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REVISED MANUSCRIPT

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Understanding differential aspects of microdiffusion (channeling) in the Coenzyme Q and Cytochrome *c* regions of the mitochondrial respiratory system.

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Abstract

Over the past decades, models of the organization of mitochondrial respiratory system have been controversial. The goal of this perspective is to assess this “conflict of models” by focusing on specific kinetic evidence in the two distinct segments of Coenzyme Q- and Cytochrome *c*-mediated electron transfer. Respiratory supercomplexes provide kinetic advantage by allowing a restricted diffusion of Coenzyme Q and Cytochrome *c*, and short-range interaction with their partner enzymes. In particular, electron transfer from NADH is compartmentalized by channeling of Coenzyme Q within supercomplexes, whereas succinate oxidation proceeds separately using the free Coenzyme Q pool. Previous evidence favoring Coenzyme Q random diffusion in the NADH-dependent electron transfer is due to downstream flux interference and misinterpretation of results. Indeed, electron transfer by complexes III and IV via Cytochrome *c* is less strictly dependent on substrate channeling in mammalian mitochondria. We briefly describe these differences and their physiological implications.

Keywords

Coenzyme Q or ubiquinone; Cytochrome c; diffusion; channeling; respiratory supercomplex.

Abbreviations

AOX, alternative oxidases; BHM, bovine heart mitochondria; CI, Complex I or NADH:ubiquinone oxidoreductase; CII, Complex II or succinate dehydrogenase; CIII, Complex III or ubiquinol:cytochrome *c* oxidoreductase; CIV, Complex IV or cytochrome *c* oxidase; CoQ, Coenzyme Q or Ubiquinone; Cyt. *c*, Cytochrome *c*; ETS, electron transport system; MFC, metabolic flux control; SC, respiratory supercomplex; SHM, swine heart mitochondria.

1. Introduction

It has been assumed for long time that CoQ and Cyt. *c* are two mobile electron-carrying components of the mitochondrial ETS. Indeed, in eukaryotic cells, CoQ-homologs act as electron shuttles between different dehydrogenases and oxidases of the inner mitochondrial membrane [1].

NADH:ubiquinone oxidoreductase and succinate dehydrogenase are major CoQ-reducing enzymes (**Fig. 1**) besides other enzyme partners (e.g. dihydroorotate dehydrogenase, choline dehydrogenase, electron-transferring flavoprotein dehydrogenase, mitochondrial glycerol-3-phosphate dehydrogenase, proline dehydrogenases 1 and 2, and sulfide:quinone oxidoreductase among others) that are involved in a range from amino acid and fatty acid oxidation to nucleotide biosynthesis, methylation, and hydrogen sulfide detoxification, but which are often omitted or considered ancillary in the context of respiratory enzymes (for review cf. [2]). Reduced CoQ (i.e., ubiquinol) acts as an electron donor to CIII as well as to AOX widespread in plants, fungi, and some protozoa [3]. Cyt. *c* molecules that reside in the mitochondrial intermembrane space transfer electrons from CIII to CIV, which catalyzes oxidation of Cyt. *c* and reduction of molecular oxygen to water.

< INSERT FIGURE 1 as a 2-column fitting image. For color reproduction online only >

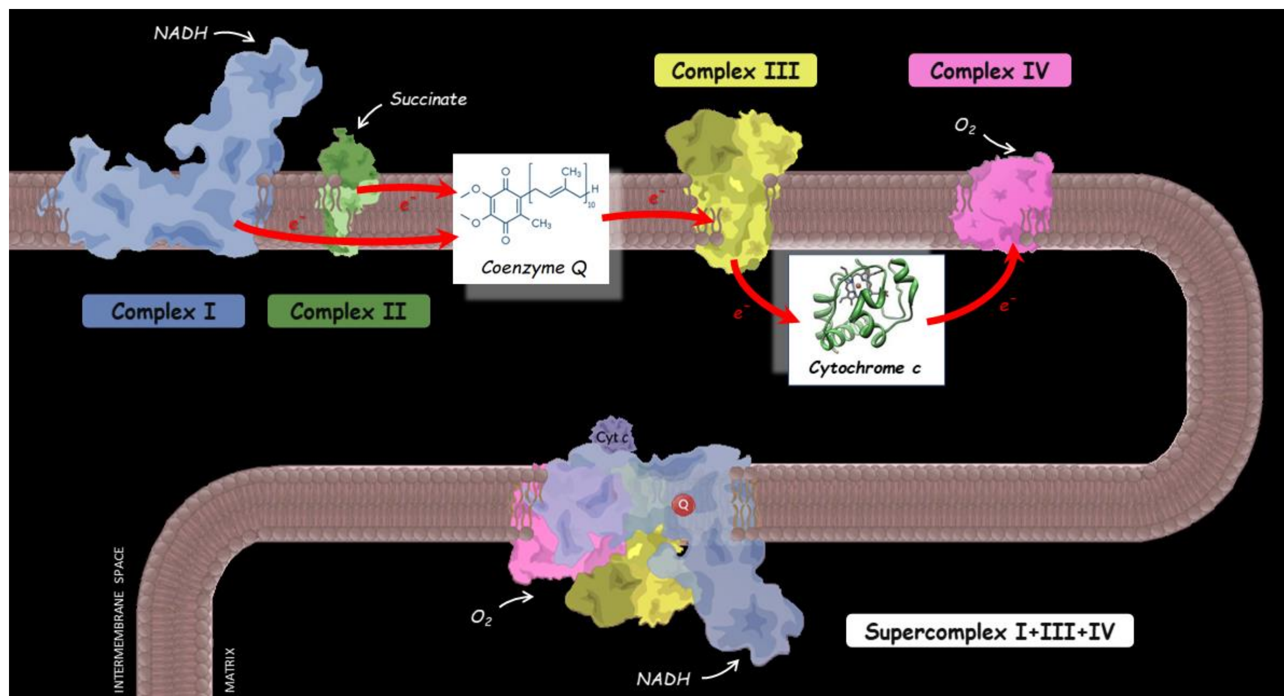

Figure 1: Schematic representation of electrons funneled to redox acceptors of the respiratory system in eukaryotic mitochondria. Most of the electrons arise from the action of dehydrogenases in catabolic pathways and are funneled to redox carriers like flavin and nicotinamide nucleotides (e.g., NADH), membrane benzoquinones (e.g. Coenzyme Q, Q), and cytochromes (e.g., Cytochrome *c*, Cyt. *c*). Complex I, Complex II, Complex III and Complex IV are the mitochondrial redox enzymes of the main ETS pathways (red arrows). These components, together with ancillary redox enzymes in the branching pathways (not shown, for review cf. [2]), pass the electrons to one another through multiple reactions in an energetically downhill sequence driven by the high reduction potential of oxygen. The demonstration that CI, CIII and CIV can assemble as specific supramolecular enzyme units, namely supercomplexes, led to postulate what rate advantage on electron transfer they may confer by substrate channeling of Coenzyme Q and/or Cytochrome *c*. The figure shows the largest SC I+III+IV (the respirosome) although other types of SCs have also been described in the literature (see text for discussion).

Because CoQ and Cyt. *c* can diffuse freely in the membrane and water phases, respectively, a functional link between the components of the respiratory system does not require a physical linkage between these complexes [4, 5]. Indeed, the random collision model of mitochondrial electron transport put forward by Hackenbrock et al. in 1986 [6] is founded on the principle that all the respiratory complexes are randomly dispersed and functionally connected by free diffusion of smaller redox components, CoQ and Cyt. *c*, the former diffusing laterally in the lipid bilayer, the latter diffusing in 3D in the intermembrane space. This model was conceived by Hackenbrock upon a large body of previous evidence, started with the isolation and reconstitution studies by Hatefi et al. in David Green's laboratory [7], followed by the careful kinetic studies of Kröger and Klingenberg [8] and by the phospholipid dilution experiments by Hackenbrock and coworkers [9].

However, since the 2000s, the demonstration that mammalian CI, CIII and CIV can be isolated and functionally resolved as specific electron-transporting supramolecular units, called supercomplexes (SCs), immediately led to postulate that such units may confer a rate advantage on electron transfer and therefore increase the efficiency of oxidative phosphorylation by substrate channeling while also diminishing the production of reactive oxygen species [10, 11, 12, 13]. Substrate channeling is the direct transfer of an intermediate between the active sites of two enzymes which catalyze reactions which occur one after the other [14]; in the respiratory chain, this means that electrons are transferred between two consecutive enzyme complexes by alternate reduction and re-oxidation of an intermediate which is not diffused in the medium. In such a case, inter-complex electron transfer cannot be distinguished from intra-complex electron transfer. Therefore, the mobile intermediates predicted to exhibit substrate-like behavior in the random collision model, i.e., CoQ and Cyt. c, would be buried in the interface between two consecutive respiratory complexes within the SC (Fig. 1).

For the sake of truth, it is worth noting that Hackenbrock himself had not excluded the idea that the respiratory complexes can assemble in one structural entity (i.e. like SCs), as in 1979 he commented that "*cytochrome c oxidase can diffuse laterally in the energy-transducing membrane, either independently of all other integral proteins or in physical union with one or more other integral proteins*", although not yet identified in his case as the "*remainder of the respiratory chain*" [15]. However, even the idea that the supramolecular organization of respiratory complexes confers a kinetic advantage, as advanced in early papers [16, 17, 18], was neglected over the years and not further explored because much of the literature was influenced by the *Random Collision Model* of electron transfer between respiratory complexes [6].

In this context, although several types of SCs have been isolated and described in the literature so far, including SCs I+III, III+IV and I+III+IV (namely, the respirasome), two conflicting models of electron transfer along the mitochondrial respiratory system — the above mentioned Hackenbrock-

inspired *pool behavior model* and the *channeling model* based on SCs structural evidence — are currently being debated by distinct cohorts of experts in the field. Both models have produced useful insights, but neither has yet provided a validated mechanistic account for the functional role of a mixed population of respiratory SCs and individual (free) complexes in the inner mitochondrial membrane. The goal of this perspective is to assess this "conflict of models" by focusing more on evaluating specific kinetic evidence in the two distinct regions of CoQ- and Cyt. *c*-mediated electron transfer. We conclude that the two regions differ fundamentally, thus making both models valid in specific conditions.

2. Studies aimed to reveal if channeling is present in the CoQ region

Our laboratory first engaged a research project to investigate the possible kinetic role of SCs: our experimental approach was based on the MFC analysis aimed to determine the rate limiting steps of a metabolic pathway [19, 20]. Kinetic testing using MFC analysis is a powerful source of information on the supramolecular organization of enzyme complexes [21].

In fact, MFC predicts that, if a metabolic pathway is composed of distinct enzymes freely diffusible in a dynamic organization, the extent to which each enzyme is rate controlling may be different and the sum of all the flux control coefficients for the different enzymes should be equal to unity. The flux control coefficient of an enzymatic step in a metabolic pathway is defined as the fractional change in the global flux through the entire pathway as induced by a fractional change in the enzyme under consideration.

On the other hand, in a SC, the entire metabolic pathway would behave as a single enzyme unit, as inter-complex electron transfer cannot be distinguished from intra-complex electron transfer, thus inhibition of any one of the SC components would elicit the same flux control near unity, and the sum of all coefficients would be >1 [22].

MFC of NADH oxidation in BHM and submitochondrial particles revealed high flux control coefficients for both CI and CIII, suggesting that the two enzymes are functionally associated as a single enzyme SC, with channeling of the common substrate, CoQ. No such functional relation was found with CII, in line with-lack of convincing structural evidence of its participation in SCs, thus no CoQ channeling in the succinate-dependent pathway was inferred. Surprisingly, CIV was also found to have a low MFC coefficient despite its large participation in the SCs architecture (e.g. SC I+III+IV and SC III+IV), so channeling vs. pool behavior in the Cyt. *c* region had not been clearly defined [19] (cf. also Section 5 and references therein).

Subsequently, MFC analysis was only scantly exploited to investigate supramolecular organzation; our group produced additional evidence of high MFC coefficients in CI-dependent respiration in potato tuber mitochondria (original experimental data in Table 1 of our review paper

[23]). These mitochondria, however, had a high MFC coefficient also for CIV, suggesting that the entire respirasome is active in channeling electrons from NADH to oxygen, by means of restricted diffusion in both the CoQ and the Cyt. *c* regions. Conversely, CII was still a complex free from any respiratory SC aggregation, and thus without CoQ channeling, even in plant mitochondria so the possibility that the CoQ pool may not be homogeneous becomes more evident in favor of two separate CoQ redox pathways in mitochondrial ETS, depending on the electron supply source.

Ten years later, Judy Hirst's laboratory [24] severely criticized our experiments and produced new evidence that electrons from NAD-dependent substrates are delivered to CoQ following the previously proposed "pool" behavior according to Kröger and Klingenberg [8]. The major experimental point was the only partial additivity they found in the simultaneous aerobic oxidation of NADH and succinate. Hirst and coll. reasoned that channeling of electrons from NADH in the CoQ region, which is what is actually inferred from our findings in the articles mentioned above, would have created a separate flux from that originating from succinate in such way to produce complete additivity when both substrates are oxidized simultaneously, but apparently this was not the result of their experiments so Hirst et al. rejected the CoQ-channeling hypothesis [24]. However, as explained in detail in Section 3, we think that their conclusion is ambiguous because in Hirst's experimental assays [24] the electrons from NADH and succinate to O_2 cross two potential pools, that of CoQ and that of Cyt c.

In a later study, Fedor and Hirst [25] found that AOX incorporated into mammalian heart coupled submitochondrial membranes strongly increases the rate of aerobic NADH oxidation in presence of KCN, indicating that the quinol generated by CI in SCs, if any, is reoxidized more rapidly in the CoQ pool by AOX than inside the SC by CIII. Based on these results, Hirst [26] concluded that CoQ diffuses freely in and out of CI-containing SCs, thus no substrate channeling occurs, showing that it is not required to support AOX-assisted respiration. As explained in Section 4, however, we think that such results express a dissociation of CoQ from the SC forced by the blocking of electron transfer through CIV induced by KCN in their assay conditions.

On the contrary, in 2016, we still obtained more kinetic evidence for channeling between CI and CIII in permeabilized BHM membranes showing complete additivity of succinate and NADH oxidation by excess exogenous Cyt. *c* (i.e NADH- and succinate-oxidoreductase activity), where the electron transfer path for the oxidation of both substrates had been functionally shortened by including only CoQ and CIII as redox intermediates while enzymatic re-oxidation of excess exogenous Cyt. *c* by oxygen in CIV was excluded in our assays, thus avoiding the bottleneck of different experiments using endogenous Cyt. *c*. (see original experimental data published in Table 2 of our review paper [27]).

Very recently, in the search for different approaches, we also found [28] that SHM reveal complete additivity of NADH and succinate oxidation investigated by oxygraphic measurements, suggesting the presence in these mitochondria of two separate independent electron fluxes to O_2 starting from NADH or from succinate (cf. sections below) and revealing that different types of mitochondria may exhibit different behaviors in their mechanism of electron transfer.

3. Coenzyme Q pool versus deceptive Cytochrome c pool in functional assays

Since the additivity experiments in both Hirst's [24] and our recent study [28] were obtained by oxygraphic measurements of oxygen consumption, they might be potentially ambiguous since they depend on integrated pathways of two subsequent intermediate pools, that of CoQ and that of Cyt. *c*. This consideration also applies to the early kinetic analysis by Kröger and Klingenberg [8] on CoQ reoxidation.

The available data suggest the scheme shown in Fig. 2 for the different options. Actually, the ambiguities are dissolved if complete kinetic additivity is found experimentally, as in SHM [28]: two independent electron fluxes underlie NADH and succinate aerobic oxidation, and structural knowledge also tells us that the flux from NADH occurs through the entire SC I+III+IV that is present in SHM [29, 30] whereas the flux from succinate exploits the pool at the CoQ level (Fig. 2A).

Conversely, when additivity of NADH and succinate aerobic oxidation is incomplete, as in BHM [24, 28, 31], the results are potentially ambiguous because they could derive from pool behavior either in the CoQ region or in the Cyt. c region or at both sites (namely, option no. 1, 2 or 3 of Fig. 2, respectively). We believe that such ambiguities due to incomplete kinetic additivity are resolved with further experimental results therefore we deduce that intermediate substrate mixing in BHM indeed occurs only at the level of Cyt. *c* (i.e. option no. 2 in Fig.2B) for the following reasons:

(*a*) The flux control analysis of Bianchi et al. [19] in BHM established that CI and CIII have high MFC coefficients, whereas CIV has a low MFC coefficient, indicating the presence of channeling between CI and CIII but not between CIII and CIV, thus ruling out the pool behavior for both CoQ and Cyt. *c* sites at the same time (i.e., ruling out an entire path based on random walks, like option no. 3, in the case of NADH as a substrate that powers a flow of electrons through CI). This property of a low MFC coefficient for CIV has been confirmed by similar results in BHM in the recent study of Nesci et al. [28]. The logical interpretation, supported by structural association of CI and CIII in BHM [27], is the presence of channeling in the SC I+III, whereas electron transfer between CIII and CIV occurs largely by diffusion at the Cyt. *c*-junction (i.e., ruling out also a path with channelled Cyt. *c*, like option no. 1, in the case of NADH as a substrate of CI)

(*b*) The experiment reported in Table 2 of Lenaz et al. [27] demonstrates a clear complete additivity of the rates of exogenous Cyt. *c* reduction by simultaneous oxidation of NADH and succinate in BHM

samples where enzymatic re-oxidation of Cyt. *c* was excluded (i.e., where NADH+succinate oxidoreductase activity was investigated instead of aerobic respiratory activity): this means that in these mitochondrial samples there are separate fluxes through the NADH- and succinate-linked segments, thus excluding the concept that they converge at the CoQ-junction (i.e., excluding both options no. 1 and no. 3 in the case of NADH as a substrate of CI).

We cannot exclude however that, also in SHM (Fig. 2A), CoQ reduced by succinate may interact with free CIII molecules, thus inducing pool behavior of Cyt. c (i.e., admitting a path like option no. 3 in addition to no. 1 in the case of succinate as a substrate in SHM, not shown).

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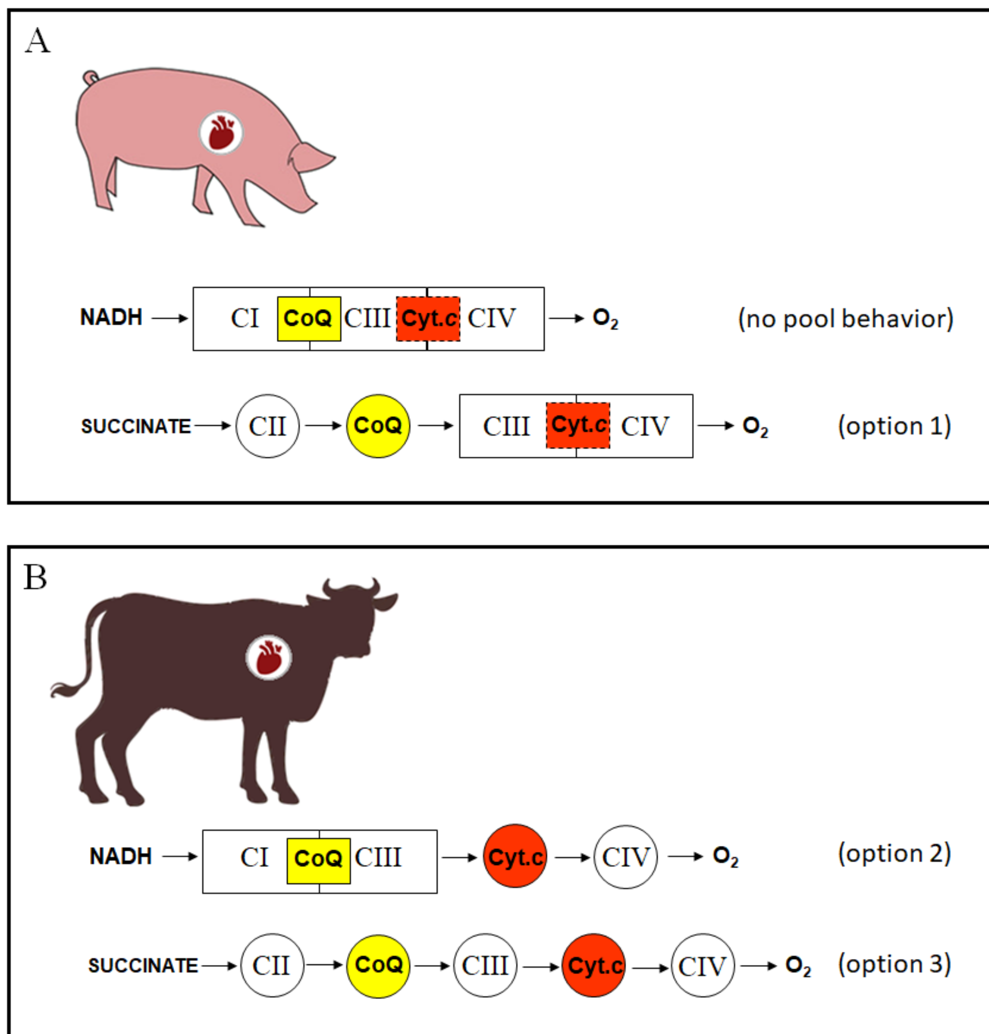

Figure 2 – Schematic representation of different possible combnations of CoQ and Cyt. c pool as suggested by functional analysis in SHM (A) and BHM (B). Arrows indicate electron transfer via random diffusion of the substrates and respiratory enzyme complexes shown in the circles whereas the rectangular boxes indicate channeling within the SC boundaries. Two separate independent electron fluxes underlie NADH and succinate aerobic oxidation in SHM while electron fluxes in BHM can converge at the Cyt. *c* pool level, but they are still separated in the CoQ region due to channeling between CI and CIII, thus ruling out the concept that electron fluxes from CI and CII converge at the CoQ-junction in mitochondria (see section 3 for discussion).

We should keep in mind that the schemes in Fig. 2 refers to the functional behavior and not the structural organization of the so-called mobile elettron carriers of the respiratory chain (i.e., CoQ and Cyt. c). This means that while channeling obviously implies that these redox substrates are compartmentalized within the constraints of stoichiometric supramolecular architectures made of lipids and partner enzyme components, conversely the random diffusion behavior of substrates does not infer that they react exclusively with enzyme partners that are separate (i.e. free complexes) and not even with enzymes forming SC protein assemblies. In other terms, the functional random diffusion behavior of substrates is still compatible with the presence of SC structural organization of respiratory enzymes in the mitochondrial membrane (see section 4 and ref. [32] for further review).

4. Dissociation equilibrium of SC-bound CoQ

The existence of a dissociation equilibrium of SC-bound CoQ with the CoQ pool was proposed by Genova and Lenaz [32] to become operative when the flux of electrons from NAD-linked substrates via CI is slowed down at the level of CIII. Actually, it cannot be overlooked that the SC-bound ubiquinone that allows the electron flow directly from CI to CIII is in dissociation equilibrium with the pool of CoQ molecules in the membrane therefore, at steady state, its amount would be dictated by the size of the membrane bulk pool itself. This equilibrium would explain previous results in the literature showing the saturation kinetics for total ubiquinone exhibited by the integrated activity of CI and CIII [33] and the decrease of respiratory activities in mitochondria fused with phospholipids with subsequent dilution of the CoQ pool [9]. To be in agreement with the experimental observation obtained by MFC analysis (cf. section 2), this proposition must, however, require that the dissociation rate constants (k_{off}) of bound CoQ be considerably slower than the rates of intercomplex electron transfer via the same bound quinone molecules within the SC I+III. In this scenario, CoQ reduced by CI within the SC is more easily reoxidized by supercomplexed CIII under normal flux conditions, when both the reducing pressure and ETS oxidation capability are adequate, because presumably the dissociation rate constant k_{off} is slower than the rate constant of CIII reduction [34]. Conversely, CoQ dissociation prevails and CoQ is dispersed in the membrane pool when CIII is inhibited and/or somehow deprived of adequate enzymatic capacity.

Indeed, even at low concentrations, Antimycin A inhibition of CIII induces loss of additivity for succinate and NADH oxidation, and induces CoQ pool function in SHM [28].

A similar rationale would explain also the above mentioned results by Fedor and Hirst [25] with AOX in BHM (cf. section 2): since they used KCN to block electron transfer through CIV, under this condition electrons are forced to follow the AOX pathway by dissociation of ubiquinol molecules from the SC I+III+IV into the CoQ pool. In favor of this interpretation, it was observed that AOX

expressed in mouse mitochondria is not engaged with CI substrates unless a respiratory inhibitor is added [35].

Structural studies by cryo-electron microscopy are also in favor of the existence of a CoQ dissociation equilibrium from SCs. The respirasome structure reported by Letts et al. [36, 37] indicates that both CoQ binding sites in CI and CIII are separated and accessible to the membrane, and are likely to provide no limit to free CoQ diffusion from/to the membrane environment.

The structural studies necessarily exhibit a static pattern that does not provide clear information on the kinetic aspects of CoQ behavior in physiological conditions. However, the CoQ molecules of the bulk of the lipid milieu in close proximity to the active sites of CI and CIII necessarily experience a facilitated interaction with these sites within the SC in competition with a longer and random diffusion path to other SCs and free respiratory complexes of the inner mitochondrial membrane compartment.

The major kinetic advantage of channeling is a reduction in bulk pool sizes and, as a result, a more rapid system response reflected in a shortened reaction time [38]. This reveals that no channeling advantage is achieved at high enzyme concentration of free enzymes (i.e., comparable to K_m) or, in the case of 'leaky' channels, where rapid equilibrium kinetic mechanisms are present. In the case of a perfect channel with no leakage and direct transfer of metabolite between adjacent enzyme active sites, the reaction time is minimized. Clearly a rate advantage depends on the system and on the experimental condition.

To settle the debate on substrate channeling and the function of SCs, new experimental frameworks are needed to further explore their physiological roles. The SC I+III_2 structure recently isolated by Letts et al. [39] from ovine heart mitochondria revealed a CI state-dependent conformational flexibility of the CIII's MT-CYB subunit in close contact to CI, indicating crosstalk between the two complexes and suggesting a more subtle functionally relevant interaction between CI and CIII in the SC, beyond their function of channeling the substrate. Such conformational crosstalk can likely play a role in well matching both CI and CIII turnover to operate at the highest rates via adequate exchange with the CoQ bulk pool in different metabolic conditions.

At the same time, the presence of two separate pathways was confirmed by a completely different approach. The experimental observation on the site-specific inhibition (both CI-linked and CII-linked respiration) elicited by guanidine derivatives, and the differential restoration of respiration induced by two classes of uncoupling agents was interpreted with the existence of two separate electron transfer pathways [40].

On the other hand, in a recent study Milenkovic et al. [41] generated homozygous knock-in mice that have normal levels of respiratory chain complexes but profoundly decreased levels of respirasomes and found that the mutant mice are healthy, with preserved respiratory chain capacity

and normal exercise performance. This study does not exclude that channeling occurs in the WT mice where SCs are present, but raises doubts on the importance of channeling for optimal electron transfer since the activities were not lowered in the knock-in mice system that they investigated, where SCs were absent.

The separation of electron fluxes in the mitochondrial ETS, however, has further physiological consequences that are described in Section 6.

5. Species variability in the cytochrome c region

Our recent results in SHM [28] reveal profound differences in the Cyt. *c* region compared to BHM. Indeed, swine mitochondria have a high flux control at the level of CIV, suggesting the existence of a stable functional SC comprising CIV and Cyt. *c* channeling, unlike bovine mitochondria. The different behavior is surprising in two closely related species (mammals). As for the composition of complexes and SCs in swine mitochondria, a study by Shimada et al. [30] compared the SC composition from different sources including bovine heart and porcine heart: the BN-PAGE studies showed a similar pattern with the presence of both a SC I+III+IV and free CIV. Therefore, the striking difference between bovine and swine CIV functional properties is not due to a hypothetical different proportion of SC-bound and free complexes.

It is ascertained in the literature that the lipid environment is required for the activity of membrane-bound enzymes (reviewed by Lenaz [42]). The lipid requirement for activity of CIV reconstituted in lipid vesicls was investigated by Vik and Capaldi [43] and by Cuyper and Joniau [44]: both studies found no evidence for headgroup specificity, although a strict requirement for tightly bound cardiolipin was highlighted. On the other hand, a strong dependency on membrane fluidity was detected, largely but not exclusively dependent on the fatty acid composition of the lipid environment [45].

The study of Shimada et al. [30] also pointed out that the phospholipid headgroup composition is very similar in beef and pig mitochondria. However, the fatty acid composition of pig tissues is characterized by a larger proportion of unsaturated fatty acids than beef tissues [46]. It is known that in ruminants dietary fatty acids undergo hydrogenation by the microbiota of the rumen [47]; nevertheless they conserve linoleate and other unsaturated fatty acids by limiting their oxidation [48]. Indeed both pig heart and beef heart mitochondria have large amounts of polyunsaturated fatty acids [49, 50].

Clearly the lipid composition data from the literature are somewhat ambiguous and do not appear to reveal striking differences between the two species considered. On the other hand, what appears to be important for the activity of membrane-boud enzymes is the physical state of the lipids [42]: membrane fluidity is affected not only by the fatty acid composition but also by other factors such as

the presence of cholesterol and the density of integral proteins around which lipids are immobilized forming a rigid annulus [51].

In the study by Nesci et al. [28] we have directly compared the Arrhenius plots of cytochrome oxidase from BHM and SHM, and found that a break, indicating a transition in the state of the lipid bilayer, occurs at higher temperature in BHM vs. SHM (i.e., 29.4°C vs 22.3°C respectively), demonstrating that SHM have higher fluidity than BHM at a given temperature. It is demonstrated in the literature that membrane lipid fluidity has a direct influence on the conformation of the active site of membrane-associated enzymes, with the result that such enzymes display a lower activation energy when the membrane lipids are comparatively more fluid, as detected by Arrhenius plots showing a break at the transition temperature below which the membrane becomes more rigid [52]. Obviously further investigation is required to show if the different fluidity is indeed responsible for the different behavior in electron transfer in SCs and free respiratory complexes from SHM and BHM, as speculated by Nesci et al. [28].

Further studies are needed to ascertain the mechanism of electron transfer in the Cyt. *c* region in different organisms, of which we have only a scattered knowledge [53]. Besides plant mitochondria [23], also yeast mitochondria appear to have a functional tight channeling at Cyt. *c*. Functional evidence for Cyt. *c* channeling was found in *S. cerevisiae* [54] mitochondria which are characterized by having all CIV molecules bound to CIII in the SC III_2+$IV_{1\text{-}2}$ [55], thus preventing electron transfer through free CIV units. The structural evidence by single particle cryo-EM sustains channeling, since the distance between the binding sites of Cyt. *c* (*i.e.*, cytochrome c_1 of CIII and the Cu_A-subunit II of CIV) is considerably shorter in the yeast SC III_2+$IV_{1\text{-}2}$ (there is no CI in this yeast species) than that in bovine mitochondria [56]. Accordingly, analyses of a yeast mutant defective in SC formation, which still contains fully functional individual complexes, showed that the lack of SC assembly delays the diffusion of cytochrome c between the separated complexes, thus reducing electron transfer efficiency [57]. Moreover, the structural and kinetic studies of Moe et al. [58] indicate that Cyt. *c* travels along a negatively charged patch on the SC surface. Thus, rather than enhancing electron transfer rates by decreasing the distance that Cyt. *c* must diffuse in three dimensions, formation of the SC $III_2IV_{1/2}$ facilitates electron transfer by two-dimensional (2D) diffusion of Cyt. *c*.

At difference with the above results, by a time-resolved study of the respiratory chain in intact cells Trouilllard et al. [59] showed that Cyt. *c* is not trapped within SCs in yeast and encounters no particular restriction to its diffusion, which further questions the functional relevance of these supramolecular assemblies.

The structural analysis of purified SCs by cryo-EM adds structural reasons for the looseness of CIII–CIV interactions in different animal species. In ovine mitochondria, Letts et al. [37] identified two distinct arrangements of SC I+III+IV: a major “tight” form and a minor “loose” form. In both

structures, the density for CIV is weaker relative to that for CI and CIII, indicating the greater conformational flexibility of CIV.

An important factor for proper assembly and funcyion of SCs is the existence of specific assembly factors, such as Rcf1 in yeast [60]. Rydström Lundin et al. [61] demonstrated that the factor Rcf1 promotes the formation of a direct electron-transfer pathway from CIII to CIV via a tightly bound Cyt. *c* in yeast. Consistently, direct channeling does not take place in a strain lacking Rcf1 and electrons are only transferred via the Cyt. *c* pool in that case.

A requirement for specific assembly factors for proper channeling from CIII to CIV is also described for mammalian mitochondria, as the COX7A2L subunit or SCAF1 (Supercomplex Assembly Factor I) protein [62]. Finally, the importance of Cyt. *c* post-translational modifications in assuring better contacts with the partner enzymes should be also taken in consideration [63]

6. Physiological implications of separate fluxes

The existence of two different functional CoQ compartments, one for NADH oxidation and another one for oxidation of succinate and other FAD-linked substrates, has deep implications for the metabolic adaptation to the feeding state. Carbohydrate oxidation through glycolysis and Krebs cycle occurs largely in a well-fed state, whereas during fasting fatty acid oxidation prevails. The use of different fuel molecules generates different proportions of NADH and $FADH_2$, which require an optimal equilibrium between the corresponding routes of electron transfer in the respiratory chain. In glucose catabolism, the oxidation of NAD-linked substrates exceeds succinate oxidation (ca. 5:1), and the electron flux must proceed mainly through SC I+III. On the other hand, metabolic adaptation of liver mitochondria to fasting forces fat mobilization and fatty acid oxidation [64], with a ratio NAD-linked to FAD-linked substrates ca 2:1.

An increase of the $FADH_2$-dependent respiration, as in fatty acid oxidation, induces saturation of the CoQ pool reoxidation capacity and promotes reverse electron transport from ubiquinol to CI [65]; the resulting local generation of superoxide triggers protein degradation of CI by oxidative damage. Thus, we can infer that CoQ redox status acts as a metabolic sensor that fine-tunes the configuration and supramolecular organization of the respiratory ETS in order to match the prevailing substrate profile [66].

Recently also Enriquez' group [67] confirmed that the SC I+III assembly allows the partial segregation of the CoQ pool and allows substrate channeling in the CoQ region. Their results with different cellular models suggest that when CI is not superassembled, as in CIII-KO cells, CoQ exists in a sole functional pool.

Why channeling at the level of Cyt. *c* may be an optional solution in mammalian mitochondria? Mammalian mitochondria have several dehydrogenases directing electrons to CoQ, however, they

usually have only one oxidase (CIV) receiving electrons from CIII via Cyt. *c*. For this reason, we may speculate that, whilst it may be useful to separate the major NADH-dependent flux through CI from those departing from succinate, fatty acid oxidation or other metabolic pathways by separating channeled and pool CoQ compartments, there is no such need for Cyt. *c* that is by and large receiving electrons univocally from CIII [68, 69]. This assumption is reinforced by the fact that CIII and CIV of plant mitochondria are structurally and functionally operating as a SC, with Cyt. *c* tightly bound in the SC, as revealed by flux control analysis and BN-PAGE [23]. Not surprisingly, plant mitochondria are characterized by a high branching also of the electron transfer pathways feeding electrons directly to Cyt. *c* [70], which requires adjustment of the different routes as a response to physiological needs, as it happens in mammalian mitochondria at the CoQ level. In plant mitochondria, segmentation might be achieved by regulating different compartments of free and bound Cyt. *c*.

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