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COMPLEX I FUNCTION IN MITOCHONDRIAL SUPERCOMPLEXES

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ABSTRACT

This review discusses the functional properties of mitochondrial Complex I originating from its presence in an assembled form as a supercomplex comprising Complex III and Complex IV in stoichiometric ratios. In particular several lines of evidence are presented favoring the concept that electron transfer from Complex I to Complex III is operated by channeling of electrons through Coenzyme Q molecules bound to the supercomplex, in contrast with the hypothesis that the transfer of reducing equivalents from Complex I to Complex III occurs via random diffusion of the Coenzyme Q molecules in the lipid bilayer. Furthermore, another property provided by the supercomplex assembly is the control of generation of reactive oxygen species by Complex I.

KEYWORDS

Mitochondria; Complex I (NADH:ubiquinone oxidoreductase); supercomplex; channeling; ROS

ABBREVIATIONS:[†]

BN-PAGE, Blue-Native polyacrylamide gel electrophoresis; CI, Complex I; CII, Complex II; CIII, Complex III; CIV, Complex IV; CL, cardiolipin; CoQ, Coenzyme Q; DB, decylubiquinone; DDM, n-Dodecyl-β-D-maltoside; FCC, flux control coefficient; R4B, crude mitochondrial fraction enriched in Complex I and Complex III; ROS, reactive oxygen species; SC, supercomplex

1. Types and stoichiometry of Complex I-containing respiratory supercomplexes

In recent years, mounting evidence that the redox complexes of the mitochondrial respiratory chain gather together in various ratios forming supra-molecular assemblies (supercomplexes, SC) induced a rethought of the textbook model of individual organization of Complex I (NADH-ubiquinone oxidoreductase, CI), Complex II (succinate-ubiquinone oxidoreductase, CII), Complex III (ubiquinol-cytochrome c oxidoreductase, CIII) and Complex IV (cytochrome c oxidase, CIV). Indeed, the 'random collision model' [1], accepted by the majority of the investigators in the late '80s of the last century, previously showed the respiratory complexes embedded in the inner mitochondrial membrane as separate, free floating entities that use ubiquinone and cytochrome c as highly diffusing electron carriers. A clear demonstration of the presence of respiratory assemblies came during the past decade from investigations in yeast and mammalian mitochondria. In particular, polyacrylamide gel electrophoresis under non-denaturing conditions (BN-PAGE) in digitonin-solubilized mitochondria of *Saccharomyces cerevisiae*, which possesses no Complex I, revealed two bands with apparent masses of ~750 and 1,000 kDa containing the subunits of complexes III and IV [2]. Similar interactions of SC were investigated in bovine heart mitochondria: Complex I-III interactions were apparent from the presence of Complex I in the form of the SC I_1III_2, which was also found further assembled into larger assemblies (respirasomes $I_1III_2IV_{1-4}$) comprising different copy numbers of Complex IV [3]. Only 14–16% of total Complex I was found in free form in the presence of digitonin so it seems likely that all Complex I is bound to Complex III in physiological conditions (i.e. in the absence of detergents). Though there was initial criticism that SC may just be an artefact observed because of mild detergents like digitonin used in the purification procedure, this has largely been abated by evidence that purified SC are stable, catalitically active stoichiometric units [2, 4].

Some SC have been purified and analysed by negative-stain electron microscopy [5, 6] and single-particle cryo-EM [7, 8, 9] . The 1.7 MDa bovine heart SC consists of one copy of Complex I, one Complex III dimer, and one Complex IV monomer. X-ray structures of the component complexes

were fitted to the 3D map to produced pseudo-atomic models of the bovine respirasome (**Figure 1**). Interestingly, in the bovine SC mutual orientation of Complex IV and Complex III differs significantly from their orientation in the yeast *S. cerevisiae* [9]. At first, this difference in orientation may be surprising, but this may be a result of significant differences in the composition of the yeast SC III_2IV_2 and the mammalian respirasome. The mammalian Complex III interacts with both Complex IV and Complex I, the latter being absent in *S. cerevisiae*.

Much work in recent years has focused on the discovery of SC assembly factors in biological systems and understanding how SC modulate the function of living cells (reviewed in [10]). By metabolic labelling of mtDNA-encoded proteins, Acín-Peréz et al. [4] demonstrated that a temporal gap of several hours occurs between the formation of free complexes and the incorporation of labelled complexes into SC. The first assembly factor identified for SC formation in mammalian mitochondria is Cox7a2l. This protein is required for the stable interaction between Complex III and Complex IV, but not for the assembly or function of either Complex III or Complex IV. Consequently, Cox7a2l has been renamed SCAF1 for SC assembly factor 1 [11]. The precise function of SCAF1 remains unknown. The current model proposes that SC assembly proceeds by the incorporation of individual subunits or modules into a partially assembled Complex I scaffold, rather than by the association of previously assembled enzymes. Perhaps respirasome assembly initially proceeds as proposed [12] and, subsequently, SCAF1 aids the dynamic exchange of Complex IV, reflecting its variable stoichiometry within SC. In contrast to the results from Lapuente-Brun et al. [11], the group of Larsson [13] surprisingly found no alteration in the supramolecular organization of complexes I, III, and IV in C57BL/6J mice, showing that different COX7a2l isoforms do not influence SC formation.

Although more proteins, e.g. Rcf1/HIG2A and Rcf2, were recently reported to mediate SC assembly in yeast and in mammals [14, 15, 16, 17], no clear demonstration has been given so far about the role of the identified assembly factors in supporting the association of Complex I to the other respiratory complexes.

– Insert Figure 1 –

Interactions between respiratory chain complexes within SC were also described to be dependent on protein-cardiolipin (CL) interactions [18]. Supercomplexes have 2-5 nm gaps at the transmembrane interfaces of individual complexes (**Figure 1**). These gaps lie within the membrane imbedded domains of the SC and are therefore most likely filled with lipids. The mammalian SC has larger gaps than the yeast SC [7, 8, 9]. In Barth syndrome patients, where cardiolipin remodelling is altered due to the mutation of the gene Tafazzin, SC are unstable, leading to the mitochondrial functional impairment that underlies the disease [19]. Direct involvement of CL in the formation of SC was demonstrated in genetically manipulated strains of *S. cerevisiae* in which the CL content can be regulated *in vivo* [20]. BN-PAGE of digitonin extracts of mitochondria revealed that Δcrd1 yeast mutants completely lacking CL, but containing elevated amounts of its precursor phosphatidylglycerol, did not form the stable SC III_2IV_2 as observed in the wild type parental strain whereas the total amount of individual respiratory complexes was not affected by the lack of CL [20, 21]. However, no experimental evidences about Complex I-containing SC could be obtained in these studies, since *S. cerevisiae* lacks Complex I.

The importance of lipid composition in the stabilization of Complex I-containing SC is supported by our results showing that reconstitution of binary CI/CIII proteoliposomes from bovine heart at high lipid to protein ratio (30:1 w:w) hampers the assembly of SC [22, 23], as outlined in the following sections. On the contrary, very preliminary data suggest that SC I_1III_2 and efficient NADH-cytochrome c reductase activity may be preserved when similar high-lipid proteoliposomes are enriched with CL (CL to Asolectin (soybean phospholipids) ratio of 20:80, w:w), resembling the 18-20% content of CL in the mitochondrial membrane [M. Kopuz, Y. Birinci, S. Nesci, G. Lenaz and M.L. Genova, unpublished data]. Presumably, protein dilution by phospholipids removes endogenous CL from the binding sites thus allowing supercomplexes to dissociate; however, the presence of CL in the phospholipids used for proteoliposomes preparation counteracts the

dissociation of endogenous CL and prevents CL dispersion in the lipid phase of the membrane. In the case of bovine respirasome it was suggested that the lipid filled space between Complex I and Complex III could also serve as a diffusion microdomain, which restricts movement of CoQ facilitating its channelling inside the SC (cf. Section 2).

Despite recent experimental advances in our capability to characterize and describe redox proteins in respiratory SC, two particular challenges need to be met: determine how the intermolecular redox reactions are coordinated with intramolecular redox reactions and how redox reactions are coordinated with other chemical events such as proton transfer or substrate binding and release (cf. [24] for recent review).

2. Evidence for channelling between Complex I and Complex III

Immediately after the discovery of SC it was proposed that the natural consequence of such assemblies is substrate channelling or enhanced catalysis in inter-complex electron transfer. Substrate channelling is the direct transfer of an intermediate between the active sites of two enzymes catalysing consecutive reactions [25]; in the case of electron transfer, this means direct transfer of electrons between two consecutive enzymes by successive reduction and reoxidation of the intermediate without its diffusion in the bulk medium.

Some evidence for possible channelling comes from the 3D structure of the mitochondrial SC $I_1III_2IV_1$ [7]; a unique arrangement of the three component complexes indicates the pathways along which ubiquinone and cytochrome c can travel to shuttle electrons between their respective protein partners [7]. In the above mentioned model, the Coenzyme Q-binding sites in Complex I and in Complex III face each other and are separated by a 13-nm gap within the membrane core of the SC. Coenzyme Q (CoQ) is likely to run a trajectory through this gap which is presumably filled with membrane lipids. Althoff and colleagues [7] also reported the presence of significant amounts of bound phospholipids in the purified SC from mammalian mitochondria and demonstrated that cardiolipin is enriched in the SC compared with bovine heart total lipid. Moreover, HPLC analysis

of the lipid extracts indicated that each SC contains at least one molecule of ubiquinol [7]. Of course, structural data alone cannot prove CoQ channelling if not supported by functional evidence; indeed the problem has been tackled in recent years (Sections 2.1-2.5).

On the basis of kinetic analysis to be discussed later in this review (cf. Section 2.3), Blaza et al. [26] claimed that SC have no specific function and only derive from the necessity to maintain a high protein concentration in the inner membrane thus avoiding nucleation and unspecific aggregation. It is certainly true that SC formation depends on the lipid concentration, being favoured at high protein to lipid ratio [22]; on the other hand, if electron transfer by channelling actually does not occur, it is difficult to understand why the active sites of interacting complexes should be located in strict vicinity, in stereospecific non-random manner (see Section 1). In the next sections we will consider the body of evidence that assigns a functional role to respiratory SC.

2.1. Metabolic flux control analysis

Although the theoretical basis of channelling was already established [27], the first demonstration of respiratory supercomplex association on a functional basis was achieved in our laboratory some years later [28, 29]. We exploited the flux control analysis and the principle that the sum of the flux control coefficients (FCC) of the individual enzymes in an integrated pathway must equal 1 unless these enzymes form supramolecular units and establish substrate channelling. In the latter case, these enzymes would be all equally rate-limiting and the sum of the control coefficients would be higher than 1 [27].

Using this principle and exploiting inhibition with specific inhibitors in order to define the extent of metabolic control exerted by each individual complex over the entire respiration, we found that both Complex I and Complex III have flux control coefficients approaching 1 in bovine heart mitochondria (**Table 1**), thus suggesting that the two complexes behave as a single enzymatic unit and that electron transfer through Coenzyme Q is accomplished by channelling between the two redox enzymes [28]. This approach is similar to that previously applied by Boumans et al. [30], who

had found that CoQ does not follow pool behaviour in yeast mitochondria unless they are treated with chaotropic agents.

In addition, from our flux control analysis using cyanide inhibition [28], Complex IV appears to be randomly distributed, or in other words that a large excess of active enzyme exists in free form in the pathway from NADH to oxygen.

Very few other studies were addressed to the functional aspects of SC using metabolic control analysis [31, 32, 33]; these studies confirmed that the respiratory chain, at least under certain conditions, is organized in functionally relevant supramolecular structures. In digitonin-permeabilized HepG2 cells, Quarato et al. [31] observed that under conditions of high membrane potential the sum of the FCC calculated for Complexes I, III and IV activities exceeded 1, supporting the proposition that they are complexed in a supramolecular unit.

In saponin-permeabilized breast and cholorectal tumor samples Kaambre et al. [32, 33] observed flux control coefficients for mitochondrial oxidative phosphorylation activities whose sum approached 4 and interpreted the data as due to the presence of supercomplex association.

Blaza et al. [26] criticize the evidence of channelling in the respiratory chain that derives from flux control analysis [28] on the basis that rotenone is competitive with CoQ and therefore inhibits Complex I to different extents in the presence of exogenous substrates. Indeed, using rotenone and CoQ_1, Blaza et al. [26] find FCC for Complex I that is certainly not valid, since it exceeds 1. The discrepancy with [28] may be ascribed to the different exogenous substrates used for Complex I assay in the two studies, i.e. CoQ_1 and decylubiquinone (DB) respectively and also to the technical difficulty of calculating FCC from the initial slopes of the inhibition curves. DB has much lower affinity than CoQ_1 for Complex I [34], thus exerting lower competition with rotenone and lower influence on the inhibition efficiency. On the other hand, we note that the FCC values found by Blaza et al. [26] using alternative inhibitors of Complex I are oddly low. Considering that in mitochondrial fragments Complex I does not share metabolic control with the membrane potential, the low FCC for Complex I may be ascribed to a limiting amount of cytochrome c in their samples,

that would shift the major control of the chain to the cytochrome c region. Significantly, in their study, addition of cytochrome c to the mitochondrial membranes raised the FCC from 0.19 to 0.67.

Finally, we emphasize that the major point discussed by [28] to demonstrate the existence of a SC I+III was the high FCC of Complex III in NADH oxidation (not measured, on the contrary, by Blaza et al. [26]), which is incompatible with a CoQ-pool model postulating Complex I and Complex III as independent molecules in the membrane. Such high FCC value of Complex III, calculated by inhibitor titration with mucidin, is not an artefact because the corresponding FCC of Complex III measured by using the same inhibitor in succinate oxidation is low (as expected from the rate-limiting role of Complex II and the lack of SC II+III). In other words, the FCC of Complex III can be close to 1 if Complex III is assembled together with the quinone reductase that precedes in the electron pathway and, in our hands, this condition is experimentally observed only for the NADH-dependent pathway involving Complex I whereas it does not occur in the case of Complex II. In addition, the FCC of Complex III in NADH oxidation is drastically decreased after dissociation of the SC I_1III_2 in a reconstituted system [22] (**Table 1** and **Figure 2**).

- Insert Table 1 -

- Insert Figure 2 -

2.2. Evidence for rate advantage

The rate of electron transfer between membrane-bound electron carriers depends on their structural organization in the membrane. If two redox enzymes are connected by a mobile redox carrier undergoing long-range diffusion in the medium, the overall reaction rate would be governed by the frequency of useful collisions between the mobile carrier and its two redox partners. On the other hand, if the whole redox system is fixed in a solid state arrangement, the frequency of encounters will be dictated only by the proximity and fixed contacts between the redox partners (channelling). **Figure 3**

schematically illustrates the difference existing between collision-based electron transfer and channelling in the respiratory chain.

- Insert Figure 3 -

2.2.1 Collision-based electron transport: the "pool" behaviour

The functional significance of a random distribution of mitochondrial complexes was supported by the kinetic analysis of Kröger and Klingenberg [35] for the enzymes connected by CoQ; they showed that steady-state respiration in submitochondrial particles from beef heart, using either NADH or succinate as electron donors, 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

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

where V_{ox} is the rate of ubiquinol oxidation and V_{red} is the rate of CoQ reduction.

This expression (the *pool equation*) was verified under a wide variety of input and output rates and establishes that CoQ distributes electrons randomly among the CoQ-reducing flavin dehydrogenases and the bc_1 complexes, behaving indeed as a laterally diffusing pool of molecules in a variety of systems [36, 37, 38, discussed by Lenaz and Genova 39, 40].

However, it is worth mentioning that most available data on CoQ pool concern succinate oxidation in submitochondrial particles, whereas fewer data are available for NADH oxidation mediated by Complex I. In bovine heart submitochondrial particles, the measured rate of electron transfer between Complex I and Complex III was found to be comparable to that calculated (V_{obs}) from the pool equation [34]. On the other hand in mitochondrial systems the rate of Complex I activity is strongly underestimated, due to the properties of CoQ analogues used as acceptors [41] so that the pool equation is not directly applicable [42].

As a consequence of this observation most calculations based on absolute values of NADH-CoQ reductase activity are to be taken with extreme caution. A very detailed analysis of the possible errors concerning the interpretation of the pool equations can be found in Lenaz and Genova [39, 40].

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.

2.2.2. Fixed assemblies: stoichiometric behaviour

Early experiments reported by Ragan and Heron [43] 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 (clearly at difference with the SC found by BN-PAGE, where Complex III is present as a dimer, cf. Section 1) and 2-3 moles of CoQ_{10} per mol of protein unit. The same authors also indicated that electron transfer between a unit of Complex I-Complex III and any extra molecules of Complexes I or III does not contribute to the overall rate of cytochrome c reduction. The reduction by NADH of the cytochrome b of mixtures of Complexes I and III is biphasic and the extents of the fast and slow phases of reduction are determined by the amount of Complex III specifically associated with Complex I.

These studies [43] were able for the first time to demonstrate the existence of a SC formed by Complexes I and III, although its formation *in vitro* requires a specific low-lipid environment.

2.2.3 Dissociation of supercomplexes shifts channelling to a less efficient pool behaviour

The previously quoted study [43] first described stoichiometric behaviour for the activity of NADH cytochrome c reductase, ascribable to the formation of a SC. However, CoQ-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 [43]. Inclusion of phospholipid into the reconstituted system may have a number of effects on the physical state of the system. Heron and co-workers [44] proposed that, 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.

Heron et al. [44] also reported that endogenous CoQ_{10} leaks out of the Complex I-III unit when extra phospholipid is present in the proteoliposomes, causing a decrease in activity that could be alleviated by adding more ubiquinone. It is likely that the function of the large amount of ubiquinone in the natural membrane may be, therefore, to maintain the CoQ_{10} content in the SC unit when it is formed.

A more direct comparison of the effect of channelling with respect to CoQ-pool behaviour was performed in a simple experimental condition in our laboratory.

A system obtained by fusing a crude mitochondrial fraction (R4B) [45] enriched in Complex I and Complex III with different amounts of phospholipids and CoQ_{10} [46] was used in our laboratory to discriminate whether the reconstituted protein fraction behaves as individual enzymes (CoQ-pool behaviour) or as assembled SC depending on the experimental distances 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 (Eq. 1 in section 2.2.1) showed overlapping results at phospholipid dilutions (w/w) from 1:10 to 1:40, i.e. for theoretical distances >50 nm. On the contrary, pool behaviour was not effective and the observed rates of NADH-cytochrome c reductase activity were higher than the theoretical values [22, 29, 46] at low protein:lipid dilution (1:1 w/w), resembling the mean nearest neighbour distance between respiratory complexes in mitochondria [47, 48, 49].

Moreover when the same proteoliposomes at 1:1 protein:lipid ratio were treated with n-Dodecyl-β-D-maltoside (DDM) to destroy the supercomplex organization, the NADH cytochrome c reductase activity fell dramatically, whereas both Complex I and Complex III individual activities were unchanged [23]; an analogous behaviour was detected by treating bovine heart mitochondria with the same detergent.

In addition, we have titrated NADH-cytochrome c reductase activity (Complex I+III) with DDM and found that the activity was significantly decreased only at concentrations of detergent >2 DDM/protein (w:w) (**Figure 4**). The decrease in NADH-cytochrome c activity was observed in the same range of DDM as dissociation of SC I_1III_2 was detected by BN-PAGE (**Figure 5**). These data can be interpreted as maintenance of CoQ channelling within the SC I_1III_2 allowing NADH oxidation to take place at high rate as long as the SC is not disassembled by high DDM. We note that the sharp increase and stabilization of activity at 0.3-1.5 DDM/protein ratios, as compared to rates at very low concentration of detergent, indicates that NADH and cytochrome c had limited access to the inner membrane of the frozen and thawed mitochondria used.

These studies demonstrate that electron transfer between Complex I and Complex III can take place both by CoQ channelling within the SC I_1III_2 and by the less efficient collision-based pool behaviour, depending on the experimental conditions. The *plasticity model* first proposed by Acín-Peréz et al. [4] accounts for a dynamic equilibrium of the respiratory complexes between free individual units floating in the lipid bilayer and supramolecular units (SC), both endowed with electron transfer activity.

Maas et al. [50] have been able to express functional alternative oxidase in a mutant of the fungus *Podospora anserina* lacking complexes III and IV. Under these conditions, unlike the mammalian enzyme, Complex I is stable and works perfectly in combination with the alternative oxidase, which is able to oxidize ubiquinol supplanting the combined role of Complex III, cytochrome c and Complex IV. This demonstrates *in vivo* that Complex I-dependent respiration does not strictly require the formation of SC I+III.

– Insert Figure 4 –

– Insert Figure 5 –

2.3. Separate pools of Coenzyme Q and/or Complex III

Gutman [36] 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, concluding 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 (**Figure 6**).

- Insert Figure 6 -

The non-homogeneity of the ubiquinone pool with respect to succinate and NADH oxidation [36] may be interpreted today in terms of compartmentalization of CoQ in the SC I+III in contrast with the free pool used for connecting Complexes II and III.

Benard et al. [51] described the existence of three different pools of CoQ during succinate-dependent steady-state respiration in rat liver and muscle mitochondria: one pool is directly utilised, another (approx. 8% in muscle and 23% in liver) is mobilized as a reserve in case of a perturbation to maintain the energy fluxes at normal values (e.g. due to inhibition of the respiratory complexes or in case of mitochondrial diseases), and a third one (approx. 79% in muscle and 21% in liver) cannot be mobilized at all. These results are compatible with CoQ compartmentalization,

although similar results with NADH oxidation were not provided in order to functionally prove that the fraction of CoQ that is not utilisable for succinate oxidation is channelled within SC I+III.

Lapuente-Brun et al. demonstrated [11] that the physical assembly between complexes I and III determines a preferential pathway for electrons mediated by a dedicated subset of CoQ molecules. According to their results, this compartmentalization prevents significant cross talk between NADH oxidation (Complex I-dependent) and succinate oxidation (dependent on Complex II) or other flavoenzyme-dependent oxidations. Those Complex III molecules ($CIII_{bound}$) that physically interact with Complex I in the formation of SC are also exclusively dedicated to NADH oxidization while those Complex III molecules ($CIII_{free}$) that are not bound to Complex I are mainly responsible for oxidization of succinate and other substrates using the free CoQ pool. When a partial loss of Complex III occurs, the Complex I-Complex III association is preferred to the free state of either Complex I or Complex III, due to a very high affinity between the two complexes. In this situation NADH oxidization catalyzed by the SC I+III is preferentially maintained despite the risk of compromising the oxidation of FAD-linked substrates [11].

The already quoted paper by Blaza et al. [26] showed that the steady-state rates of aerobic NADH and succinate oxidation were not additive in bovine heart submitochondrial particles; moreover, the rates of cytochromes b_H, b_L, c and c1 reduction in the same cyanide-inhibited particles were similar if the reductant was either NADH or succinate or a mixture of the two substrates. Blaza et al. interpreted the results as a demonstration that a single pool of CoQ molecules exists that receives electrons indifferently from Complex I and Complex II. In this scenario, $CIII_{free}$ and $CIII_{bound}$ are able to equally receive electrons from the CoQ pool. However, it is worth noting that the experiments of Blaza et al. showing lack of additivity for succinate and NADH oxidation were performed for steady state oxidation by oxygen, thus comprising also the steps of cytochrome c and Complex IV reduction and re-oxidation. On the contrary, our preliminary study of the competition of succinate and NADH oxidation by exogenous cytochrome c in KCN-inhibited mitochondria at steady-state shows that the rates are additive in permeabilised

mitochondria in the presence of exogenous cytochrome c and KCN, which shortened the electron transfer pathway for both oxidation reactions by including only CoQ and Complex III as redox intermediates (**Table 2**).

- Insert Table 2 -

The disagreement between our results and those of Blaza et al. may be due to cytochrome c being rate-limiting in Blaza's experimental system and provoking a bottleneck step both in NADH and in succinate oxidation when electrons compete for a common pool of cytochrome c and Complex IV molecules in free form. Indeed most cytochrome c and Complex IV are free and able to receive electrons from both NADH and succinate with only a small portion of Complex IV dedicated to NADH (i.e. forming the respirasome I_1III_2IV), as demonstrated by our flux control analysis data [28] that showed no channelling in the cytochrome c region. As a consequence of this bottleneck, the fluxes through the NADH:O_2 and succinate:O_2 pathways would be necessarily smaller with one substrate than with both substrates together, but without having a complete additive effect since they are largely linked to the same cytochrome c population. However, when we use *exogenous cytochrome c* as final electron acceptor under saturating conditions, the NADH and succinate pathways become clearly distinguishable because of their different CoQ pools.

We propose that the lack of additivity found by Blaza et al. [26] for the reduction of *endogenous cytochromes b and c* by succinate and NADH has a different explanation; as suggested by us, the CoQ molecules in the SC are in a dissociation equilibrium with the molecules in the pool, albeit with a slower turnover than the channelled redox reactions that are of the order of few ms. For this reason, channelling occurs at steady state, but it is inevitable that in cyanide-inhibited mitochondria the bound CoQ molecules would exchange with those in the pool in the experimental times (several seconds) of Blaza et al.; indeed, from a scrutiny of Figure 3 in the paper by Blaza et al. [26], it is possible to observe that the initial rates of cytochromes reduction (i.e. data taken at the

shortest experimental times) appear to be higher when both substrates are added, as expected from separate CoQ pools.

Thus we are convinced that the evidence for existence of channelling of CoQ between Complex I and Complex III is overwhelming, although under particular conditions also collision-based electron transfer is possible.

2.4. The case of reverse electron transfer

The hyperbolic relation experimentally found by Gutman [36] between succinate oxidase and the rate of reverse electron transfer from succinate to NAD^+, involving sequential interaction of complexes II and I by means of CoQ (cf. Figure 6), is in complete accordance with the pool equation. This observation poses a particularly puzzling question [40] about how ubiquinol reduced in the membrane pool by Complex II interacts with the CoQ binding site in Complex I, since Complex I is totally engaged in the SC. The same dilemma applies to the NADH-fumarate reductase activity that also involves interaction of Complex I and Complex II. In view of the recent progress in the knowledge of the detailed atomic structure of Complex I [52, 53], the previous view that two different routes may exist for forward and reverse electron transfer within Complex I [54] is no longer tenable as such, unless we consider two different conformations, of which the one present during reverse electron transfer makes the CoQ site more accessible to the pool.

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 that, according to Piccoli et al. [55], might be the physiological signal causing the structural reorganization of the respiratory complexes. The *plasticity model* hypothesis (Section 2.2.3) suggests that the SC I+III would dissociate its constituting complexes under high $\Delta\mu_{H+}$ condition, and this would no longer limit the access from the CoQ pool to the binding site in Complex I.

This model is apparently incompatible with the observation reported by Gutman [36] that NADH-fumarate reductase, that occurs at low membrane potential, shares the same pathway of the

reverse reduction of NAD^+ by succinate. We have to keep in mind, however, that the rate of NADH-fumarate reductase is one order of magnitude lower than the rate of NADH-cytochrome c reductase and that of NAD reduction by succinate, therefore it might well be within the time range of a dynamic equilibrium between CoQ in the SC and CoQ in the membrane pool.

We cannot however exclude on a clear experimental basis that the pathways linking Complex II and Complex I during either direct or reverse electron transfer may require the interaction of the CoQ pool with Complex I within the SC. If this is true, we should conclude that Complex I in the SC is somewhat accessible to the CoQ pool. This accessibility, however, would be no proof against the existence of channelling between Complex I and Complex III.

2.5. Concluding evidence about channelling

As we described in the previous sections of this paper, the major observations supporting the notion that supercomplex association determines channelling in the CoQ region are the following: (a) rate advantage of NADH-cytochrome c reductase when SC are present; (b) both Complex I and Complex III are rate-limiting as measured by flux control analysis; (c) evidence for two compartments of CoQ in experiments of competition of NADH and succinate oxidation.

One main point favouring controversy if not recognized is the dynamic character of CoQ bound within the SC, that is in dissociation equilibrium with the free pool of CoQ in the membrane~~;~~ . According to our hypothesis, all or most of the CoQ molecules are channelled from Complex I to Complex III during electron transfer at steady state; however, when electron transfer is slow or blocked by an inhibitor, the relevance of CoQ dissociation from SC /to the pool becomes significant. This notion raises the puzzling question whether the mentioned low flux control coefficients found by Quarato et al. [31] in state-4 (i.e. low-rate respiration) were due to the predominance of CoQ dissociation over CoQ channelling rather than to the physical disassembly of SC. To this respect, the plasticity model [4], would be a functional rather than structural feature of the respiratory chain, at least in the CoQ region. It is desirable that studies on the dissociation

constants of CoQ from SC will give an answer to this scientific dilemma in the future. In addition, the rate of NADH-fumarate reductase [26, 36] may represent an indirect parameter of the dissociation turnover of CoQ from SC to the pool.

3. Supercomplex assembly and ROS generation: a device for control of ROS levels?

The generation of ROS by isolated mitochondria may account for up to 2-3% of oxygen consumed under particular conditions that may not be found physiologically [56] , depending also on the type of tissue (see Panov et al. [57]) and on the substrate employed. For example, succinate is important for ROS production in brain, heart, kidney, and skeletal muscle, while fatty acids are major generators of ROS in kidney and liver [58].

The factors controlling mitochondrial ROS levels are linked to their rate of generation and of removal. The redox potential of the NAD^+/NADH couple and the proton-motive force act as powerful regulators of the steady-state concentration of redox species responsible for electron leaking and ROS generation [59, 60]. In turn, these forces are regulated by the redox supply to the respiratory chain, by the degree of coupling and/or by physio-pathological constraints to electron transfer, such as enzyme phosphorylation, cytochrome c removal, Complex IV inhibition, oxygen concentration etc.

The "Redox-Optimized ROS Balance hypothesis" (R-ORB) described by Aon et al. [61] attempts to explain at a mechanistic level the link between mitochondrial respiration and ROS emission. The hypothesis is based on the observation that ROS levels (as the net result of production and scavenging) attain a minimum when mitochondria maximize their energetic output (i.e. maximal state-3 respiration) at intermediate values of the redox environment between fully oxidized and fully reduced redox couples, such as NADH/NAD^+ and GSH/GSSG. These redox carriers are more oxidized than the corresponding values in state-4 respiration. On the other hand, ROS overflow will occur at both highly reduced or highly oxidized redox environment, albeit governed by the extent of proton leak and by the compromised scavenging capacity, respectively.

Cortassa et al. [62]confirmed that mitochondria are able to keep ROS emission to a minimum likely compatible with signalling, while maximizing their energetic output, as they assessed by the analysis of mitochondrial respiration, ROS emission, and the redox environment in isolated guinea pig heart mitochondria under forward electron transport.

In addition, mitochondrial ROS release is modulated by a series of nuclear encoded proteins such as by p53, p66Shc, the Bcl-2 family and Romo-1 [63] and in response to external stimuli, such as TNFα [64], hypoxia [65], serum deprivation [66], oxidative stress (the so-called ROS-induced ROS release [67]).

An implication of supercomplex organization as the missing link between oxidative stress and energy failure was first suggested by our speculation [39] that dissociation of SC I_1III_2 occurs under conditions of oxidative stress, with loss of facilitated CoQ channelling, thus causing electron transfer to depend upon less efficient collisional encounters of the free ubiquinone molecules with their partner complexes.

As a consequence, the alteration of electron transfer may elicit further induction of ROS generation. Following this line of thought, the different susceptibility of different types of cells and tissues to ROS damage [68] may be interpreted in terms of extent and tightness of supercomplex organization in their respiratory chains and capability of the respiratory enzymes to hinder reaction of auto-oxidizable prosthetic groups with oxygen. Disorganization of the supramolecular architecture of the respiratory chain would both decrease NAD-linked respiration and ATP synthesis and increase the capacity of producing superoxide by destabilised Complex I and Complex III.

The actual shape of the SC $I_1III_2IV_1$ from bovine heart [69] suggests a slightly different conformation of bound Complex I showing a higher bending of the matrix arm towards the membrane (and presumably Complex III), in line with the notion that Complex I may undergo important conformational changes [70]. Since, however, subsequent studies at higher resolution have failed to show conformational changes [7, 8], further studies are needed to solve this issue.

Nevertheless the observed destabilization of Complex I in the absence of SC may render the 51 kDa subunit containing the FMN more prone to interact with oxygen.

On the basis of their studies on rat brain mitochondria oxidizing different substrates, Panov et al. [57] suggested that the assembly of Complex I into SC prevents excessive superoxide production during oxidation of NAD-linked substrates because efficient CoQ channelling helps maintaining the chain in the oxidized state (see also below), whereas on succinate oxidation the backward electron flow keeps the centres in Complex I more reduced and prone to produce superoxide; to this purpose it is interesting to note that Complex II does not aggregate to form SC.

In a recent study, Maranzana et al. [23] obtained the first direct demonstration that loss of supercomplex organization causes a dramatic enhancement of ROS generation by Complex I. In a model system of reconstituted binary Complex I/Complex III at high lipid to protein ratio (30:1), where formation of the SC I_1III_2 is prevented, the generation of superoxide is several-fold higher than in the same binary system reconstituted at a 1:1 ratio, which is rich in supercomplexes **(Figure 7)**. In agreement with this finding, dissociation of the SC I_1III_2 after treatment with the detergent DDM induces a strong enhancement of ROS generation both in proteoliposomes and in mitochondrial membranes.

- Insert Figure 7 -

It is worth noting that, in the experiments reported by Maranzana et al. [23], ROS production is investigated in the presence of inhibitors (mucidin and rotenone) that prevent electron transfer to any respiratory intermediate, therefore the redox centres in Complex I are maximally reduced both in the presence and in the absence of SC. Consequently, the above mentioned reasoning by Panov et al. [57], giving emphasis to the relief of the redox pressure in the super-assembled respiratory chain, cannot be taken as the only explanation of the role of SC in regulating ROS formation by Complex I.

Several additional observations in cellular and animal models link together SC dissociation and enhanced ROS production.

A strong decrease of high molecular weight SC correlating with higher ROS generation was observed in mouse fibroblasts expressing the activated form of the k-ras oncogene, in comparison with wild type fibroblasts [71, 72].

Diaz et al. [73] showed that diminished stability of SC is associated with increased levels of ROS in mouse lung fibroblasts lacking the Rieske iron-sulphur protein of Complex III and hence devoid of Complex I-containing SC.

The cardiolipin defect in Barth syndrome, a cardio-skeletal myopathy with neutropenia which is characterized by respiratory chain dysfunction, results in destabilization of SC by weakening the interactions between respiratory complexes. Remarkably, hydroethidine staining revealed higher basal levels of superoxide production in lymphoblasts from patients, compared to control cells [19, 74].

The availability of cardiolipin-deficient yeast mutants, although lacking Complex I, provided the opportunity to demonstrate alterations in the stabilization of SC similar to those found in Barth syndrome and exhibited increased protein carbonylation, an indicator of ROS [75]. The increase in ROS is most likely not due to defective oxidant defence systems, since the CL mutants do not display sensitivity to other oxidants like paraquat, menadione or hydrogen peroxide (H_2O_2).

The evidence that Complex I-containing SC may physiologically exist in dynamic equilibrium with isolated Complex I [4] raises the puzzling question that also ROS generation may be subjected to physiological changes. It is tempting to suggest that these changes may be aimed to regulation of ROS levels in the cell, in view of their well-documented role in cellular redox signalling.

4. Conclusions

The functional properties of Complex I cannot be solely described by the study of the isolated enzyme, since its physiological assembly with respiratory complexes III and IV confers new properties that depend on the structural and kinetic interactions between these enzymes. The level of knowledge of such new properties is still low, and many issues are controversial. This review has promoted increasing evidence that CoQ channelling occurs in the electron transfer between Complex I and Complex III and that the association of Complex I with Complex III limits ROS generation by Complex I. It is possible that the supramolecular structure may also affect other properties such as proton translocation [76].

Finally, the fixed constraints existing between individual complexes when assembled in SC may impose stoichiometric ratios that depend on the metabolic conditions, thus directing and controlling entire metabolic pathways [11].

5. Acknowledgements

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FIGURE LEGENDS

Figure 1 – Bovine Mitochondrial Supercomplex $I_1III_2IV_1$ A) Fitted model by single particle cryo-EM. (PDB ID: 2YBB) [7]; B) Side view and C) view from the intermembrane space (IMS) showing two CL molecules (in yellow) in the cavity of each monomer of Complex III formed by cytochromes c_1 and b. MA denotes the mitochondrial matrix. Image taken from [18], with permission.

Figure 2 - Supramolecular organization of respiratory Complex I and Complex III in R4B 1:1 and R4B 1:30 proteoliposomes. (A) R4B 1:1 and **(B)** R4B 1:30 samples were separated by 2D BN/SDS-PAGE after solubilisation with digitonin at a detergent to protein ratio of 8 (w:w) and resolved by western blotting followed by immunodetection using monoclonal antibodies specific for single respiratory subunits. The images shown in the picture were obtained *in camera* by double overlaying exposures to the antibody against the NDUFA9 (39kDa) subunit of Complex I and, in sequence (not postproduction computer-graphic overlay), to the antibody against the Rieske protein (22kDa) of Complex III. *Arrows* point to monomeric Complex I. The *upper panel* of the figures schematically shows the position of SC I_1III_2, Complex I (CI) and dimeric Complex III (CIII) in the 1D BN-gel prior to second dimension electrophoresis (2D SDS-PAGE). R4B, mitochondrial fraction enriched in Complex I and Complex III. Image taken from [23] with permission.

Figure 3 - Schematic models showing ubiquinone as a mediator of the electron transport in the respiratory chain. (*Upper panel*) Pool behaviour: Coenzyme Q as a freely diffusible interconnecting mediator (Q pool) among the electron donors (Complex I) and the acceptors (Complex III). Interaction of antimycin (AA) with the respiratory chain causes the "nonlinear" inhibition curve of substrate oxidation (due to multiple choices for quinol oxidation by the residual active molecules of Complex III, as indicated also by the dotted arrows). (*Lower panel*)

Supercomplex arrangement: the serial mediation by ubiquinone (Q) only within the I–III supramolecular assembly would result in a linear relation of the respiratory activity to antimycin. $V_0 = V_{obs}$ in the absence of antimycin. See Lenaz and Genova [39] for a more detailed analysis. Reproduced from Lenaz and Genova [39] with permission.

Figure 4 – Functional association of Complex I and Complex III in BHM. Detergent dependence of NADH-cytochrome *c* reductase. After freeze-thawing twice, aliquots of mitochondria were assayed in the absence and in the presence of detergent (DDM). NADH-cytochrome c activity at saturated concentration of both substrates was measured by the change of differential absorbance of cytochrome c at λ=550 minus 540 nm. Data points are multiple results of repeated titration experiments. The average value at zero concentration of detergent, corresponding to 100% activity rate in the graph, is 0.356±0.031 (μmoles cytochrome *c* reduced/min/mg protein). Experimental details as described in Maranzana et al. [23]. We note that the sharp increase and stabilization of activity at 0.3-1.5 DDM/protein ratios, as compared to rates at very low concentration of detergent, indicates that NADH and cytochrome c had limited access to the inner membrane of the frozen and thawed mitochondria used.

Figure 5 – Structural association of Complex I and Complex III in BHM. Three dimensional view of the immunodetection after 2D BN/SDS-PAGE and Western blotting of mitochondria *(control)* solubilised by digitonin (8 g:g protein) or *(DDM 0.3-2)* by increasing amounts of DDM, as indicated by labels in the picture. Most Complex I was found assembled (I_{SC}) into high molecular weight SC comprising Complex III (III_{SC}) both in control samples and after low DDM solubilisation (DDM/protein ratio <1.5 w:w). On the contrary, significant quantitative dissociation of SC I_1III_2 into individual enzyme complexes (i.e. 86% of total Complex I as free units) was obtained by solubilisation at DDM/protein ratio of 2 g:g. Experimental details according to Maranzana et al. [23] as described in the legend of Figure 2.

Figure 6 - Postulated interactions between Complex II and Complex I. *left:* Reduction of NAD^+ by succinate by energy-dependent reverse electron transfer and forward electron transfer from succinate dehydrogenase to oxygen; *middle:* NADH-fumarate reductase; *right:* as a comparison, it is shown the interaction of Complex I with Complex III within the SC.

Figure 7 - Production of ROS by mitochondrial Complex I in different situations where supercomplexes are maintained or disassembled. The percentage value of ROS production measured in all the samples listed in the table (*lower panel*) is plotted in the graph (*upper panel*). The ratio of bound Complex I versus total Complex I was determined by densitometric analysis of immunoblots obtained after 2D BN/SDS-PAGE. The NADH-stimulated production of ROS was measured as the relative fluorescence intensity of dichlorofluorescein in the presence of 1.8 μM mucidin and 4 μM rotenone, and expressed as percentage value of the corresponding reference samples in the absence of rotenone. In the case of BHM, the existence of endogenous systems operating to reduce ROS levels in the mitochondrial sample might have counteracted the dramatic effects of the complete dissociation of Complex I, thus leading to a twofold only increase of the measured ROS production. Proteoliposomes, R4B 1:1 w:w (cf. text for details); DIL, dilution at high lipid to protein ratio (30:1 w:w); DDM, dodecyl maltoside. Modified from Genova and Lenaz [77] with permission.

Table 1 - Flux control coefficients of respiratory complexes over NADH oxidase and succinate oxidase activity.

Sample	Respiratory activity	Complex I	Complex II	Complex III	Ref.
BHM	NADH oxidase	1.06	n.a.	0.90	[28]
	Succinate oxidase	n.a.	0.88	0.34	[28]
R4B 1:1	NADH-cytochrome c oxidoreductase	0.93	n.a.	0.73	[72]
R4B 1:30	NADH-cytochrome c oxidoreductase	0.92	n.a.	0.15	[72]

BHM, bovine heart mitochondria; R4B, mitochondrial fraction enriched in Complex I and Complex III reconstituted in proteoliposomes at different protein:phospholipid ratios (w:w); n.a., not applicable in this sample.

Table 2 - NADH and succinate oxidation by exogenous cytochrome c in cyanide-inhibited BHM.

Substrate as electron donor	**Rate of cytochrome c reduction** µmoles/min/mg protein
NADH	0.356 ± 0.031 (5)
Succinate	0.591 ± 0.060 (5)
NADH+Succinate	0.883 ± 0.071 (5)

NADH-cytochrome c oxidoreductase activity and succinate-cytochrome c oxidoreductase activity were assayed spectrophotometrically in frozen and thawed BHM at 30°C by monitoring the reduction of 50 µM cytochrome c in the presence of 1 mM KCN and 75 µM NADH or/and 26 mM succinate. When applicable, the dehydrogenase of Complex II was activated by pre-incubation of the mitochondrial samples with succinate at 37°C for 5 min. Data are mean values ± standard deviation of separate experiments, as indicated by the numbers in round brackets.

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HIGHLIGHTS

This review critically examines the functional properties of Complex I within the respiratory supercomplex assembly. In particular, evidence is presented about:

- occurrence of Complex I-containing respiratory supercomplexes in mitochondria;
- channelling of Coenzyme Q (ubiquinone) between Complex I and Complex III;
- modulation of ROS production by the supercomplex assembly.

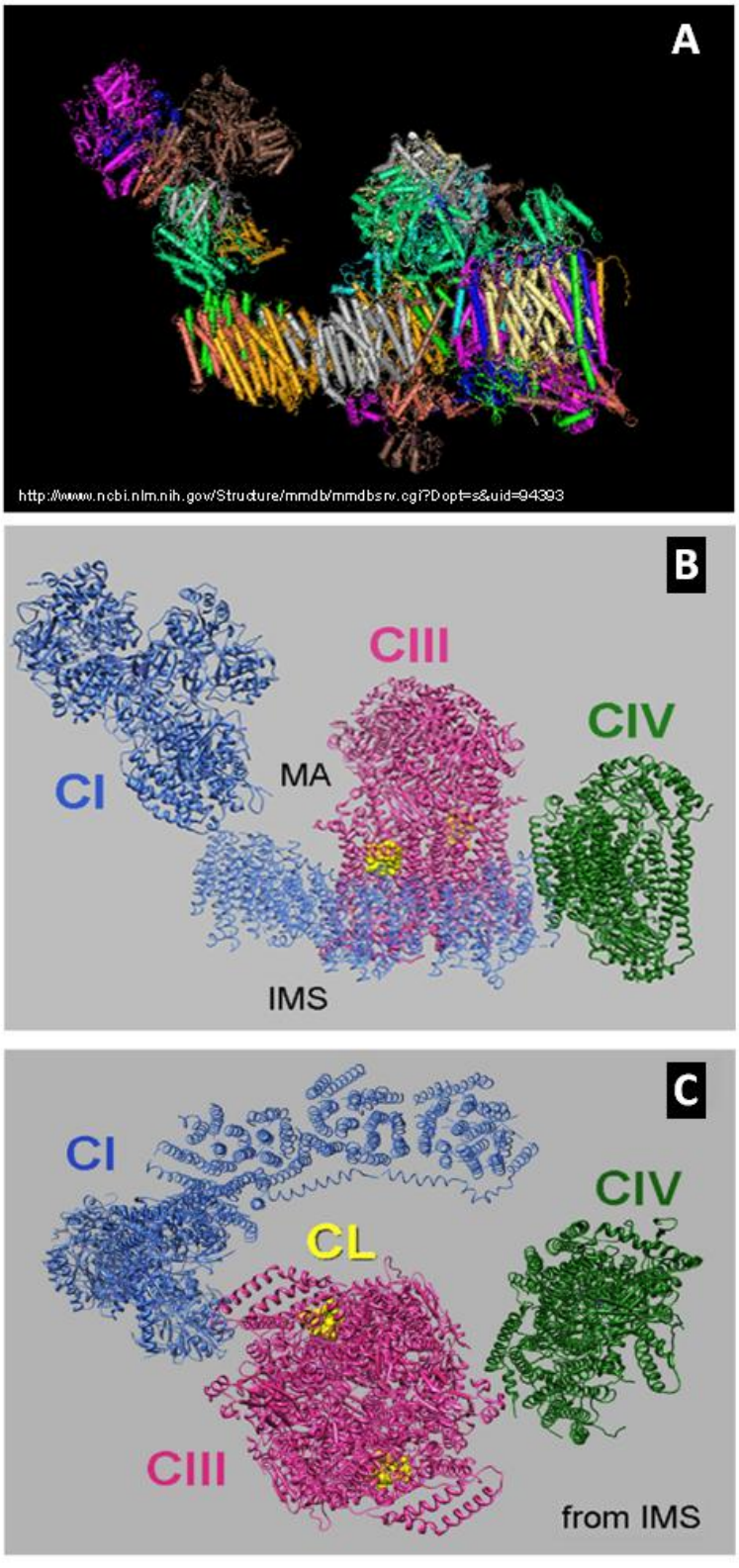
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Figure 1

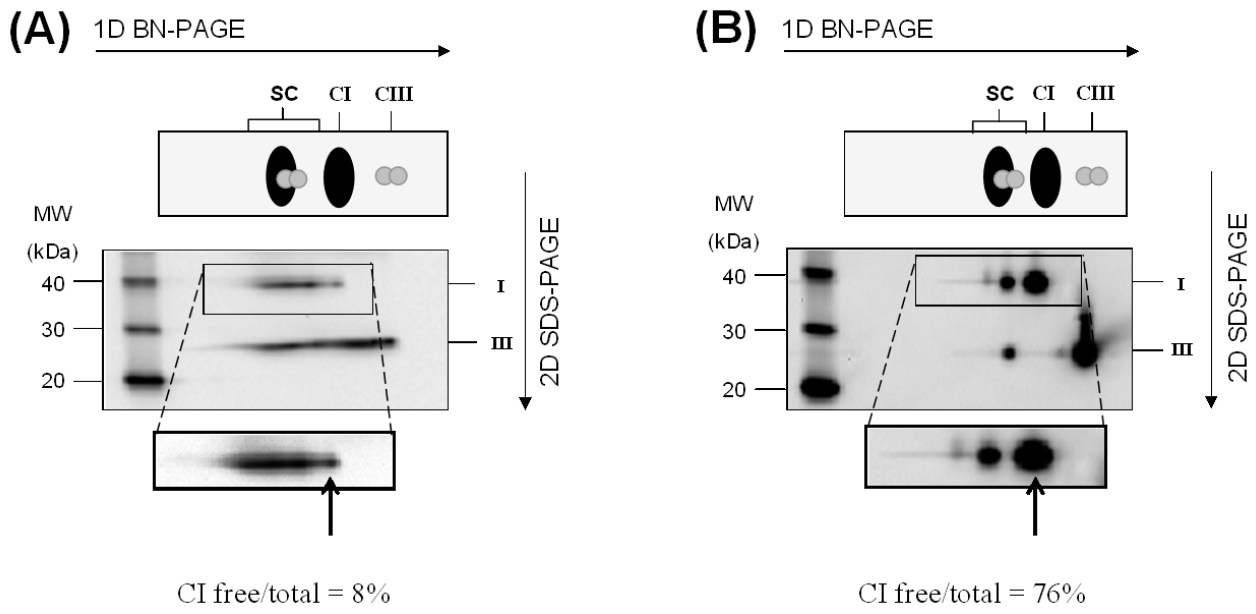


Figure 2

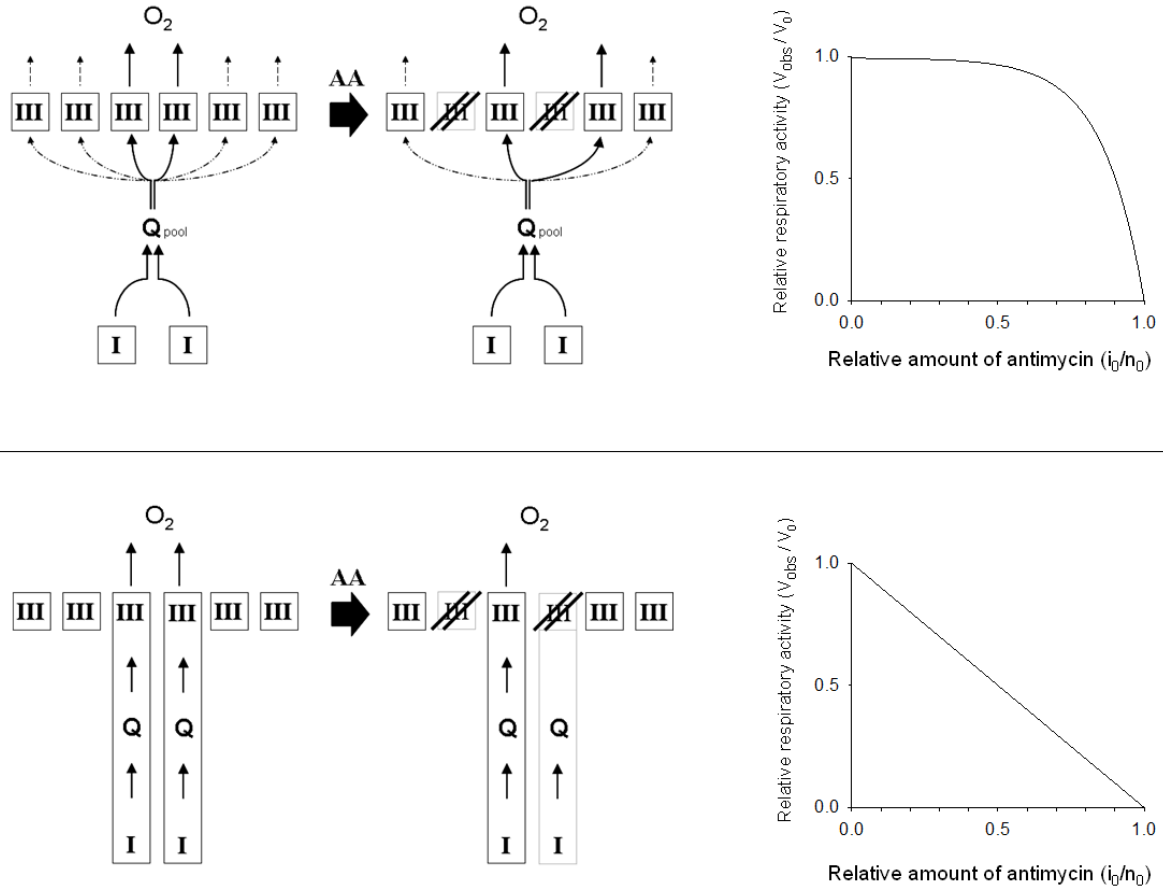


Figure 3

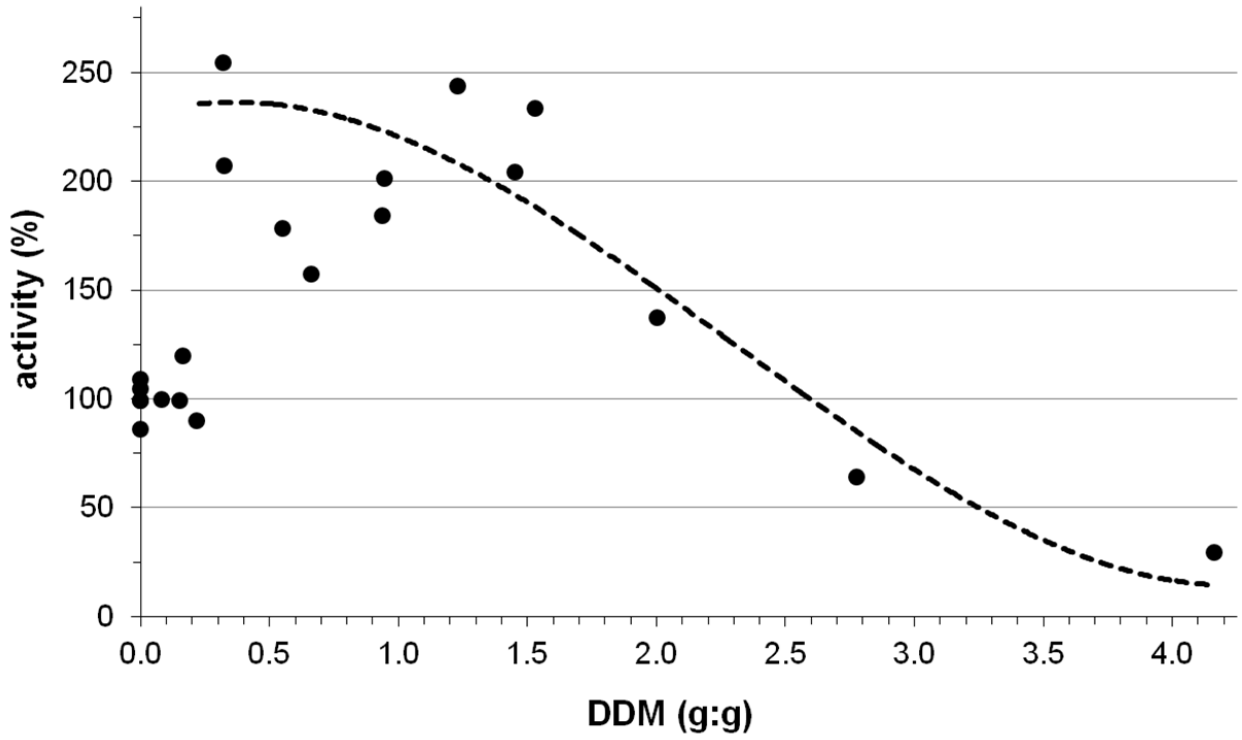


Figure 4

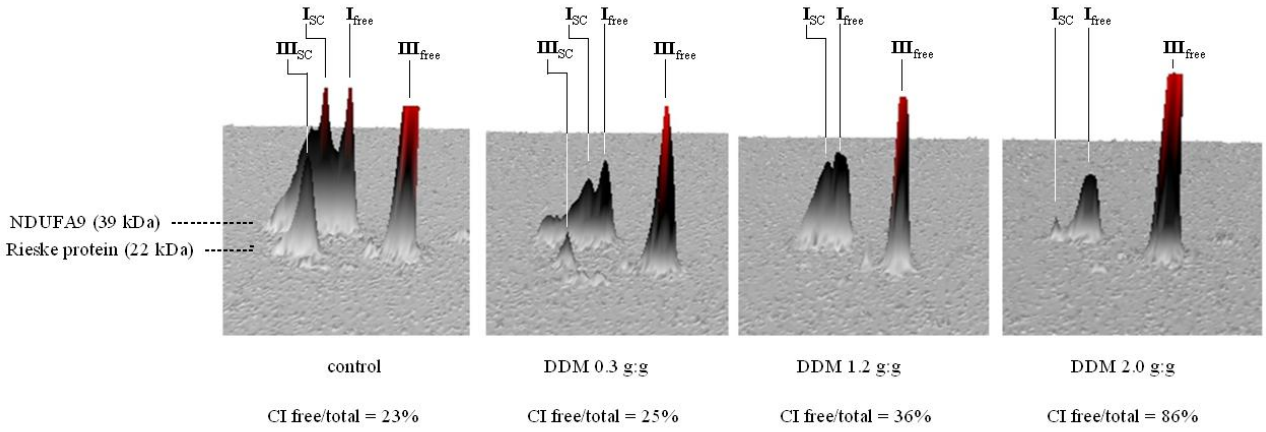


Figure 5

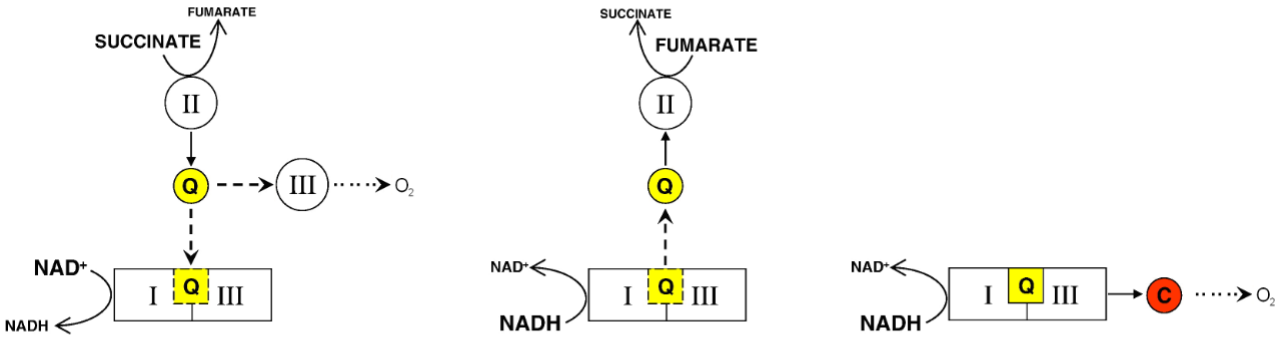
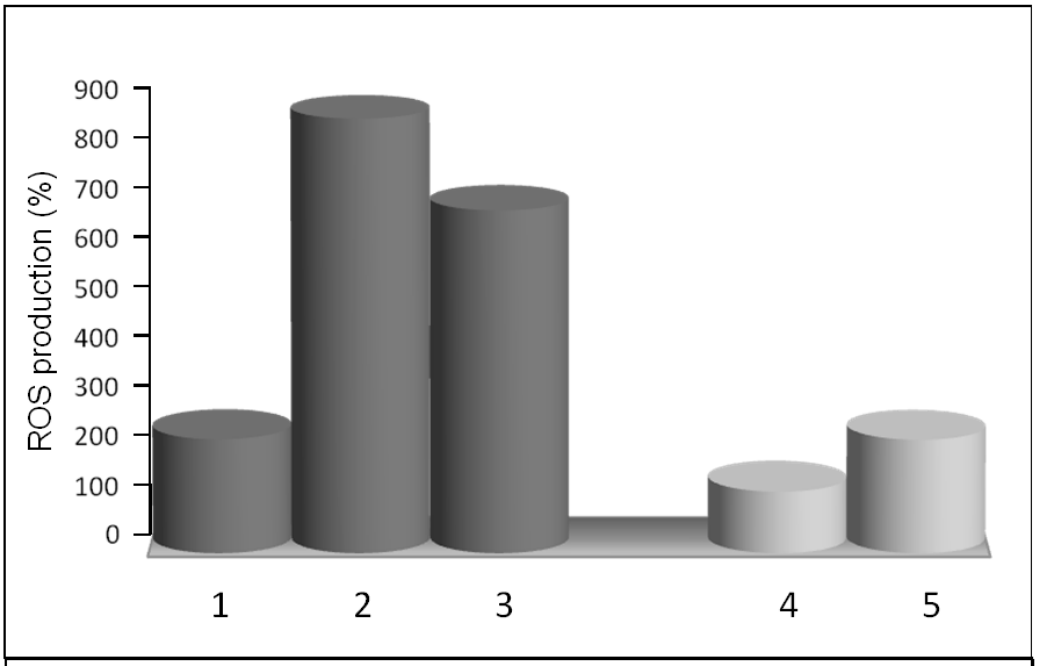


Figure 6



Sample ID Complex I	Relative amount (%) (% of total)	ROS production of bound
1 proteoliposomes	92	224±83
2 proteoliposomes DIL	24	851±243
3 proteoliposomes +DDM	21	672±257
4 BHM	87	121±20
5 BHM +DDM	2	223±86

Figure 7

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