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Respiratory Chain Supercomplexes: Structures, Function and Biogenesis

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Abstract

Over the past sixty years, researchers have made outmost efforts to clarify the structural organization and functional regulation of the complexes that configure the mitochondrial respiratory chain. As a result, the entire composition of each individual complex is practically known and, aided by notable structural advances in mammals, it is now widely accepted that these complexes stablish interactions to form higher-order supramolecular structures called supercomplexes and respirasomes. The mechanistic models and players that regulate the function and biogenesis of such superstructures are still under intense debate, and represent one of the hottest topics of the mitochondrial research field at present. Noteworthy, understanding the pathways involved in the assembly and organization of respiratory chain complexes and supercomplexes is of high biomedical relevance because molecular alterations in these pathways frequently result in severe mitochondrial disorders. The purpose of this review is to update the structural, biogenetic and functional knowledge about the respiratory chain supercomplexes and assembly factors involved in their formation, with special emphasis on their implications in mitochondrial disease. Thanks to the integrated data resulting from recent structural, biochemical and genetic approaches in diverse biological systems, the regulation of the respiratory chain function arises at multiple levels of complexity.

Keywords

Respiratory Chain Function; Supercomplexes; Respirasome; Assembly Factors

Introduction

Recent investigations have shed light into the sophistication surrounding the biogenesis of the oxidative phosphorylation (OXPHOS) system. In mammals, the OXPHOS system is formed by five multiprotein enzyme complexes and two mobile electron carriers embedded

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in the inner mitochondrial membrane. The first four enzyme complexes (CI–CIV) comprise the mitochondrial respiratory chain (MRC), which facilitates electron transfer from reducing equivalents to molecular oxygen coupled to the generation of a proton gradient across the inner membrane that will be used by the ATP synthase (complex V) to drive ATP synthesis. In addition to their structural subunits, the biogenesis of these five hetero-oligomeric enzymatic complexes requires an extensive number of ancillary factors to coordinate subunits maturation, incorporation of prosthetic groups and assembly into the holoenzymes. The discovery that some MRC enzymes physically interact to form a variety of supramolecular structures called supercomplexes (SCs) and respirasomes, and the existence of SC-specific assembly factors involved in their assembly, has put the spotlight on the structural and functional properties of the SCs, and on the regulatory pathways involved in their biogenesis. It is currently debated whether SCs play a relevant functional role in cellular bioenergetics, in the formation of reactive intermediates, or in the stabilization of the individual MRC complexes. Since genetic alterations in MRC subunits and assembly factors often lead to severe encephalomyopathies and neurodegenerative disorders, a full understanding of the structural organization and biosynthetic regulation of the MRC is essential to understand the molecular mechanisms underlying mitochondrial pathology. In this review we will explore the current knowledge on mammalian respiratory SCs, integrating the historical perspective with the most recent structural findings, and putting this information in the context of mitochondrial disease.

1. Mitochondrial respiratory chain, a long-lasting journey

1.1. Composition and function of the respiratory chain

The production of adenosine 5'-triphosphate (ATP), the key energy source of the cell, through aerobic substrate oxidation is the main function of the mitochondrial metabolism. In the late 50s, several redox enzymes and prosthetic groups responsible for the classic mitochondrial electron transfer chain were defined [1] followed by their reconstitution in the early 60s [2]. The overall respiratory chain activity was postulated as a sequential transfer of electrons between four major multi-enzymatic complexes dispersed in the inner mitochondrial membrane (IMM): NADH dehydrogenase:ubiquinone oxidoreductase (complex I, CI), succinate:ubiquinone oxidoreductase (complex II, CII), ubiquinol:cytochrome *c* oxidoreductase or cytochrome *bc1* complex (complex III, CIII), and cytochrome *c* oxidase (complex IV, CIV). In addition, the electron transfer was ensured by the diffusion of two mobile components acting as co-substrates: the lipophilic ubiquinone, also designated as coenzyme Q (CoQ), embedded in the membrane lipid bilayer, and the hydrophilic heme protein cytochrome *c* (cyt*c*) located on the external surface of the IMM [3,4]. Altogether, these components form the mitochondrial respiratory chain (MRC) where cellular respiration takes place. Organic nutrients are catabolized into small electron donor molecules, NADH₂ and FADH₂, which transfer the electrons to CI and CII, respectively. CoQ uptakes the electrons from both sources, transferring them to dimeric CIII (CIII₂), then to cyt*c* and finally to CIV, that yields the electrons to molecular oxygen. This electron flux is coupled to a proton pump from the matrix to the intermembrane space through complexes I, III and IV, which generates an electrochemical gradient across the IMM that provides the

necessary free energy for the ATP synthase (complex V, CV) to synthesize ATP through the mechanism known as oxidative phosphorylation [5].

1.2. Models for the structural organization of the respiratory chain

Despite the well-known functional relevance of the respiratory chain, the structural organization of its components remains unclear.

1.2.1. Solid-state Model—The pioneering spectrophotometric studies of Chance and Williams represented the MRC as a solid state assembly of prosthetic groups that carry out sequential redox reactions in a protein matrix [1]. The evidences in favour of this “rigid” or “solid-state model” were based on the isolation of CI–CIII and CII–CIII active units in a stoichiometric molar ratio of 1:1 during intermediate purification steps of the individual enzymes, that were interpreted as secondary enzymatically active complexes [6]; and on the reconstitution of a “repeating unit of electron transfer” containing all MRC complexes from bovine heart mitochondria [7]. This model implied the notion of permanently-bound CoQ to the MRC units. Accordingly, vesicle reconstitution experiments showed stoichiometric associations between CI and CIII₂ at high protein concentrations with no exchange between free and bound ubiquinone, i.e., no ‘CoQ-pool’ behaviour [8]. Only when sufficient lipid was added the CoQ-pool behaviour was restored, but this was interpreted as the movement of complex-associated CoQ rather than of free CoQ [9]. Later studies in *Saccharomyces cerevisiae* provided evidence that CoQ and cytc only diffused freely along the membrane upon the addition of chaotropic agents, suggesting that the respiratory chain in yeast also behaves as one functional unit that at least comprises complexes III and IV with bound CoQ and cytc [10].

1.2.2. Liquid-state Model—The general vision gradually evolved into a “random collision”, “fluid” or “liquid-state” model, proposed by Hackenbrock and co-workers, that pictured all membrane proteins and redox components that catalyse electron transport and ATP synthesis in constant and independent diffusional motion, where electron transfer to place through diffusion-based collisions among the redox partners [11]. Evidence in favour of the “liquid-state model” arose from kinetic analyses proposing not only that electron transfer in the CoQ and cytc regions obeyed a pool behaviour in mammalian mitochondria, but also that it followed saturation kinetics with regards to CoQ and cytc concentrations [3,12]. Furthermore, enzymatic activities were retained upon isolation of the individual OXPHOS complexes [2] and the use of fluorescent antibodies against CIII₂ and CIV caused independent aggregation of these complexes, suggesting that they diffuse laterally in the membrane plane independent of one another [13].

1.2.3. Dynamic aggregate or Plasticity Model—The two previous models were proposed for the way in which MRC components interact to accomplish a maximal-efficient electron transfer, representing extreme examples where intermediate modes of operation are feasible. Alternative studies by Ferguson-Miller and collaborators that analysed the role of lateral diffusion of cytc in electron transfer within native mitochondrial membranes, gave rise to the “dynamic aggregate” model [14]. In this model, a dynamic equilibrium exists between freely diffusing and associated-forms of the MRC components, all active in electron

transfer. This new representation of the respiratory chain reconciled the two classical models, as it incorporated transient aggregates as well as free lateral diffusion of redox components to account for the electron transport rates. The reversible formation of specific MRC aggregates additionally offered a mechanism for localized proton flow and the possibility of regulating the direction and efficiency of electron transfer [15,16]. This idea was disregarded in favour of the “fluid” model until new data from two groups, based on the use of blue native polyacrylamide gel electrophoresis (BN-PAGE) developed by Schägger and collaborators, showed the co-existence of individual MRC complexes together with their inter-associations in supramolecular assemblies that were termed supercomplexes (SCs) [17,18]. Experimental evidences demonstrated that SCs are evolutionarily conserved stable structures in both prokaryotes and eukaryotes, and not random associations of MRC complexes [19,20], and recent high-resolution cryo-electron microscopy (cryo-EM) analyses showed the detailed structural architecture of mammalian respiratory SCs (Figure 1) [21–23]. The coexistence of individual MRC complexes and SCs thus supports the principles of the “dynamic aggregate” model, which was lately renamed as the “plasticity” model by Enríquez and co-workers [24,25]. In its current form, this model proposes that the switch between freely moving MRC complexes to fixed SCs would optimize electron flux from different substrates (NADH and FADH₂), adapting the efficiency of the respiratory chain to changes in cellular metabolism via partitioned CoQ and cytc pools [26]. The catalytic relevance of this hypothesis, based on BN-PAGE and oxygen consumption analyses in mouse cells depleted of MRC complexes, was however questioned by more direct spectroscopic and kinetic experiments in yeast and bovine submitochondrial particles showing that: a) the metabolic pathways for NADH and succinate oxidation occur through a single and universally accessible CoQ-pool [27], and b) that cytc does not encounter major barriers to its free diffusion [28]. Moreover, this model was proposed on the basis of differences in the steady-state levels of respiratory chain structures on BN-PAGE gels, and therefore, the reversible and dynamic association/dissociation events –the so-called “plasticity”- between the individual complexes and the respirasomes remains to be proven. The prospective evolution of the “plasticity” model will thus rely on the extensive characterization of the structural, functional and kinetic properties of the SCs upon variations in physiological conditions [29,30].

2. Structural architecture of respiratory chain supercomplexes

2.1. Types of respiratory chain supercomplexes

The notion of “supercomplexes” first appeared upon the observations of preferential associations between bacterial MRC complexes [31,32]. Respiratory chain SCs of different compositions and stoichiometries were later reported by means of BN-PAGE analyses of mitochondrial fractions solubilized with the mild detergent digitonin. This method allows the retention of labile supramolecular assemblies of membrane protein complexes that would otherwise be dissociated (Figure 2A). The solubilization of OXPHOS complexes from yeast and bovine heart mitochondria using varying digitonin-to-protein ratios and subsequent BN-PAGE allowed to separate assorted types of stoichiometric associations of complexes I, III and IV within the molecular mass range from ~750 to ~2100 kDa, in an overall 1:3:6 stoichiometry [33]. Additional BN-PAGE, single-particle electron microscopy

(EM) and cryo-electron tomography studies, consistently reported specific associations between CI, CIII₂ and CIV in a wide range of organisms [19]. It is worth mentioning that for a long time, many researchers attributed the appearance of SCs on BN-PAGE gels to protein aggregation as a consequence of detergent solubilisation. However, the migrations of these bands in the gels are consistently reproducible, as shown by many different laboratories in the last 17 years, and well-defined structures from these bands extracted from the gels are now available.

The composition and abundance of the respiratory chain SCs may vary among organisms and tissues depending on the metabolic and physiological conditions [17,26,34–40], as well as on the lipid content of the mitochondrial inner membrane [41–44]. In most CI-containing eukaryotes, CI primarily interacts with CIII₂ and CIV to form the most abundant SC, I + III₂ + IV₁, to which additional CIV monomers are added to form SCs I + III₂ + IV_{2–4}. These structures are known as the respirasomes [17], since they contain all the components required to transfer electrons from NADH to molecular oxygen. Additionally, CI associates with CIII₂ to form SC I + III₂; and CIII₂ binds one to two CIV monomers to assemble SCs III₂ + IV_{1–2}. In mammals, most CI, ~40–50% of CIII₂ and ~20–30% of CIV are localized in the largest SCs I + III₂ and I + III₂ + IV_{1–4} (Figure 2B). CIII₂ and CIV may also interact to form SC III₂ + IV₁ that scarcely represents 5–10% of the total MRC structures [33,45]. In higher plants mitochondria, CIV-containing SCs are barely detectable, and SC I + III₂ is the predominant macrostructure [46]. In *S. cerevisiae*, which lacks CI, two bands of ~750 and ~1000 kDa corresponding to SCs III₂ + IV₁ and III₂ + IV₂, respectively, are predominant [17,18]. These variations in the relative abundance of SCs may reflect different stoichiometries of CI, CIII₂ and CIV among organisms. Regarding CII and CV, these enzymes form oligomers that do not interact with complexes I, III and IV under normal physiological conditions [17,33,45,47–50].

Based on the differential solubilisation of MRC complexes and SCs on BN-PAGE, a higher organization level of SCs in “respiratory strings” was proposed for mammals and yeast [51], where respirasomes would be interconnected by CIV tetramers at regular intervals, thus generating linear assemblies of respiratory SCs. The observation of CI dimers (CI₂) in *Yarrowia lipolytica* [52] redefined this model as “respiratory patches” generated by the interactions between two CI monomers within adjacent respiratory strings. An alternative model for plants based on single-particle EM, proposed the repetition of I₂III₂IV₂ units into a respiratory string mediated by the interaction of two neighbouring CIV through a dimeric interface [53], which differed from the previous model by the lower abundance of CIV copies. The structural and functional characterization of SCs among different species would benefit very much from *in situ* studies without disrupting the mitochondrial membranes with detergents; for example, using fluorescence life-time imaging, as recently published [54].

2.2. Structural properties of respiratory chain supercomplexes

2.2.1. Supercomplexes I + III₂ and III₂ + IV_{1–2}—The first structure of SC I + III₂ from *Arabidopsis thaliana* mitochondria was determined at 18 Å resolution [46], revealing the lateral association of CIII₂ to the membrane-embedded distal part of CI. However, the lack of an atomic CI structure failed to decipher the precise subunit interactions within this SC.

Recently, the architectures of the mammalian SC I+III₂ were resolved at high resolution in ovine (Figure 1A) [22] and bovine [55]. In these structures, contacts between CI and CIII₂ were similar to the ones detected for SC I+III₂+IV₁ (described in Section 3.2.2.).

A pseudo-atomic model at 15Å resolution of yeast SCs III₂+IV₁₋₂ [56] revealed that CIII₂ is attached to the convex side of two CIV monomers, leaving the opposite interfaces open for CIV dimerization. Cardiolipin and phosphatidylethanolamine lipids were identified at the CIII₂-CIV interface. This structure also revealed cytc bound to CIII₂, which moves and rotates within a distance of 40Å (~4 nm) to mediate electron transfer between CIII₂ and CIV. Later studies based on 3D-cryo-EM maps specified the distance between the cytc binding sites of CIII₂ and CIV as ~6nm, considered to be sufficiently short to enable the channelling of cytc between these complexes [57], although this was previously refuted [28]. The yeast CIII₂ subunits cytochrome *b*, cytochrome *c*₁, Qcr6 (Hinge protein), Qcr7, Qcr8 and Qcr9 were identified at the CIII₂-CIV interface, as well as the CIV subunits CoxI, CoxII, CoxIII, CoxIV, CoxVIc, CoxVIIa and CoxVIIc [56]. However, direct evidence supporting precise subunit interactions was missing. Due to its low relative abundance, SCs III₂+IV₁₋₂ from higher organisms await structural characterization.

2.2.2. Respirasome or supercomplex I+III₂+IV₁—In mammalian mitochondria, I+III₂+IV₁ is the most abundant SC. The first 3D maps of bovine heart SC I+III₂+IV₁ [47,50,58,59] revealed the lateral binding of CIII₂ to the middle part of the CI membrane arm, with CIV positioned in the distal tip of CI while laterally interacting with CIII₂, and cardiolipin molecules filling the gaps between the transmembrane domains at the interfaces between the individual complexes [47,59]. The recent characterization of the atomic structure of mammalian CI by cryo-EM [60,61] represented a major step forward that enabled to obtain high-resolution projection maps of the mammalian respirasomes [21–23,55]. Yang and collaborators solved the conformation of porcine SC I+III₂+IV₁ [21,23], where CIV would loosely bind CI and CIII₂. In addition, Letts *et al.* [22] distinguished two architectures for the ovine respirasome, a major “tight” and a minor “loose” conformations, where CIV would contact both CI and CIII₂ within the tight form, but only CI within the loose form (Figures 1B–1C). Sousa *et al.* also resolved two classes of SC I+III₂+IV₁ in bovine [55]. The tight form of the ovine respirasome was essentially identical to the bovine respirasome class 1. However, the bovine respirasome class 2 differed in the conformational flexibility of CIII₂, as this rotates by 25° relative to CI while CIV remains unchanged. The bovine respirasome additionally showed clear density in one of the two membrane extrinsic iron-sulphur domains of CIII₂, suggesting that only one CIII monomer would be active [55]. The heterogeneity among these structures deserves further consideration [30], as it could reflect the existence of independent structural entities resulting from the dynamic association and dissociation of the MRC complexes in response to, e.g., tissue-specific phospholipid environments or ROS levels.

The respirasome-bound CI is more compact than free CI due to its associations with CIII₂ and CIV [60,61]. Although several interaction points exist between CI, CIII₂ and CIV, the most extensive and stable interactions take place between CI supernumerary subunits (absent in bacteria) and CIII₂ at two major points: CI subunits NDUFA11 (B14.7 in bovine) and NDUFB4 (B15) directly interact with CIII₂ subunit UQCRQ at the matrix and inner

membrane interface, and CI subunits NDUFB4 and NDUFB9 (B22) bind CIII₂ subunits UQCRC1 and UQCRFS1 in the matrix [22,23]. Another important interaction occurs between CI subunit NDUFB7 (B18) and subunit UQCRH on CIII₂. Both subunits contain disulphide bonds, suggesting that redox regulation might modulate the interactions between MRC complexes [22]. Since the long helix of NDUFB7 is poised at the interface of the three complexes, it may also interact with CIV through the COX7A and COX8B subunits at the intermembrane space and inner membrane interface [22,23]. CIV is less tightly bound to the respirasomes and major contacts differ among structures, reflecting its varying location. There is a close association of CIV subunit COX7C and ND5 on CI, as well as an interaction of COX7A on CIV with CIII₂ subunits UQCR11, UQCRC1 and UQCRB at the matrix and inner membrane interface [22,23]. This interaction between CIII₂ and IV seems to swing away in the loose respirasome form, where only COX7A would contact CI through subunit ND5 [22]. It must be noted that the differences that exist between the structural models presented from ovine/bovine/porcine probably rely on the species-specific protein sequences of the MRC subunits. Therefore, structural variations in the supercomplexes and respirasomes from other species, such as rodents and human, appear well possible depending on the degree of conservation of the specific protein domains and residues that promote the interactions within these structures.

2.3. Effect of cardiolipin on the stabilization of supercomplexes

Supporting the idea that phospholipids mediate protein–protein interactions in the inner mitochondrial membrane, cardiolipin molecules were detected within yeast SCs III₂+IV₁₋₂ [57], where they stabilize these structures [41,42]. Moreover, studies in lymphocytes from patients with Barth syndrome, a mitochondrial disorder in which cardiolipin levels are drastically reduced due to mutations affecting Tafazzin (an enzyme involved in cardiolipin maturation), revealed the specific destabilization of SC I+III₂+IV₁ [62]. A pluripotent stem cell model system of this disorder later confirmed the role of cardiolipin content for SC stabilization [63]. Consistent with these observations, the atomic structure of the respirasome revealed clear gaps between CI, CIII₂ and CIV that were occupied by cardiolipin molecules to further stabilize the respirasome [23,59]. Although cardiolipin is considered to stabilize SCs, phosphatidylethanolamine, another phospholipid of the inner membrane, seems to exert the opposite effect [43]. Therefore, differences in the balance between phospholipid species may contribute to the specific reorganization of the MRC complexes and SCs.

3. Functional roles of the supercomplexes

The discovery of SCs represents a great progress in the study of the functional and structural properties of the MRC, and their existence should provide functional advantages that remain far to be fully-understood.

3.1. Catalytic enhancement of the electron flux through substrate channelling

The arrangement into SCs was initially proposed to maximize the efficiency of the electron flux across the MRC [17]. Indeed, spectrophotometric assays of the MRC activities of isolated SCs from bovine heart mitochondria showed that CI in SC I+III₂ displays about half

the activity of that in SC I+III₂+IV₁, suggesting that the full respirasome was the most active unit [50]. Substrate channelling was proposed as a possible mechanism to explain the increased rates of electron transfer within SCs based on flux control analyses of the MRC complexes in bovine heart mitochondria [64]. The authors suggested that CI and CIII₂ behave as a single enzymatic unit, where electron transfer through CoQ is accomplished by channelling between the two redox enzymes without following a pool behaviour, in agreement with other reports in yeast [10] and bovine mitochondria [8]. Following studies that analysed the roles of CoQ and *cytc* in the attenuation of CIII₂ and CIV pharmacological inhibition on the respiratory flux supported the dynamic compartmentalization of the respiratory substrates [65], as well as studies based in the competition of substrates for NADH and succinate oxidation [26]. The proposal that SCs may provide distinct electron translocation pathways through the partition of CoQ into different pools to mediate metabolic adaptation [26], was questioned by kinetic and flux control studies showing that the metabolic pathways for NADH and succinate oxidation comprise different CoQ redox steady states, but communicate and converge on a single non-partitioned CoQ pool [27,30]. It has also been argued that the *cytc* pool is equally compartmentalized [26], but studies monitoring the reduction potential of CIII upon addition of NADH, succinate or both [27], evidenced against *cytc* partitioning. In agreement, time-resolved spectroscopic analysis of *cytc* oxidation in intact yeast cells showed that *cytc* is not trapped within SCs and, therefore, there are no restrictions that limit its diffusion [28]. Moreover, the respirasome structures showed no evidence of a protein-mediated substrate channel connecting the CoQ binding sites of CI and CIII₂ [22], since both active sites are open to the membrane and separated by 10 nm, as also evidenced for *cytc*, in agreement with previous studies that questioned substrate channelling based on the distances between the substrates binding sites on SCs [47]. Therefore, the function of a direct catalytic role for mitochondrial SCs remains questionable [30].

3.2. Assembly and stability of complex I

Experimental evidence accumulated on respiratory chain disease models, suggests that the formation of mammalian SCs confer stability to their individual components, and most particularly to CI. The first description of a patient with progressive exercise intolerance due to a nonsense mutation in the CIII₂ subunit gene *MT-CYB* associated with a combined enzyme deficiency of complexes I and III [66], was followed by a more extensive study showing that genetic alterations leading to a loss of CIII₂ prevented respirasome formation and led to the secondary loss of CI [67]. Further studies confirmed that not only the structural integrity of CIII₂ [68], but also that of CIV [69–71], were essential to maintain the stability of mammalian CI. Despite these evidences, ostensible CI functional alterations are relatively infrequent to most patients presenting with CIII₂ or CIV enzyme deficiencies [72,73], indicating that only severe structural alterations of these two complexes induce a parallel CI dysfunction. On the contrary, a dramatic decrease in CI levels do not generally lead to CIII₂ and CIV functional defects in mammals [67]. In agreement, the depletion of 28 different CI accessory subunits in human HEK293T cells showed a loss of SCs I+III₂ and I+III₂+IV₁ with no alterations in the steady-state levels of CIII₂ or CIV [74].

The specific dependence of CI stability on the respirasome biosynthesis has important repercussions for our understanding of the respiratory chain disorders. In this regard, two main hypotheses are currently debated to explain the integration and stabilization of CI into SCs (Figures 3 and 4, see Section 5.1.). However, the fact that CI is purified and remains active in its free form adds to the debate whether CI stability may rely on additional molecular mechanisms, like alterations in the mitochondrial membrane potential [49] or ROS levels (see Section 4.3.). In this regard, the absence of respirasomes due to the lack of *cytc* [75], CIII₂ or CIV induced reverse electron transport from reduced CoQ to CI, triggering local superoxide generation and CI degradation [76].

3.3. Modulation of ROS production

Because CI and CIII₂ constitute the main redox centres responsible for oxygen reduction to superoxide [77,78], it has been hypothesized that their arrangement into SCs could minimize ROS production. Measurements in bovine heart mitochondria provided the first demonstration that the disruption of SC I+III₂ enhanced the generation of superoxide from CI [79], and a direct correlation between ROS levels and CI dissociated from SCs was established in neurons and astrocytes [80]. According to the ovine respirasome structure [22], the two CoQ binding cavities on CIII₂ are arranged in such way that the symmetry of CIII₂ is broken, and this asymmetry could limit ROS production at the expense of maximal activity.

Although decreased ROS production may be a functional consequence of SCs, oxygen levels can also modulate the assembly of these superstructures and the efficiency of mitochondrial respiration. For instance, during prolonged hypoxia, potato mitochondria showed a rearrangement of CI from SC I+III₂ to its free form [36]. Or studies in mouse fibroblasts lacking the Rieske Fe-S protein of CIII₂ showed that enhanced ROS disrupted SCs and produced a deleterious effect on the stability of complexes I and IV [81]. One way the cells adapt to hypoxic conditions is by building a more efficient respiratory chain through CIV, a key enzyme composed of oxygen-regulated subunit isoforms in yeast and mammals [82–84]. Variations in oxygen levels may thus affect the assembly state of CIV and its incorporation into respirasomes. Aging, a fundamental biological process that affects all eukaryotic lives, is generally attributed to increased oxidative damage induced by ROS, and this is usually accompanied by a decay of SCs levels [85]. Therefore, the interplay between oxygen levels, superoxide production, association and dissociation of SCs and impaired respiration, arises as a crucial regulatory process in health, disease and ageing.

4. Biogenesis of respiratory chain supercomplexes

4.1. The respirasome assembly pathway

The precise mechanisms that regulate the biosynthesis of mammalian mitochondrial SCs remain unsolved. Two models have been proposed to explain the association of CI with complexes III and IV to form the respirasome [45,86]. The first model (Figure 3) proposes that CI gets fully-assembled prior to its binding to SCs [86]. Time-course incorporation analyses of the 13 radiolabelled mitochondrial-encoded polypeptides into complexes and SCs showed the existence of a temporal gap between the formation of the complexes and

their co-localization in SCs, suggesting that SCs originate by the direct association of single preassembled complexes [24]. Recent proteomics studies based on BN-PAGE and complexome profiling of CI intermediates upon mitochondrial translation inhibition with chloramphenicol, agreed that CI was independently assembled before SC formation [86]. In contrast, previous studies from the same group in *Ndufs4*-KO mice had shown that CI lacking its functional NADH-binding (N) module is associated with CIII₂ in a partially-assembled SC [87], suggesting that the biogenesis of SCs does not necessarily require the preassembly of individual complexes. The possibility that the formation of the respirasome could be achieved instead through a coordinated association of submodules and free subunits was supported by the observation in *Neurospora crassa* that the assembly of SC I+III₂ occurs before the individual CI is formed [88], and by studies showing that, in mitochondria from patients with chronically reduced CIV levels, newly-imported COX subunits preferentially integrate into SCs [89,90]. In accordance with this idea, we analysed the formation of SC assembly intermediates by reversibly depleting control cell lines of OXPHOS complexes by long treatment with doxycycline, a reversible inhibitor of mitochondrial translation. Results led us to propose a second model (Figure 4) that involves the sequential binding of subcomplexes from CIII₂ and CIV to an almost complete CI scaffold that lacks the N catalytic module, which would be incorporated at the end of the assembly process to ensure the activation of the respirasome once all the essential structural components are present [45]. This model implies that the SCs constitute the structural units where CI gets fully-assembled and activated, providing a basis to explain the relevance of the respirasomes for CI stability [67] and why certain mutations in CIII₂ or CIV-associated genes lead to a combined enzyme deficiency of CI [45]. The differences between both CI assembly models [45,86] could be attributed to technical differences related to the use of two different mitochondrial translation inhibitors, the length of the inhibition periods, or the time gaps between the collected samples, which probably affect the balances between the synthesis of individual MRC subunits and their kinetics of incorporation into the different MRC structures. Although the first model [86] supports the dynamic exchange of CI between its free form and a SC-associated form, the fact that “free CI” is underrepresented in mammalian tissues under mild purification conditions [33] mainly agrees with our statement that SCs provide a scaffold for the full-assembly and stability of CI. Additionally, our model supports the idea that, when “unable to associate to / or once dissociated from” SCs, free CI is prone to degradation by, i.e. locally-produced superoxide [76,79]; moreover, it does not exclude the dynamic exchange of CIII₂ and CIV once the respirasome assembly has been completed (being compatible with the variable stoichiometry of CIV within SCs), and it allows for the dynamic replacement *on site* of specific CI reassembled modules to avoid futile continuous cycles of turnover and *de novo* synthesis of this large complex. In this regard, we agree with the previous reports where a CI intermediate lacking the N catalytic module is stably associated in SCs [55,87,91]. Extensive structural studies in cellular and animal models of respiratory chain disease are necessary to clarify the intriguing mechanisms that govern the stepwise biogenesis of the mitochondrial respirasome.

4.2. Supercomplex assembly factors

An important issue is the regulatory function that chaperones or assembly factors play on the assembly of SCs and respirasomes. Here we will discuss the roles of the two best-studied protein types defined as SC assembly factors: COX7A2L and the RCFs.

4.2.1. COX7A2L/COX7RP/SCAFI—An interesting debate concerns the regulatory role of the protein COX7A2L/COX7RP in the formation and stabilization of mitochondrial SCs. COX7A2L was first reported to be present in the respirasomes and SC III₂+IV₁ but not in CIII₂ or free CIV, therefore constituting the first SC-specific assembly factor that was renamed SCAFI [26]. Enríquez and co-workers discovered that certain wild-type mouse strains widely used in biological research, e.g. C57BL/6J and BALB/c, were homozygous for a 6 bp deletion in the *Cox7a2l* gene and expressed a short, unstable COX7A2L isoform that failed to support CIV association into SCs, thereby promoting differences in mitochondrial respiration rates and ATP production. The authors proposed that COX7A2L is a SC-specific assembly factor that adapts respirasomes formation and mitochondrial function to metabolic variations [26,29]. This hypothesis was challenged by Larsson and co-workers, who demonstrated normal respirasome formation and respiratory chain function in liver and heart mitochondria from mice strains bearing the truncated COX7A2L isoform [92], which was conclusively supported by other studies [93–97]. A recent work from our laboratory showed that COX7A2L is neither essential for respirasome formation in human cell lines [98]. This protein is not uniquely found associated in SCs, since it independently interacts with both CIII₂ and free CIV to promote the stabilization of SC III₂+IV₁, results later supported by other groups [84,96,99]. In the absence of CI and CIV, COX7A2L remains associated with CIII₂-containing SCs, thereby showing a preferential association for CIII₂ [98]. The fact that the formation/stabilization of mammalian SC III₂+IV₁ is regulated by a specific protein in a respirasome-independent manner implies the co-existence of independent regulatory mechanisms for the biogenesis and turnover of different SC structures that deserve further attention.

Variations observed among tissues in the relative distribution of BN-PAGE bands above the canonical respirasome (SC I+III₂+IV₁) in mice strains bearing the short COX7A2L isoform [94–96], opened the possibility that the assembly of specific SCs could be regulated in a tissue-specific manner. The high sequence similarity between COX7A2L and tissue-specific isoforms of the CIV subunit COX7A, led to speculate that COX7A2L may replace COX7A in SCs, acting as a bridge to stabilize the interaction between CIII₂ and CIV [22]. Mass spectrometric analyses of SCs from CD1 mice mitochondria (that express the long COX7A2L isoform), revealed that COX7A2 was present almost exclusively in CIV_{1–2}, whereas COX7A2L was present in SCs III₂+IV and I+III₂+IV_{1–4} [84]. These observations suggested that the homologous region of COX7A2L could displace COX7A from CIV to form a tight CIII₂-CIV interface within SCs, while in the absence of COX7A2L, respirasomes would form with a weaker CIII₂-CIV interface solely stabilized by interactions with CI. This possibility remains to be confirmed by higher resolution cryo-EM density maps of the “tight” and “loose” respirasome structures that allow identification of the individual residues [22].

4.2.2. Rcf 1–3—Three independent groups identified two respiratory complex factors (Rcf1 and Rcf2) that control the formation and stabilization of yeast SC III₂+IV₁₋₂ [100–102]. Rcf1 and Rcf2 belong to the conserved *hypoxia induced gene 1* (Hig1) protein family, and both are CIV-binding proteins that may also interact with CIII₂. Regarding Rcf1, deletion mutants (*Rcf1*[−]) showed an impaired incorporation of Rcf2, and subunits Cox13 and Cox12 (Cox6a and Cox6b in mammals, respectively) into CIV [101,102], resulting in defective CIV activity, decreased SC III₂+IV₁₋₂ levels and increased ROS production [100,102]. Remarkably, SC III₂+IV was detectable in the *Rcf1*[−]/*Rcf2*[−] double mutants, indicating that the core of CIV could still bind CIII₂ in the absence of these proteins [101]. These results, as well as recent kinetic and spectroscopic studies in *Rcf1*[−] and *Rcf2*[−] mutants [103], revealed the presence of at least two different enzymatically-active CIV forms, which differ in their protein composition and are independently incorporated into SCs, maybe to adapt the respiratory function to different physiological conditions. Rcf1 interacts with the Aac2 protein [101], a member of the ADP/ATP carrier protein family that facilitates the equimolar exchange of ATP for ADP across the inner membrane [104]; therefore, changes on the conformational state of the Aac2 protein could influence the binding of Rcf1 to CIV and the regulation of its activity [101]. Latest studies showed that Rcf1 interacts with CIV to regulate late stages of its assembly process [105], suggesting that it functions in the assembly of individual CIV rather than playing a direct role in SC III₂+IV₁₋₂. Rcf1 eukaryotic homologs include two variants, *HIGD1A* and *HIGD2A*, that display the broadest expression pattern in mammals [100]. Silencing of *HIGD1A* in mice C2C12 cells did not induce structural alterations on mammalian SCs [100]; instead, this protein was shown to bind CIV in early assembly stages [107], and to upregulate CIV activity under hypoxic cellular stress [106]. However, *HIGD2A* knockdown caused the depletion of SC III₂+IV₁ and of CIV-containing bands above the SC I+III₂+IV₁ without altering free CIV levels [100], suggestive of a true SC stabilizing role. Rcf2 is found in yeast species, and it is specifically required for CIV assembly [101,102]. Recent studies showed that Rcf2 is processed into a stable C-terminal fragment that remains associated with CIV and SCs, and a labile N-terminal fragment that displays high sequence similarity with another mitochondrial protein, Rcf3 [107]. Rcf2 and Rcf3 associate predominantly with CIV and CIV-containing SCs in the inner membrane, and in the absence of any of these proteins, CIV activity is increased. However, *Rcf2*[−]/*Rcf3*[−] double mutants displayed a decrease in CIV activity that resulted in the loss of yeast respiratory growth, indicating an overlapping function of both proteins. Whether the Rcf proteins are truly SC assembly factors is a matter of discussion, as they primarily assist CIV assembly rather than the biogenesis of SCs. However, the fact that all three Rcf proteins remain associated with CIII₂ in the absence of a functional CIV [107], open new prospects regarding their functional implications, as well as those of their human orthologues, in the assembly of SCs.

5. Conclusions and Perspectives

The structural and functional organization of the respiratory chain has been long debated. Although the existence of specific associations of complexes I, III and IV into higher-order structures (i.e., respirasomes and intermediate SCs I+III₂ and III₂+IV₁₋₂) is generally accepted, their functional relevance remain unsolved. Recent structural advances in the

composition and organization of the mammalian respirasomes represent a significant step forward to understand the functional consequences of the dynamic rearrangements between individual MRC complexes and SCs, as well as their implications in the regulation of the respiratory chain function in different physiological conditions. This is of particular importance for the understanding of the molecular mechanisms underlying respiratory chain disorders and ageing, where a general deterioration of SCs formation is commonly observed. The following research excitingly point towards the decryption of the specific interactions that govern the bindings among the individual complexes in a variety of superstructures, and to unravelling the molecular mechanisms and players that regulate SCs formation.

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ABBREVIATIONS

MRC	mitochondrial respiratory chain
CoQ	coenzyme Q or ubiquinone
cytc	cytochrome <i>c</i>
SC	supercomplex
CI–CIV	respiratory chain complexes I to IV
CIII₂	dimeric CIII
CV	ATP synthase
BN-PAGE	blue native gel electrophoresis
EM	electron microscopy

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HIGHLIGHTS

- The respirasome is the most abundant supercomplex in mammalian mitochondria.
- The functional consequences of the supercomplexes remain questioned.
- There are two current hypotheses for the biogenesis of the respirasome.
- The biogenesis of SC III₂+IV is independent from the respirasome.
- The recent structures of the respirasomes represent an important novelty.

Figure 1. Architectures of the mammalian respirasomes

Side views along the membrane from (A) Supercomplex I+III₂, (B) the tight respirasome, and (C) the loose respirasome, according to the structures proposed by Letts *et al.* [22].

Images were obtained from the RCSB Protein Data Bank in combination with the NGL viewer. The structural models of CI, CIII₂, and CIV are colored in red, turquoise, and navy blue, respectively. The transmembrane region is indicated by two dashed lines. M, matrix; IM, mitochondrial inner membrane; IMS, intermembrane space.

Figure 2. Distribution of human respiratory chain complexes in supercomplexes

(A) Mitochondria isolated from cultured 143B cells (1) and cybrids (2) were analysed by BN-PAGE in combination with CI *in-gel* activity (IGA) assay and western blot with antibodies against the indicated MRC subunits. (B) Most CI (blue), ~half of CIII₂ (green) and ~20–30% of CIV (red) are localized in the respirasome (I+III₂+IV₀₋₁). SC III₂+IV₁ represents ~5% of the total amount of MRC structures, as well as CIV dimers (IV₂). CII (II) is not present in SCs. Free CI (light blue) requires to be associated in supercomplexes to minimize destabilization and ROS generation [29].

Figure 3. Respirasome biogenesis through the direct association of fully-assembled respiratory chain complexes

This model [24,86] proposes that the mammalian respirasome (I+III₂+IV₁) originates by the direct association of single preassembled CI (blue), CIII₂ (green) and CIV (red). The assembly pathways of the individual MRC complexes are depicted according to established models [72,86,108], and SC III₂+IV₁ is formed independently of the respirasomes [98]. Supercomplex assembly factors COX7A2L and HIGD2A are marked in green/red.

Figure 4. Respirasome biogenesis through the stepwise association of partially-assembled respiratory chain complexes and submodules

This model [45] proposes the sequential and coordinated association of submodules and free subunits from CIII₂ (green) and CIV (red) to a CI-scaffold (blue) that lacks the N catalytic module, which is incorporated at the latest assembly stage to ensure respirasome activation in the presence of all the necessary structural components. The assembly pathways of individual CIII₂ and CIV are depicted according to established models [72,108], and SC III₂+IV₁ is formed independently of the respirasomes [98]. Supercomplex assembly factors COX7A2L and HIGD2A are marked in green/red.