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Minireview

Is supercomplex organization of the respiratory chain required for optimal electron transfer activity?

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ABSTRACT

The supra-molecular assembly of the main respiratory chain enzymatic complexes in the form of "supercomplexes" has been proved by structural and functional experimental evidence. This evidence strongly contrasts the previously accepted Random Diffusion Model stating that the complexes are functionally connected by lateral diffusion of small redox molecules (i.e. Coenzyme Q and cytochrome *c*). This review critically examines the available evidence and provides an analysis of the functional consequences of the intermolecular association of the respiratory complexes pointing out the role of Coenzyme Q and of cytochrome *c* as channeled or as freely diffusing intermediates in the electron transfer activity of their partner enzymes.

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1. Introduction

The electron transfer chain consists of four major multi-subunit complexes designated as NADH:CoQ reductase (Complex I), succinate:CoQ reductase (Complex II), ubiquinol:cytochrome *c* reductase (Complex III) and cytochrome *c* oxidase (Complex IV). The best fit unit stoichiometry between complexes in beef heart mitochondria is 1.1 Complex I: 1.3 Complex II: 3 Complex III: 6.7 Complex IV [1]. In addition there are 0.5 ATP synthase (also called Complex V) and 3–5 units of the ADP/ATP translocase (catalyzing the equimolar exchange of ADP and ATP across the inner membrane) for each cytochrome oxidase, and there is one $NADH/NADP^+$ transhydrogenase per Complex I [2]. Indeed, wide differences in cytochromes, Coenzyme Q and pyridine nucleotide contents of mitochondria from different species, as well as from different organs of the same species, have been reported [3–6]; data in the literature indicate that even the molar ratios of the respiratory components vary significantly.

2. Supra-molecular organization of the mitochondrial respiratory chain

2.1. Electrophoretic evidence

The model of a random distribution of mitochondrial complexes (Random Diffusion Model), with electron transfer ensured by collisional interactions of small connecting molecules (Coenzyme Q and cytochrome *c*) [7] has been recently revised when Schägger [1,8,9] found structural evidence by Blue-Native electrophoresis (BN-PAGE) of specific associations in yeast and mammalian mitochondria, and introduced the model of the "respirasome", confirming earlier observations in favor of specific inter-complex interactions (cf. [10] for an extended list of references). Concomitantly, biochemical evidence for homooligomeric ATP synthase competent for ATP hydrolytic activity has been provided [11–13] and recently specific associations of ATP synthase with other OXPHOS components building up "ATP synthasomes" have been proposed [14].

Respiratory supercomplexes were investigated in bovine heart mitochondria: Complex I–III interactions were apparent from the presence of a I_1III_2 supercomplex, which represents about 17% of total Complex I of mitochondria, that was found further assembled into two additional major supercomplexes (respirasomes) comprising different copy numbers of Complex IV ($I_1III_2IV_1$ and $I_1III_2IV_2$ contain 54% and 9% of total Complex I, respectively). Only 14–16% of total Complex I was found in free form in the presence of digitonin [1]; so it seems likely that all Complex I is bound to Complex III in physiological conditions (i.e. in the

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absence of detergents). Knowing the accurate stoichiometry of oxidative phosphorylation complexes according to Schägger and Pfeiffer [1], the average ratio I:III is 1.1:3, therefore it is plausible that approximately one-third of total Complex III in bovine mitochondria is not bound to monomeric Complex I. The fraction of Complex IV in free form represents >85% of total cytochrome oxidase of mitochondria. Associations of Complex II with other complexes of the OXPHOS system could not be identified under the conditions of BN-PAGE so far.

Based on this procedure, the existence of respirasome-like supercomplexes was also reported for bacteria [15], fungi [16] and higher plant mitochondria [17,18] as well as for rat [19] and human mitochondria [20]. However, the overall electrophoretic band pattern of the digitonin-solubilized mitochondria differs among the species and the tissues and the physiological conditions, showing high molecular weight assemblies that are identified as OXPHOS supercomplexes of different composition ($I_1III_2IV_{0-4}$ and III_2IV_{1-2}). Nevertheless, the I_1III_2 supercomplex proved to be especially stable and highly represented.

Fig. 1 exhibits a typical gel obtained from bovine heart mitochondria (A) and two gels from mitochondria isolated from human cultured cells derived from a papillary thyroid tumor (B) and from an oncocytic thyroid tumor (C). Note in A the confirmed presence in bovine mitochondria of several assemblies comprising complexes I, III and IV in different proportions, with most Complex I in associated form and large amounts of free Complex IV; a Complex V dimer co-migrates with a complex I_1III_2 assembly. The mitochondria from cultured cells are peculiar in showing no detectable associated form of

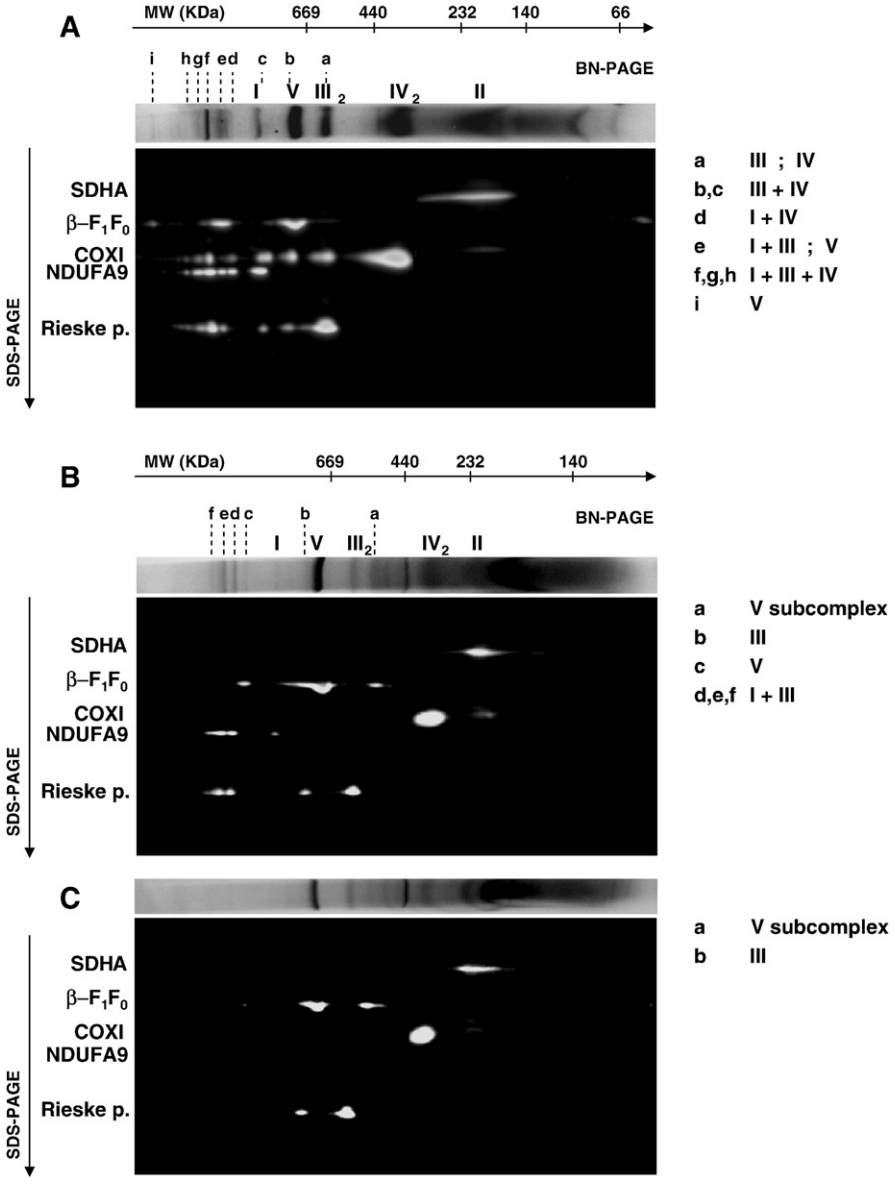

Fig. 1. Two-dimensional separation of OXPHOS supercomplexes of bovine and human mitochondria. Mitochondria were obtained from bovine heart mitochondria (A) and from two human thyroid cells lines: the non-oncocytic cell line TPC-1 derived from human papillary thyroid tumor (B) and the oncocytic carcinoma cell line XTC.UC1 derived from metastasis in the mammary gland of an oncocytic thyroid tumor (C), respectively. (see Ref. [21] for experimental details on the cell lines and preparation of mitochondria). Supercomplexes separation was achieved by 1D Blue-Native electrophoresis followed by 2D SDS-PAGE. Solubilization of membranes was accomplished by treatment of isolated mitochondria with digitonin (digitonin: protein weight ratio 6:1) at 4 °C. The 1D electrophoretic bands (upper lanes) were Coomassie blue stained, whereas the 2D gels were blotted onto nitrocellulose membrane and then exposed to a cocktail of monoclonal antibodies (MitoSciences Inc., Eugene, OR, USA) specific for single subunits of each OXPHOS complex, as follows: NDUFA9 (39 kDa) of Complex I, SDHA (70 kDa) of Complex II, Rieske protein (22 kDa, apparent molecular weight is 30 kDa) of Complex III, COX-I (57 kDa, apparent 45 kDa) of Complex IV, β-subunit (52 kDa) of the F_1F_0-ATPase. Detection of primary antibodies was achieved using a secondary goat anti-mouse IgG_{H+L} antibody labeled with horseradish peroxidase (Molecular Probes, Eugene, OR, USA) and a chemiluminescent technique based on the ECL™ Western Blotting Detection Reagent Kit (Amersham Biosciences, Piscataway, NJ, USA). The molecular mass scale of the 1D electrophoresis was drawn on the basis of standard proteins (HMW calibration kit for Native electrophoresis, Amersham Biosciences). The component complexes in the bands (a through i) are listed on the right of the figure; the list discriminates plausible co-migration (;) from assembly (+) on the basis of the molecular weights.

Complex IV when experimental conditions for mitochondrial solubilization, including the digitonin:protein ratio, are identical to those applied for bovine heart mitochondria. Nevertheless, the digitonin concentration may be critical for detection of Complex IV in supercomplexes [8]. At difference with the papillary tumor, the oncocytic cell line lacks intact functional Complex I [21] and shows no associated form of respiratory complexes. It appears from comparison of these lines that the presence of fully assembled Complex I forming the I–III supercomplexes is not a requisite sufficient to induce participation of Complex IV in a supra-molecular assembly.

The ordered association of mitochondrial respiratory complexes has been revealed also by single particle electron microscopic analysis [22,23] (see also Section 2.2).

2.2. Kinetic evidence

Although the structural evidence for the existence of specific associations increases [22–25] there has been very little functional evidence of the existence of supercomplexes, if we except a study in yeast [26] showing that the pool function of ubiquinone [27–29] investigated by inhibitor titration was lost upon addition of chaotropic agents.

We have tested the model kinetically by exploiting flux control analysis that studies the extent of control that an enzyme exerts on a metabolic pathway [30]. The flux control coefficient represents the fractional change of total activity of a pathway induced by a fractional change of the individual enzyme in the pathway. Such a change is usually obtained by stepwise addition of a specific inhibitor. A flux control coefficient approaching 1 means that the change of the individual step induces the same change on the whole pathway, so the enzyme is totally rate-limiting; a flux control coefficient approaching zero means that the enzyme has no control on the whole pathway and its activity is in large excess. A more evident way of showing flux control is by means of threshold plots [31], where the residual activity of the total pathway is plotted as a function of the extent of inhibition of the individual step.

Using specific inhibitors of each enzyme complex, we have applied flux control analysis on each segment of the respiratory chain of mitochondria from different sources.

In frozen and thawed bovine heart mitochondria (BHM) as well as in open sub-mitochondrial particles (SMP_{BHM}) [32,33], where the system is simplified by the absence of membrane potential and ATP synthesis and by the lack of requirement of the carrier systems for substrates, we surprisingly found that both Complex I and Complex III are almost completely controlling aerobic NADH oxidation [34,35], whereas Complex IV exerts a lower metabolic control (Table 1).

The most reasonable explanation is that Complex I and Complex III behave as a single enzyme, forming a supercomplex with metabolic channeling (i.e. direct interaction without diffusion-coupled steps) of the connecting intermediate, Coenzyme Q. This is contrary to the common view of electron transfer [7,36], but is in line with the recent structural findings by Schägger and Pfeiffer [1]. At difference with electrophoretic evidence, however, the kinetic evidence suggests that only Complex I and III appear to form a supercomplex, while Complex IV seems to behave independently. The reasons for this apparent discrepancy are discussed in Section 6.2. On the other hand, Complex II is rate-limiting over succinate oxidation (Table 1), but there appears to be no channeling between Complexes II and III (cf. Ref. [35]).

In permeabilized mitochondria from freshly harvested potato tubers, where no activity of the so called alternative oxidase (AOX) is present at the level of ubiquinone [37], inhibitor titration experiments on the rotenone-sensitive and rotenone-insensitive respiration indicate that Complex III and IV are involved in the formation of a supercomplex assembly comprising Complex I (Table 1), whereas the alternative dehydrogenases, as well as the molecules of Complex II, are considered to be independent structures within the inner mitochondrial membrane (Genova M.L. et al., manuscript in preparation).

The first three-dimensional map of a bovine respiratory supercomplex was recently determined [23] and the positions and orientations of all the individual complexes within the 3D map were assigned unambiguously. According to the authors, the putative ubiquinone binding site of Complex I faces the corresponding binding site of Complex III, suggesting short diffusion distances within the supercomplex and supporting the notion that a more efficient electron transfer may occur when the respiratory complexes are assembled together (Fig. 2).

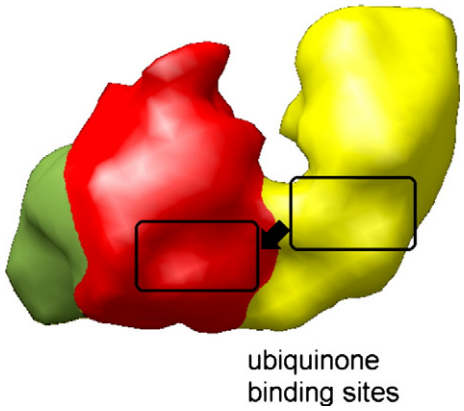

Fig. 2. Side view along the membrane plane of the 3D map of supercomplex $I_1III_2IV_1$. The individual complexes in the supercomplex are shown in yellow (Complex I), red (Complex III dimer) and green (Complex IV). The putative ubiquinone interaction sites in Complex I and in Complex III are marked in black. The positions of the Q-binding sites are only indicative. Modified from Schäfer et al. [23]; the 3D map was taken from the EM database (http://www.ebi.ac.uk/msd/index.html) with the accession number EMD-1318.

3. Determinants and consequences of intermolecular assembly

In a previous report [34] we demonstrated that dilution with phospholipids of a mitochondrial fraction enriched in Complexes I and III determines adherence to pool behavior for CoQ but only at dilution protein:phospholipids higher than 1:5, whereas at lower phospholipids content the turnover of NADH:cytochrome *c* reductase is higher than expected by the pool equation.

This means that membrane lipids may modulate inter-complex association; it is not unlikely that besides the quantity of lipids also their composition may affect supercomplex formation. It has been shown that cardiolipin is required for specific association between complexes [38]; a decrease in cardiolipin content, such as due to peroxidation by mitochondrial ROS production following decreased electron transfer of Complex I [39] and Complex III [40], might deeply affect inter-complex assembly. Indeed, preliminary results from our laboratory show that peroxidation of the lipids used to reconstitute the

Table 1
Flux control coefficients (C_i) of the respiratory complexes involved in NADH and succinate oxidation in bovine heart mitochondria (BHM) and in potato tuber mitochondria (POM)

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

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

mitochondrial fraction enriched in Complexes I and III (see above) induces dissociation of the complexes even at high lipid to protein ratios.

The issue of the organization of the respiratory chain is not trivial in concern of mitochondrial diseases: Schägger et al. [20] have recently published evidence that mutations of Complex III affect the assembly and stability of Complex I subunits. Analysis of the state of supercomplexes in human patients with an isolated deficiency of single complexes [20] and in cultured cell models harboring cytochrome *b* mutations [41,42] also provided evidence that the formation of respirasomes is essential for the assembly/stability of Complex I. Genetic alterations leading to a loss of Complex III prevented respirasome formation and led to secondary loss of Complex I, therefore primary Complex III assembly deficiencies were presented as Complex III/I defects. Conversely, Complex III stability was not influenced by the absence of Complex I. D'Aurelio et al. [42] studied mtDNA complementation 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 containing a distinct homoplasmic mutation in a subunit of Complex III, cytochrome *b*. Upon cell fusion, respiration was recovered in hybrid cells, indicating that mitochondria fuse and exchange genetic and protein materials. The recovery of mitochondrial respiration correlated with the presence of supra-molecular structures (supercomplexes) containing Complexes I, III and IV; critical amounts of Complexes III or IV are therefore required in order for supercomplexes to form and provide mitochondrial functional complementation. From these findings, supercomplex assembly emerged as a necessary step for respiration, its defect setting the threshold for respiratory impairment in mtDNA mutant cells.

Moreover, the different tissue distribution of pathological aspects of mitochondrial cytopathies has been attributed, besides to different degrees of heteroplasmy, also to different extents of flux control by individual complexes of oxidative phosphorylation [43]; in view of the results discussed above, it is tempting to speculate that different supercomplex organizations may be present in individual tissues.

4. Are supercomplexes present in mitochondria under native conditions?

The electrophoretic demonstration of the existence of respiratory supercomplexes requires the use of digitonin that alters the membrane and destroys the permeability barriers; on the other hand, our initial demonstration by flux control analysis that a Complex I–III assembly exists in respiring sub-mitochondrial particles was done under non-phosphorylating conditions in a system lacking possible rate-limiting steps external to the respiratory chain per se (dehydrogenases, substrate carriers, membrane potential, ATP synthase, adenine nucleotide carrier).

We have obtained recent evidence of the existence of a Complex I–III assembly by flux control analysis in rat liver mitochondria under phosphorylating conditions (state 3). The threshold plots of rotenone inhibition of Complex I and of mucidin inhibition of Complex III are both linear (Fig. 3), indicating the existence of a functional supercomplex. No information about the presence of supra-molecular assemblies can be inferred based on the plots obtained in state 4 (controlled respiration) because it is known that in this condition the proton leak across the inner membrane becomes the rate-limiting step: therefore a very low metabolic control over respiration is exerted by the enzyme complexes regardless of their assembly status.

5. Role of the Coenzyme Q pool

Can we reconcile the extensive evidence formerly gained in favor of the pool function of CoQ between Complexes I and III with the more recent evidence favoring the existence of supercomplexes? The subject has been discussed in a previous review [28] where it was reasoned that the pool equation of Kröger and Klingenberg [29], establishing the total observed rate of electron transfer (V_{obs}) as a function of rate of reduction (V_{red}) and of reoxidation (V_{ox}) of the CoQ pool,

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

can be verified only for comparable rates of CoQ reduction and oxidation: in such case the resulting total rate is much lower than the individual rates, otherwise the observed total rate approaches that of the slower process and the result could not be distinguished from the kinetics when channeling occurs in a supercomplex.

If we consider bound CoQ as stoichiometric with one site in the complexes that have been shown to contain bound CoQ (I, II, III) [44–46], in beef heart mitochondria we come up to no more than 0.35 nmol/mg protein, that would increase to ca. 0.5 nmol assuming more than one site to be fully occupied in Complex I and Complex III. Since the total CoQ content is higher than 3 nmol/mg [2,3], we must assume that most CoQ (>84%) is free in the bilayer.

A great deal of biophysical data in the literature shows that CoQ is dispersed in the phospholipids and is freely mobile by lateral diffusion; however, most of these data were obtained for CoQ inserted in liposomes (e.g. Ref. [47,48]). Nevertheless, some indirect evidence for the existence of a mobile CoQ pool in the mitochondrial inner membrane lipids stems from studies showing that CoQ homologues are endowed with lateral diffusion also when added to natural mitochondrial membranes [7,49].

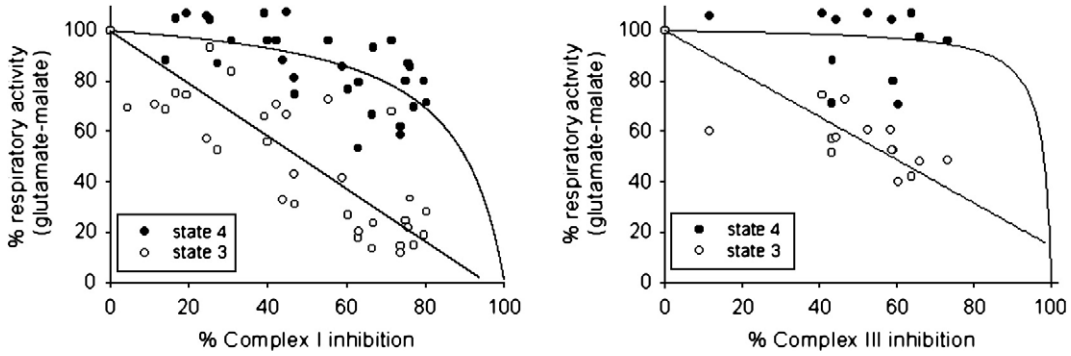

Fig. 3. Threshold plots of state 3 (open circles) and state 4 (filled circles) respiration in rat liver mitochondria. Rat liver mitochondria were isolated according to the procedure of Kun et al. [62], with minor modifications as described elsewhere [63]. Respiration was followed in the presence of 6 mM glutamate and 6 mM malate with and without addition of 0.6 mM ADP (state 3 and state 4, respectively), using an oxygraph apparatus equipped with a Clark-type electrode [63]. Specific activity of Complex I and Complex III was determined spectrophotometrically as in Refs. [35] and [63]. Each data point comes from experimental titration curves and represents the percent rate of the examined respiratory activity as a function of the percentage of Complex I inhibition for the same rotenone concentration (left panel) and of Complex III inhibition for the same mucidin concentration (right panel). The curves through the data points were drawn by linear or non-linear regression fitting (SigmaPlot, Jandel Scientific). The linearity of the plots under state 3 conditions indicates that both Complex I and Complex III are rate-limiting (metabolic control coefficients approaching 1, see Section 2.2), thus suggesting the presence of a functional assembly.

Thus, we may conclude that most certainly a mobile pool of CoQ exists in the inner mitochondrial membrane, and that this pool is in equilibrium with protein-bound CoQ. On the other hand, the bound inter-complex quinone that allows electron flow within the I–III supercomplex may well be in dissociation equilibrium with the CoQ pool, so that its amount, at steady state, would be dictated by the size of the pool: this equilibrium would explain the saturation kinetics for total ubiquinone exhibited by the integrated activity of Complex I and Complex III [50] and the decrease of respiratory activities in mitochondria fused with phospholipids with subsequent dilution of the CoQ pool [51]. To be in agreement with the experimental observation obtained by metabolic flux analysis, this proposition must however require that the dissociation rate constants of bound CoQ be considerably slower than the rates of inter-complex electron transfer via the same bound quinone molecules. In order to explain the high apparent K_m found for CoQ in NADH oxidase activity [50] also CoQ binding to Complex I must be slow [52] (cf. Fig. 5 in Ref. [10]).

A major function of the CoQ pool, therefore, must be to drive binding into sites formed at the border between adjacent Complexes I and III in order to assure correct channeling of electrons form one to the other complex. Since the dissociation constant of CoQ from the supercomplex must be high, as inferred from the apparent K_m of the integrated activity and discussed above, any decrease of CoQ concentration in the pool would decrease the amount of bound CoQ and therefore induce a fall of electron transfer. By this way, free CoQ behaves as a reservoir for binding to the I–III supercomplexes; in addition, free CoQ may be a reservoir also for other functions believed to require CoQ binding to specific proteins, such as uncoupling proteins [53] and the permeability transition pore [54,55].

Furthermore, other electron transfer activities, such as succinate oxidation, are the result of interaction of complexes that do not show association either by BN-PAGE or by kinetic flux control analysis: these activities necessarily need collisional interactions of CoQ molecules from the pool with both Complexes II and III also randomly dispersed in the bilayer. Other activities such as glycerol-3-phosphate dehydrogenase, ETF dehydrogenase, dihydroorotate dehydrogenase, that are likely to be in minor amounts and strongly rate-limiting in integrated electron transfer, are probably dictated by interaction through the CoQ pool. The only direct study addressed to this problem [56] demonstrated that in brown fat mitochondria the inhibition curve of glycerol phosphate:cytochrome *c* reductase is sigmoidal in the presence of myxothiazol and antimycin, suggesting the presence of a homogeneous CoQ pool between glycerol phosphate dehydrogenase and Complex III.

Also reverse electron transfer from succinate to NAD^+, involving sequential interaction of Complexes II and I by means of CoQ, must take place by collisional interactions in the CoQ pool, since no association exists between Complexes I and II. Gutman [57] has provided evidence that energy-driven reverse electron transfer from succinate to NAD^+ indeed follows pool behavior.

This observation poses a particularly puzzling question: if Complex I is completely or almost completely associated with Complex III, and the interaction of CoQ in the pool with the quinone-binding site in common between the two enzymes is necessary, then how can $CoQH_2$ reduced by Complex II interact from the pool with the CoQ site in Complex I at a rate compatible with the steady state kinetics of reverse electron transfer? The intriguing idea that Complex I may possess two different quinone-binding sites for direct and for reverse electron transfer respectively is compatible with the proposal by Grivennikova et al. [58] that two different routes exist for forward and reverse electron transfer within the complex. These two sites might become alternatively accessible depending on the magnitude of the membrane potential. Alternatively one should postulate that the association rate constant of reduced CoQ from the pool to Complex I in the supercomplex should be sufficiently fast to be compatible with the turnover of reverse electron transfer. It must be noted that the ATP-driven reverse electron transfer from succinate to NAD^+ occurs in the presence of a high mitochondrial transmembrane protonmotive force that, according to [59], might be the physiological signal and, at the same time, the trigger causing the structural reorganization of the enzymatic complexes of the mitochondrial OXPHOS system. The model hypothesis depicted by Piccoli et al. from data on cytochrome oxidase [59] might be extended to other enzymes of the respiratory chain, suggesting that also the I–III supercomplexes would dissociate its constituting complexes under high $\Delta\mu_{H^+}$ condition, and this would no longer limit the access to the CoQ binding site in Complex I.

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

6. Is there evidence for substrate channeling?

6.1. Coenzyme Q

A ubiquinone/supercomplex ratio of 10–30 mol/mol was calculated in a chromatographic preparation of a supra-molecular assembly of NADH oxidase ($I_1III_4IV_4$) isolated from *Paracoccus denitrificans* membranes [15]. Based on the phospholipid determination, the ubiquinone content accounts for a 8-fold increase in the respirasome compared to native membranes. To our knowledge, this is the only evidence reported so far that seems to indicate specific enrichment of ubiquinone in a supercomplex. According to Stroh et al. [15], NADH:cytochrome *c* oxidoreductase activity and turnover numbers for the respiratory enzymes, as measured in the sample, suggest almost optimum electron transfer from Complex I to Complex III. However, NADH oxidase activity (Complexes I, III and IV) and ubiquinol oxidase activity (Complexes III and IV) were reduced by 74%. This seems to indicate that electron transfer between Complex III and Complex IV was impeded, in agreement with partial loss of cytochrome c_{552} (approx. 40–80% of total) during isolation.

The ubiquinone enrichment in the isolated supercomplex raises the question whether electron transfer within the assembly is indeed occurring via channeling of electrons within single ubiquinone molecules connecting the active sites of both enzymes or rather via a compartmented ubiquinone pool localized within the neighboring membrane environment of the enzymes; since either solution implies electron transfer between a single Complex I unit and a single Complex III dimer, it is likely that these two mechanisms would be kinetically indistinguishable.

Either electron channeling or the action of a localized pool are bound to elicit rates above those dictated by diffusion and collisions between randomly dispersed enzymes. The increase of electron transfer activity between Complex I and Complex III (Table 2) to rates above those predicted by the pool equation [29] was demonstrated by us in proteoliposomes enriched in Complex I and III activities at a protein to phospholipid ratio of 1:1 (when kinetic testing according to flux

Table 2
Experimental and calculated values of NADH:cytochrome *c* reductase in a mitochondrial R4B protein fraction[a] diluted with different levels of phospholipids

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

Values in brackets indicate multiple experiments.
[a] Fraction R4B from bovine heart mitochondria was fused with phospholipids (Asolectin) and Coenzyme Q_{10} by cholate dilution [61].
[b] The theoretical distances between Complex I and Complex III were calculated according to [7].
[c] C_I and C_{III} are the metabolic flux control coefficients of Complex I and Complex III, respectively, over NADH:cytochrome *c* oxidoreductase activity in the proteoliposomes.
[d] NADH:cytochrome *c* reductase activity as calculated from the pool equation [29] using experimental values of NADH:CoQ reductase and ubiquinol:cytochrome *c* reductase activity as V_{red} and V_{ox}, respectively.

control analysis indicates the presence of supercomplexes I–III) but not at a ratio of 1:30 (when the supercomplex is dissociated); this is a demonstration that supercomplex formation indeed enhances the rate of electron transfer above that occurring via a ubiquinone pool in the membrane [34].

6.2. Cytochrome c

Despite the constant finding of Complex IV in a supercomplex also comprising Complexes I and III in bovine heart mitochondria by electrophoretic techniques [1], flux control analysis in the same mitochondria fails to detect the presence of a functional Complex III–IV assembly; in these mitochondria cytochrome *c* oxidase activity is never rate-limiting over NADH oxidation and the threshold plots are strongly biphasic, indicating that electron transfer occurs via a random pool of cytochrome *c* molecules. The pool behavior of cytochrome *c* may be due to rapid exchange of bound and pool cytochrome *c* molecules. The presence of a large excess of free cytochrome oxidase is in line with this conclusion and in addition strongly implies that a specific association of Complexes III and IV is not required for electron transfer. The nature of the method employed does not allow to ascertain whether additional electron transfer occurs by channeling within those Complex IV units that are bound in a respirasome. Nevertheless, the loss of cytochrome *c* found in the purification of the respirasome in *P. denitrificans* together with the fall of ubiquinol oxidase activity [15] strongly suggests that this loss may not be artifactual but must reflect a prevalent electron transfer between Complexes III and IV via random diffusion of cytochrome *c* in the intermembrane space. In other words electron channeling may occur but is not required for electron transfer in this region. This notion is also supported by preliminary data from our laboratory indicating that no traces of cytochrome *c* bound to supercomplexes I–III–IV can be detected by BN/SDS-PAGE in bovine heart mitochondria. According to these premises, the reason for association of Complex IV in a respirasome may be rather structural than kinetic.

The supercomplex occurring in potato tuber mitochondria, where flux control analysis reveals that Complexes I, III and IV are all rate-limiting (see Section 2.2), suggests that also association of Complex IV is functionally relevant in these mitochondria; accordingly, BN-electrophoresis shows that most Complex IV molecules might be present within I–III–IV supercomplexes (cf. [64]).

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References

[1] H. Schägger, K. Pfeiffer, The ratio of oxidative phosphorylation complexes I–V in bovine heart mitochondria and the composition of respiratory chain supercomplexes, J. Biol. Chem. 276 (2001) 37861–37867.
[2] R.A. Capaldi, Arrangement of proteins in the mitochondrial inner membrane, Biochim. Biophys. Acta 694 (1982) 292–306.
[3] M. Battino, E. Ferri, R.F. Villa, A. Gorini, J.F.R. Huertas, P.C. Fiorella, M.L. Genova, G. Lenaz, M. Marchetti, Natural distribution and occurrence of coenzyme Q homologues, Membrane Biochem. 9 (1990) 179–190.
[4] P.V. Blair, T. Oda, D.E. Green, Studies on the electron transfer system. LIV Isolation of the unit electron transfer, Biochemistry 2 (1963) 756–764.
[5] D.E. Green, D.C. Wharton, Stoichiometry of the fixed oxidation-reduction components of the electron transfer chain of beef heart mitochondria, Biochem. Z. 338 (1963) 335–348.
[6] T.E. King, K.S. Nickel, D.R. Jensen, Iron, copper, cytochrome, and lipid contents of heart muscle preparation and heart mitochondria, J. Biol. Chem. 239 (1964) 1989–1994.
[7] C.R. Hackenbrock, B. Chazotte, S.S. Gupte, The random collision model and a critical assessment of diffusion and collision in mitochondrial electron transport, J. Bioenerg. Biomembranes 18 (1986) 331–368.
[8] H. Schägger, K. Pfeiffer, Supercomplexes in the respiratory chains of yeast and mammalian mitochondria, EMBO J. 19 (2000) 1777–1783.
[9] C.M. Cruciat, S. Brunner, F. Baumann, W. Neupert, R.A. Stuart, The cytochrome *bc*1 and cytochrome *c* oxidase complexes associate to form a single supercomplex in yeast mitochondria, J. Biol. Chem. 275 (2000) 18093–18098.
[10] G. Lenaz, M.L. Genova, Kinetics of integrated electron transfer in the mitochondrial respiratory chain: random collisions vs. solid state electron channeling, Am. J. Physiol. Cell Physiol. 292 (2007) C1221–C1239.
[11] F. Krause, N.T.H. Reifschneider, S. Goto, N.A. Dencher, Active oligomeric ATP synthases in mammalian mitochondria, Biochem. Biophys. Res. Commun. 329 (2005) 583–590.
[12] I. Arnold, K. Pfeiffer, W. Neupert, R.A. Stuart, H. Schägger, Yeast mitochondrial F1F0-ATP synthase exists as a dimer: identification of three dimer-specific subunits, EMBO J. 17 (1998) 7170–7178.
[13] G.M. Boyle, X. Roucou, P. Nagley, R.J. Devenish, M. Prescott, Identification of subunit g of yeast mitochondrial F1F0-ATP synthase, a protein required for maximal activity of cytochrome *c* oxidase, Eur. J. Biochem. 262 (1999) 315–323.
[14] C. Chen, Y.H. Ko, M. Delannoy, S.J. Ludtke, W. Chiu, P.L. Pedersen, Mitochondrial ATP synthasome: three-dimensional structure by electron microscopy of the ATP synthase in complex formation with carriers for Pi and ADP/ATP, J. Biol. Chem. 279 (2004) 31761–31768.
[15] A. Stroh, O. Anderka, K. Pfeiffer, T. Yagi, M. Finel, B. Ludwig, H. Schägger, Assembly of respiratory complexes I, III, and IV into NADH oxidase supercomplex stabilizes complex I in *Paracoccus denitrificans*, J. Biol. Chem. 279 (2004) 5000–5007.
[16] F. Krause, C.Q. Scheckhuber, A. Werner, S. Rexroth, N.H. Reifschneider, N.A. Dencher, H.D. Osiewacz, Supramolecular organization of cytochrome *c* oxidase- and alternative oxidase-dependent respiratory chains in the filamentous fungus Podospora anserina, J. Biol. Chem. 279 (2004) 26453–26461.
[17] H. Eubel, J. Heinemeyer, S. Sunderhaus, H.P. Braun, Respiratory chain supercomplexes in plant mitochondria, Plant Physiol. Biochem. 42 (2004) 937–942.
[18] F. Krause, N.H. Reifschneider, D. Vocke, H. Seelert, S. Rexroth, N.A. Dencher, "Respirasome"-like supercomplexes in green leaf mitochondria of spinach, J. Biol. Chem. 279 (2004) 48369–48375.
[19] N.H. Reifschneider, S. Goto, H. Nakamoto, R. Takahashi, M. Sugawa, N.A. Dencher, F. Krause, Defining the mitochondrial proteomes from five rat organs in a physiologically significant context using 2D Blue-Native/SDS-PAGE, J. Proteome Res. 5 (2006) 1117–1132.
[20] H. Schägger, R. de Coo, M.F. Bauer, S. Hofmann, C. Godinot, U. Brandt, Significance of respirasomes for the assembly/stability of human respiratory chain complex I, J. Biol. Chem. 279 (2004) 36349–36353.
[21] E. Bonora, A.M. Porcelli, G. Gasparre, A. Biondi, A. Ghelli, V. Carelli, A. Baracca, G. Tallini, A. Martinuzzi, G. Lenaz, M. Rugolo, G. Romeo, Defective oxidative phosphorylation in thyroid oncocytic carcinoma is associated with pathogenic mitochondrial DNA mutations affecting complexes I and III, Cancer Res. 66 (2006) 6087–6096.
[22] N.V. Dudkina, H. Eubel, W. Keegstra, E.J. Boekema, H.P. Braun, Structure of a mitochondrial supercomplex formed by respiratory-chain complexes I and III, Proc. Natl. Acad. Sci. U. S. A. 102 (2005) 3225–3229.
[23] E. Schäfer, N.A. Dencher, J. Vonck, D.N. Parcej, Three-dimensional structure of the respiratory chain supercomplex $I_1III_2IV_1$ from bovine heart mitochondria, Biochemistry 46 (2007) 12579–12585.
[24] H. Eubel, L. Jansch, H.P. Braun, New insights into the respiratory chain of plant mitochondria. Supercomplexes and a unique composition of complex II, Plant Physiol. 133 (2003) 274–286.
[25] K. Pfeiffer, V. Gohil, R.A. Stuart, C. Hunte, U. Brandt, M.L. Greenberg, H. Schägger, Cardiolipin stabilizes respiratory chain supercomplexes, J. Biol. Chem. 278 (2003) 52873–52880.
[26] H. Boumans, L.A. Grivell, J.A. Berden, The respiratory chain in yeast behaves as a single functional unit, J. Biol. Chem. 273 (1998) 4872–4877.
[27] G. Lenaz, Quinone specificity of complex I, Biochim. Biophys. Acta 1364 (1998) 207–221.
[28] G. Lenaz, A critical appraisal of the mitochondrial coenzyme Q pool, FEBS Lett. 159 (2001) 151–155.
[29] A. Kröger, M. Klingenberg, The kinetics of the redox reactions of ubiquinone related to the electron-transport activity in the respiratory chain, Eur. J. Biochem. 34 (1973) 358–368.
[30] A. Kacser, J.A. Burns, Molecular democracy: who shares the controls? Biochem. Soc. Trans. 7 (1979) 1149–1160.
[31] R. Rossignol, M. Malgat, J.P. Mazat, T. Letellier, Threshold effect and tissue specificity. Implication for mitochondrial cytopathies, J. Biol. Chem. 274 (1999) 33426–33432.
[32] R. Fato, M. Cavazzoni, C. Castelluccio, G. Parenti Castelli, G. Palmer, M. Degli Esposti, G. Lenaz, Steady-state kinetics of ubiquinol-cytochrome *c* reductase in bovine heart submitochondrial particles: diffusional effects, Biochem. J. 290 (1993) 225–236.
[33] M. Hansen, A.L. Smith, Studies on the mechanism of oxidative phosphorylation VII. Preparation of a submitochondrial particle (ETP_H) which is capable of fully coupled oxidative phosphorylation, Biochim. Biophys. Acta 81 (1964) 214–222.
[34] C. Bianchi, R. Fato, M.L. Genova, G. Parenti Castelli, G. Lenaz, Structural and functional organization of Complex I in the mitochondrial respiratory chain, BioFactors 18 (2003) 3–9.
[35] C. Bianchi, M.L. Genova, G. Parenti Castelli, G. Lenaz, The mitochondrial respiratory chain is partially organized in a supercomplex assembly: kinetic evidence using flux control analysis, J. Biol. Chem. 279 (2004) 36562–36569.
[36] G. Lenaz, Role of mobility of redox components in the inner mitochondrial membrane, J. Membr. Biol. 104 (1988) 193–209.

[37] C. Affourtit, K. Krab, A.L. Moore, Control of plant mitochondrial respiration, Biochim. Biophys. Acta 1504 (2001) 58–69.
[38] M. Zhang, E. Mileykowskaya, W. Dowhan, Gluing the respiratory chain together. Cardiolipin is required for supercomplex formation in the inner mitochondrial membrane, J. Biol. Chem. 277 (2002) 43553–43556.
[39] G. Paradies, G. Petrosillo, M. Pistolese, N. Di Venosa, A. Federici, F.M. Ruggiero, Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart: involvement of reactive oxygen species and cardiolipin, Circ. Res. 94 (2004) 53–59.
[40] G. Petrosillo, F.M. Ruggiero, N. Di Venosa, G. Paradies, Decreased complex III activity in mitochondria isolated from rat heart subjected to ischemia and reperfusion: role of reactive oxygen species and cardiolipin, FASEB J. 17 (2003) 714–716.
[41] R. Acin-Perez, M.P. Bayona-Bafaluy, P. Fernandez-Silva, R. Moreno-Loshuertos, A. Perez-Martos, C. Bruno, C.T. Moraes, J.A. Enriquez, Respiratory complex III is required to maintain complex I in mammalian mitochondria, Mol. Cell. 13 (2004) 805–815.
[42] M. D'Aurelio, C.D. Gajewski, G. Lenaz, G. Manfredi, Respiratory chain supercomplexes set the threshold for respiration defects in human mtDNA mutant cybrids, Hum. Mol. Genet 15 (2006) 2157–2169.
[43] W.S. Kunz, Different metabolic properties of mitochondrial oxidative phosphorylation in different cell types — important implications for mitochondrial cytopathies, Exp. Physiol. 88 (2003) 140–154.
[44] A.D. Vinogradov, V.D. Sled, D.S. Burbaev, V.G. Grivennikova, I.A. Moroz, T. Ohnishi, Energy-dependent Complex I-associated ubisemiquinones in submitochondrial particles, FEBS Lett. 370 (1995) 83–87.
[45] G.Y. Lee, D.Y. He, L. Yu, C.A. Yu, Identification of the ubiquinone-binding domain in QPs1 of succinate-ubiquinone reductase, J. Biol. Chem. 270 (1995) 6193–6198.
[46] B.L. Trumpower, New concepts on the role of ubiquinone in the mitochondrial respiratory chain, J. Bioenerg. Biomembranes 13 (1981) 1–24.
[47] R. Fato, M. Battino, M. Degli Esposti, G. Parenti Castelli, G. Lenaz, Determination of partition and lateral diffusion coefficients of ubiquinones by fluorescence quenching of *n*-(9-anthroyloxy)stearic acids in phospholipid vesicles and mitochondrial membranes, Biochemistry 25 (1986) 3378–3390.
[48] S.S. Gupte, E.S. Wu, L. Hoechli, M. Hoechli, K. Jacobson, A.E. Sowers, C.R. Hackenbrock, Relationship between lateral diffusion, collision frequency, and electron transfer of mitochondrial inner membrane oxidation-reduction components, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 2606–2610.
[49] K. Rajarathnam, J. Hochman, M. Schindler, S. Ferguson-Miller, Synthesis, location, and lateral mobility of fluorescently labeled ubiquinone 10 in mitochondrial and artificial membranes, Biochemistry 28 (1989) 3168–3176.
[50] E. Estornell, R. Fato, C. Castelluccio, M. Cavazzoni, G. Parenti Castelli, G. Lenaz, Saturation kinetics of coenzyme Q in NADH and succinate oxidation in beef heart mitochondria, FEBS Lett. 311 (1992) 107–109.
[51] H. Schneider, J.J. Lemasters, C.R. Hackenbrock, Lateral diffusion of ubiquinone during electron transfer in phospholipid- and ubiquinone-enriched mitochondrial membranes, J. Biol. Chem. 257 (1982) 10789–10793.
[52] M.L. Genova, C. Bianchi, G. Lenaz, Supercomplex organization of the mitochondrial respiratory chain and the role of the coenzyme Q pool: pathophysiological implications, BioFactors 23 (2006) 1–16.
[53] K.S. Echtay, E. Winkler, M. Klingenberg, Conezyme Q is an obligatory cofactor for uncoupling protein function, Nature 408 (2000) 609–613.
[54] J.S. Armstrong, M. Whiteman, P. Rose, D.P. Jones, The Coenzyme Q10 analog decylubiquinone inhibits the redox-activated mitochondrial permeability transition: role of mitochondrial complex III, J. Biol. Chem. 278 (2003) 49079–49084.
[55] L. Walter, L.H. Miyoshi, X. Leverve, P. Bernard, E. Fontaine, Regulation of the mitochondrial permeability transition pore by ubiquinone analogs. A progress report, Free Rad. Res. 36 (2002) 405–412.
[56] H. Rauchova, M. Battino, R. Fato, G. Lenaz, Z. Drahota, Coenzyme Q pool function in glycerol-3-phosphate oxidation in hamster brown adipose tissue mitochondria, J. Bioenerg. Biomembranes 24 (1992) 235–241.
[57] M. Gutman, in: G. Lenaz, Q. Coenzyme (Eds.), Kinetic analysis of electron flux through the quinones in the mitochondrial system, Wiley, Chichester U.K., 1985, pp. 215–234.
[58] V.G. Grivennikova, R. Roth, N.V. Zakharova, C. Hagerhall, A.D. Vinogradov, The mitochondrial and prokaryotic proton-translocating NADH:ubiquinone oxidoreductases: similarities and dissimilarities of the quinone-junction sites, Biochim. Biophys. Acta 1607 (2003) 79–90.
[59] C. Piccoli, R. Scrima, D. Boffoli, N. Capitanio, Control by cytochrome c oxidase of the cellular oxidative phosphorylation system depends on the mitochondrial energy state, Biochem. J. 396 (2006) 573–583.
[60] L. Ernster, G. Dallner, Biochemical, physiological and medical aspects of ubiquinone function, Biochim. Biophys. Acta 1271 (1995) 195–204.
[61] G. Lenaz, R. Fato, S. Di Bernardo, D. Jarreta, A. Costa, M.L. Genova, G. Parenti Castelli, Localization and mobility of coenzyme Q in lipid bilayers and membranes, BioFactors 9 (1999) 87–93.
[62] E. Kun, E. Kirsten, W.N. Piper, Stabilization of mitochondrial functions with digitonin, Methods Enzymol. 55 (1979) 115–118.
[63] B. Ventura, M.L. Genova, C. Bovina, G. Formiggini, G. Lenaz, Control of oxidative phosphorylation by Complex I in rat liver mitochondria: implications for aging, Biochim. Biophys. Acta 1553 (2002) 249–260.
[64] H. Eubel, J. Heinemeyer, H.P. Braun, Identification and characterization of respirasomes in potato mitochondria, Plant Physiol. 134 (2004) 1450–1459.