_{red} = V_{ox}$. In the real situation, we have to consider that there is an excess of Complex III dimers not involved in the supercomplex formation [8] which would not significantly contribute to determination of flux control coefficients, since the shapes of the titration curves are compared as normalized percentage values; however it cannot be excluded that they would contribute to the determination of V_{ox} in Eq. (2), since the experimental rates of ubiquinol–cytochrome *c* reductase activity are obtained in the presence of saturating amounts of exogenously added quinol and might include both free Complex III and Complex III in the supercomplex. As in the example shown above, application of Eq. (2) under such a condition of V_{ox} overestimation would raise the calculated V_{obs}, that would approach the true high rate of channelling in the supercomplex, and would erroneously indicate the presence of a pool behaviour as a consequence of the apparent strict correspondence between the experimental and the calculated rate values.

Let us consider again the example where NADH–CoQ reductase activity is 0.5 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ and ubiquinol–cytochrome *c* reductase activity is 2.5 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$. Then suppose that, in the latter rate value, 1.0 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ would be the activity of bound Complex III, whereas the excess of free Complex III would have 1.5 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$. As a consequence:

1. for channelling, the expected rate for the integrated activity NADH–cyt. *c* would be 0.5 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ (same as V_{red}, the rate-limiting step);
2. whereas we would obtain $V_{obs} = 0.33\ \mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$, when applying the pool equation (Eq. (2)) only to Complex III involved in the supercomplex;
3. and (erroneously) $V_{obs} = 0.42\ \mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$ (much similar to channelling), when applying the pool equation to all the Complex III molecules.

It is also worth mentioning that the demonstration of pool behaviour by the convex hyperbolic inhibition curve of NADH or succinate oxidase activity in the presence of antimycin [2] may be complicated by the cooperativity of antimycin inhibition in the dimer of Complex III [97,98], so that other inhibitors should be preferred [14]. Indeed, in bovine heart SMP we found a convex hyperbolic inhibition curve of ubiquinol–cytochrome *c* reductase by antimycin, that was overlapped by a similar shaped inhibition curve of either NADH– or succinate–cytochrome *c* reductase. On the other hand, the ubiquinol–cytochrome *c* reductase inhibition curve by either myxothiazol or mucidin was hyperbolic; so was the inhibition curve of NADH–cytochrome *c* reductase (unpublished observations).

(b) Electron transfer in the CoQ region follows saturation kinetics with respect to CoQ.

The hyperbolic relation of V_{obs} on total CoQ concentration in the membrane (Eq. (3)) indicating saturation kinetics has been obtained by solvent extraction of CoQ followed by reconstitution [79]; thus the design of the experiment allows considerable time to elapse between the CoQ incorporation in the membrane and the activity assays. If binding of inter-complex quinone occurs, thus allowing electron channelling directly from Complex I to Complex III, but the bound quinone is in dissociation equilibrium with the CoQ pool, then its amount, at steady state, would be dictated by the size of the pool (i.e. the greater the pool the more the bound molecules); this equilibrium would explain the saturation kinetics for total ubiquinone exhibited by the integrated activity of Complex I and Complex III [79]. To be in agreement with the experimental observation obtained by metabolic flux analysis, this proposition must however require that the dissociation of bound CoQ be considerably slower than its inter-complex electron transfer activity. Moreover, in order to explain the high apparent K_m found for CoQ in NADH oxidase activity [79], also the rate constant for CoQ binding to Complex I in the supercomplex must be slow [51].

(c) The integral proteins of the inner membrane are randomly distributed in the bilayer and phospholipid dilution of the mitochondrial membrane proteins slows down electron transfer.

Freeze-fracture electron microscopy of giant mitochondria (mega-mitochondria obtained by cuprazone treatment) showed that the intramembrane particles (IMP) are randomly distributed in the inner membrane [99]. It is possible that weak hydrophobic forces keeping together the respirasomes are broken by the freeze-fracture technique. Close inspection of the fractured faces in the electron micrographs [100], however, shows long-range random distribution of the IMP but in the short range it reveals apposition of small clusters of IMP in the fractured planes, hinting at possible associations of the integral proteins, that were not considered by the authors. In a subsequent study by rapid-freeze deep-etch technique of the inner membranes of *Paramecium* it was not possible to obtain fracture faces in the tubular cristae [101]; however the study revealed projections of 9 nm diameter, identified as F1-ATPase, located entirely out of the cristae towards the matrix; these particles were arranged in non-random tightly ordered pattern, contrasting the idea of a random distribution of membrane components; moreover other regularly arranged projections to the matrix, 13 nm wide and 13 to 22 nm long, were seen aligned at regular intervals and were identified as Complex I, possibly in a dimeric state. This study revealed a high ordered state of the proteins in the mitochondrial cristae, at difference with what previously suggested. In a recent study Thomas et al. [102] by combining freeze-fracture and other types of EM also found regular rows of ATP synthase dimers in yeast mitochondrial membranes.

The decrease of respiratory activities in mitochondria fused with phospholipids [80] and the restoration of activity by incorporating excess CoQ can be easily explained by dilution of the CoQ pool together

Table 2
Experimental and calculated values of NADH:cytochrome *c* reductase in a mitochondrial R_4B protein fraction[a] diluted with different levels of phospholipids

Protein/PL[a] (w:w)	Distance[b] (nm)	C_I[c]	C_{III}[c]	NADH–cyt. *c* (μmol/min/mg protein)	V_{obs} (calculated)[d]
1:1	18	0.9	0.7	0.472 ± 0.091 (7)	0.210
1:30	97	0.9	0.2	0.273 ± 0.028 (4)	0.236

Values in brackets indicate multiple experiments.
(Reprinted from [58], Copyright 2008, with permission from Elsevier.)
[a] Fraction R_4B from bovine heart mitochondria was fused with phospholipids (Asolectin) and Coenzyme Q_{10} by cholate dilution (cf. ref. [91]).
[b] The theoretical distances between Complex I and Complex III were calculated according to [5].
[c] C_I and C_{III} are the metabolic flux control coefficients of Complex I and Complex III, respectively, over NADH:cytochrome *c* oxidoreductase activity in the proteoliposomes.
[d] NADH:cytochrome *c* reductase activity as calculated from the pool equation (Eq. (2)) using experimental values of NADH:CoQ reductase and ubiquinol:cytochrome *c* reductase activity as V_{red} and V_{ox}, respectively.

with the proteins when the phospholipid to protein ratio increases: such condition closely corresponds to point (b), thus we may easily explain these results with dissociation of CoQ from the I–III supercomplex after membrane dilution with phospholipids. In other words, the CoQ concentration in the lipids decreases by dilution of the membrane and, consequently, dissociation of bound CoQ is favoured therefore its concentration in the supercomplex falls below that yielding V_{max}, and is of course restored by incorporation of exogenous CoQ.

At high phospholipid to protein ratio, however, actual dissociation of the supercomplexes assembly takes place as we have demonstrated by measuring the rates of electron transfer and by flux control analysis by fusing a crude mitochondrial fraction (R_4B) [103] enriched in Complex I and Complex III with different amounts of phospholipids and CoQ_{10} [70,104,105] (Table 2). The comparison of the experimentally determined NADH–cyt. *c* reductase activity with the values expected by theoretical calculation applying the pool equation showed overlapping results at phospholipid dilutions (w/w) from 1:10 on, i.e. for distances >50 nm; on the contrary, at shorter distances between Complex I and Complex III that resemble the mean nearest neighbour distance between respiratory complexes in mitochondria [106,107], pool behaviour was not effective anymore [104,105]. In the two experimental models, kinetic testing according to the Metabolic flux Control Analysis validated the hypothesis of a random organization at low protein content (long distances) and of a functional association between Complex I and Complex III at high protein content (short distances) [70].

8.1. An additional comment to this critical analysis

Of course, the functional relevance of super-complex organization in electron transfer may vary with the physiological conditions in the intact cells: clearly under physiological conditions the respiration rate is under control of energy consuming processes (respiratory control). Under prevalent State 4 conditions (high ATP/ADP ratio), the electrochemical proton gradient is largely the rate-limiting step, so that operation of the respiratory chain as tunnelling or diffusion may be of minor importance for the flux control. On the other hand, the situation may be dramatically different under conditions of high energy demand (State 3) or under uncoupling conditions; the latter may be more important in vivo than originally thought: it has been suggested that muscle and liver mitochondria may dissipate 30–50% of the electrochemical potential as heat [108] through action of the uncoupling proteins [109].

9. Function of the Coenzyme Q pool

Despite the above considerations, a great deal of data in the literature, reviewed in the previous sections, indicates that most

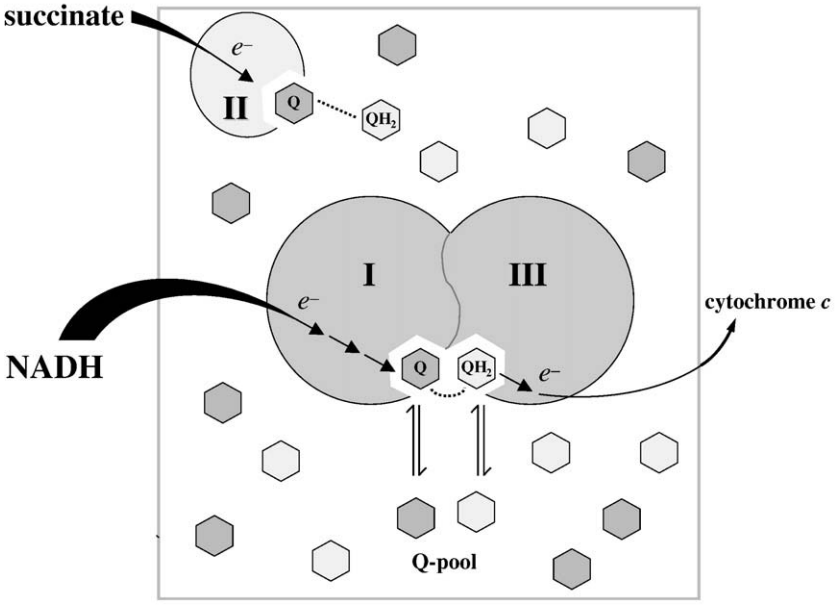

Fig. 2. Possible mechanism for the dissociation of the bound inter-complex quinone with the CoQ pool. Kinetic evidence for intermediate channelling in the I + III supercomplex requires the dissociation rate constants of ubiquinone and ubiquinol to be considerably slower than the rates of electron transfer via the same quinone molecules bound in the supercomplex. Quinone interactions with Complex II are assumed to follow pool behaviour. (Reprinted from G. Lenaz, R. Fato, G. Formiggini and M.L. Genova, The role of Coenzyme Q in mitochondrial electron transport, Mitochondrion 7S 8-33, Copyright 2007, with permission from Elsevier.)

certainly a mobile pool of CoQ exists in the inner mitochondrial membrane, and that this pool coexists with protein-bound CoQ. As discussed above, a major function of the CoQ pool must be to drive binding into sites formed at the border between adjacent Complexes I and III in order to assure correct channelling of electrons form one to the other complex; any decrease of CoQ concentration in the pool would decrease the amount of bound CoQ and therefore induce a fall of electron transfer in the supercomplex (Fig. 2).

By this way, free CoQ behaves as a reservoir for binding to the I–III supercomplex; in addition, free CoQ may be a reservoir for other functions believed to require CoQ binding to specific proteins, such as uncoupling proteins [110] and the permeability transition pore [111,112], and it also represents the main antioxidant species in the inner mitochondrial membrane [113].

Certainly, the CoQ pool is required for electron transfer from Complex II to Complex III: as mentioned in the previous section, Complex II kinetically follows pool behaviour in reconstitution experiments [79] and in intact mitochondria [84], in complete accordance with the lack of super-complexes found by both BN-PAGE [8] and flux control analysis [10].

Furthermore, other activities such as glycerol-3-phosphate dehydrogenase, ETF dehydrogenase, dihydroorotate dehydrogenase, that are likely to be in minor amounts and strongly rate-limiting in integrated electron transfer, are probably dictated by interaction through the CoQ pool. The only direct study addressed to this problem [114] demonstrated that in brown fat mitochondria the inhibition curve of glycerol phosphate–cytochrome *c* reductase is sigmoidal in the presence of myxothiazol and antimycin, suggesting the presence of a homogeneous CoQ pool between glycerol phosphate dehydrogenase and Complex III.

Also reverse electron transfer from succinate to NAD^+, involving sequential interaction of Complexes II and I by means of CoQ, must take place by collisional interactions in the CoQ pool, since no aggregation was demonstrated between Complexes I and II. The hyperbolic relation experimentally found by Gutman between the rate of reverse electron transfer and succinate oxidase [11] is in complete accordance with the pool equation (cf. also Fig. 1).

This observation poses a particularly puzzling question [51]: if all or most Complex I units are associated with Complex III, and the interaction of CoQ in the pool with the quinone-binding site in common between the two enzymes is necessarily slow, then how can $CoQH_2$ reduced by Complex II interact from the pool with the CoQ site in Complex I at a rate compatible with the steady state kinetics of reverse electron transfer? The intriguing idea that Complex I may possess two different quinone-binding sites for direct and for reverse electron transfer respectively is compatible with the proposal [115] that two different routes exist for forward and reverse electron transfers within Complex I. These two sites might become alternatively accessible depending on the magnitude of the membrane potential. Fig. 3 schematically depicts how CoQ in the pool, once reduced by Complex II, would on its hand reduce Complex I under conditions of high membrane potential and drive reverse electron transfer within the Complex to reduce NAD^+ to NADH. In this hypothetical scheme, the CoQ molecule(s) buried in the I–III supercomplex and also in equilibrium with the pool (cf. Fig. 2) would not be in direct communication with the CoQ molecules driving reverse electron transfer.

An alternative possibility, however, may be envisaged. It must be noted that the ATP-driven reverse electron transfer from succinate to NAD^+ occurs in the presence of a high mitochondrial transmembrane protonmotive force; according to Piccoli et al. [116], this force might be the physiological signal and, at the same time, the trigger causing the structural reorganization of the enzymatic complexes of the mitochondrial OXPHOS system. The model hypothesis depicted by Piccoli et al. from the data on cytochrome oxidase might be extended to other enzymes of the respiratory chain, suggesting that also the I–III supercomplex would dissociate its constituting complexes under high $\Delta_{\mu H}+$ condition, and this would no longer limit the access to the CoQ binding site in Complex I.

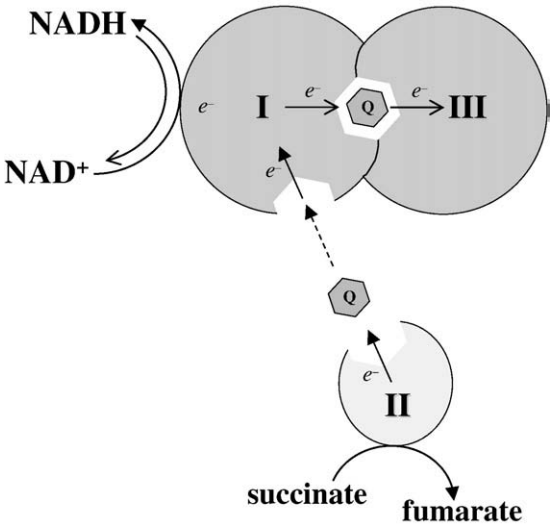

Fig. 3. Schematic representation of forward and reverse electron transfers within Complex I. The intriguing idea that Complex I may possess two different quinone-binding sites is discussed in the text. The arrows represent the direction of electron fluxes but are not indicative of any specific route within the complexes. The possible interaction of the bound inter-complex quinone with the CoQ pool was omitted for clearness (cf. Fig. 2).

10. Conclusions

The substantial contribution of Achim Kröger to understanding the function of Coenzyme Q in mitochondrial electron transfer is still valid today, 35 years after his seminal publications. First, the methodology he developed together with his collaborators is still useful to discriminate the presence of a diffusible intermediate in electron transfer; in particular the inhibitor titration that they developed in their second paper [2] is practically coincident with the use of inhibitor titration in metabolic control analysis for establishing the extent of control exerted by an individual step on the whole pathway.

Although electron transfer between Complex I and Complex III may not be best described by the pool equation and it is likely that substrate channelling of CoQ must take place, the pool equation seems to represent the best description of the mechanism of electron transfer from Complex II and other flavin-linked dehydrogenases to Complex III (or from any dehydrogenase, including Complex I, to the alternative oxidase when present). In addition, the CoQ pool operates in reverse electron transfer from Complex II to Complex I (energy-dependent reduction of NAD^+ by succinate). Moreover, the concept of a CoQ pool is still crucial in presence of CoQ channelling from Complex I to Complex III in determining the steady state equilibrium between bound and free ubiquinone. To this respect, further insight into the mechanisms of electron transfer in energy-conserving membranes cannot avoid considering the pool equation and its derivations as an indispensable tool of evaluation and discrimination.

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Family, spending pleasant hours and exchanging opinions on science and music.

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