



## Review

Functional role of mitochondrial respiratory supercomplexes<sup>☆</sup>

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## ABSTRACT

Recent experimental evidence has replaced the random diffusion model of electron transfer with a model of supramolecular organisation based upon specific interactions between individual respiratory complexes. These supercomplexes were found to be functionally relevant by flux control analysis and to confer a kinetic advantage to NAD-linked respiration (channelling). However, the Coenzyme Q pool is still required for FAD-linked oxidations and for the proper equilibrium with Coenzyme Q bound in the supercomplex. Channelling in the cytochrome c region probably also occurs but does not seem to confer a particular kinetic advantage. The supramolecular association of individual complexes strongly depends on membrane lipid amount and composition and is affected by lipid peroxidation; it also seems to be modulated by membrane potential and protein phosphorylation. Additional properties of supercomplexes are stabilisation of Complex I, as evidenced by the destabilising effect on Complex I of mutations in either Complex III or IV, and prevention of excessive generation of reactive oxygen species. The dynamic character of the supercomplexes allows their involvement in metabolic adaptations and in control of cellular signalling pathways. This article is part of a Special Issue entitled: Dynamic and ultrastructure of bioenergetic membranes and their components.

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## 1. Introduction

Until the end of the past century, the most widely accepted view of the oxidative phosphorylation system in mitochondria envisaged a random organisation of the respiratory chain complexes according to the random diffusion model of Hackenbrock et al. [1] which, in turn, is based on the previous enzymatic studies in the Enzyme Institute in Wisconsin [2]. The year 2000 represented a drastic change that has switched the current understanding to an organisation in supramolecular assemblies called *supercomplexes* or *respirasomes*. Analysis of a multitude of data in the literature obtained by Blue-Native gel electrophoresis (BN-PAGE) unambiguously reveals that the complexes involved in supramolecular association are the three proton-translocating enzymes: Complex I (NADH:Coenzyme Q reductase), Complex III (ubiquinol:cytochrome c reductase) and Complex IV (cytochrome c oxidase). Supercomplexes with various configurations and stoichiometries occur in mitochondria from different sources (cf. [3–11]).

Furthermore, there are suggestions that even higher levels of organisation may exist (megacomplexes or respiratory strings) [12,13].

The structural evidence for supercomplex association is not matched by a corresponding knowledge of their function(s), and several questions are still open concerning the functional role of such supramolecular entities. In particular, is the supramolecular organisation of the respiratory chain such to confer a real kinetic advantage on electron transfer? Or is it just a structural device stabilising the respiratory proteins but without a clear functional role in electron transfer? This review will examine experimental evidence pertaining to the functions of supercomplexes.

## 2. Respiratory supercomplexes are functional

If we look back to the early studies on the isolation and purification of respiratory complexes, we can find observations leading against their random distribution; in fact, during the intermediate purification steps of the individual enzymes, Complex I–Complex III units [14–16] and Complex II–Complex III units [17] were obtained; similarly in chloroplasts also cytochrome *b*<sub>6</sub>f-photosystem I units were described [18]. The natural conclusion is that such units may be preferentially associated in the native membrane. These units possessed the activities expected from integration of the respective individual complexes, and can now be viewed as *bona fide* supercomplexes.

Because early reports of combined complexes used bile salts, which can lead to protein aggregations, and associations of complexes were not detected by antibodies within the membranes [19], not many researchers paid attention to a potential supramolecular organisation of the respiratory chain. Stable supercomplexes of Complex III and IV isolated from *Paracoccus denitrificans* [20], thermophilic *Bacillus* PS3

Abbreviations: AOX, alternative oxidase; BN-PAGE, Blue-Native gel electrophoresis; CI, Complex I; CII, Complex II; CIII, Complex III; CIV, Complex IV; CJ, cristae junction; CoQ, Coenzyme Q; Ubiquinone; CREB, response element binding protein; ETF, electron transfer flavoprotein; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species

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[21] and thermoacidophilic archaeon *Sulfolobus* [22] seemed to be a special feature of these bacteria. Ozawa et al. [23] proposed the existence of a stable solid-state assembly of the entire OXPHOS system; on the other hand, Hochman et al. [24] suggested the existence of dynamic aggregates in equilibrium with freely diffusing complexes. Due however to the lack of clear experimental evidence, these hypotheses were usually overlooked.

The first clear experimental demonstration of supercomplexes by BN-PAGE following mild digitonin solubilisation of mitochondria [3,25] pointed out that several types of association are present between respiratory complexes I, III and IV. Among the various types of association, the I<sub>1</sub>III<sub>2</sub>IV<sub>1</sub> combination was denominated *respirasome* because considered the minimal unit to perform complete respiration from NADH to oxygen. These units possessed the in-gel activities of the individual complexes, however the first demonstration that they are indeed functional and capable of full activity came from the chromatographic isolation of a functional respirasome from *P. denitrificans* [26]. The respirasome contained complexes I, III and IV in a 1:4:4 stoichiometry and was enriched in Coenzyme Q (CoQ) with respect to the original membrane, but was considerably deficient in cytochrome c<sub>552</sub>; for this reason, although the activity of NADH cytochrome c reductase was close to that expected from the rate-limiting activity of Complex I, the whole NADH oxidase was strongly reduced with respect to the expected rate.

The respiratory activity of a respirasome isolated from mammalian mitochondria was reported by Acín-Pérez et al. [27]. They demonstrated that BN-PAGE bands containing both CoQ<sub>9</sub> (the major CoQ homologue in rodents) and cytochrome c showed complete NADH oxidase activity, as measured using a Clark electrode. A mixture of the bands of the individual complexes I, III, and IV, was inactive. This study demonstrated that supercomplexes not only are real entities, but are competent in respiration, and therefore their role must be consistent with their activity, i.e. they must provide some advantage in electron transfer.

### 3. Supercomplex association provides a kinetic advantage

Immediately after the discovery of supercomplexes 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 [28]; 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. In such a case, inter-complex electron transfer becomes indistinguishable from intra-complex electron transfer, so that the so-called mobile intermediates, predicted to exhibit substrate-like behaviour in the classic view of the random collision model, would rather be buried in the interface between the two consecutive complexes.

Some evidence for possible channelling comes from the 3D structure of the mitochondrial supercomplex I<sub>1</sub>III<sub>2</sub>IV<sub>1</sub> [29]; 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 [29]. 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 supercomplex. Coenzyme Q is likely to run a trajectory through this gap which is presumably filled with membrane lipids. Althoff and colleagues also reported the presence of significant amounts of bound phospholipids in the purified supercomplex from mammalian mitochondria and demonstrated that cardiolipin is enriched in the supercomplex compared with bovine heart total lipid. Moreover, HPLC analysis of the lipid extracts indicated that each supercomplex contains at least one molecule of ubiquinol [29].

#### 3.1. Supercomplex association enhances the rate of electron transfer in the Coenzyme Q region

The rate of electron transfer between membrane-bound electron carriers depends on their structural organisation 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 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). Let us analyze separately the two situations with respect to the mitochondrial respiratory chain. Fig. 1 schematically illustrates the difference existing between collision-based electron transfer and channelling.

##### 3.1.1. Collision-based electron transport: the “pool” behaviour

The functional significance of a random distribution of mitochondrial complexes had been supported by the kinetic analysis of Kröger and Klingenberg [30] for the enzymes connected by Coenzyme Q; 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 ( $V_{\text{obs}}$ ) will be determined by the redox state of the quinone and proportional to either reduced ( $Q_{\text{red}}$ ) or oxidised ( $Q_{\text{ox}}$ ) CoQ concentration:

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

where  $V_{\text{ox}}$  is the rate of ubiquinol oxidation,  $V_{\text{red}}$  is the rate of CoQ reduction and  $Q_{\text{t}}$  is total CoQ concentration (reduced plus oxidised).

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

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

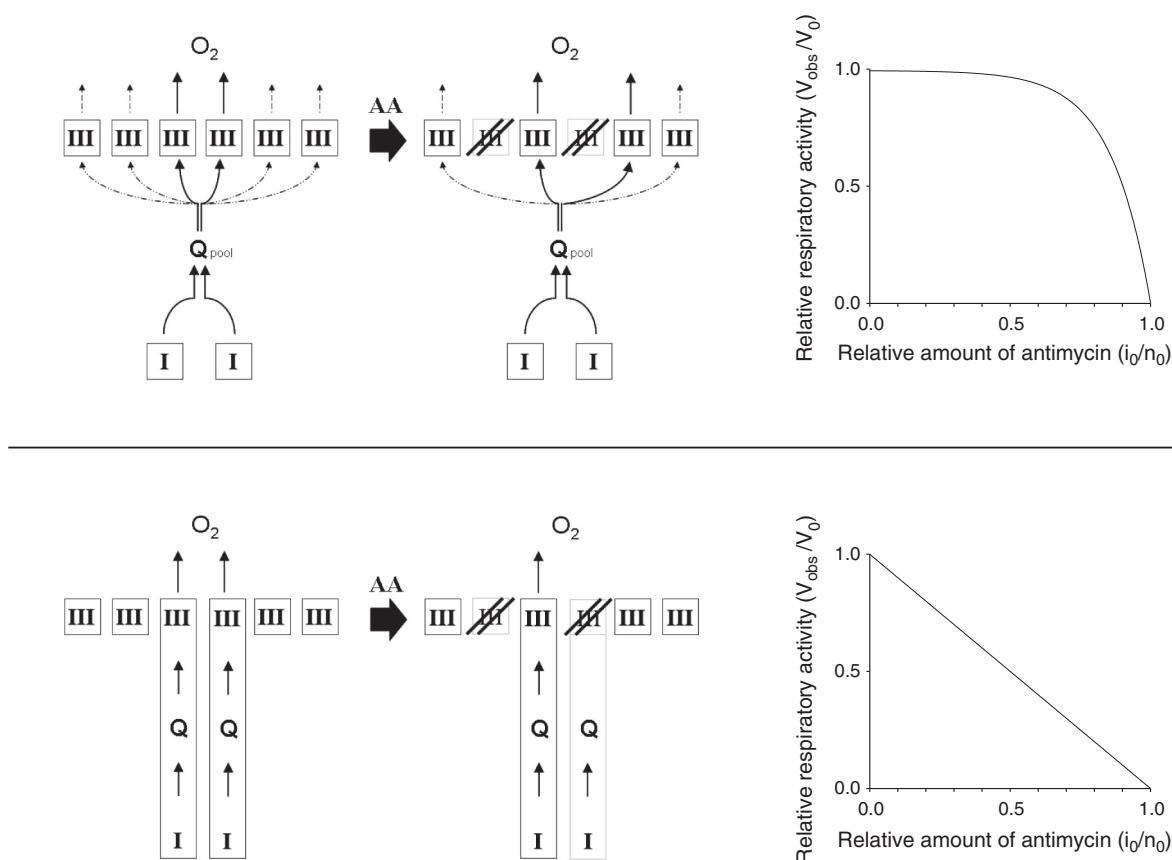
This expression was verified under a wide variety of input and output rates and establishes that CoQ distributes electrons randomly among the CoQ-reducing flavin dehydrogenases and the bc<sub>1</sub> complexes, behaving indeed as a laterally diffusing pool of molecules in a variety of systems [31–33], discussed by Lenaz and Genova [6,34].

Further evidence was provided by the characteristic effect of changing  $V_{\text{red}}$  or  $V_{\text{ox}}$  on inhibitor titration curves [35]. In the case of the CoQ pool and titration of Complex III by antimycin, a convex hyperbolic relationship exists between the integrated oxidation rate ( $V_{\text{obs}}$ ) and the fraction ( $x$ ) of Complex III inhibited by antimycin:

$$V_{\text{obs}} = [V_{\text{red}} \cdot V_{\text{ox}} \cdot (1-x)]/[V_{\text{red}} + V_{\text{ox}} \cdot (1-x)] \quad (3)$$

On the contrary, a linear relationship is expected in the case of a stoichiometric association between Complex III and the CoQ-reducing enzymes. However, the convexity of the inhibition curve in a system obeying to pool behaviour is also a function of the ratio between  $V_{\text{red}}$  and  $V_{\text{ox}}$  [35] so that pool behaviour may not be easily recognised and experimentally distinguished from CoQ-channelling in a system where  $V_{\text{red}} \gg V_{\text{ox}}$ .

Most available data on CoQ pool concern succinate oxidation in submitochondrial particles, whereas fewer data are available for NADH oxidation. In bovine heart submitochondrial particles, the observed rate of electron transfer between Complex I and Complex III was found to be comparable to that calculated from the pool equation [36]. In other mitochondrial systems the rate of Complex I activity is strongly underestimated, due to the properties of CoQ analogues used as acceptors [37] so that the pool equation is not directly applicable



**Fig. 1.** 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). In agreement with Eq. (3), the relation between the respiratory activity ( $V_{obs}/V_0$ ) and the amount of antimycin relative to the antimycin-titer ( $i_0/n_0$ ) depends on the ratio  $V_{red}/V_{ox}$ ; the most hyperbolic curve is observed with the smallest value (i.e.  $V_{red}/V_{ox} = 0.05$ ). (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. Reproduced from Lenaz and Genova [6] with permission.

[38]. 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 [6,34].

A study dealing with presence of diffusible intermediates in the respiratory chain in intact phosphorylating mitochondria was accomplished by the double inhibitor titration technique [39] by Stoner [40] who showed that in intact coupled mitochondria in state 3 (i.e. synthesizing ATP), inhibition of Complex III with myxothiazol makes succinate oxidase less sensitive to the Complex II inhibitor 3'-hexylcarboxin, in accordance with the existence of a freely diffusible intermediate between the two steps [39]. Unfortunately in his study Stoner did not perform similar titrations for search of a diffusible intermediate between Complexes I and III.

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

Although the pool equation has been developed for CoQ between dehydrogenases and  $bc_1$  complexes, the same reasoning is valid for cytochrome c that shares with ubiquinone the postulated role of mobile substrate-like component of the respiratory chain [41].

Nevertheless, the role of cytochrome c mobility between Complex III and Complex IV is even more intriguing than that of ubiquinone [42]. Hackenbrock et al. [1] observed that under physiological conditions of 150 mM ionic strength, cytochrome c is readily dissociated from the

membrane and undergoes three-dimensional diffusion in the intermembrane space [43,44]. In reconstituted enzyme systems in presence of excess cytochrome c, “pool behaviour” is followed [41]; likewise, the rotation rates of cytochrome c oxidase reconstituted in lipid vesicles are not influenced by simultaneous incorporation of the reductase [45], suggesting that the two enzymes act as independent entities.

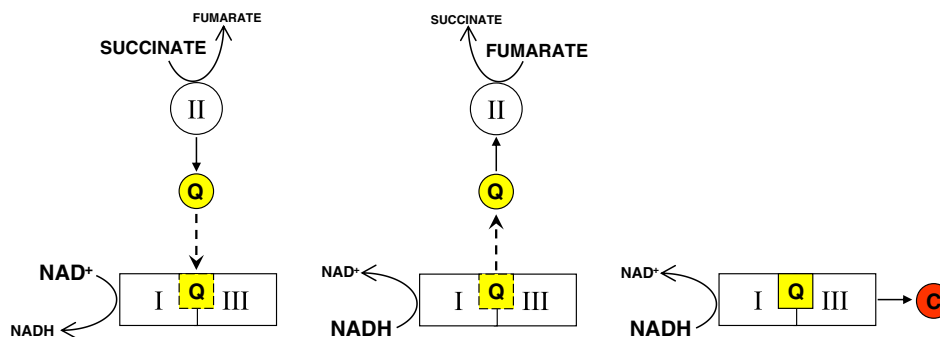
### 3.1.2. Deviations from pool behaviour

The universal validity of the pool equation has been questioned in the past, since deviations from pool behaviour were described [8,32].

Gutman [31] 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 (Fig. 2).

The non-homogeneity of the ubiquinone pool with respect to succinate and NADH oxidation [31] may be interpreted today in terms of compartmentalisation of CoQ in the I–III supercomplex in contrast with the free pool used for connecting Complexes II and III.

Kröger and Klingenberg [30,35] already noticed that 10–20% of CoQ in submitochondrial particles is not reduced by any substrate. More



**Fig. 2.** Postulated interactions between Complex II and Complex I. *Left:* Reduction of  $\text{NAD}^+$  by succinate by energy-dependent reverse electron transfer; *middle:* NADH-fumarate reductase; *right:* as a comparison, it is shown the interaction of Complex I with Complex III within the supercomplex. See text for explanations.

recently Benard et al. [46] described the existence of three different pools of CoQ in rat liver and muscle mitochondria: one pool is utilised during succinate-dependent steady-state respiration, another (approx. 8% in muscle and 23% in liver) is mobilised 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 mobilised at all. These results are compatible with CoQ compartmentalisation, 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 supercomplex I–III.

Recently, it was demonstrated [47] that the physical assembly between complexes I and III determines a preferential pathway for electrons mediated by a dedicated subset of CoQ molecules. This compartmentalisation 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 that physically interact with Complex I in the formation of supercomplexes are also exclusively dedicated to NADH oxidation ( $\text{CI}_{\text{NADH}}$ ) while those Complex III molecules that are not bound to Complex I are mainly responsible for oxidation of succinate and other substrates using the free CoQ pool. Complex I has a very high affinity for Complex III, so that this association is preferred to the free state of either Complex I or Complex III when a partial loss of Complex III occurs. In this situation NADH oxidation is preferentially maintained despite the risk of compromising the oxidation of FAD-linked substrates [47].

### 3.1.3. Fixed assemblies: stoichiometric behaviour

Early experiments reported by Ragan and Heron [48] 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 supercomplexes found by BN-PAGE, where Complex III is present as a dimer) and 2–3 moles of  $\text{CoQ}_{10}$  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.

Activation-energy measurements for NADH-cytochrome c oxidoreductase activity showed that oxidoreduction of endogenous  $\text{CoQ}_{10}$  proceeds somewhat differently from the oxidation and the reduction of exogenous quinone homologues, supporting the idea that the mobility of  $\text{CoQ}_{10}$  in the Complex I–III unit is highly restricted.

These studies were able for the first time to demonstrate the existence of a supercomplex formed by Complexes I and III, although its

formation *in vitro* requires a specific low-lipid environment that apparently 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 organisation of the matrix [49,50] may keep the integral proteins in a clustered immobilised arrangement thus favouring segregation of most of the phospholipids into separated patches.

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### 3.1.4. Dissociation of supercomplexes shifts channelling to a less efficient pool behaviour

The previously described study [48] first described stoichiometric behaviour for the activity of NADH cytochrome c reductase, ascribable to the formation of a supercomplex. 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 [48] to levels comparable to those of natural mitochondrial membranes. Inclusion of phospholipid into the reconstituted system may have a number of effects on the physical state of the system. Heron and co-workers have 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. [51] also reported that endogenous  $\text{CoQ}_{10}$  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. It is likely that the function of the large amount of ubiquinone in the natural membrane may be, therefore, to maintain the  $\text{CoQ}_{10}$  content in the supercomplex unit when it is formed.

As a conclusion of these studies, Heron et al. [51] proposed a model by which electron transfer occurs only through stoichiometric Complex I–Complex III units, which, however, are formed and re-formed at rates higher than the rate of electron transfer. This model would explain the adherence to CoQ-pool behaviour of the activity in native membranes (but see Section 3.1.1 for the criticisms to pool behaviour observations in NADH oxidation).

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

A system, obtained by fusing a crude mitochondrial fraction ( $\text{R}_4\text{B}$ ) [52] enriched in Complex I and Complex III with different amounts of phospholipids and  $\text{CoQ}_{10}$  [53] was used in our laboratory to discriminate whether the reconstituted protein fraction behaves as individual enzymes (CoQ-pool behaviour) or as assembled supercomplexes 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. (2) in Section 3.1.1) showed overlapping results at phospholipid dilutions (w/w) from 1:10 on, 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 [53–55] at low protein:lipid dilution (1:1 w/w), resembling the mean nearest neighbour distance between respiratory complexes in mitochondria [56,57,42].

Moreover when the same proteoliposomes at 1:1 lipid:protein ratios were treated with dodecyl maltoside to destroy the supercomplex organisation, the NADH cytochrome c reductase activity fell dramatically, whereas both Complex I and Complex III individual activities were unchanged [58]; an analogous behaviour was detected by treating bovine heart mitochondria with the same detergent.

Additional evidence for channelling between complexes I and III derives from the demonstration that selective decrease of Complex III content under a given threshold induces a preferential deficiency in the transfer of electrons between complexes II and III while the transfer of electrons between Complex I and III remains unaltered [47].

These studies clearly demonstrate that electron transfer between Complex I and Complex III can take place both by CoQ channelling within the I<sub>1</sub>III<sub>2</sub> supercomplex and by the less efficient collision-based pool behaviour, depending on the experimental conditions.

Recently, evidence has been obtained that electron transfer may occur *in vivo* in the absence of supercomplexes.

Maas et al. [59] have been able to express functional alternative oxidase (AOX) in mammalian cells lacking complexes III and IV. Under these conditions, Complex I is stable and works perfectly in combination with AOX, which is able to oxidise ubiquinol supplanting the combined role of Complex III, cytochrome c and Complex IV (reported by J.A. Enriquez and G. Lenaz, submitted paper). This demonstrates *in vivo* that Complex I-dependent respiration does not strictly require supercomplex I–III formation.

In addition to that, further evidence exists that supercomplexes may be physiologically dissociable units (see Section 3.6, Evidence for the dynamic nature of supercomplexes).

### 3.2. Evidence for channelling in the CoQ region was first obtained by flux control analysis

The assumption that supercomplex association has the major function of conferring a rate advantage by substrate channelling arose together with their discovery [3]. Although the theoretical basis of channelling was already established [60], the first demonstration of respiratory supercomplex association on a functional basis was achieved in our laboratory some years later [54,61]. We exploited the flux control analysis and the principle that the sum of the flux control coefficients 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 [60].

Using this principle and exploiting inhibition with specific inhibitors, in bovine heart mitochondria we found that both Complex I and Complex III have flux control coefficients approaching 1, suggesting that they behave as a single enzymatic unit, so that electron transfer through Coenzyme Q is accomplished by channelling between the two complexes. This approach is similar to that previously applied by Boumans et al. [62], 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 [61], 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.

Surprisingly, very few other studies were addressed to the functional aspects of supercomplexes using metabolic control analysis [63–65]; these studies confirmed that the respiratory chain, at least under certain conditions, is organised in functionally relevant supramolecular structures. Quarato et al. [63] in digitonin-permeabilised HepG2 cells observed that under conditions of high membrane potential the sum of the flux control coefficients calculated for Complexes I, III and IV activities exceeded 1, supporting the proposition that they are complexed in a supramolecular unit (see Section 3.6.3 for further speculation on the role of membrane potential).

In saponin-permeabilised breast and colorectal tumor samples Kaambre et al. [64,65] observed flux control coefficients for mitochondrial oxidative phosphorylation activities whose sum approached 4, in contrast with lower values for normal tissues; they also interpret the data as due to the presence of supercomplex association.

### 3.3. Complex II and other CoQ-reducing enzymes use the CoQ pool

The CoQ pool is certainly required for electron transfer from Complex II to Complex III: in bovine heart mitochondria and submitochondrial particles, Complex II kinetically follows pool behaviour in reconstitution experiments [66] and also in intact mitochondria [40]; this is in complete accordance with the lack of Complex II-containing supercomplexes as found by both BN-PAGE [25]. Flux control analysis [61] confirmed that Complex II is the only rate-limiting step in succinate oxidation, and both Complex III and Complex IV have low flux control coefficients at difference with NADH oxidation (Section 3.2). The existence of small amounts of supercomplexes including Complex II molecules, described by Acín-Pérez et al. [27] in mouse cell lines and in mouse liver mitochondria, does not contradict the knowledge that most of succinate oxidation takes place by the CoQ pool between Complexes II and III. An exception is the study of Boumans et al. [62] in yeast, showing that succinate oxidation did not obey pool behaviour unless the mitochondria were treated with chaotropic agents.

The CoQ pool behaviour must fully apply to other enzymes that deliver electrons to CoQ (succinate dehydrogenase, glycerol-3-phosphate dehydrogenase, electron transfer flavoprotein (ETF)-ubiquinone oxidoreductase, dihydroorotate dehydrogenase, choline dehydrogenase, sulfide CoQ reductase and proline dehydrogenase). These enzymes are likely to be in minor amounts and strongly rate-limiting in integrated electron transfer [67]. Few direct studies are however available on possible associations of these enzymes. The only direct study addressed to this problem [68] demonstrated that in brown fat mitochondria the inhibition curve of glycerol phosphate-cytochrome c reductase is sigmoidal in the presence of myxothiazol and antimycin, suggesting the presence of a homogeneous CoQ pool between glycerol phosphate dehydrogenase and Complex III.

Also the hyperbolic relation experimentally found by Gutman [31] between succinate oxidase and the rate of reverse electron transfer from succinate to NAD<sup>+</sup>, involving sequential interaction of complexes II and I by means of CoQ, is in complete accordance with the pool equation. This observation poses a particularly puzzling question [6] about how ubiquinol reduced by Complex II interacts from the pool with the CoQ site in Complex I, since Complex I is totally engaged in the supercomplex. The same dilemma applies to the NADH fumarate reductase activity that also involves interaction of Complex I and Complex II (Section 3.1.2). In view of the recent progress in the knowledge of the detailed atomic structure of Complex I [69,70], the previous view that two different routes may exist for forward and reverse electron transfer within Complex I [71] 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<sup>+</sup> occurs in the presence of a high mitochondrial transmembrane protonmotive force that, according to Piccoli et al. [72], might be the physiological signal causing the structural reorganisation

of the respiratory complexes. The model hypothesis suggests that the I–III supercomplex 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 (cf Section 3.6.3). This model is however incompatible with the observation reported by Gutman [31] that NADH fumarate reductase, that occurs at low membrane potential, shares the same pathway of the reverse reduction of  $NAD^+$  by succinate (Section 3.1.2). According to this consideration, the scheme in Fig. 2 shows that the pathways linking Complex II and Complex I during either direct or reverse electron transfer most probably require the interaction of the CoQ pool with Complex I within the supercomplex. If this is true, we must conclude that Complex I in the supercomplex is somewhat accessible to the CoQ pool.

### 3.4. Saturation kinetics of Coenzyme Q

A finding that in the past was believed to strengthen the validity of the random diffusion model is the observed saturation kinetics of CoQ not only in succinate oxidation but also in NADH oxidation. As previously described, Complex I is almost totally associated in a supercomplex with Complex III, with electron channelling of bound CoQ in the boundary between the two complexes, while the CoQ pool is directly required for electron transfer from Complex II to Complex III.

The finding that Complex I is almost totally associated in a supercomplex with Complex III seems to exclude a role for the CoQ pool in physiological electron transfer between these two complexes. Surprisingly, strong evidence exists that NADH cytochrome c reductase activity follows saturation kinetics with respect to CoQ.

The relation between electron transfer rate and CoQ concentration was seen for NADH and succinate oxidation in reconstituted systems [66] and in phospholipid-enriched mitochondria [73]. 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 [66], corresponding to a  $Q_t$  concentration of 4–10 mM in the lipid bilayer. The  $K_m$  for CoQ<sub>10</sub> of NADH-cytochrome c reductase was found to be much higher than that of succinate-cytochrome c reductase. A direct study on a reconstituted mitochondrial fraction containing Complexes I and III showed that the experimental rate of NADH cytochrome c reductase was hyperbolically related to the content of CoQ<sub>10</sub>, with an apparent  $K_m$  in the same range as in mitochondria [53].

Analysis of the literature shows that the physiological CoQ content of several types of mitochondria is in the range of the  $K_m$  for NADH oxidation, and therefore not saturating for this activity [74]. However, this does not exclude that free CoQ in the pool is also necessary for proper channelling between the Complexes. In fact, the bound inter-complex quinone that allows electron flow directly from Complex I to Complex III must be in dissociation equilibrium with the CoQ pool, so that its amount, at steady state, would be dictated by the size of the pool: this equilibrium explains the saturation kinetics for total ubiquinone exhibited by the integrated activity of Complex I and Complex III and the decrease of respiratory activities in mitochondria fused with phospholipids with subsequent dilution of the CoQ pool. To be in agreement with the experimental observation obtained by metabolic flux analysis, this proposition requires that the dissociation rate constants ( $k_{off}$ ) of bound CoQ be considerably slower than the rates of inter-complex electron transfer via the same bound quinone molecules [9,10]. The high apparent  $K_m$  for CoQ<sub>10</sub> in NADH oxidation is in line with this postulation. The observation by Schneider et al. that dilution of the inner membrane proteins with phospholipids lowers electron transfer and the effect is reversed by CoQ addition is also in line with this concept [73]. Earlier studies by Heron et al. 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 [51]. 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.

The existence of I–III super-complexes where only inter-complex bound CoQ is active by channelling electrons from Complex I to Complex III is apparently incompatible with a dose-dependent effect of exogenously administered CoQ<sub>10</sub> as in several clinical studies [10]; however the notion that inter-complex bound CoQ is in chemical equilibrium with CoQ in the pool is sufficient to explain the improved cell bioenergetics upon addition of exogenous CoQ. It is expected that even a slight decrease of CoQ content in the membrane is sufficient to dissociate part of the quinone from the supercomplex thus decreasing the rate of electron channelling.

### 3.5. Electron transfer through cytochrome c

The flux control studies in our laboratory clearly established that CI and CIII behave as a functional supercomplex in bovine heart and rat liver mitochondria, whereas Complex IV does not since it has a low flux control coefficient, despite the BN-PAGE showing that a fraction of Complex IV is physically associated in the respirasome in the same mitochondrial samples [61,55,67].

The reason for this discrepancy is not clear. It is true that most of CIV appears to be free in the BN-gels (cf. Section 2), and one might consider that the activity in the respirasome is masked by the excess of active enzyme in free form; however, the problem is rather difficult to examine critically. In fact, the experiments of Bianchi et al. [61] show that NADH oxidase activity responds to cyanide inhibition with a lower slope than cytochrome c oxidase. Assuming that (i) all CIV units, either free or bound in the respirasome, have the same sensitivity to  $CN^-$  and that (ii) the probability of the inhibitor to bind the enzyme is the same in both assay conditions, then the percent decrease of activity would be the same for NADH oxidase and for cytochrome c oxidase if electron transfer occurred by cytochrome c channelling in the respirasome, giving a flux control coefficient close to unity. Since this is not the case, we must conclude that either CIV in the supercomplex has a lower sensitivity to cyanide or that cytochrome c can escape from the respirasome and rely on an excess of free CIV units which can be randomly reduced. The latter means that the free molecules of CIV are involved in electron transfer from NADH, and implies that the molecules in the supercomplex do not receive electrons from CIII by channelling of cytochrome c [10]. Of course, we are aware that branched pathways are difficult to approach by metabolic control analysis and that measurements in isolated mitochondria by inhibitor titration of the relevant enzymes inevitably involves using assay conditions (i.e. substrate concentrations) that are not necessarily intended to reproduce *in vivo* conditions.

Moreover, the non-artifactual nature of the lack of functional channelling shown by the flux control coefficients in mammalian mitochondria is supported by the fact that in potato tuber mitochondria we clearly showed that the flux control coefficients approach 1 for each one of the three complexes (CI, CIII, CIV); in such mitochondria therefore the respirasome seems to be completely functional in channelling [67]. The reason for the difference may be in a tighter binding of cytochrome c in the respirasome. Likewise in *S. cerevisiae* mitochondria Boumans et al. [62] found evidence for cytochrome c channelling; accordingly, single particle cryo-electron microscopy revealed that the supercomplex consisting of a III<sub>2</sub>IV<sub>2</sub> unit (since there is no Complex I in this yeast species) is arranged in such way that the distance between the binding sites of cytochrome c, i.e. cytochrome c1 in CIII and subunit II of CIV (containing the CuA), is considerably shorter than in bovine mitochondria [75]. To further confuse the issue, it is also worth to note that Trouillard et al. [76] recently demonstrated in yeast mitochondria that the time-resolved oxidation of cytochrome c by CIV is a random process not involving substrate compartmentalisation.

The purified supercomplex I<sub>1</sub>III<sub>2</sub>IV<sub>1</sub> obtained from bovine heart [29] and some putative respirasomes isolated from rat liver mitochondria

[27] also showed to contain traces of cytochrome c; therefore we cannot definitively exclude that substrate channelling on this level occurs also in mammalian mitochondria.

Using a different approach, Enriquez recently demonstrated that at least part of Complex IV forms a functional supercomplex with channelling of cytochrome c [47]. In particular, they showed that when Complex IV is allowed to participate in supercomplexes by the presence of SCAF1, a significant proportion of Complex IV activity is not utilised by glucose driving respiration. Moreover, they demonstrated in isolated liver mitochondria that the assembling of Complex IV in supercomplexes defines three types of Complex IV, one dedicated exclusively to receive electrons from NADH oxidation (forming supercomplex I + III + IV), another dedicated to receive electrons from FAD-dependent enzymes (forming supercomplex III + IV) and a third major one that is in free form and that is able to receive electrons from both NADH and FADH<sub>2</sub> oxidation.

On the contrary, if Complex IV is maintained permanently detached from supercomplexes by elimination of SCAF1, the maximum respiration activity of intact cells under glucose-rich medium parallels the maximum respiration activity obtained in the same cells by feeding directly electrons to Complex IV. In this case, since all Complex IV is in free form and electron transfer takes place via diffusion of the cytochrome c pool, this experiment demonstrates that the cytochrome c pool function is also very efficient.

Fig. 3 schematically shows different types of supercomplexes and the postulated different mechanisms of electron transfer.

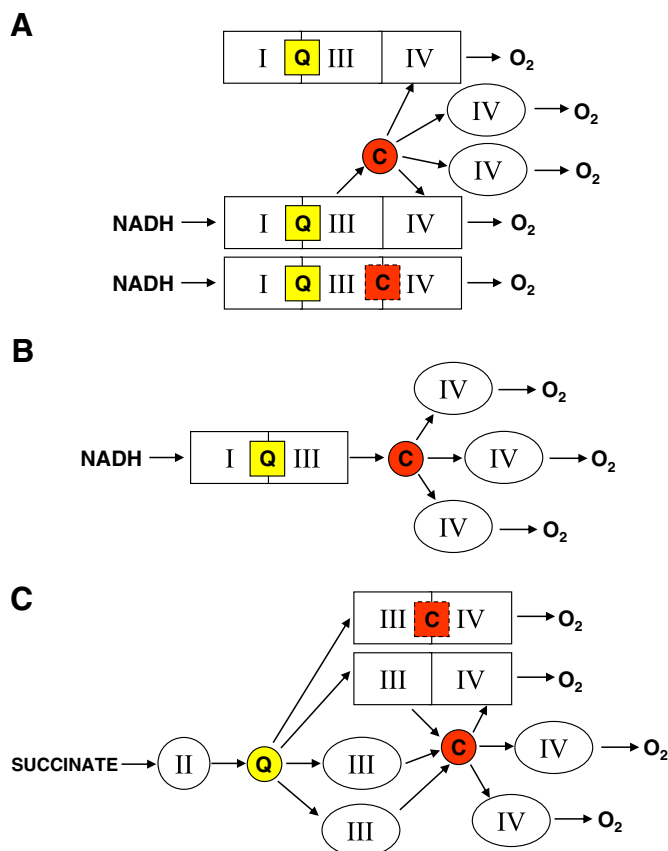
### 3.6. Evidence for the dynamic nature of supercomplexes

When Acín-Pérez et al. [27] demonstrated that purified respirasomes (i.e. supercomplex I<sub>1</sub>III<sub>2</sub>IV<sub>1</sub>) were able to respire in a Clark's electrode, they also observed other types of associations, as I + III or III + IV, which were formed regardless the presence of the third partner of the respirasome, strongly suggesting that they can also be present *in vivo*. Therefore they concluded that the variety of associations between respiratory complexes was larger than the respirasome and that the free complexes likely co-exist with supercomplexes. In this context they proposed an integrated model, the *plasticity model*, for the organisation of the mitochondrial electron transport chain. The previous opposed models, solid vs. fluid, would be two extreme allowed and functional situations of a dynamic range of molecular associations between respiratory complexes [27]. The stoichiometry of the complexes and the variable stability of different free vs. associated structures under different physiological conditions would determine a variety of different structural options. The plasticity model and the dynamics of mitochondrial supercomplexes are widely discussed by J.A. Enriquez in this special issue.

A fundamental prediction of the plasticity model is that, *in vivo*, the mitochondrial respiratory chain should be able to work both when supercomplexes are present and when the formation of supercomplexes is prevented. Indeed, several studies *in vitro* support the view that electron transfer in the respiratory chain can occur in absence of supercomplexes, as extensively described in Section 3.1.

The early studies of Hackenbrock in the 80s showed that the intramembrane particles are randomly distributed in the plane of the inner membrane where they diffuse with relatively high diffusion coefficients in the range of  $10^{-10}$  cm<sup>2</sup>s<sup>-1</sup> [77,1]. Those accurate studies cannot be dismissed as artefacts, therefore they must be explained within the frame of a dynamic equilibrium between supercomplexes and randomly diffusing individual complexes. To this respect, the time window of the experimental approaches is of paramount importance and must be taken in account.

The first attempt to follow the temporal dynamics of the respiratory complexes *in situ* was made by Muster et al. [78] who suggested that supercomplex dissociation into a fully random distribution may not be a fast event.



**Fig. 3.** Postulated organization of the respiratory chains in mammalian mitochondria. In (A) are represented respirasomes (I–III–IV) and free cytochrome c oxidase (IV) molecules; electron transfer between CI and CIII always occurs by CoQ (Q) channelling, whereas fast dissociation of cytochrome c (C) from CIII may also allow electron transfer by random collisions of free cytochrome c with CIV, either free or bound in other respirasomes (cf. Section 3.5). In (B) is a scheme of the possible function of the supercomplex formed by CI and CIII; in this case it is postulated that free cytochrome c collides with uncomplexed CIV units. In (C) is depicted the mechanism of electron transfer from succinate via free CII and the CoQ pool. This model may also apply to other enzymes feeding electrons to the CoQ pool (see text for details). In this case, the CoQ pool interacts with either free CIII or CIII/CIV associations. The CIII units are shown as monomers for sake of simplicity. Reproduced from Genova and Lenaz [10] with permission.

In a very recent study Wilkens et al. [79] studied the impact of mitochondrial fusion/fission on the reorganisation of oxidative phosphorylation (OXPHOS) complexes in the inner mitochondrial membrane of different HeLa cells tagged with fluorescent proteins (GFP and DsRed-HA). After cell fusion by polyethylene glycol treatment, redistribution of the tagged OXPHOS complexes was followed by means of immunoelectron microscopy, two color super-resolution fluorescence microscopy and single molecule tracking. In contrast to outer membrane and matrix proteins, which mix quickly and homogeneously upon mitochondrial fusion, the mixing of inner membrane proteins was decelerated. The authors suggest that the composition of the cristae is highly preserved during fusion of mitochondria and that cristae with mixed OXPHOS complexes are slowly and successively formed by restricted diffusion of the inner membrane proteins into the existing cristae. Significantly, Complex II was found very mobile, showing the highest abundance in close proximity to the cristae junction (CJ) and a significant quota of molecules also localised in the inner boundary membrane facing the outer mitochondrial membrane. On the contrary, the diffusion of the other complexes was more restricted along the intruding cristae membrane microcompartment distal from the CJ. In particular, the comparison of the localisation profiles of OXPHOS complexes I, III and IV in the inner membrane compartments clearly showed an overlap: 80% of CI coincided with 80% of CIII and CIV, thus supporting



the idea of a physical assembly constituted by the three enzymes. Interestingly the diffusion coefficients reported by Wilkens et al. [79] are of the order of those reported by Hackenbrock et al. [1].

Some other observations in the literature indirectly support the view that the supercomplex organisation may not be fixed but in equilibrium with randomly dispersed complexes in living cells under physiological conditions. Besides phospholipid composition that may change by genetic or dietary reasons in a relatively long time-scale, some biochemical parameters at a shorter time-scale have been suggested to affect the supramolecular structure of the respiratory complexes. These parameters are the mitochondrial membrane potential and the phosphorylation state of the protein subunits of the complexes.

### 3.6.1. Lipid content and composition affect supercomplex association

Lipids are essential determinants of supercomplex association [6], however they can modify the nature and extent of the associations only in long time-scales, since they are affected by the organism species, by the tissue, and within a given tissue and species by diet and lifestyle.

We will only briefly summarise the influence that lipids have on supercomplexes, since this subject is partly covered by G. Paradies in this special issue.

The forces responsible for supercomplex association appear to strongly depend on the lipid content and composition and likely on the shape of the inner mitochondrial membrane [6]; in particular the studies reported in Section 3.1.3 by Ragan [48,51] and from our laboratory [55] have shown that reconstitution of a binary I/III mitochondrial protein fraction at a high lipid to protein ratio (>10:1) prevents formation of the I–III supercomplex, as shown by BN-PAGE and kinetic studies [55]. In other words, it appears that dilution of the proteins with an excess of phospholipids may weaken the forces holding together the respiratory complexes.

Among lipids, cardiolipin and phosphatidyl ethanolamine are crucial for mitochondrial functions; they are both non-bilayer-forming phospholipids, due to their small polar heads compared with the bulky nonpolar tails [80,81]. The phospholipids in closest vicinity to the protein surface, as well as those in the free bilayer, are highly mobile and free to exchange, but cardiolipin is tightly bound being more likely buried within the protein complexes [82–84]. The absolute requirement of cardiolipin for the activity of cytochrome c oxidase, Complex I and Complex III suggests that this phospholipid plays a crucial role in the coupled electron transfer process [85,86].

There are now extensive indications that cardiolipin stabilises respiratory supercomplexes as well as the individual complexes. Preliminary results from our laboratory suggest that reconstitution of binary I/III proteoliposomes (see above) at high lipid to protein ratio albeit enriched with 20% cardiolipin (w:w), resembling the percent content of cardiolipin in the mitochondrial membrane, can restore efficient NADH-cytochrome c reductase activity, which is presumably achieved by preservation of CoQ<sub>10</sub> channelling in the preparation [M. Kopuz and Y. Birinci, unpublished data].

More evidence concerning this subject has been collected for the III<sub>2</sub>–IV<sub>2</sub> supercomplex in yeast mutants lacking cardiolipin [87,88]. The putative direct protein–protein interaction of cytochrome c oxidase and Complex III in yeast is proposed to involve also one molecule of cardiolipin and one of phosphatidylethanolamine, tightly bound in a cavity of the membrane imbedded domain of Complex III [83]. It has been suggested that these two phospholipids can provide a flexible linkage between the interacting subunits of Complexes III and IV as well as with the ADP/ATP carrier that is also known to exist in physical association with the III–IV supercomplex [89]. Site-directed mutagenesis investigations [90] indicated that cardiolipin stabilises supercomplex formation by neutralising the charges of lysine residues in the interaction domain of Complex III with cytochrome c oxidase.

In addition the stability and assembly of Complex IV was found to be reduced in yeast cells lacking Taz1 [91], the orthologous of human tafazzin, an acyl transferase involved in the synthesis of mature

tetralinoleyl cardiolipin [92]: mutations of tafazzin in humans result in Barth syndrome, a cardio-skeletal myopathy with neutropenia, characterised by respiratory chain dysfunction. Significantly, the cardiolipin defect in Barth syndrome results in destabilisation of the supercomplexes by weakening the interactions between respiratory complexes [93]. More recently, Gonzalez et al. [94] confirmed that in immortalised lymphoblasts from Barth's syndrome patients the amount of supercomplexes is decreased, as well as the amount of individual complexes I and IV; these changes were compensated by increasing mitochondrial mass.

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 [95–97], reviewed by Paradies et al. [98]. Genova et al. [55] demonstrated by flux control analysis that the maintenance of a I–III supercomplex in proteoliposomes is abolished if lipid peroxidation is induced by 2,2'-azobis-(2-amidinopropane) dihydrochloride before reconstitution. Evidently, the distortion of the lipid bilayer induced by peroxidation and the alteration of the tightly bound phospholipids determine dissociation of the supercomplex originally present in the preparation.

In contrast with cardiolipin depletion that destabilises supercomplexes, a recent study reported that phosphatidyl ethanolamine depletion tends to favour the formation of larger supercomplexes between CIII and CIV in *S. cerevisiae* mitochondria [99]. The reason why cardiolipin and phosphatidylethanolamine, both non-bilayer forming phospholipids, behave in an opposite way on supercomplex stability has been ascribed to the different charge, being the former an anionic phospholipids and the latter zwitterionic [99].

### 3.6.2. Protein factors affecting supercomplex association

Besides the several assembly factors that have been described to be required for the biogenesis and assembly of the supercomplexes (see review by Enriquez in this special issue), up to now only a single protein has been recently described that may be involved in the reversible regulation of supercomplex association and activity: Hatle et al. [100] identified MCJ/DnaJC15 as a distinct cochaperone that localises at the mitochondrial inner membrane, where it interacts preferentially with Complex I. MCJ impairs the formation of supercomplexes and functions as a negative regulator of the respiratory chain. Knocking out the expression of MCJ leads to increased Complex I activity, mitochondrial hyperpolarisation, and enhanced ATP production, in line with an enhanced association of respiratory Complex I into supercomplexes. In this study Hatle et al. [100] speculate that MCJ may be involved in the balance between Complex I active and inactive forms; in addition MCJ decreases Complex I activity by dissociating the supercomplexes.

### 3.6.3. Membrane potential

A study of the influence of the mitochondrial trans-membrane potential ( $\Delta\mu_{H^+}$ ) on the flux control exerted by cytochrome c oxidase on the respiratory indicated that, under conditions mimicking state 4 respiration in intact cells and isolated mitochondria, the control strength of the oxidase is decreased in respect to endogenous state 3 respiration [72]. More recently the extension of these studies to the other proton-translocating respiratory complexes (I and III) revealed that under conditions of low potential the sum of the flux control coefficients exceeded 1, whereas at high potential the coefficients were much lower [63].

Although the interpretation of the results in such a complex system is very difficult, the authors suggest that such a change in control strength might be featured in terms of supercomplex plasticity. In particular, the results showing that the sum of the flux control coefficients of Complexes I, III, and IV exceeds 1 in state 3, were compatible with a respirasome organisation at lower membrane potential and with a random organisation of cytochrome c oxidase with respect to other complexes at high membrane potential (state 4); since the respiratory



rate is high in state 3 conditions, the supercomplex organisation would produce an extra advantage by raising the rate by channelling.

We extended the study in actively phosphorylating human hepatoma HepG2 cells under conditions in which the electrical ( $\Delta\Psi$ ) and chemical ( $\Delta\text{pH}$ ) components of  $\Delta\mu_{\text{H}^+}$  were selectively modulated by addition of ionophores. We concluded that  $\Delta\Psi$  is predominantly responsible for the tight control exerted by cytochrome c oxidase over endogenous respiration whereas  $\Delta\text{pH}$  seems irrelevant in this respect [101].

Although the molecular mechanisms leading to the assembly/disassembly of the supercomplexes have been not yet defined, it is not inconceivable that, given the membrane-integrated nature of the single complexes, electrostatic/hydrophobic interactions may enter into play in response to  $\Delta\mu_{\text{H}^+}$ . In this respect, recent investigations have revealed a surprising interplay between pH gradients, lipid composition and packing and membrane cristae shape [102,103].

In potato tuber mitochondria it was found that hypoxia dissociates the supercomplex I–III<sub>2</sub>–IV into individual Complex I plus Complex III–IV units [104]; it was shown that the change was due to the acidification of the medium in hypoxia and that acidification could reproduce the same effect of hypoxia; moreover the effect was not due to changes in membrane potential.

### 3.6.4. Post-translational changes

It is now established that some of the mitochondrial complexes are subjected to reversible phosphorylation and dephosphorylation [105,106]. Mitochondria contain protein kinases and phosphatases and both serine/threonine phosphorylation [107,108] and tyrosine phosphorylation [107,109] of mitochondrial proteins are important in regulation of activity of these organelles.

Phosphorylation of Complex I has been shown to modify the activity of the enzyme and its ROS generating capacity [110–114]. Cyclic AMP-dependent phosphorylation of the 18 kDa subunit of Complex I, encoded by the nuclear NDUF54 gene, is required for import of the subunit; modulation of subunit phosphorylation by intramitochondrial protein kinase A and phosphoprotein phosphatase contributes to the stability of Complex I and stimulation of its activity [115]. It is tempting to speculate that the increase of activity of Complex I and the decrease of ROS generation by phosphorylation may be, in part, the result of enhanced stability of the I–III supercomplex, however no studies to date have established any correlation between them. Phosphorylation of Complex III subunits has also been reported although its functional role remains undetermined [116].

Complex IV is a target of the intra-mitochondrial cAMP-PKA signalling pathway [117,118].

It is tempting to speculate that endocrine alterations may affect the assembly state of Complex IV, by hyper- or hypo-phosphorylation of some subunits in the complex [10, J.A. Enriquez and G. Lenaz submitted paper]. Indeed, cAMP- and PKA-dependent phosphorylation of Complex IV in heart mitochondria [119] was found to be higher in free Complex IV not associated in supercomplex than in the bound enzyme, suggesting that phosphorylation prevents supercomplex association. Nevertheless, caution must be exerted in the interpretation of the role of post-translational changes in supercomplex formation, since no causal correlation has yet been established.

### 3.7. Mechanism of electron transfer in the supercomplexes

The mechanism of electron transfer in supercomplexes is still uncertain. Ideally, we should have a detailed knowledge of the molecular structure of the interacting sites, and this knowledge is still lacking. Obviously we may have the extremes from close docking of the active sites with real interprotein tunnelling, up to relatively long distances that may be covered either by important conformation changes or by restricted diffusion (microdiffusion) of the mobile components within the space between the two active sites; all of these alternatives have

in common obligate channelling between two fixed sites, so that even the last situation, microdiffusion, would be quite distinguishable from pool behaviour. In the latter case, the interaction of the mobile component may stochastically occur with a great number of possible sites located on several different protein targets which can be reached by random diffusion [10]. In the interaction between Complex I and Complex III within a supercomplex, if the sites are connected by CoQ microdiffusion, it is possible that it takes place within a lipid milieu, although we cannot exclude that the sites are put together by movement of CoQ on the protein or by movement of the protein itself.

In the 3D structure of the mitochondrial supercomplex I<sub>1</sub>III<sub>2</sub>IV<sub>1</sub> which was recently reported by Althoff et al., 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 [29]. In the above mentioned model (cf. also Section 3), one of the Complex III monomers faces the lipid bilayer while the other is surrounded by Complex I. Althoff and colleagues also reported the presence of significant amounts of bound phospholipids in the purified respirasome from mammalian mitochondria and estimated that roughly 300 lipid molecules would fit the gap that extends between complexes I, III, and IV on the level of the membrane-embedded portion of the supercomplex [29] (cf. also Dudkina et al. [120] and the article by N.V. Dudkina in this special issue).

There are recent indications that supercomplex association may provide further kinetic advantages besides substrate channelling. The study of Schaefer et al. [121] showed that a supercomplex comprising cytochrome c oxidase (I<sub>1</sub>III<sub>2</sub>IV<sub>1</sub>) had higher Complex I and Complex III activities than the supercomplex devoid of the terminal oxidase (I<sub>1</sub>III<sub>2</sub>); evidently, the presence of Complex IV modifies the conformation of the partner complexes in such a way to enhance their catalytic activity. Likewise, Hildebrandt [122] showed that supercomplex dissociation abolishes the protective effect of dehydroascorbic acid on sulphide toxicity to cytochrome c oxidase, suggesting a conformational effect of supramolecular association on the allosteric properties of cytochrome c oxidase.

Too little is known as yet about the possibility that protein–protein interactions within the supercomplex I–III–IV might affect the subunits that appear to be important in the proton-pumping mechanism of Complex I, Complex III and Complex IV. The redox potential span which provides the driving force for proton translocation across the inner mitochondrial membrane is subjected to the steady state reduction level of the electron donors/acceptors as catalyzed by the respiratory supercomplex. It would be of interest to know whether the supercomplex association may improve the proton pumping capability of the respiratory chain by increasing the redox potential span of the substrates that are channeled within the protein assembly itself, in comparison to the homogenous donor/acceptor pool. However, to our knowledge, a thermodynamic analyses that specifically addresses this problem in the respiratory supercomplex is still lacking, possibly because it is challenging to measure the redox potentials of the ubiquinol/ubiquinone and ferro/ferricytochrome c couples directly in the supercomplex.

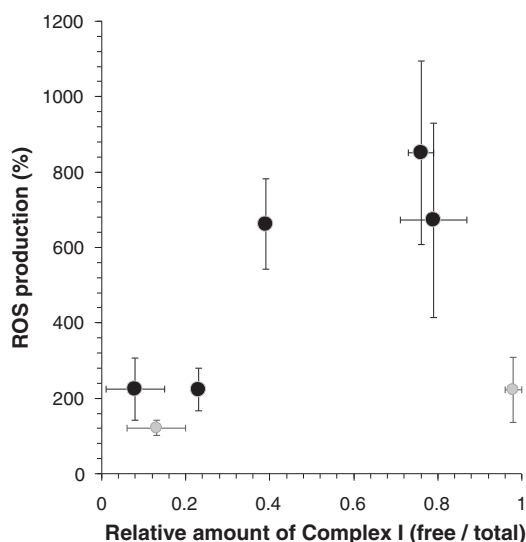
## 4. Supercomplex association limits production of reactive oxygen species from Complex I

The possibility that supercomplex assembly prevents excessive ROS generation from Complex I has been advanced on theoretical grounds [123–125] but in a complex biological system it is difficult to ascertain a clear relationship of cause to effect between supercomplex alteration and ROS production, since each of the two phenomena may influence the other one. On the contrary, in a recent study [58] we induced a primary condition in mitochondrial samples by which we could enhance free Complex I with respect to Complex I bound to the supercomplex; we obtained the first direct demonstration that loss of supercomplex organisation causes an enhancement of ROS generation by Complex I

itself. In the above study we have shown an enhanced ROS generation by Complex I in two experimental systems in which the supramolecular organisation of the respiratory assemblies was destroyed by: (i) treatment either of bovine heart mitochondria or liposome-reconstituted supercomplex I–III with dodecyl maltoside; (ii) reconstitution of Complexes I and III at high phospholipids to protein ratio (Fig. 4).

The ROS increase does not appear to be the mere result of Complex I damage as a possible consequence of the treatment in the presence of detergents, since the NADH-ubiquinone redox activity is only slightly depressed, though Complex I is mostly in its free form. Moreover, the hypothetical reasoning that facilitation of electron flow by substrate channelling within the respirasome helps maintaining the redox components of the complexes in the oxidised state, thus limiting ROS formation, cannot be the only explanation. In fact, in our study [58], ROS production is investigated in the presence of inhibitors (mucidin and rotenone) that prevent electron transfer to any possible acceptor, so that we can guess that the redox centers in Complex I are maximally reduced both in the situations where supercomplexes are maintained and in the situation where Complex I is free.

Two potential sites for oxygen reduction exist in Complex I, represented by FMN [126] and iron–sulfur cluster N2 [127] or the (likely) ubisemiquinone associated with this cluster [128], which might distinctly prevail in generating ROS under different conditions. N2 would be a predominant source of ROS in membrane particles containing super-assembled Complex I whereas FMN would become exposed to oxygen only when Complex I is dissociated from Complex III [3]. Indeed, the actual shape of the  $I_1III_2IV_1$  supercomplex from bovine heart [121] suggests a slightly different conformation of Complex I in the supercomplex, showing a smaller angle of the matrix arm with the membrane arm and a higher bending toward the membrane (and presumably Complex III) which may limit exposure of the redox centers of Complex I to oxygen. On the contrary, the observed destabilisation of Complex I in the absence of supercomplex may render the 51 kDa subunit containing the FMN more “loose” allowing it to interact with oxygen [129].



**Fig. 4.** Production of ROS by mitochondrial Complex I in different situations where supercomplexes are maintained or disassembled. The percent value of ROS production is plotted in the graph against the corresponding ratio of free Complex I versus total Complex I. The statistical analysis of the data using the Pearson's parametric test indicates a positive correlation ( $r = 0.884$ ,  $p < 0.05$ ) between ROS generation and relative amount of free Complex I in the R4B proteoliposomes and in the SMP samples (black symbols). The BHM samples (gray symbols) were not included in the correlation analysis because the existence of endogenous antioxidant systems operating to reduce ROS levels in the mitochondria might have counteracted the dramatic effects of the complete dissociation of Complex I, thus leading to a two-fold only increase of the measured ROS production. SMP, submitochondrial particles. Figure reproduced from Maranzana et al. [58].

This in vitro study is supported by several observations in cellular and animal models linking together supercomplex dissociation and enhanced ROS production.

In mouse fibroblasts expressing the activated form of the k-ras oncogene we had previously observed a strong decrease of high molecular weight supercomplexes correlating with higher ROS generation in comparison with wild type fibroblasts [130,67]. Moreover, enhanced ROS generation and oxidative stress were found in yeast mutants lacking the supercomplex assembly factor Rcf1 and thus devoid of supercomplexes III–IV [131–133]; since the yeast *S. cerevisiae* lacks Complex I, in this case we may consider the origin of the extra ROS being presumably free Complex III, while supposing that other potential sources of ROS (i.e. external alternative NADH dehydrogenase) are not affected in the Rcf1 mutants.

Numerous pathological states are accompanied by enhanced generation of ROS [134] and both mitochondria and other systems, such as plasma membrane NADPH oxidase, have been implicated as the sources of ROS. In an experimental model of heart failure, the decrease of oxidative phosphorylation has been associated with a decrease of respiratory supercomplexes [135,178,179].

Lymphoblasts from patients affected by Barth Syndrome, due to genetic loss of tafazzin, an enzyme involved in cardiolipin remodelling, have altered mitochondrial supercomplexes [93]; likewise, in a yeast experimental model of tafazzin mutation, Chen et al. [136] observed an increased oxidative stress in response to ethanol.

Aging is also accompanied by a decline of supercomplex association [137,138]; despite some uncertainties and challenges, aging is generally associated with increased ROS and oxidative damage (for a recent review cf. Cui et al. [139]). Gómez and Hagen [140] reason that age-associated destabilisation of respiratory supercomplexes may be important for the development of the mitochondrial aging phenotype by means of impaired bioenergetics and enhanced ROS production; in addition Frenzel et al. [138] on the basis of the 3D-structures of supercomplexes and the close spatial arrangement of the respective electron carrier binding sites [121] conclude that less superoxide radical formation is expected to occur in supercomplexes than in randomly distributed individual complexes. These latter studies, however, fail to show which is the causing event (i.e. supercomplex dissociation causing ROS increase or, alternatively, ROS increase causing supercomplex dissociation) or even if they are independent phenomena.

## 5. Supercomplex association is required for the stability of Complex I

Since the discovery of supercomplexes it was noticed that the stability of Complex I is strongly decreased when it is not assembled in the respirasome.

The first chromatographic isolation of a complete respirasome ( $I_1III_4IV_4$ ) from digitonin-solubilised membranes of *Paracoccus denitrificans* indicated that Complex I is stabilised by assembly into the NADH oxidase super-complex 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 CoQ reductase activity [26].

Analysis of the state of supercomplexes in human patients with an isolated deficiency of Complex III [141,142] and in cultured cell models harbouring Complex III [117], Complex IV [143] or cytochrome c [144] depletion, lead to propose that the formation of respirasomes may be essential for the assembly/stability of Complex I. Genetic alterations leading to a loss of Complex III led to secondary loss of Complex I, therefore primary Complex III assembly deficiencies presented as Complex III/I defects [117,145].

D'Aurelio et al. [146] studied the complementation of mitochondrial DNA (mtDNA) in human cells by fusing two cell lines, one containing a homoplasmic mutation in a subunit of respiratory chain Complex IV, COX-I, and the other with a distinct homoplasmic mutation in a subunit

of Complex III, cytochrome b. Upon cell fusion, respiration was recovered in correlation with the presence of supercomplexes containing complexes I, III and IV. From these findings, supercomplex assembly was proposed as a necessary step for respiration (Fig. 5).

In a different study, deletion of the *ndufs4* gene, encoding an accessory subunit of Complex I, in mouse tissues results in decreased stability and activity of Complex I: however, the portion of Complex I that is still active is bound in the supercomplexes, indicating that the supramolecular association improves the stability of Complex I [147].

Conversely, mutations of Complex I had controversial effects, since in some studies they did not affect the amount of other complexes [142,148], while in others they significantly reduced the amounts of Complexes III and IV [117,149–151]. The reason for this discrepancy is not known, but might be related to the specificity of the mutation affecting subunits of Complex I involved in the contacts with the other complexes.

Animal models of the effects of Complex III and Complex IV mutations on Complex I should prove useful for a better understanding of the role of supercomplexes. In one study, knocked down predicted homologues of COX-IV and COX-Va in the nematode *Caenorhabditis elegans* showed that intrinsic Complex I enzymatic activity is dependent on the presence of Complex IV, despite no overall decrease in the amount of Complex I [152]. In a further study in *C. elegans* the same group showed that Complex III defects inhibit Complex I by several different mechanisms involving supercomplex destabilisation [153]. Mutant analysis revealed that Complex III affects supercomplex I–III–IV formation by acting as an assembly/stabilising factor. Also, a mtDNA mutation affecting Complex III, *ctb-1*, inhibits Complex I function by weakening the interaction of Complex IV in supercomplex I–III–IV. Other Complex III mutations inhibit Complex I function either by decreasing the amount of Complex I (*isp-1*), or its assembly as the most active supramolecular form, the I–III–IV supercomplex (*isp-1;ctb-1*).

It is suggested that allosteric interactions involve all three complexes within the supercomplex and are necessary for maximal enzymatic activities.

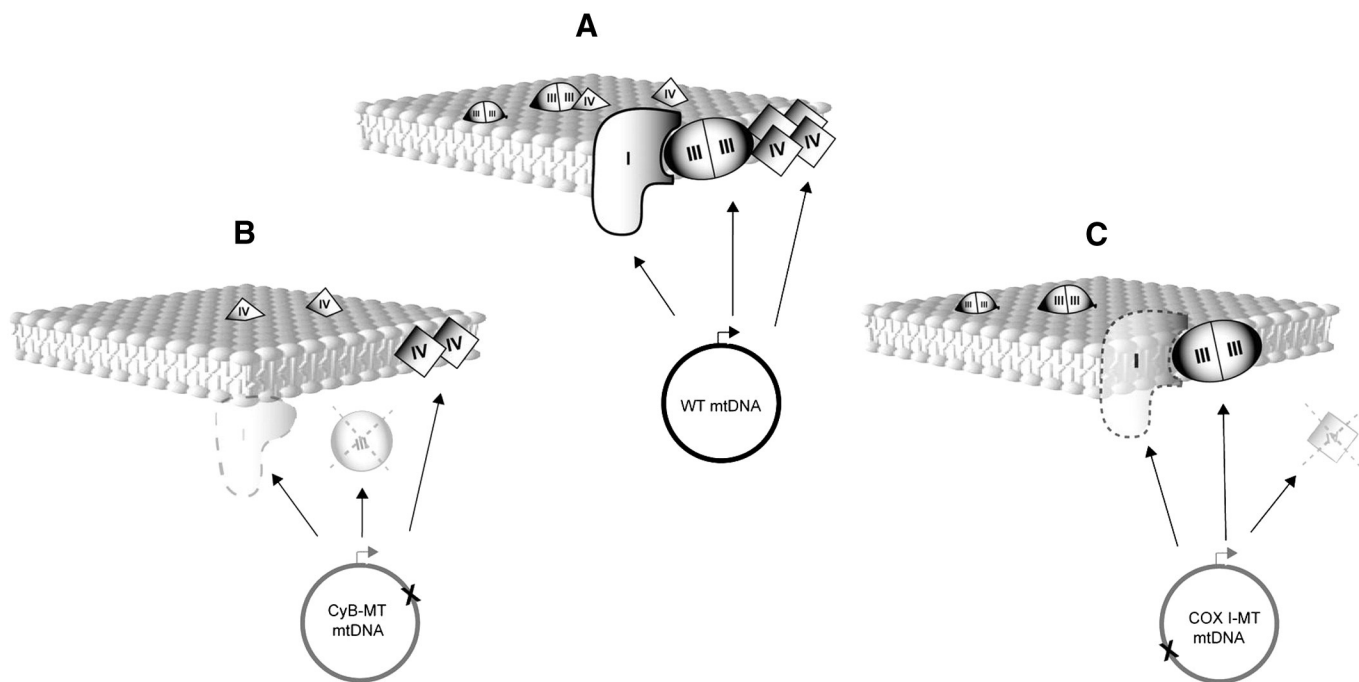
Is the stabilisation of Complex I by its association in a supercomplex a primary effect on assembly during biogenesis or is it a secondary effect on the stability of the Complex after its assembly? It is possible that both events may occur.

Moreno-Lastres et al. [154] report that at early supercomplex assembly stages Complex I is neither fully assembled nor active when it binds to other respiratory chain complexes, since it lacks part of the N-module catalytic subunits, thus suggesting that the supercomplex constitutes the structural unit where Complex I gets to its complete assembly.

On the other hand, Acín-Peréz et al. [27,141] report that the assembly of supercomplexes is temporally delayed from the full assembly of the individual complexes and that association of Complex I and Complex III is devoted to the storage and preservation of Complex I since the enzyme stability and not its normal assembly is compromised in the absence of Complex III.

Overall, we can suggest the following course of events: misassembled CIII prevents formation of supercomplex; the lack of the supercomplex association induces an enhanced ROS generation from Complex I (see Section 4), with consequent Complex I damage: Complex I is vulnerable to oxidative stress [155], and it was found that its activity is strongly decreased by ROS through the peroxidation of cardiolipin [156].

The fragility of free Complex I is not the result of inherent instability of the enzyme, since the isolated Complex I is stable and active for long time, as evidenced by the early studies of Hatefi and coworkers [15] and also by our analysis of Complex I activity in proteoliposomes [58]. However, Complex I shows some fragility due to its sensitivity to the increased production of ROS during the activity of the respiratory chain.



**Fig. 5.** Model of respiratory chain complexes structure and assembly in WT and mtDNA mutant cells. (A) In WT mammalian cells, respiratory chain complexes are organised in supramolecular structure (supercomplexes) composed of monomeric Complex I, dimeric Complex III and mono-, di-, tri- or tetrameric Complex IV. The supercomplexes coexist with a pool of partially assembled supercomplexes ( $\text{III}_2 + \text{IV}$ ) and individual complexes (dimeric Complex III and monomeric Complex IV), indicating that a functional 'solid-state' model, like the supercomplexes, can exist in equilibrium with randomly organised, enzymatically active, isolated complexes. (B) In 100% CyB-MT cybrids, the complete loss of Complex III results in disassembly and degradation of Complex I; only Complex IV, mostly organised in a monomeric form, remains in the mitochondrial inner membrane. (C) In 100% COX I-MT cybrids, the total loss of Complex IV results in a partial decrease of Complex I. The residual Complex I is completely assembled with Complex III; Complex III dimers are unaffected by the COX I mutation. From this model, it is clear that Complex III constitutes the structural core to which Complex I and Complex IV bind to form a stable supramolecular structure. Complex I, because of its instability, cannot exist as an individual complex. Reproduced from D'Aurelio et al. [146] with permission.

A recent paper on fibroblasts lacking the Rieske iron sulphur protein [157] suggests that the physical association of Complex I into supercomplexes per se would explain all the above results. Thus it points out to a different mechanism for Complex I destabilisation in the absence of the Complexes III or IV, i.e. in an enhanced ROS generation (cf previous section) supporting the current knowledge that Complex I is particularly sensitive to ROS damage.

Since ROS also damage lipids, and in particular cardiolipin, this series of events can create a vicious circle of oxidative stress and supercomplex disorganisation (Fig. 6) [3].

It is also noteworthy that some structural alterations of Complex III are compatible with the supercomplex assembly but they can still induce increased levels of ROS. Recently Ghelli et al. [158] characterised the biochemical properties of cybrids carrying a human cytochrome b missense mutation m.15579A > G, which substitutes the Tyr 278 with Cys (p.278Y > C) that was identified in a patient with severe exercise intolerance and multisystem manifestations. This mutation does not induce disassembly of the supercomplex containing Complex I and no subunit assembly defect of Complex III was reported in the mutant cybrids. However, decreased amounts of dimeric Complex III, drastic loss of its specific activity and enhanced ROS production were observed with the mutated as compared to wild type cybrids. Evidently, there must be specific structural consequences of Complex III mutations in order to disassemble the I<sub>1</sub>III<sub>2</sub>IV supercomplex with consequent destabilisation of Complex I. Similarities of the major findings of the human mitochondrial model and its counterparts in bacteria (Y302C *Rhodobacter capsulatus*) and in yeast (Y279C *S. cerevisiae*) are remarkable [159], including decreased catalytic activity of Complex III, enhanced ROS production and ensuing cellular responses and damages.

## 6. Advantages of a dynamic superassembly in metabolic regulation

The supercomplex organisation of the mitochondrial electron transport chain may have deeper functional consequences than expected. Rate changes that depend on the compartmentalisation of the respiratory assemblies may be expected to occur according to the substrate availability. These changes may address metabolic pathways to preferential routes. Moreover, the possibility to modulate ROS production and ATP levels by changing the supramolecular organisation may contribute to the control of the cellular activity by interfering with redox-sensitive and energy-sensitive signal transduction pathways.

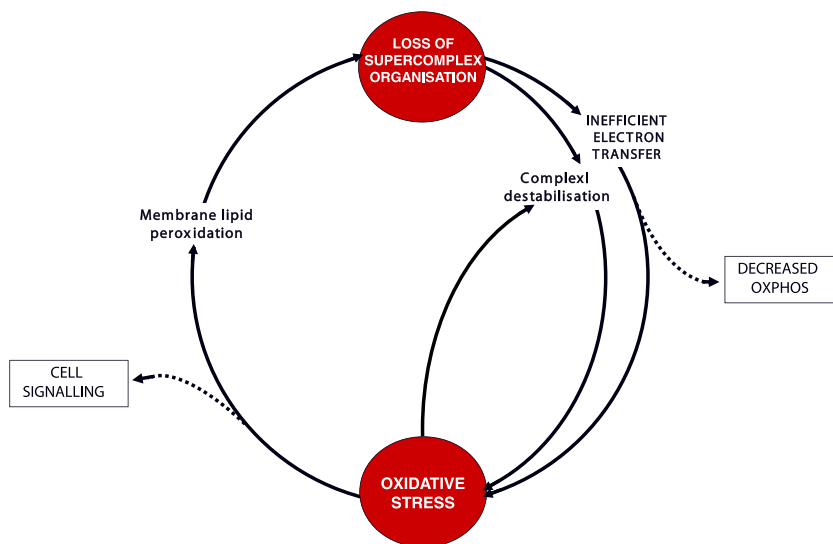
### 6.1. Metabolic adaptations

According to Enriquez [47] the supercomplex organisation of the respiratory chain by its dynamic character is able to optimise the use of available substrates. Glucose utilisation as a fuel contributes largely to feed electrons to the respiratory chain through NADH and Complex I, and less through FAD-linked substrates (succinate, glycerol-3-phosphate), whereas fatty acid oxidation feeds more electrons through a FAD-linked pathway (ETF dehydrogenase). The major difference between these two pathways is that NADH delivers electrons through the respirasome, whereas FAD-linked oxidations use the CoQ pool. Adjustment of respiratory superassembly would provide a mechanism to ensure efficient oxidation of available substrates. To test this, Lapuente-Brun et al. [47] analyzed respiration capacity through the FAD and NADH routes in liver mitochondria from CD1 and C57Bl/6j mice fed ad libitum or fasted for 18 h to activate fatty acids degradation. In CD1 mitochondria, fasting reduced maximal Complex I and Complex I + III activities without influencing Complex II or Complex II + III, but this effect was not seen in C57Bl/6j mitochondria. Accordingly, only in CD1 mitochondria, the proportion of CIII assembled with CI (NADH route) is reduced by fasting.

Adaptation to mitochondrial respiratory substrates that generate different proportions of NADH and FADH<sub>2</sub> (as when mitochondria rely on fatty acids rather than glucose during fasting) requires adjustments to the capacity for electron transport via the NADH and FAD routes. Regulated modification of the proportion of respiratory supercomplexes allows this adaptation [47], but how this change is produced and what would happen if this adaptation were impeded remain to be investigated. At present no known molecular mechanism can be invoked to explain the exquisite regulation of the balance between NADH/Complex III (Complex III dedicated to Complex I) and FAD/Complex III. Further understanding of this behaviour is of paramount importance.

The modulation of supercomplex assembly may have further consequences resulting from rate modulation in the respiratory chain. Dissociation of supercomplexes would help keep NADH oxidation low and hence a high NADH/NAD<sup>+</sup> ratio within mitochondria. This may help a metabolic switch towards biosynthetic pathways, e.g. by elevating mitochondrial citrate levels and thus favouring citrate export and lipid biosynthesis. At the same time the increased reducing power will also favour biosynthetic pathways.

The possibility of organised multienzyme systems that may allow NADH to be transferred directly from its partner dehydrogenase to



**Fig. 6.** Scheme showing how the loss of supercomplex organisation may be involved in a vicious circle of oxidative stress and energy failure. ROS production by Complex I is enhanced as a consequence of supercomplex disassembling. Membrane phospholipid peroxidation and consequent further loss of supercomplex organisation may occur due to mitochondrial oxidative stress, thus perpetuating the vicious circle. Depending on the amount produced, ROS can also operate as signalling molecules from mitochondria to the cell. See text for explanations.



Complex I without dissociation into the bulk phase is still unclear. It has been observed that several mitochondrial soluble  $\text{NAD}^+$ -dependent dehydrogenases such as malate dehydrogenase,  $\beta$ -hydroxyacyl-CoA dehydrogenase,  $\alpha$ -ketoglutarate dehydrogenase complex, and the pyruvate dehydrogenase complex specifically associate with Complex I [160–162]. However, evidence for an effective NADH oxidation mechanism via substrate channelling, compared to free diffusion of the substrate, remains controversial [163].

## 6.2. ROS modulation of signalling pathways

In addition to a means of modulating the oxidative phosphorylation machinery, the plasticity of the respiratory chain supercomplexes (cf. Section 3.6) can be envisaged as a possible mechanism of intracellular signalling. In the latter case, the regulatory effect of the dynamic association of supercomplexes over the production of ROS (cf. Section 5) would be of great importance. In fact, ROS act as damaging agents and also operate as signalling molecules from mitochondria to the cell, depending on the amount produced [164–166].

Our understanding of the signalling pathways modulated by ROS has expanded in recent years, thus providing the answer to the apparent paradox between the specificity that is required for signalling and the reactive nature of ROS that renders them indiscriminate and potentially lethal oxidants. Specificity in intracellular signalling is generally achieved through the non-covalent binding of a ligand to its cognate receptor by virtue of the complementarity of macromolecular shapes. By contrast, ROS molecular recognition occurs at the atomic level and they operate in signalling mainly through chemical reactions with specific sulfhydryl groups of target proteins involved in signal transduction pathways [167]. These ROS signals are generally not primary agonists but are superimposed co-signals that may allow the integration of cellular activities by recruiting, timing, and tuning growth-factor signalling pathways in accordance with the metabolic state of the cell. The paradigm of mitochondrial signalling leading to changes in nuclear gene expression is relatively novel and is considered “mitochondrial retrograde signalling” [168]. The retrograde signalling pathway interacts with several other signalling pathways, such as target of rapamycin (TOR) and ceramide signalling. All of these pathways respond to stress, including metabolic stress [169]. ROS can be part of an important retrograde signal by stimulating the antioxidant response element (ARE) of cytoprotective genes. One notable example is Nrf2 that, in the presence of ROS, is translocated from the cytoplasm to the nucleus. There, it binds the ARE of genes involved in the antioxidant response like heme oxygenase and inducers of mitochondrial biogenesis such as NRF-1 [170].

A large body of data in the literature suggests that mitochondrial biogenesis and re-shaping of the mitochondrial network are tuned to accommodate the energy demand in tissues with variable metabolic activity, or during development (for review, see [171]). Tasseva et al. [172] recently reported of mitochondrial alterations that occur in a cell model with moderate depletion of mtPE, as a result of RNAi silencing of phosphatidylserine decarboxylase and the consequent diminished biosynthesis of PE. The authors demonstrate that this cell condition impairs the supramolecular organisation of CI and CIV in form of respiratory supercomplexes. Interestingly, mitochondria are extensively fragmented and their ultrastructure is grossly aberrant, despite normal or even increased levels of mitochondrial fusion proteins in the mtPE-deficient cells and no reduction in mitochondrial membrane potential. The results of Tasseva et al. [172] suggest a possible link between respiratory chain supercomplexes, cell metabolism and mitochondrial morphology. However, to gain further insight into this subject it has become critical to decipher the stimuli, the pathways, and the physiology behind mitochondrial adaptation. Benard et al. [173] showed that a parallel increase in mitochondrial biogenesis and mitochondrial fission occurs when the level of Complex IV inhibition reaches a sufficient (high) degree that initiates a drop in mitochondrial respiratory rate [174]. This metabolic condition is obtained in human fibroblasts grown

in the presence of potassium cyanide in order to mimic an OXPHOS defect, as in Benard's experimental model, but a similar condition occurs when efficient electron channelling within the supercomplexes is lost (cf. previous sections). The retrograde activation of mitochondrial biogenesis observed by Benard et al. [173] upon cell energy deprivation involves the increase in cytosolic NO concentration, as a consequence of upregulated endothelial NO synthase (eNOS) by the cAMP response element binding protein (CREB). This condition is blocked by inhibition of NO production. While the downstream signalling pathway that connects NO to mitochondrial biogenesis is already deciphered [175], the molecular determinants linking the respiratory chain to CREB activation remain poorly understood. However, it is worth noting that the group of Arnould analyzed the mitochondria-to-nucleus communication in cells grown in the presence of the respiratory chain Complex III inhibitor antimycin A [176], or in conditions of mitochondrial protein synthesis suppression [176], and of mitochondrial DNA depletion [177]. In all cases, their studies revealed the activation of CREB consecutive to mitochondrial bioenergetic stress.

Mitochondrial dysfunction and deficiency of respiratory supercomplexes are correlated to mitochondrial morphology/quality maintenance also in the development of the mitochondrial aging phenotype [137,138,140] and in the etiopathology of several diseases, like Parkinson's and Alzheimer's disease, cardiac dysfunction, diabetes and cancer [93,178,179].

Much of the work dedicated to deciphering the mammalian retrograde response has utilised cancer cell lines reflecting the fact that the mitochondrial defects have been associated with many types of cancers. Wallace provides an excellent review [180], outlining multiple examples of mitochondrial genetic and metabolic defects leading to altered nuclear gene expression and tumorigenic progression.

MtDNA mutations in genes encoding Complex I subunits ND4, ND5 and ND6 have been found in various cancer cells [181]. In cell lines carrying a heteroplasmic ND5 mutation similar to a natural ND5 mutation found in colorectal cancer [182], Sharma and coll. [183] investigated the role of Complex I in tumorigenesis and the effects of rescued function of Complex I by introducing the gene of the yeast alternative NADH:quinone oxidoreductase (NDI1) into cells. They found that, with the restoration of respiration and mitochondrial functions, the tumorigenic potential could be reversed. They also demonstrated that such regulation is mediated by metabolic alterations (i.e. 32% decrease in the  $\text{NAD}^+/\text{NADH}$  ratio in Complex I defective cells compared with control) and by changes in ROS production that modulate the v-AKT murine thymoma viral oncogene (AKT) pathway during Complex I stress. In Sharma's study, the mutant cells exhibited increased oxidative stress because of a genetic defect in Complex I, but there is no indication about how exactly the respiratory chain is organised in the cells. However, elevated ROS levels and altered redox status are common characteristics of many tumors; in some cases, a deep implication of the supercomplex organisation of the respiratory chain was also suggested [184].

It is easy to foresee that specific metabolic signals may arise in the cell in response to a tuned production of ROS from the mitochondrial respiratory chain, as a consequence of the controlled dynamics of supercomplex assembling/disassembling [26] at different physiological conditions.

## 7. Concluding remarks

In the recent years our view of the mitochondrial OXPHOS system has changed to a more integrated approach to mitochondrial structure and function within the cell. This new understanding has opened unexpected perspectives particularly in relation to the role that mitochondria exert both in cell physiology and in pathological changes. On one side, mitochondria are recognised to be central in the pathways of intracellular communication; on the other, the field of mitochondrial medicine is now growing exponentially, shedding light on the pathogenesis of diseases in almost every branch of pathology. In this scenario, the

supramolecular structure of the respiratory chain has acquired a strong position, in view of the multiple role that supercomplex association exerts in mitochondria. Unfortunately, the structural evidence for supercomplex association is not matched by a corresponding knowledge of their function(s), and several questions are still open concerning the functional role of such supramolecular entities. In this review we have attempted to point out the major findings related to the functional role of respiratory supercomplexes. Although the overall picture is not yet clear, what is emerging is a dynamic pattern opening fascinating research avenues in the field of metabolic control and regulation of signalling pathways.

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