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Review

Mitochondrial Complex I: Structural and functional aspects

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Abstract

This review examines two aspects of the structure and function of mitochondrial Complex I (NADH Coenzyme Q oxidoreductase) that have become matter of recent debate.The supramolecular organization of Complex I and its structural relation with the remainder of the respiratory chain are uncertain. Although the random diffusion model [C.R. Hackenbrock, B. Chazotte, S.S. Gupte, The random collision model and a critical assessment of diffusion and collision in mitochondrial electron transport, *J. Bioenerg. Biomembranes* 18 (1986) 331–368] has been widely accepted, recent evidence suggests the presence of supramolecular aggregates. In particular, evidence for a Complex I–Complex III supercomplex stems from both structural and kinetic studies. Electron transfer in the supercomplex may occur by electron channelling through bound Coenzyme Q in equilibrium with the pool in the membrane lipids. The amount and nature of the lipids modify the aggregation state and there is evidence that lipid peroxidation induces supercomplex disaggregation. Another important aspect in Complex I is its capacity to reduce oxygen with formation of superoxide anion. The site of escape of the single electron is debated and either FMN, iron–sulphur clusters, and ubisemiquinone have been suggested. The finding in our laboratory that two classes of hydrophobic inhibitors have opposite effects on superoxide production favours an iron–sulphur cluster (presumably N2) is the direct oxygen reductant.The implications in human pathology of better knowledge on these aspects of Complex I structure and function are briefly discussed.

Keywords: Complex I; Supercomplex; Coenzyme Q; ROS; Rotenone; Stigmatellin

1. Introduction

Complex I is a very large enzyme catalyzing the first step of the mitochondrial electron transport chain [1,2]. The enzyme oxidizes NADH transferring electrons to Ubiquinone (Coenzyme Q, CoQ), a lipid soluble electron carrier embedded in the lipid bilayer of the inner mitochondrial membrane. The total number of subunits in the bovine enzyme is 46 [3] for a molecular mass of about 1000 kDa. Seven subunits are the products of the mitochondrial genome [4,5] and correspond to hydrophobic components named ND1–ND6 and ND4L. The minimal active form of the enzyme is that found in bacteria, composed of 14 subunits, all of which are homologous to their mitochondrial counterparts, while all other subunits are called "accessory subunits" and their role in the mitochondrial enzyme is not clear. From structural and phylogenetic considerations, the enzyme is envisaged to consist of three different sectors: a dehydrogenase unit and a hydrogenase-like unit constituting the peripheral arm protruding into the matrix, and a transporter unit deeply embedded in the membrane [6,7].

The enzyme is endowed with several prosthetic groups: FMN is the entry point for electrons from NADH, that are then transferred to the iron–sulphur clusters [8]. Enzymes from different sources have different numbers of iron–sulphur clusters, most of which share the same midpoint potential and are called "isopotential" clusters. Two clusters present different characteristics: N1a, that is of the kind Fe_2S_2, has the lowest

Abbreviations: AIF, apoptosis-inducing factor; BN-PAGE, blue-native polyacrylamide gel electrophoresis; CIA30, Complex I intermediate-associated protein30; CIA84, Complex I intermediate-associated protein84; CL, cardiolipin; CoQ, Coenzyme Q; DCIP, dichlorophenol indophenol; DPI, diphenylene iodonium; EPR, Electron Paramagnetic Resonance; LHON, Leber's Hereditary Optic Neuropathy; mtDNA, mitochondrial DNA; NDUFAF1, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex assembly factor 1; NDUFS4, NADH dehydrogenase (ubiquinone) Fe–S protein 4; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species

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midpoint potential (E_m=−370 mV) and N2, that is of the kind Fe_4S_4, resides at the interface between the 22 kDa subunit PSST and the 49 kDa subunits [9] and has the highest midpoint potential (E_m between −150 mV and −50 mV), presenting EPR magnetic interactions with the ubisemiquinone radicals; for these reasons, it is considered to be the direct electron donor to ubiquinone [10]. N2 iron–sulphur cluster is most likely located in the connection between the peripheral and the membrane arm. The magnetic interaction with the semiquinone radical, corresponding to a distance of about 10 Å [11,12], suggests that the ubiquinone headgroup could somehow reach up into the peripheral arm as recently assumed by Brandt et al. [13] , who have hypothesized an amphipathic 'ramp' guiding ubiquinone into the catalytic site. Recently the arrangement of iron–sulphur clusters in the hydrophilic domain of Complex I from *T. thermophilus* has been determined by X-ray crystallography, showing a linear chain of all clusters except N1a and N7 [14]. A schematic picture of the enzyme is depicted in Fig. 1.

Complex I is inhibited by more than 60 different families of compounds [15] starting from Rotenone, the prototype of this series, to a number of synthetic insecticides/acaricides. These inhibitors were grouped into three classes based on their effects on the kinetic behaviour of the enzyme: Class I/A (the prototype of which is Piericidin A), Class II/B (the prototype of which is Rotenone) and Class C (the prototype of which is Capsaicin). Nevertheless from kinetic studies it was not possible to assign different binding sites for these three classes of inhibitors: it is commonly accepted that they share the same hydrophobic large pocket in the enzyme [16].

Complex I is also involved in the formation of the transmembrane proton gradient with a stoichiometry of 4 $H^+/2e^-$. The limited knowledge about the structure and the function of Complex I makes it difficult to predict the mechanism by which this respiratory chain complex uses redox energy to translocate protons across the inner mitochondrial membrane (for a review, see [17]).

Besides its well-known redox role in the electron transport chain, Complex I is considered one of the main sites of reactive oxygen species (ROS) production: electron leaks at Complex I can release single electrons to oxygen and give rise to superoxide anion. The mechanism of superoxide production by Complex I is not clear, probably for lack of knowledge of the exact sequence of the electron carriers and how electron transfer is coupled to proton translocation. The major sites of ROS production in the mitochondrial electron transport chain have been localized in Complex I and Complex III [18]; while the site of electron escape in Complex III has been identified in the so called center "o", the direct oxygen reductant site in Complex I is not known with certainty [19].

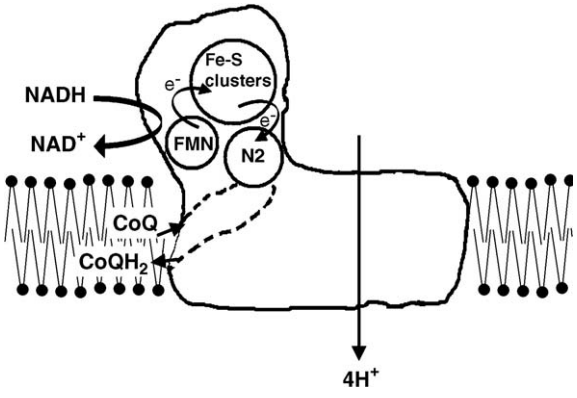

Fig. 1. Schematic picture of Complex I in the inner mitochondrial membrane. The figure is freely redrawn from Brandt et al. [13].

The notion of Complex I as an individual enzyme stems out of its isolation as a discrete lipoprotein unit by detergent fractionation [20]. In spite of the above prevalent view, two extreme conditions can be envisaged on theoretical grounds, and have indeed been proposed, for the organization of the respiratory chain [21]. In the first view, the chain is organized in a *liquid state*. The large enzymatic complexes are randomly distributed in the plane of the membrane, where they move freely by lateral diffusion. Ubiquinone and cytochrome *c* are also mobile electron carriers, whose diffusion rate is faster than that of the bulkier protein complexes. Alternatively, the components of the chain are envisaged to be in the form of aggregates, ranging from small clusters of few complexes to the extreme of a *solid-state* assembly. The aggregates may be either permanent or transient, but their duration in time must be larger than any electron transfer turnover in order to show kinetic differences from the previous model. Recent structural and kinetic evidence strongly suggests that Complexes I and III form stable functional supercomplexes [22,23].

In this mini-review, we focalize on two aspects of Complex I: (a) its relationship with the remainder of the respiratory chain, analyzing available evidence for the existence of a super-complex I–III; (b) the mechanism of electron transfer in the acceptor quinone region of the Complex, exploited by elucidation of the mechanism of oxygen reduction to superoxide radical. Both aspects are particularly relevant to human pathology, and some hints on pathological implications will be given in this review.

2. Supramolecular organization of Complex I

2.1. Structural and kinetic evidence for supercomplexes

A great deal of structural, biophysical and enzyme kinetics studies in the past have addressed the problem of the supramolecular organization of the respiratory chain, until the random collision model proposed by Hackenbrock [24] was accepted by the majority of investigators in the field, in spite of several findings raising doubts on the universal validity of the model.Experimental evidence pertaining to the problem of supramolecular organization with pros and cons the random collision model and related references is exhibited in Table 1.

The random collision model was proposed on the basis of direct investigation of the mobility of mitochondrial components by either electrophoretic relaxation or FRAP (fluorescence recovery after photobleaching), yielding lateral diffusion coefficients in the range between 10^{-9} and 10^{-10} cm^2/s for mitochondrial membrane complexes [24] and higher than 10^{-9} cm^2/s for CoQ [24,25]. The first proposal that CoQ functions as a mobile electron carrier was given by Green [20] on the basis of the isolation of discrete lipoprotein complexes of the respiratory chain, of which the

Table 1
Structural and kinetic evidence related to the organization of the mitochondrial respiratory chain

Experimental analysis	Evidence	Reference	
Ultrastructural morphological appearance of the inner mitochondrial membrane	Supercomplex assembly	S. Fleischer et al. (1967)	[146]
	Random distribution	E. Sowers & C.R. Hackenbrock (1981)	[147]
	Random distribution	C.R. Hackenbrock et al. (1986)	[24]
	Supercomplex assembly	T. Ozawa et al. (1987)	[34]
Isolation of enzyme complexes	Supercomplex assembly	Y. Hatefi et al. (1962)	[148]
	Supercomplex assembly	A. Yu & L. Yu (1980)	[149]
	Supercomplex assembly	E.A. Berry & B.L. Trumpower (1985)	[150]
	Supercomplex assembly	T. Ozawa et al. (1987)	[34]
	Supercomplex assembly	N. Sone et al. (1987)	[151]
	Supercomplex assembly	T. Iwasaki et al. (1995)	[152]
Reconstitution of respiratory complexes in lipid vesicles	Supercomplex assembly	C.I. Ragan & C. Heron (1978)	[57]
	Supercomplex assembly	C. Heron et al. (1978)	[61]
Mobility of mitochondrial complexes (electrophoretic relaxation, ER; FRAP)	Random distribution	S. Kawato et al. (1981)	[153]
	Random distribution	E. Sowers & C.R. Hackenbrock (1981, 1985)	[154,155]
	Random distribution	H. Schneider et al. (1982)	[33]
	Random distribution	S.S. Gupte & C.R. Hackenbrock (1988)	[156]
	Random distribution	G. Lenaz (1988)	[21]
Kinetic analysis of steady state respiration	Random distribution	A. Kröger & M. Klingenberg (1973)	[26]
	Random distribution	R.J. Froud & C.I. Ragan (1984)	[157]
	Random distribution	M. Gutman (1985)	[27]
	Random distribution	C.I. Ragan & I.R. Cottingham (1985)	[28]
	Random distribution	R. Fato et al. (1996)	[112]
	Supercomplex assembly	C. Bianchi et al. (2003)	[66]
Saturation kinetics of electron transfer	Random distribution	B. Norling et al. (1974)	[158]
	Random distribution	H. Schneider et al. (1982)	[33]
	Random distribution	G. Lenaz et al. (1986, 1989, 1997)	[25,30,159]
	Random distribution	E. Estornell et al. (1992)	[29]
Double inhibitor titration	Random distribution for Complex II–III, Supercomplex assembly for Complex III–IV	B. Stoner (1984)	[58]
Calorimetric and spin label EPR analysis of the interaction between complexes	Supercomplex assembly for Complex II–III	S.H. Gwak (1986)	[160]
Gel electrophoresis	Supercomplex assembly	C.M. Cruciat et al. (2000)	[37]
	Supercomplex assembly	H. Schägger et al. (2001, 2004)	[39,44]
	Random distribution for Complex II	H. Schägger & K. Pfeiffer (2001)	[39]
	Random distribution for Complex II	H. Eubel et al. (2003)	[47]
	Supercomplex assembly	H. Eubel et al. (2004)	[46]
	Supercomplex assembly	R. Acin-Perez et al. (2004)	[48]
	Supercomplex assembly	N.V. Dudkina et al. (2005)	[45]
Flux control analysis	Supercomplex assembly	H. Boumans et al. (1998)	[35]
	Supercomplex assembly	C. Bianchi et al. (2004)	[23]
	Random distribution for Complex II and IV		

quinone was a substrate in excess concentration over the prosthetic groups in the complexes, and was subsequently supported by the kinetic analysis of Kröger and Klingenberg [26]; 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 described by the *pool equation* [26]

$$V_{obs} = (V_{red} \cdot V_{ox})/(V_{red} + V_{ox}). \quad (1)$$

This expression was verified under a wide variety of input and output rates and establishes that CoQ distributes electrons randomly among the dehydrogenases and the bc_1 complexes, behaving indeed as a freely diffusible intermediate. 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 [27,28].

If the CoQ concentration is not saturating for the activity of the reducing and oxidizing enzymes, the equation is modified [28] by feeding it in the Michaelis–Menten equation for enzyme kinetics, taking into account total CoQ concentration [Q_t], the individual V_{max} of the dehydrogenase and Complex III and their dissociation constants for reduced and oxidized CoQ, respectively. 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} [25].

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 [29], corresponding to a Q_t concentration of 4–10 mM in the lipid bilayer. 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} [30]. Analysis of the literature shows that the physiological CoQ content of several types of mitochondria [31] is in the range of the K_m for NADH oxidation, and therefore not saturating for this activity.

The relation between electron transfer rate and CoQ concentration was observed in reconstituted systems and in phospholipid-enriched mitochondria for NADH oxidation [32,33].

In spite of this evidence, the idea of preferential associations and of specific aggregates of electron transfer complexes has never been abandoned (cf. [34–36]).

More recently, Cruciat [37] and Schägger [38,39] have produced convincing evidence of multicomplex units in yeast and mammalian mitochondria introducing a quantitative approach: a mild one-step separation protocol for the isolation of membrane protein complexes, namely blue native polyacrylamide gel electrophoresis (BN-PAGE). In bovine heart mitochondria Complex I–III, interactions were detected from the presence of high molecular weight aggregates suggesting the existence of a supercomplex containing one copy of Complex I and two copies of Complex III, i.e., I_1III_2, that was found further assembled into two major supercomplexes (respirasomes) comprising different copy numbers of Complex IV ($I_1III_2IV_2$ and $I_1III_2IV_4$). Only 14–16% of total Complex I was found in free form in the presence of digitonin [39]; it seems therefore likely that all Complex I is bound to Complex III in physiological conditions (i.e., in the absence of detergents). Knowing the accurate stoichiometry of oxidative phosphorylation complexes according to [39], the average ratio I:II:III:IV:V is 1.1:1.3:3.0:6.7:3.5: it is then plausible that approximately one third of total Complex III in bovine mitochondria is not bound to monomeric Complex I. Associations of Complex II with other complexes of the OXPHOS system could not be identified under the conditions of BN-PAGE so far.

BN-PAGE has become a popular experimental strategy for the structural analysis of the protein-complex composition of the respiratory chain in different systems. Based on this procedure, the existence of respirasome-like supercomplexes was also reported for bacteria [40], fungi [41] and higher plant mitochondria [42,43] as well as for human mitochondria [44].

The I–III supercomplex proved to be especially stable. Depending on the plant investigated, 50–90% of Complex I forms part of the I_1III_2 supercomplex in *Arabidopsis* [45], potato [46], bean and barley [47] upon solubilisation of isolated mitochondria by nonionic detergents; whereas Complex IV-containing supercomplexes are of low abundance (bound Complex IV is <10%) and Complex II clearly is not associated, even in plants.

The first chromatographic isolation of a complete respirasome ($I_1III_4IV_4$) from digitonin-solubilised membranes of *Paracoccus denitrificans* indicated that Complex I is stabilized by assembly into the NADH oxidase supercomplex, since attempts to isolate Complex I from mutant strains lacking Complexes III or IV led to the complete dissociation of Complex I under the conditions of BN-PAGE. Reduced stability of Complex I in those mutant strains was also apparent from an almost complete loss of NADH: ubiquinone oxidoreductase activity when the same protocol as for parental strain was applied [40].

Analysis of the state of supercomplexes in human patients with a primary Complex III deficiency [44] and in cultured cell models harbouring cytochrome b mutations [48] also provided evidence that the formation of respirasomes is essential for the assembly/stability of Complex I. Genetic alterations leading to a loss of Complex III prevented respirasome formation and led to secondary loss of Complex I, therefore primary Complex III assembly deficiencies presented as Complex III/I defects. Conversely, Complex III stability was not influenced by the absence of Complex I.

A question that remains to be solved is how the influence of Complex III on Complex I is exerted. In this respect, deeper analytical research is needed to clearly explain the structural linkage between the two enzymes in the supercomplex assembly.

Recently, Dudkina et al. [45] presented a model for how complexes I and III are spatially organized within a stable I_1III_2 supercomplex purified from isolated *Arabidopsis* mitochondria. Structural characterisation by single-particle Electron Microscopy at a resolution of 18 Å revealed a specific lateral association of dimeric Complex III to the tip of the hydrophilic portion of Complex I within the membrane plane. Because negative stain does not penetrate the hydrophobic parts, direct evidence of the membrane-embedded architecture is lacking. Although it is not known which subunits of Complex I constitute the tip, 3D modelling indicated that subunit 9 of Complex III is closest to the interface; whereas the very bulky matrix-protruding domains of both complexes appear in one another's vicinity but are not (strongly) interacting.

Future investigations of the exact composition of Complex I and of its assembly process will probably enhance our understanding of the natural form of the I–III supercomplex. Indeed, it is known that mitochondrial Complex I assembles through the combination of evolutionary conserved modules [49] and that the precise hierarchy through which each of the nascent subunits interact, refold, and self-assemble to generate multipart building blocks can be modulated by several proteins chaperones [44,50,51] It is quite conceivable that, analogous to recent examples of assembly factors for Complex I (i.e., CIA30, CIA84, NDUFAF1, AIF, NDUFS4), additional proteins are involved in the stabilization of Complex I assembly intermediates and it has been proposed that even Complex III subunits may act in the maturation of Complex I. If this process is impeded, because Complex III is absent, Complex I is directed to degradation [48].

Kinetic testing using metabolic flux control analysis is a powerful source of information on the supramolecular organization of enzyme complexes [52,53]. 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. 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 oxidative phosphorylation apparatus (i.e., ATP synthase, membrane

potential, and carriers), such as open non-phosphorylating submitochondrial particles (SMP), 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 [54].

Inhibitor titration experiments in isolated mitochondria from the yeast *Saccharomyces cerevisiae* led to the conclusion that the respiratory chain consists of supermolecular assemblies. Under physiological conditions, neither ubiquinone nor cytochrome *c* exhibits pool behaviour implying that the respiratory chain in yeast is one functional and physical unit, all respiratory complexes having a control coefficient of one on respiration [35].

The problem was also addressed in mammalian and in plant mitochondria. The flux control coefficients of the complexes involved in NADH oxidation (I, III, IV) and in succinate oxidation (II–IV) were investigated in our laboratory using bovine heart mitochondria and submitochondrial particles devoid of substrate permeability barriers. Both Complex 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 appears randomly distributed, although it is possible that if any stable interaction with Complex IV exists, 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 toward Complexes III and IV [23].

Respiration has special features in plant cells; the mitochondrial respiratory chain is branched at the level of ubiquinone owing to the presence of at least five additional so-called "alternative" oxidoreductases which participate in electron transfer [55,56]. Inhibitor titration experiments on the rotenone-sensitive and rotenone-insensitive respiration in permeabilized mitochondria from freshly harvested potato tubers (alternative oxidase absent) indicate that Complex III and IV are involved in the formation of a supercomplex assembly comprising Complex I, whereas the alternative dehydrogenases, as well as the molecules of Complex II, are considered to be independent structures within the inner mitochondrial membrane (unpublished observations from our laboratory).

2.2. Supercomplexes and the Coenzyme Q pool

A critical appraisal of the supercomplex model requires its reconciliation with previous kinetic and morphological evidence that was largely in favour of a random distribution of the complexes.

Morphological evidence by freeze-fracture electron microscopy [24,33] always showed the majority of the intramembrane particles to have a random distribution, with little evidence for organized aggregations; the size of the particles does not seem to be compatible with that of large super-complexes. Nevertheless, it is not possible from microscopy alone to attain quantitative evaluation of the size of the proteins visualized as intramembrane particles. The presence of very large aggregates of the respirasome type seems to be excluded by the freeze-fracture electron microscopic appearance, however smaller supercomplexes such as Complex I–Complex III units would probably be indistinguishable from Complex I monomers. One possible explanation would be that the manipulations required for freeze-fracture microscopy dissociate large, relatively unstable, assemblies allowing detection of only individual complexes or smaller aggregates.

The pool equation is only valid if CoQ behaves as a homogeneous diffusible pool between *all* reducing enzymes (V_{red}) and *all* oxidizing enzymes (V_{ox}) [27]: is this compatible with the existence of preferential associations? Stoichiometric channelling of CoQ between Complex I and Complex III [34,39] would exclude the bulk of the CoQ pool from kinetic determination and would therefore be incompatible with the pool behaviour. Thus, in presence of preferential associations, the pool equation would be experimentally validated only if the rate of association/dissociation of the complexes were *faster* than the rate of electron transfer between complexes and CoQ molecules in the pool.

On the other hand, the bound inter-complex quinone that allows electron flow directly from Complex I to Complex III may well be in dissociation equilibrium with the CoQ pool, so that its amount, at steady state, would be dictated by the size of the pool, i.e., by CoQ concentration: this equilibrium would explain the saturation kinetics for total ubiquinone exhibited by the integrated activity of Complex I and Complex III [29] and the decrease of respiratory activities in mitochondria fused with phospholipids with subsequent dilution of the CoQ pool [33]. To be in agreement with the experimental observation obtained by metabolic flux analysis, this proposition must however require that the dissociation rate constants (k_{off}) of bound CoQ be considerably *slower* than the rates of inter-complex electron transfer (for CoQ reduction by Complex I and its reoxidation by Complex III) via the same bound quinone molecules (Fig. 2). To this purpose, Ragan and Heron [57] in reconstitution experiments of Complexes I and III at different stoichiometries demonstrated that CoQ reduced by Complex I in a supercomplex does not interact at significant rates with free Complex III units by indicating that its dissociation in the pool, if any, must be very slow.

In order to explain the high apparent K_m found for CoQ in NADH oxidase activity [29] also k_{on} for CoQ binding to Complex I must be slow.

On the contrary, Complex II kinetically follows pool behaviour in reconstitution experiments [29] and in the double inhibitor titration experiments [58], in complete accordance with the lack of supercomplexes found by both BN-PAGE [22] and flux control analysis [23]. Since a substantial amount of Complex III molecules appear to be not associated with Complex I, it is likely that $CoQH_2$ reduced by Complex II preferentially reacts with those free complexes.

Other enzymes necessarily needing collisional interactions of CoQ molecules from the pool with Complex III, although no direct demonstration exists, may well be glycerol-3-phosphate dehydrogenase, ETF dehydrogenase, dihydroorotate dehydrogenase, that are likely to be in minor amounts and strongly rate-limiting in integrated electron transfer.

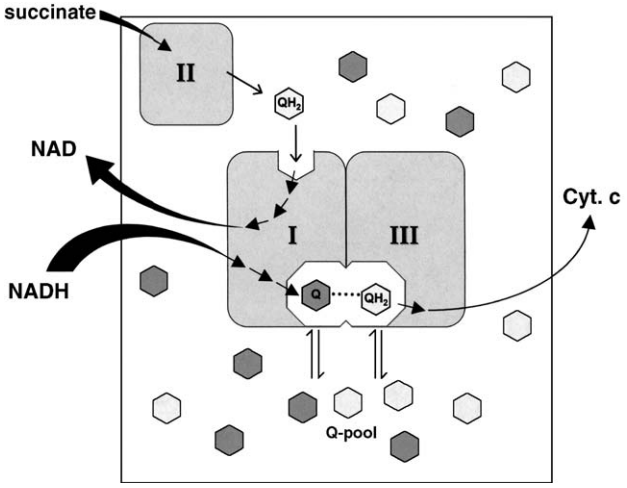

Fig. 2. Scheme of a possible mechanism for the dissociation equilibrium of the bound inter-complex quinone with the CoQ pool. In order to conciliate functional evidences for substrate channelling and saturation kinetics for total ubiquinone exhibited by the integrated activity of Complex I and Complex III, the dissociation rate constants of bound inter-complex ubiquinone (Q) and ubiquinol (QH_2) are assumed to be considerably slower than the rates of electron transfer via the same quinone molecules in the I–III supercomplex. The existence of an alternative site of QH_2 binding in Complex I is hypothetically envisaged for reverse electron transfer from succinate to NAD^+ (cf. text for comments).

For the same reason, 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 exists between Complexes I and II (cf. Fig. 2). This observation poses a particularly puzzling question: if Complex I is completely or almost completely associated with Complex III [22,39], and the interaction of CoQ with the quinone-binding site in common between the two enzymes is necessarily slow (see above), 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 by Vinogradov [59] that two different routes exist for forward and reverse electron transfer within the Complex. These two sites might become alternatively accessible depending on the magnitude of the membrane potential. Alternatively, one should postulate that the association rate constant of *reduced* CoQ from the pool to Complex I in the supercomplex should be sufficiently fast to be compatible with the turnover of reverse electron transfer.

The free diffusing CoQ is also likely to represent the main antioxidant species in the inner mitochondrial membrane, where it can break up the radical chain reaction of lipid peroxidation [60].

2.3. Role of lipids in supercomplex formation and dissociation

Early experiments reported by Ragan and Heron [57] provided evidence that purified Complex I and Complex III, when mixed as concentrated solutions in detergent and then co-dialysed, combine reversibly in a 1:1 molar ratio to form a Complex I–III unit (NADH-cytochrome *c* oxidoreductase) that contains equimolar FMN and cytochrome c_1 and 2–3 mol of Ubiquinone-10 per mol of protein unit. Transfer of reducing equivalents to or from this unit by extra Complex I or Complex III molecules is slow and does not contribute to the overall rate of electron transfer from NADH to cytochrome *c*. Moreover, activation-energy measurements for NADH-cytochrome *c* oxidoreductase activity showed that oxidoreduction of endogenous Ubiquinone-10 proceeds somewhat differently from the oxidation and the reduction of exogenous quinones, supporting the idea that the mobility of Ubiquinone-10 in the Complex I–III unit is highly restricted and suggesting that CoQ_{10} is effectively trapped between the component complexes in an environment that may be partly protein and partly derived from the lipid annuli of those complexes.

However, Q-pool behaviour could be restored and Complex I and Complex III could be made to operate independently of each other by raising the concentrations of phospholipid and ubiquinone (approx. a 2-fold and a 6-fold increase, respectively) in the concentrated mixture [61]. Inclusion of phospholipid into the reconstituted system may have a number of effects on the physical state of the system. Heron et al. [61] have proposed that the principal difference lies in the relative mobility of the reconstituted complexes; when phospholipid in excess of that needed to form an annulus is absent, relative mobility is lost and complexes are frozen in their Complex I–III assembly favouring a stable orientation of the site of reduction of ubiquinone with respect to the site of oxidation. Apparently, the idea that the respiratory complexes are associated in fixed assemblies may sound incompatible with the presence of phospholipids in the natural inner mitochondrial membrane; nevertheless it is worth mentioning that inner–outer membrane contacts and the quasi-solid organization of the matrix [62] may keep the integral proteins in a clustered immobilized arrangement thus favouring segregation of most of the phospholipids into separated patches.

Heron and coworkers also reported that endogenous Ubiquinone-10 leaks out of the Complex I–III unit when extra phospholipid is present, causing a decrease in activity that could be alleviated by adding more ubiquinone [61]. It is likely that the function of the large amount of ubiquinone in the natural membrane may be, therefore, to maintain the ubiquinone-10 content in the supercomplex unit when it is formed.

An analogous system, obtained by fusing a crude mitochondrial fraction (R_4B) enriched in Complex I and Complex III with different amounts of phospholipids and CoQ_{10}, was used to discriminate whether the reconstituted protein fraction behaves as individual enzymes (Q-pool behaviour) or as assembled supercomplexes depending on the average distances experimentally induced between the intramembrane particles. The comparison of the experimentally determined NADH-cytochrome *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, whereas at shorter distances between Complex I and Complex III, resembling the mean nearest neighbour distance between respiratory complexes in mitochondria [63,64], pool behaviour was not effective any more [65,66]. In the two experimental models, kinetic testing according to the Metabolic flux Control Analysis

validated the hypothesis of a random organization at high phospholipids content and of a functional association between Complex I and Complex III at low phospholipids content (unpublished results from our laboratory).

The formation of the supercomplex I–III is conditioned by the lipid component, but the role played by the lipid environment, in terms of its chemical composition, is not completely known.

All purified preparations of mitochondrial electron transfer complexes are isolated as lipoprotein complexes, the extent of associated lipid depending upon the particular method used for isolation. Complete extraction of phospholipid from the lipoprotein complexes revealed a gross phospholipid composition reflecting that for the mitochondrial inner membrane; predominant phospholipids present include cardiolipin, phosphatidylcholine, phosphatidylethanolamine and lesser amounts of neutral lipids and phosphatidylinositol [67].

Two roles of phospholipid have been distinguished: (i) a dispersive solubilisation effect that can be duplicated by appropriate detergents; and (ii) a catalytic effect that can be specifically fulfilled only by cardiolipin [68–71]. Indeed, there are yet two more possible roles that may need to be met, particularly in the case of Complex I and Complex III. These roles might be to provide a sufficiently lipophilic environment for the interaction of the lipophilic electron carrier, ubiquinone, and to participate in linking together components of the respiratory chain.

The phospholipids in closest vicinity to the protein surface, as well as those in the free bilayer, are actually highly mobile and free to exchange, but cardiolipin was indicated as tightly bound being more likely buried within the protein complexes [72–74]. The absolute requirement of cardiolipin (CL) for cytochrome oxidase, Complex I and Complex III activities suggests that this phospholipid plays a crucial role in the coupled electron transfer process [69], but recent results seem also to indicate that cardiolipin stabilizes respiratory chain supercomplexes as well as the individual complexes. The availability of a CL-lacking yeast mutant (Δ*crd1* null) provided the opportunity to demonstrate that mitochondrial membranes still contained the III_2–IV_2 supercomplex, but that it was significantly less stable than supercomplexes in the parental strain. The other phospholipids, that increase in the mutant in substitution of lacking CL, including phosphatidylethanolamine and phosphatidylglycerol, could not substitute for cardiolipin in preventing dissociation of supercomplexes, showing 90% of the individual homodimers of Complex III and IV not organized into supercomplex under BN-PAGE conditions [75,76]. The putative direct protein–protein interaction of cytochrome oxidase and Complex III is proposed to involve the domain formed by transmembrane helices of cytochromes b and c_1 and the core components of Complex IV, namely COX1, COX2 and COX3 [75]. In the structure of yeast, the membrane imbedded domain of Complex III forms a depression and two phospholipid molecules, i.e., cardiolipin and phosphatidylethanolamine, are tightly bound in this cavity [74], suggesting that they can provide a flexible linkage between the above mentioned subunits of Complexes III and IV.

It is well documented that exposure of mitochondria to reactive oxygen species (ROS) can affect the respiratory activity via oxidative damage of cardiolipin which is required for the optimal functioning of the enzyme complexes [77–79]. Circumstantial evidence from our laboratory suggests that dissociation of respiratory supercomplexes occurs in proteoliposomes, due to lipid peroxidation induced by 2,2′-azobis-(2-amidinopropane)dihydrochloride (AAPH) before reconstitution of a protein fraction enriched with Complex I and III (R_4B) into the phospholipid vesicles (unpublished data).

2.4. Pathological implications

The involvement of mitochondria in a variety of pathological aspects and in aging [80–84] has been largely ascribed to their central role in production of Reactive Oxygen Species (ROS) and to the damaging effect of ROS on these organelles. In particular, damage to mitochondrial DNA (mtDNA) would induce alterations of the polypeptides encoded by mtDNA in the respiratory complexes, with consequent decrease of electron transfer activity, leading to further production of ROS, and thus establishing a vicious circle of oxidative stress and energetic decline [85,86]. This fall of mitochondrial energetic capacity is considered to be the cause of aging and age-related degenerative diseases [83,84,86,87]. This vicious circle might be broken by agents capable to prevent a chain reaction of ROS formation and damage, such as CoQ in its reduced form [60].

The observation that lipid peroxidation disrupts the aggregation of Complexes I and III (see above) has profound pathophysiological implications, since ROS produced by the mitochondrial respiratory chain induce a progressive peroxidation of mitochondrial phospholipids [60], and in particular of cardiolipin [77,78] in aging and ischemic diseases, with demonstrated decreased activity of both Complexes I and III [79,88]. It is tempting to speculate that under the above conditions, a dissociation of Complex I–III aggregates occurs, with loss of facilitated electron channelling and resumption of the less efficient pool behaviour of the free ubiquinone molecules. Although no direct demonstration exists yet, dissociation of supercomplexes might have further deleterious consequences, such as disassembly of Complex I and III subunits and loss of electron transfer and/or proton translocation; we could not even exclude that the consequent alteration of electron transfer may elicit further induction of ROS generation. The observation that Complex III alterations prevent proper assembly of Complex I has therefore deep pathological implications beyond the field of genetic mitochondrial cytopathies.

Following this line of thought, the different susceptibility of different types of cells and tissues to ROS damage may depend, among other reasons, on the extent and tightness of super-complex organization of their respiratory chains, that depend on their hand on phospholipids content and composition of their mitochondrial membranes.

3. Mechanism of superoxide production by Complex I

3.1. On the site of univalent oxygen reduction

Complex I is generally considered as the major enzyme contributing to generation of ROS in mitochondria; the site of

univalent oxygen reduction in Complex I is still controversial and the reason is in part in the scant knowledge of the mechanism of electron transfer within the enzyme prosthetic groups (for reviews on ROS production by mitochondria cf. ref. [19,89–94]). The physiological relevance of ROS generation by Complex I as well as by different mitochondrial sites is still uncertain and is even questioned by some investigators [95].

Recently, using different Complex I inhibitors to functionally dissect the enzyme, it was suggested that an iron–sulphur cluster, presumably N2 [96,97], but also N1a [98] could be the site of electron leak; however, also ubisemiquinone [99,100] and FMN [101–106] were proposed as electron donors to oxygen. In addition, it was found that the production of reactive oxygen species (ROS) is enhanced in defective Complex I [18], suggesting that structural modifications of the enzyme may play a crucial role in the ROS production process.

The superoxide production by Complex I is higher during the reverse electron transport from succinate to NAD^+ [92,100,107–110], whereas during the forward electron transport it is much lower. Reverse electron transfer-supported ROS production requires high membrane potential and is inhibited by uncouplers and by processes dissipating membrane potential [108–111]. Rotenone has been found to enhance ROS formation during forward electron transfer [96,97] and to inhibit it during reverse electron transfer [98,100,105].

3.1.1. Novel findings from our laboratory

We have exploited the ability of Complex I to transfer electrons directly to molecular oxygen with the aim to elucidate not only the site of electron escape in Complex I but also the mechanism of electron transfer inside the enzyme. To this purpose, we have tested the effects of different inhibitors on the radical production from Complex I, detected by fluorescence of dichlorofluorescin diacetate (DCFA), that is deacylated by mitochondria and submitochondrial particles. The findings provide evidence on a strikingly differential effect of two classes of Complex I inhibitors, based on their ability to affect oxygen radical production by the enzyme (unpublished results from our laboratory; cf. Proceedings of the Symposium "Bari International Conference on Mitochondria, from molecular insight to physiology and pathology, Abstract L5-4).

(1) Class A inhibitors, that induce a strong increase in the ROS production from Complex I.
(2) Class B inhibitors, that completely prevent ROS production from the enzyme.

Class A inhibitors include Rotenone, Piericidin A, Rolliniastatin-1 and -2, but also myxothiazol, while Class B includes Stigmatellin, Capsaicin, Mucidin at high concentration, and also short ubiquinone analogues such as Coenzyme Q_2. Accurate controls have excluded for these compounds a generic effect as free radical scavengers. The effects of inhibitors on ROS formation and DCIP reduction (see below) are summarized in Table 2.

Most of Class B inhibitors are also classical Complex III inhibitors acting at the so called center "o" where they block electron transfer from ubiquinol to the Rieske protein; other inhibitors of this class are short chain quinones like CoQ_2, known to be poor electron acceptors from Complex I on which they exert an inhibitory effect ascribed to the quinol form [112].

Table 2
Effect of Complex I inhibitors on the rates of ROS formation and DCIP reduction [a]

Inhibitor	Concentration (μM)	ROS generation	DCIP reduction
Rotenone	1	++++	Inhibition
Rolliniastatin-1,-2	30	++	ND
Piericidin A	30	++	ND
Myxothiazol	50	++	ND
Stigmatellin	50	−	No inhibition
Mucidin	60	−	ND
Capsaicin	60	−	ND
Rotenone+ Stigmatellin	1+50	−	Inhibition

[a] Assays performed in bovine heart submitochondrial particles. ROS formation was measured in particles supplemented with NADH both fluorimetrically by the DCFDA assay [161] and spectrophotometrically using Amplex Red [162]. DCIP reduction was measured in a dual wavelength spectrophotometer at 600–700 nm using an extinction coefficient of 21 mM^{-1} cm^{-1}, after subtraction of the DPI-insensitive rate (corresponding to the DCIP reduction by FMN in Complex I). ND=not determined.

Starting from available knowledge from the literature and from the results described in this work, we can propose the following. Class A inhibitors prevent access of the physiological CoQ_{10} from the CoQ ramp [13] to its reduction site, thus allowing the CoQ reductant to release one electron to oxygen instead. On the other hand, Class B inhibitors appear to directly act on the site of oxygen reduction.

3.1.2. Identification of the site of superoxide production

The identification of the oxygen reducing site has been the subject of extensive investigation, and several prosthetic groups in the enzyme have been suggested to be the direct reductants of oxygen (Table 3).

3.1.2.1. Flavin. A major candidate as the electron donor to oxygen has been proposed to be FMN [101,102,105]; the rationale for such identification has been that diphenylene iodonium (DPI), an inhibitor of Complex I at the FMN region, blocks reverse electron transfer-supported ROS formation [101]; however, DPI also inhibits NADH-supported ROS formation ([101], and unpublished data from our laboratory). Recently Brandt [106] showed that ROS production was still present in a mutant Complex I from *Yarrowia lipolytica* lacking iron sulphur cluster N2, concluding a direct involvement of FMN in this activity. On the other hand, Ohnishi and coworkers [99] showed that DPI enhances ROS production in the reverse electron transfer, while inhibiting it in the forward electron transfer. The loss of ROS detection in presence of DPI seems to exclude any involvement of FMN in ROS production to advantage of a direct involvement of iron–sulphur clusters. In fact DPI inhibits the iron–sulphur clusters reduction while the reduced state of protein-bound FMN is stabilized [113]. Indeed, the FMN involvement in ROS production still remains an open question and the discrepancies in the literature should be at least in part ascribed to difficulty in achieving complete inhibition of the NADH-O_2 activity: the inhibition of Complex I activity was never more than 80–85%, allowing a residual electron flux to iron sulphur clusters. Herrero

Table 3
Postulated sites of superoxide generation in Complex I

Site of ROS production	Detection method	Experimental evidences	References
FMN	Fluorescence determination of p-HPAA in presence of HRP Spectrophotometric determination of adrenaline oxidation to adrenochrome	H_2O_2 production during reverse electron transfer is inhibited by DPI.	[101]
	Spectrophotometric determination of acetylated cytochrome *c* reduction	Rotenone and piericidin prevent formation of iron–sulfur center N-2-associated ubisemiquinone while stimulating superoxide generation.	[105]
	Spectrophotometric determination of acetylated cytochrome *c* reduction	Wild type and a mutant Complex I (lacking a detectable iron–sulfur cluster N2) from *Yarrowia lipolytica* exhibit the same rate of ROS production.	[106]
Iron–Sulphur clusters/ ubisemiquinone	Fluorescence determination of Amplex Red oxidation in presence of HRP	In forward electron transfer ROS production is enhanced by rotenone and piericidin A and inhibited by DPI and ethoxyformic anhydride. In reverse electron transfer DPI, ethoxyformic anhydride and Piericidin A enhance ROS production, suggesting that the major site of superoxide generation is protein-associated ubisemiquinones spin-coupled with cluster N2.	[99]
	Spectrophotometric determination of adrenaline oxidation to adrenochrome	The rotenone-stimulated superoxide production is inhibited by both *p*-chloromercuribenzoate and ethoxyformic anhydride indicating that oxygen radical generator is located between the ferricyanide and the ubiquinone reduction site (N2).	[97]
	Spectrophotometric determination of adrenaline oxidation to adrenochrome	*p*-hydroxy-mercuribenzoate inhibits ROS generation while inhibitors acting as quinone antagonists enhance it. CoQ depletion does not inhibit ROS production, in agreement with N2 as ROS production site.	[96]
	Fluorescence determination of Amplex Red oxidation in presence of HRP	The redox properties of reduced FMN exclude it as the ROS generator. The ROS production enhanced by rotenone and inhibited by *p*-chloromercuriobenzoate is consistent with a ROS site located on N-1a centre.	[98]
	Fluorescence determination of p-HPAA in presence of HRP	ROS production in reverse electron transport is strongly dependent on ΔpH. Between the three ubisemiquinone species found inside the enzyme only the SQ_{Nf} exhibits sensitivity to ΔpH, so it might be the direct reductant of oxygen in Complex I.	[100]
	Fluorescence determination of DCFA	Differential action of two classes of Complex I inhibitors on ROS production in SMP treated with NADH.	R. Fato, C. Bergamini, G. Lenaz (unpublished)

p-HPAA = *p*-hydroxyphenylacetic acid.
HRP = horseradish peroxidase.
DPI = Diphenyleneiodonium.
DCFA = Dichlorofluorescin diacetate.

and Barja [97] found that ROS production in forward electron transfer in Complex I was also inhibited by ethoxyformic anhydride, an inhibitor of iron sulphur clusters, clearly excluding FMN as the site of oxygen reduction. In addition, the studies by Lambert and Brand [100] and by Ohnishi et al. [99] also exclude FMN as the reductant of oxygen, pinpointing a site close to or coincident with the CoQ-binding site (see below). Our unpublished study also excludes flavin as the site of oxygen reduction, since it would be incompatible with our present finding of the differential action of two classes of inhibitors both acting downstream of the iron sulphur clusters in the enzyme. Nevertheless, a major role can be envisioned for FMN in the formation of radical species by Complex I in the presence of physiological hydrophilic quinones (i.e., cathecolamine-derived oxidative products). The mechanism through which adrenochrome was shown to enhance the formation of ROS by Complex I is a multiple-step process involving a site situated upstream in the redox-active chain of the enzyme, likely coincident with a FMN, since the reaction is insensitive to both rotenone and p-hydroxymercuribenzoate [114].

3.1.2.2. Ubisemiquinone. One of the possible candidates is the ubisemiquinone species. Brand [100] excludes any site upstream of the quinone/semiquinone couple itself on the basis of the significant differences found in the stimulating effects of rotenone, piericidin and myxothiazol on ROS production: since all these are inhibitors of the CoQ site, the sites upstream of CoQ should have been affected to the same extent by the different inhibitors. However, with the same reasoning, it is not possible to exclude the electron donor(s) to CoQ, such as N2, that share the CoQ site. Ohnishi et al. [99] reach similar conclusion from the differential effects of rotenone and piericidin in both forward and reverse electron transfer, and conclude that cluster N2 and/or ubisemiquinones bound to cluster N2 may

be the electron donor(s) to oxygen. From the EPR data reported by the Onhishi group [11] it appears that Complex I inhibitors such as Rotenone and Piericidin A turn off the EPR signal from the semiquinones species. Unfortunately there is no available evidence about the effects of the other Complex I inhibitors on the EPR semiquinone signals. From our unpublished results on the ROS production it appears that inhibitors known to shut down the semiquinone signal are also most efficient in the direct transfer of electrons to molecular oxygen. These results would suggest that the endogenous semiquinone formed during the redox cycle of the enzyme is not involved in ROS production. This conclusion is also in line with a previous report showing that in CoQ-depleted mitochondria Complex I is able to produce oxygen radicals at a rate comparable with the enzyme in non-extracted mitochondria [96].

3.1.2.3. Iron sulphur clusters. Another major candidate as the direct oxygen reductant is the iron sulphur cluster N2; according to Brandt [115,116] this site is localized at the interface between the matrix site and the membranous part of the enzyme. The recent crystallographic identification of the steric location of all iron sulphur clusters of the bacterial enzyme [14] allows to locate N2 more precisely, closer to the membrane sector of the enzyme than previously suggested.. Because of its midpoint potential higher than that of the other clusters, N2 is considered as the direct electron donor to the ubiquinone. It is commonly accepted that Complex I inhibitors share the same hydrophobic large pocket binding site in the enzyme [16] and, according to the structural model proposed by Brandt et al. [17], this pocket could be the amphipathic 'ramp' guiding ubiquinone into the catalytic site. In this picture Rotenone and related inhibitors would prevent the quinone access to the catalytic site, but would not prevent the reduction of N2 cluster.

The electron transfer from NADH to ubiquinone in Complex I requires the presence of at least eight iron–sulphur clusters, seven of which are well protected from reacting with oxygen with the exception of N2. From structural and functional studies, the iron–sulphur cluster N2 seems to be localized in a region that should be accessible to protein bound ubisemiquinones, to H^+ ions and to water, hence this region should be also accessible to molecular oxygen. On the other hand, the mid point potential of cluster N2 is around −0.15 to −0.05 V [117] and therefore, it is compatible with the reduction of oxygen to superoxide anion (mid point potential for the couple superoxide/oxygen is −0.14 V [99]. The correct value of the midpoint potential for the superoxide/oxygen couple [99] makes less stringent the identification of a group having lower potential such as cluster N1a [98] and flavin itself (see above).

Our unpublished results outlined above seem to agree with the hypothesis indicating the cluster N2 as the direct reductant of molecular oxygen. Anyway, during normal redox cycle, the electron leak from Complex I is very low: it can be increased by the presence of Class A inhibitors while it is not related to the reduced state of the enzyme. In fact in presence of 1.8 μM Mucidin, that inhibits Complex III and prevents radical formation from it without affecting the Complex I activity, and at saturating concentrations of NADH (condition that allows the full reduction of all redox centres in Complex I as well as the reduction of the quinone pool [26]), the superoxide production was enhanced only to a minor extent. On the other hand, when Mucidin was used at 60 μM concentration, we could achieve full inhibition of the NADH-CoQ_1 activity together with a full inhibition of ROS production even in presence of Class A inhibitors. These results suggest that the presence of Class B inhibitors is sufficient to prevent the electron escape from Complex I even in the presence of Class A inhibitors. It might be guessed that they induce in the enzyme a conformational change that makes the reducing center more accessible to molecular oxygen, whereas Class B inhibitors would either directly block this reducing center, or induce a conformational change making it less accessible.

3.1.3. Mechanism of electron transfer in the CoQ region of Complex I

Two minimal schemes of electron transfer in Complex I are compatible with these findings (Fig. 3A, B). In a linear scheme (Fig. 3A), the electron donor to oxygen is presumably Fe–S cluster N2, whose reduction by the preceding cluster is inhibited by stigmatellin whereas its reoxidation is inhibited by rotenone. In a bifurcated scheme (Fig. 3B), the first electron is delivered to CoQ with formation of semiquinone in a rotenone sensitive way; semiquinone is then reduced to quinol by N2 in a stigmatellin-sensitive way. N2 is also the donor to oxygen; rotenone does not prevent delivery of one electron to N2 and then to oxygen, while stigmatellin prevents electron delivery to either

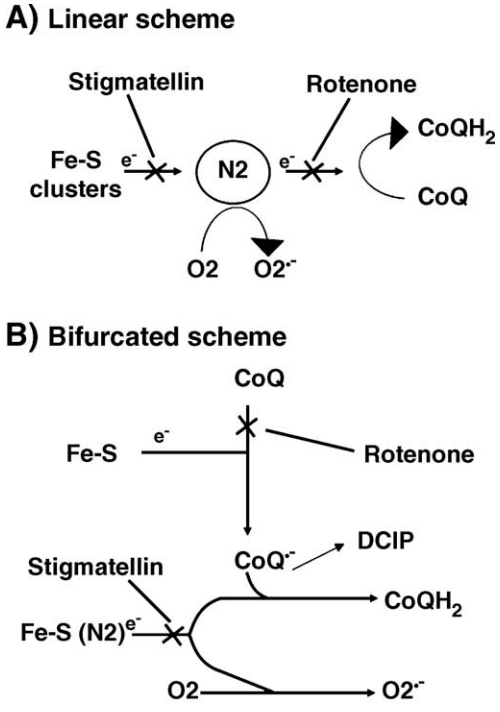

Fig. 3. Hypothetical mechanism of electron transfer in the quinone acceptor region of Complex I indicating the site of oxygen reduction to superoxide. Scheme A would explain the differential action of hydrophobic inhibitors (cf. text for explanations) but is not compatible with the effects of the inhibitors on DCIP reduction nor is in line with current knowledge on their sites of action. Scheme B better explains the findings of the effects of the inhibitors on both oxygen and DCIP reduction. Cf. text for detailed discussion.

CoQ or oxygen. The bifurcated scheme appears more in line with the position of the stigmatellin-inhibition site downstream with respect to the rotenone site, since the behaviour of stigmatellin is shared by reduced quinone analogs [13].

A further confirmation of this scheme derives from the effect of inhibitors on reduction of the acceptor dichlorophenol indophenol (DCIP) (unpublished results from our laboratory). Some DCIP is reduced at the level of FMN, since there is a component insensitive to DPI; another component is sensitive to DPI and must be reduced at the level of CoQ. In fact both hydrophilic and hydrophobic quinones enhance DPI-sensitive DCIP reduction. The reduction is inhibited by rotenone but only slightly by stigmatellin.

These findings demonstrate that DCIP is reduced at a site situated between the rotenone and the stigmatellin inhibition sites, a further indication for a split pathway of electrons at the CoQ binding site. According to the scheme presented in Fig. 3B, DCIP would be reduced by ubisemiquinone, since its formation is rotenone sensitive but stigmatellin insensitive.

The presence of a bifurcated pathway for CoQ reduction has to be reconciled with the linear pathway of electrons along the series of iron sulphur clusters as demonstrated by the crystallographic study of Sazanov [14]; our interpretation is not in contrast with the existence of a linear pathway, because the two electrons delivered to CoQ for its complete reduction could well be provided alternatively by two different clusters (or even by the same cluster) if a suitable conformational change occurs after the first electron delivery in order to provide a gating mechanism for the second electron.

3.2. Implications for human pathology

Humans are exposed to a great number of Complex I inhibitors, since numerous insecticides and pesticides belong to this category [13,118]. The aetiology of Parkinson's disease, though uncertain, may include chronic exposure to such compounds either in adulthood or during development [119,120], in association with genetic susceptibility [121–124]; epidemiological studies [124–127], the effects of inhibitors in animal models of the disease [128–131] and the known involvement of Complex I defect in Parkinson patients [132] support the idea that environmental exposures and genetic mitochondrial dysfunction may interact and result in neurodegeneration. The ability of some of these inhibitors to elicit generation of excess oxygen radicals may aggravate the damage induced by inhibition of electron transfer. Thus, knowledge of the capacity of the long list of compounds acting as Complex I inhibitors to induce oxidative stress has important practical implications.

Genetic alterations of Complex I subunits may alter electron transfer mimicking the effect of a Complex I inhibitor: thus, some alterations may induce overproduction of ROS while others might depress the oxygen radical production. Several examples of enhanced ROS production in genetic defects of Complex I are known in the literature, particularly for nuclear genes mutations [133,134], whereas the effect of mitochondrial gene mutations is less clear [135–137]; recently, cybrids carrying the LHON 14487 ND6 mutation were shown to undergo a ROS overproduction [138]. Also, physiological states, such as subunit phosphorylation, may modify the ROS generating capacity of Complex I [139,140]. It is therefore tempting to speculate that endocrine alterations may affect the capacity of ROS formation by hyper- or hypo-phosphorylation of the Complex.

Mitochondrial DNA mutations have been consistently found in cancerous cells [84,141]; they have been found to be associated with enhanced ROS production, and ROS act both as mutagens and cellular mitogens [142]; thus the involvement of mtDNA mutations in cancer may well be of pathogenic importance [84].

In a recent study, we have shown that a cell line of a malignant thyroid oncocytoma, characterized by abnormal mitochondrial proliferation [143], contains a mutation of mitochondrial DNA preventing expression of subunit ND1 [144]. These cells exhibit a dramatic decline of ATP synthesis supported by NAD-dependent substrates, while in the mitochondria isolated from these cells the Complex I activity is strongly depressed (unpublished observations). Accordingly, the cell line produces much higher amounts of ROS compared with a line derived from a non-oncocytic thyroid tumor [145]. It is relevant to quote the recent finding [146] that Complex I defects induce mitochondrial outgrowth as a consequence of increased ROS production. It is tempting to speculate that the abnormal mitochondrial proliferation characteristic of these tumours is due to stimulation by ROS of mitochondrial proliferation.

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