Biochimica et Biophysica Acta 1787 (2009) 672–680

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Supramolecular organization of ATP synthase and respiratory chain in mitochondrial membranes

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ARTICLE INFO

Article history:
Received 28 November 2008
Received in revised form 29 December 2008
Accepted 29 December 2008
Available online 8 January 2009

Keywords:
ATP synthase
Respiratory chain
Supramolecular organization
Protein–protein interaction
Mitochondria

ABSTRACT

Mitochondrial ATP synthase is mostly isolated in monomeric form, but in the inner mitochondrial membrane it seems to dimerize and to form higher oligomeric structures from dimeric building blocks. Following a period of electron microscopic single particle analyses that revealed an angular orientation of the membrane parts of monomeric ATP synthases in the dimeric structures, and after extensive studies of the monomer–monomer interface, the focus now shifts to the potentially dynamic state of the oligomeric structures, their potential involvement in metabolic regulation of mitochondria and cells, and to newly identified interactions like physical associations of complexes IV and V. Similarly, larger structures like respiratory strings that have been postulated to form from individual respiratory complexes and their supercomplexes, the respirasomes, come into the focus. Progress by structural investigations is paralleled by insights into the functional roles of respirasomes including substrate channelling and stabilization of individual complexes. Cardiolipin was found to be important for the structural stability of respirasomes which in turn is required to maintain cells and tissues in a healthy state. Defects in cardiolipin remodeling cause devastating diseases like Barth syndrome. Novel species-specific roles of respirasomes for the stability of respiratory complexes have been identified, and potential additional roles may be deduced from newly observed interactions of respirasomes with components of the protein import machinery and with the ADP/ATP translocator.

1. Introduction

The oxidative phosphorylation system in the inner mitochondrial membrane of higher eukaryotes comprises five major membrane protein complexes, the mitochondrial complexes I–V [1]. Complexes I–IV constitute the respiratory chain which is completed by complex V, the ATP synthase. Respiratory complexes I and II (NADH:ubiquinone oxidoreductase and succinate:ubiquinone oxidoreductase) pass electrons from the redox substrates NADH and succinate onto complex III, the ubiquinol:cytochrome *c* oxidoreductase, and finally to cytochrome *c* oxidase that reduces oxygen to water. This electron transport is coupled to an outward transport of protons across the inner mitochondrial membrane generating an electroosmotic proton gradient across the inner membrane. This gradient is then used by complex V to synthesize ATP from ADP and phosphate.

Mitochondrial ATP synthase was depicted for decades as a monomeric multiprotein complex although electron microscopic studies often showed mitochondrial membranes studded with closely neighbouring F_1-headpieces. This close packing was explained by the high abundance of F_1F_O ATP synthase. Dimeric and oligomeric structures were noticed only in 1989 when Allen et al. identified helical twin rows of F_1-headpieces winding around tubular cristae of *Paramecium* mitochondria [2]. Around one decade later, dimeric ATP synthase could be isolated with high yield from yeast [3] using blue-native electrophoresis (BNE, Ref. [4]). Higher oligomeric structures of ATP synthase can now be isolated ideally by clear-native electrophoresis (CNE, Refs. [5,6]). Proposed functional roles of oligomeric ATP synthase are stability advantages [3] and a special role for cristae formation as postulated by R.D. Allen in 1995 [7]. In fact, studies of the mitochondrial morphology of yeast mutants with destabilized ATP synthase dimers confirmed the concept that dimeric and oligomeric ATP synthase is involved in determining mitochondrial cristae morphology [8].

Models for the structural organization of respiratory chain complexes I–IV historically oscillated between two extremes, a "solid state" model [9] with the respiratory complexes I–IV arranged in an orderly sequence, and a "liquid state" model (see Ref. [10] for the *Random Collision model* by Hackenbrock et al.) that envisioned individual complexes diffusing laterally in the membrane and independently of one another. The solid state model was initially supported by the isolation or reconstitution of stoichiometric assemblies of two or more complexes [11–16] and by some kinetic analyses (see [17] for review). The liquid state model was essentially based on the isolation of functional individual complexes [1], on the failure of electron microscopic and liposomal fusion studies to identify associations of complexes [18], and on the pool behaviour of

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0005-2728/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2008.12.016

ubiquinone and cytochrome *c* in bovine mitochondria [19,20]. Direct association of respiratory complexes seemed not required for effective electron flow, so that the liquid state model gradually became widely accepted. The isolation of stable supercomplexes of respiratory complexes III and IV from certain bacteria [21–23] could not challenge this view. The paradigm of how the mitochondrial respiratory chain is organized changed however, when a variety of respiratory supercomplexes, stoichiometric assemblies of respiratory complexes I, III, and IV, also termed respirasomes, could be isolated with high yield [24,25]. Recently it has been proposed that the assembly of respiratory complexes into supercomplexes is just the first step in the formation of much larger supramolecular structures like respiratory strings [26]. As possible functional roles of respiratory supercomplexes catalytic enhancement, substrate channelling, stabilization of mitochondrial complexes by association to supercomplexes, and sequestration of reactive intermediates were proposed [24]. Some of these suggested functions have been verified experimentally: for example stabilization of complex I by supercomplex formation was shown for a bacteria [27] and also for human cells and skeletal muscle biopsies of patients with mitochondrial disorders [28,29]. Substrate channelling of electrons between complexes I and III has been verified by flux control studies [30].

Here we summarize recent progress in the analysis of the supramolecular organization of complexes in the inner mitochondrial membrane and the characterization of isolated fragments of these supramolecular structures. We discuss the functional advantages of the supramolecular organization, the potential roles of recently identified associated proteins of mitochondrial complexes, and the lipid and protein components linking the individual complexes and supercomplexes together.

2. Oligomeric ATP synthase

Subunit composition of monomeric and dimeric yeast and bovine ATP synthase, assembly factors, and the newly identified associated proteins AGP and MLQ have been summarized recently [31]. There is profound knowledge on the mechanism of ATP synthase and the structural properties of the monomeric enzyme as exemplified by some milestones in the field ([32–37], see also Ref. [38] for a recent review). However, experimental evidence for the functional roles of dimeric and oligomeric ATP synthase is just beginning to emerge.

2.1. Evidence for dimeric and oligomeric mitochondrial ATP synthase

First visualization of oligomeric ATP synthase in mitochondrial membranes from *Paramecium multimicronucleatum* was achieved in 1989 by Allen et al. [2] using rapid-freeze deep-etch electron microscopy. The appearance of double rows of putative F_1-heads of ATP synthase suggested that dimeric ATP synthase was the building block for the oligomeric structures. Ten years later, dimeric ATP synthase could be isolated with high yield from yeast and bovine mitochondria [3,24]. Rows of presumed F_1-heads were also seen by electron cryo-tomography of *Neurospora crassa* mitochondria [39] and dimer ribbons of putative ATP synthase seemed to shape the bovine and rat liver inner mitochondrial membrane [40]. Rows of dimeric particles with the expected size of ATP synthase have also been detected in yeast mitochondrial membranes using atomic force microscopy [41]. None of the above studies, however, used specific antibodies in order to verify the observed particles as ATP synthases. This might be less critical for electron microscopic studies that can rely on electron microscopy of isolated monomeric and dimeric ATP synthase [42–46] but seems ambiguous for the atomic force microscopy study [41].

For isolation of oligomeric ATP synthase by native electrophoresis it was important to use very low amounts of the mild detergent digitonin to solubilize the membranes. Separated oligomeric forms of ATP synthase were then visualized in the native gels by in-gel ATP hydrolysis assays [5,6]. The almost exclusive isolation of even-numbered oligomers like tetramers, hexamers and octamers suggested again that oligomeric ATP synthase is formed from dimeric building blocks [5]. Using a higher digitonin/protein ratio for membrane solubilization induces breakdown of the oligomers into dimers and monomers.

2.2. Electron microscopic analyses of isolated dimeric ATP synthase

Electron microscopic structures of dimeric ATP synthase are available from the alga *Polytomella* [43], from bovine heart [44], and from yeast [45,46]. Depending on the analyzed species, the membrane parts of both monomers are joined at angles of 35–90° [43–45] or 55–142° [46], supporting the postulate that dimeric ATP synthase is involved in bending membranes and in generating cristae morphology [8,40]. An angle of around 70° between the two monomeric ATP synthases in the dimeric enzyme from *Polytomella* keeps the F_1-head pieces well separated excluding direct F_1–F_1 interactions. The dimerization interface in *Polytomella* therefore must be formed by the membranous F_O-parts (marked yellow and pink in Fig. 1A). The model of dimeric ATP synthase, as presented in Fig. 1A, is largely based on structural information for the monomeric enzyme [42,47–49] and the recently proposed dimerization via subunit a [50]. The model for further association of the dimeric enzyme complex into oligomeric structures (Fig. 1B, C) is largely based on cryo-electron microscopic studies of mitochondria [40], and on the roles of subunits e and g [51–56]. Two alternative modes to assemble dimers further into oligomeric structures are conceivable: (i) linkage of membrane domains e.g. via subunits e and g (open and filled black circles in Fig. 1B), and (ii) linkage of the F_1-parts e.g. by dimeric inhibitor protein $(IF_1)_2$. However, assignments of proteins like subunits e, g, and IF_1 to dimerization interfaces are speculative, since specific antibodies have not been used so far. Dimer ribbons of ATP synthase that shape mitochondrial cristae and small mitochondrial vesicles [40] are drawn schematically in Fig. 1C. The dimer ribbons seem to assemble from two dimer types assigned as “true dimer” and “pseudo dimer” [45]. The “true dimer” or “dimeric building block” [5,31,45,50] is viewed from the front (Fig. 1A) and from the top, i.e. looking at the inner mitochondrial membrane from the matrix side (Fig. 1B). It seems to be characterized by large angles (70–90°) between the ATP synthase monomers. “Pseudo dimers” seem to be characterized by small angles (exemplified by a 20° angle in Fig. 1C). “Pseudo dimers” may arise from decaying dimer ribbons when the interface between two adjacent dimers in the direction of oligomerization is preserved but the monomer–monomer interfaces in the dimeric building blocks are broken.

Bovine ATP synthase dimers show a small angle (around 40°) between the two ATP synthase monomers and contacts between two F_1-headpieces were observed [44]. It is not known at present whether “pseudo dimers” were a result of the specific isolation protocol used or whether the small angle can be explained by species-specific variation of angles in the dimeric building blocks. Studies of the yeast dimeric enzyme using the detergent digitonin [45] revealed two angles around 35–40° and 70–90° suggesting that both dimer types can be isolated from oligomeric yeast ATP synthase. Other studies using the detergents BigCHAP and digitonin to solubilize yeast mitochondria showed that the isolated dimers displayed a wide variability of angles between the two monomers (55–142°) with the majority of the particles displaying an angle around 90° [46]. This suggested that detergents contribute considerably to the pleomorphism of the isolated dimers, and provides an alternative explanation for the observed small angle dimers instead of assuming “pseudo dimers” with fixed angles.

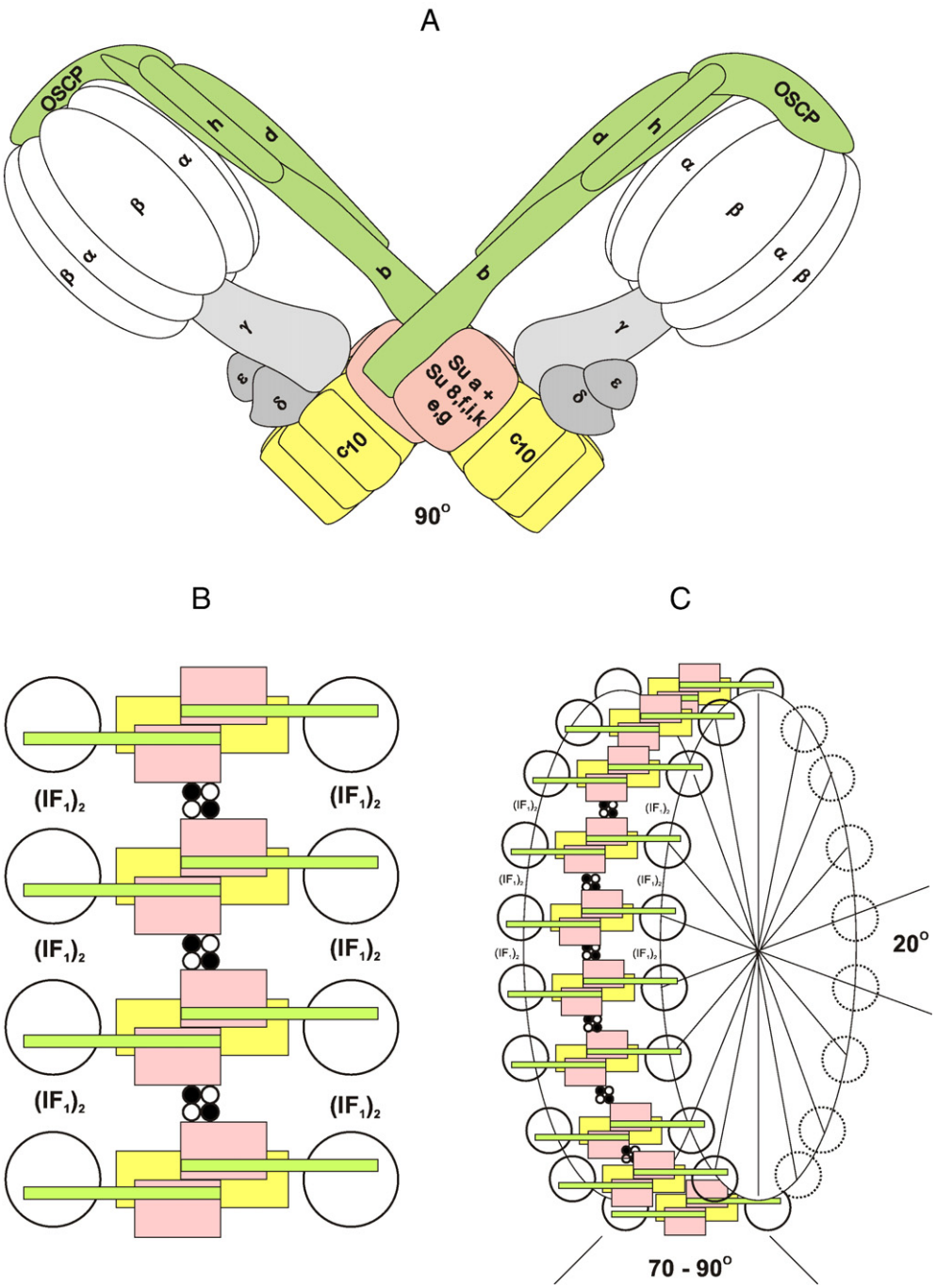

Fig. 1. Model of the structural organization of mitochondrial ATP synthase. (A) Model of dimeric ATP synthase viewed as cross-section through the inner mitochondrial membrane. The model is based on structural information for the monomeric enzyme [42,47–49] and on dimerization via dimeric a-subunit [50]. The model for further association of the dimeric building blocks into higher oligomeric structures (B, C) is viewed from the matrix side looking at the inner mitochondrial membrane. It is largely based on cryo-electron microscopic studies of mitochondria [40] and on the roles of subunits e and g [51–56]. Oligomerization of dimeric ATP synthase seems to involve subunits e and g (open and filled black circles in B). Dimeric inhibitor protein $(IF_1)_2$ is discussed as a potential linker of F_1-headpieces. Large angles (70–90°) in figure parts A and C have been proposed to occur between the monomeric ATP synthases in the dimeric building blocks or "true dimers". Small angles (exemplified by a 20° angle in figure part C) may characterize the interface between neighbouring dimers, the "pseudo dimers".

2.3. Interfaces in dimeric and oligomeric ATP synthases

Subunits e and g have been identified early as subunits important to stabilize dimeric yeast ATP synthase, since monomeric but almost no dimeric ATP synthase could be isolated by BNE from null mutants of both subunits [3]. Subunits e and g are important for the stability of dimers but are not essential for their formation in the membrane, since association of monomers has been demonstrated in the mitochondrial membranes of yeast null mutants of these subunits by FRET analyses [51]. In accordance with this result, significant amounts of dimeric ATP synthase could be isolated from these mutants by CNE which is milder than BNE [50]. The domains of subunits e and g that are important for the stabilization of the dimer and presumably also for the oligomerization of ATP synthase were studied by the groups of Jean Velours and Rosemary Stuart [52–56]. Other subunits that are found in the

same monomer–monomer interface and help to stabilize dimeric ATP synthase are subunits b, i, and h (see [31] for review). Recently, it has been postulated that the highly hydrophobic mitochondrially encoded a-subunit is most important for dimerization, since dimeric a-subunit associated with two c-rings ($c_{10}a_2c_{10}$) has been isolated from dimeric ATP synthase. This suggests that the dimeric a-subunit is the bridging module between two monomers of ATP synthases [50].

The interface of dimers in extended assemblies of oligomeric ATP synthase is less well characterized. Subunits e and g are candidate proteins for the dimer–dimer interface in addition to their role in stabilizing the monomer–monomer interface, since oxidation of mitochondrial membranes generated disulfide-bridged g–g and e–e homo-dimers with oligomeric ATP synthase but not with the dimeric form [52,55]. Another candidate to link dimers together and to induce oligomerization of ATP synthase is the natural inhibitor protein IF_1 from bovine mitochondria or the homologous Inh1 protein in yeast. These inhibitor proteins are especially interesting, since IF_1 has been shown to link soluble bovine F_1-subcomplexes in a pH dependent manner [57,58]. Therefore, it seemed conceivable that IF_1 might also be able to link holo-ATP synthases in a pH-regulated way. ATP synthase activity and mitochondrial morphology might thus be regulated by the oligomeric state of ATP synthase. This idea was not supported by studies of yeast strains with deletion of the inhibitor protein Inh1 and the associated proteins Stf1, Stf2, and Sfl2. Inhibitor and associated proteins were neither required for dimerization of ATP synthase [59] nor for oligomerization [50]. However, the situation in bovine and yeast mitochondria might be different. A recent report postulated that IF_1 promotes oligomerization of liver ATP synthase [60] but the observed effects are rather small compared to the effects of unintented experimental variation that can be expected with the actual detergent/protein ratio used. Another recent paper reports on the regulation of mitochondrial structure and function by the inhibitor protein IF_1 [61]. Specifically, the density of mitochondrial cristae was found to increase with IF_1 overexpression which was described to promote formation of dimeric ATP synthase and ATP synthase activity. Therefore, IF_1 was proposed to regulate mitochondrial function and structure under both physiological and pathological conditions. However, the identification and quantification of dimeric ATP synthase complexes in this study seems questionable, since it is well known that under the given conditions the combination of dodecylmaltoside and BNE would dissociate almost all dimeric ATP synthase into the monomeric form.

Further candidates potentially involved in oligomerization are subunits f and subunit 8 that had not been identified in the monomer–monomer interface, and the carriers for adenine nucleotides and inorganic phosphate that have been described to form a supercomplex together with ATP synthase, the so-called ATP synthasome [62,63]. Assuming that major amounts of ATP synthase are assembled in ATP synthasomes, the previously described dimer ribbons of ATP synthase [40] or helical double rows of ATP synthase [2] should rather be regarded as oligomeric ATP synthasomes. Another type of physical interaction with complex V, namely between cytochrome caa_3 (complex IV) and F_1F_O-ATP synthase (complex V) of an alkaliphilic *Bacillus* has been demonstrated in a reconstituted system and points to a sequestered proton transfer during oxidative phosphorylation at high pH [64]. Direct physical interaction of complexes V and IV was also suggested by the surprising effects of alterations introduced in the gamma subunit of yeast ATP synthase. These alterations did not severely affect the amount and the catalytic activity of complex V but dramatically reduced the level and activity of complex IV by about 90% [65] which is similar to the effects of deletion of complex V subunits e and g [3,66]. Since the monomeric, dimeric, or oligomeric state of the ATP synthase and the protein levels of subunits e and g have not been analyzed in this study [65], it is unclear at present whether there is a correlation between the loss of oligomerization of complex V and the loss of complex IV.

2.4. Functional roles of oligomeric ATP synthase

Mitochondrial ATP synthase can be isolated as a monomeric, fully active and oligomycin-sensitive enzyme. Dimerization and oligomerization of ATP synthase are therefore not required for catalytic activity, but several possible reasons for the formation of these supramolecular structures are conceivable:

(i) Dynamic oligomerization is a regulatory principle to reduce ATP synthase activity in metabolic situations with low electron-transfer activity and low mitochondrial membrane potential [67]. It has been proposed that the modulated oligomeric ATP synthase activity by inhibitor protein IF_1, may participate in the conservation of ATP at the expense of the mitochondrial redox potential and may be involved in protecting against ischemic injury [61]. Inhibitor proteins might preferentially bind to oligomeric ATP synthase structures, so that oligomerization is the regulated first step, followed by binding of inhibitor proteins and loss of catalytic activity. However, oligomerization of ATP synthase does not depend on the presence of the natural inhibitor protein in yeast [50,59] suggesting that this mechanism may only apply for mammalia.

(ii) Connecting two stator parts in the dimeric enzyme can stabilize the holo-enzyme structure, in particular since dynamic rotor/stator interactions must continuously be closed and opened which facilitates dissociation of protein components and modules.

(iii) Another structural function of dimerization and oligomerization was introduced by R.D. Allen [7]: “as dimers link together into a band, a rigid arch may form that protrudes from the planar surface carrying the membrane with it”. Eventually, this would favour cristae formation and stability which in fact was experimentally verified [8]. Angular association of two ATP synthase monomers to a dimeric enzyme, which seems to induce bending of membranes, was also demonstrated directly in the membrane [40]. Multiple dimers that are arranged as dimer ribbons at the apex of cristae membranes therefore might induce a strong local curvature and are postulated to alter the local pH gradient by 0.5 units. This local proton trap has been proposed to ensure effective ATP synthesis under proton-limited conditions [40].

(iv) Supramolecular structures of respiratory chain complexes, ADP/ATP translocator, and ATP synthase and ordered arrangements relative to each other may favour fast metabolite/substrate channelling and/or efficient cooperation of complexes. In this way the supramolecular organization of the ATP synthase would indirectly affect the overall flux through the respiratory chain and result in a lower membrane potential. Stable association of ATP synthase to oligomeric structures could thus be essential to maintain bioenergetically fully competent mitochondria [67].

(v) Efficient cooperation of complexes IV and V has recently been suggested by the physical association of cytochrome caa_3 (complex IV) and F_1F_O-ATP synthase (complex V) of an alkaliphilic *Bacillus* [64]. The postulated sequestration of proton transfer seems favourable for oxidative phosphorylation at high pH and revives discussions about the proton path for optimal efficiency of energy coupling [64]. Direct physical interaction of complexes V and IV also seems important for the assembly/stability of complex IV [65]. Since the effects of specific alterations of the ATP synthase gamma subunit resemble the effects of deletion of subunits e and g [3,66], loss of oligomerization of complex V and loss of complex IV may correlate.

3. Suprastructures of respirasomes

The structures and functions of individual respiratory complexes I–IV have been studied in detail [68–71]. Supramolecular organization of these complexes into respirasomes and even larger structures adds further benefits for the optimal function of mitochondria and seems important to keep cells and organisms in healthy condition.

3.1. Respirasomes and higher oligomeric structures in mitochondrial membranes

The terms respirasome and respiratory supercomplex are synonyms for stoichiometric associations of respiratory chain complexes; e.g. a III_2IV_1-respirasome contains dimeric complex III and monomeric complex IV. Many mammalian and plant supercomplexes contain respiratory complex I, a dimer of complex III and up to 4 copies of complex IV ($I_1III_2IV_{0-4}$). Depending on the species analyzed the interactions of complexes are highly or moderately detergent-sensitive so that the mildest detergents and/or very low detergent/protein ratios have to be used for isolation. Following the first isolation of respiratory supercomplexes from yeast and bovine mitochondria [24,25], respiratory supercomplexes were identified and isolated from a vast variety of sources as summarized in a recent review on BNE and CNE [72]. The role of respiratory supercomplexes as functional NADH oxidases has been thoroughly studied using the bacterial $I_1III_4IV_4$-supercomplex from *Paracoccus denitrificans* [27]. This supercomplex contained around 10 molecules of ubiquinone and high amounts of phospholipid. Therefore, it was not surprising that the NADH: cytochrome *c* reductase activity (complex I + III) was close to the theoretically obtainable activity (1.5 μmol min^{-1} mg^{-1} instead of 2.2 μmol min^{-1} mg^{-1}). NADH oxidase activity (complex I + III + IV), however, was reduced by 74%. This loss of activity could be explained by a 40–80% loss of cytochrome c_{552} from the bacterial supercomplex during isolation [27]. Recently, the role of respiratory supercomplexes as functional NADH oxidases has also been verified for a mammalian mitochondrial supercomplex [73].

Several reasons led to the postulate that respirasomes are not the largest assemblies of respiratory complexes in the membrane but just the building blocks for higher oligomeric structures or megacomplexes:

(i) Mitochondrial complexes can be solubilized differentially. Complexes V and II from yeast and bovine mitochondrial membranes could be selectively and almost quantitatively solubilized under conditions that kept respiratory complexes III and IV, and in bovine mitochondria also complex I entirely in the sediment after centrifugation [3,74]. This suggested that complexes III and IV in yeast and complexes I, III, and IV in bovine mitochondria were associated to highly oligomeric structures that remained in the sediment after centrifugation because they were many times larger than soluble respirasomes with masses in the 1–3 MDa range. Suprastructures of respiratory supercomplexes have not been demonstrated unambiguously so far but several lines of evidence suggest a linear supramolecular assembly of alternating $I_1III_2IV_4$ and III_2IV_4 supercomplexes in mammalian mitochondria as depicted in the first "respiratory string model" (Fig. 2A) [26].

(ii) The respiratory string model also fits to the helical arrangement of particles observed by electron microscopy in *Paramecium* mitochondria [2]. Particles that lined up in regular intervals into helical structures winding around tubular mitochondrial cristae were tentatively assigned as respiratory complex I by R.D. Allen. Since we learned that respiratory complexes I, III, and IV assemble into respirasomes [24–29,75–80] it was tempting to speculate that the observed particles contained complex I as the structural core of respirasomes and that these respirasomes were linked to the observed helical structures or "respiratory strings".

(iii) Since dimeric bovine complex IV forms tetramers under certain conditions [Schägger, unpublished results], this suggested that respirasomes may be linked by tetrameric complex IV domains to form extended respiratory strings [26]. This supramolecular organization is in agreement with a previously described patchwork arrangement of respiratory complex I following fusion of mitochondria in human cells [81].

In the yeast *Saccharomyces cerevisiae* respiratory complex I is missing and dimeric complex III can bind only two copies of complex IV which is by a factor of two less than in mammalia. We propose that $IV_1III_2IV_1$-particles, as visualized in [82], associate into respiratory strings, as depicted in Fig. 1B, since dimeric yeast complex IV is identified in BN gels [5], and complexes V and II can be selectively and almost quantitatively solubilized under conditions that force respiratory complexes III and IV completely into the sediment after centrifugation.

Species-dependent links via dimeric complex I in addition to the complex IV-links seem conceivable, since putative complex I-dimers and/or I_2III_2-supercomplexes have been observed in 2-D BNE/SDS gels of mitochondria of two filamentous fungi [79–80], and particles that most likely represented $I_2III_2IV_2$-supercomplexes were identified by electron microscopic single particle analysis of solubilized potato mitochondria [83]. Based on these $I_2III_2IV_2$ units, an alternative respiratory string model for plants has been presented [83]. This model (Fig. 2C) seems similar to the proposed respiratory string model for yeast (Fig. 2B) except for the presence of complex I. It differs from the respiratory string model for mammalia (Fig. 2A) by direct complex I–complex I interactions and by the lower complex IV copy number which resembles the situation in yeast.

3.2. Electron microscopic studies of isolated respirasomes

2-D electron microscopic analyses of respiratory supercomplexes are presently available for the yeast *S. cerevisiae* [82], for potato [83], *Arabidopsis thaliana* [84], *Zea mays* [85], and for bovine heart [86]. Recently the first 3-D map of a respiratory supercomplex, the bovine $I_1III_2IV_1$-supercomplex has been determined [87].

The I_1III_2-supercomplex containing dimeric complex III, monomeric complex I, and trimeric γ-carbonic anhydrase (most likely as a heterotrimer) is the major supercomplex isolated from *A. thaliana* and *Z. mays* [84,85]. This type of supercomplex but without γ-carbonic anhydrase is also found in mammalian mitochondria as the "core-supercomplex" to which one copy of monomeric complex IV binds so tightly that the $I_1III_2IV_1$-supercomplex becomes the major species. Binding of this single copy of complex IV is stabilized by direct binding interactions with both, complex III and complex I [24,86,87]. Association of up to a total of four complex IV monomers is possible, but binding of the additional copies seems much more detergent-sensitive, since the fraction of isolated supercomplexes decreases considerably with the copy number of complex IV. The interface between complexes III and I is not exactly known except that membrane domains of both complexes are involved. Recent evidence from electron microscopic data on the bovine supercomplex suggests that interacting domains are similar in plants and mammalia [88]. Opposite orientations of complex IV binding have been suggested for the bovine $I_1III_2IV_1$-supercomplex and the yeast III_2IV_1 and $IV_1III_2IV_1$ supercomplexes. However, we think that the current resolution is not sufficient to draw this conclusion, since the species-specific differences between complex IV from yeast and mammalia are too large: yeast complex IV exists only in a monomeric state, except maybe in the proposed respiratory strings where two complexes IV from neighbouring supercomplexes can associate (Fig. 2B). In contrast, bovine complex IV can be isolated and crystallized as a dimer [89] and the ratio relative to complex III is

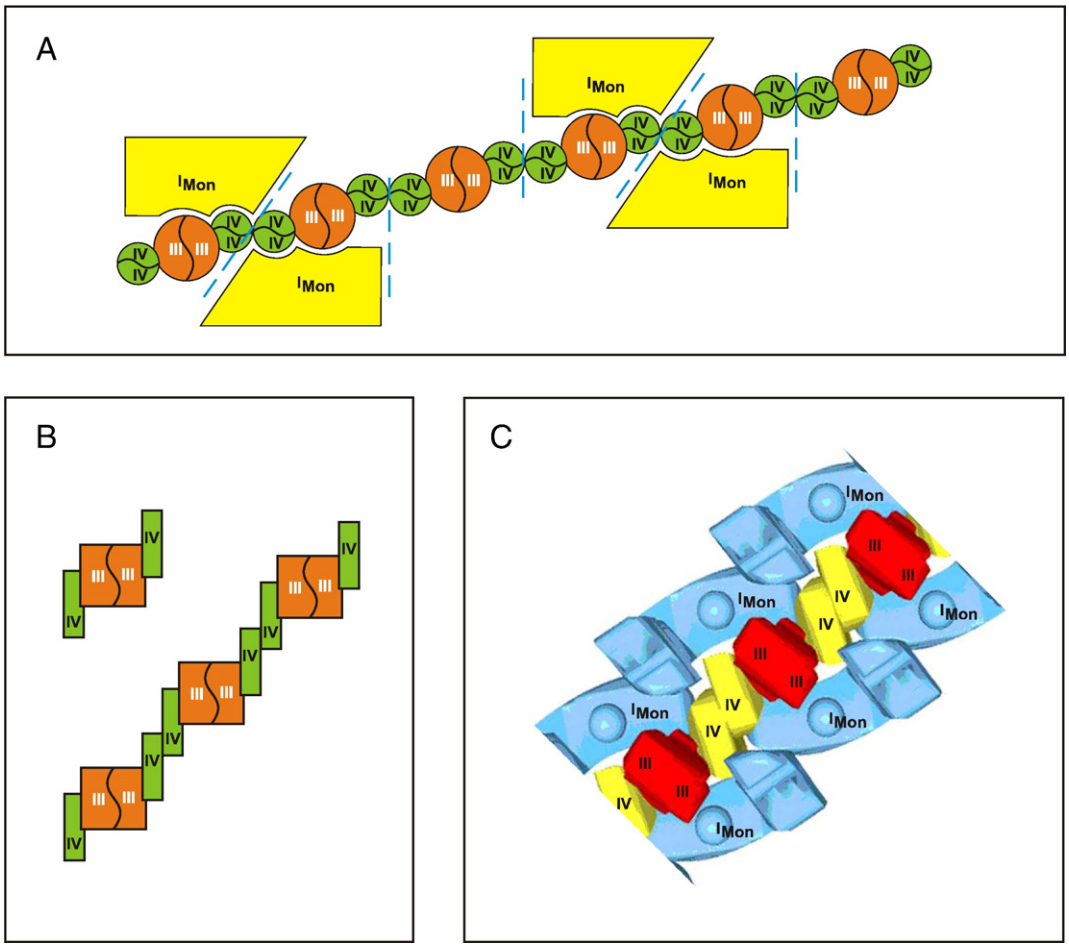

Fig. 2. Models for the species-specific association of respirasomes into respiratory strings. Complex III is generally marked red. Monomeric complex I (I_{Mon}) is marked yellow in A and blue in C. Complex IV is marked green in A and B, and yellow in C. (A) Respiratory string model for mammalian mitochondria characterized by tetrameric complex IV linkers (from [26] Biochim. Biophys. Acta 1757 (2006) 1066–1072, with permission). The building blocks, i.e. the larger and smaller supercomplexes containing and not containing complex I, are separated by dashed blue lines. (B) The yeast respiratory string model is characterized by a lower complex IV:complex III ratio and dimeric instead of tetrameric complex IV linkers. (C) Respiratory string model for potato mitochondria (modified from Ref. [83] Biochim. Biophys. Acta 1787 (2009) 60–67, with permission) resembling the yeast model (B) except for the involvement of complex I.

2:1 which is two-fold higher than in yeast. Up to two complex IV-dimers bind to the I_1III_2 core-supercomplex to generate the largest $I_1III_2IV_4$ supercomplexes [24]. More detailed structural and functional information beyond the electron microscopic data [82] is available only for the yeast respiratory supercomplexes.

3.3. Growth-dependent variation of yeast respirasomes

Yeast mitochondria, unlike mitochondria from most other sources, do not possess respiratory complex I and their complex IV content varies greatly with growth conditions. After fermentative growth on glucose medium for prolonged periods, for example, the mitochondrial content of respiratory complex III can be very low compared to non-fermentative conditions. The content of respiratory complex IV is even more dramatically reduced and often hardly measurable. Analysis of this situation by BNE reveals low amounts of monomeric complex IV (IV_1), much higher amounts of dimeric complex III (III_2) which is a structural and functional dimer, low amounts of the "small" respiratory supercomplex (III_2IV_1), and vanishing amounts of the "larger" supercomplex (III_2IV_2) containing two copies of complex IV [24]. However, if grown under non-fermentative conditions on glycerol/ethanol or lactic acid media, yeast cells require optimal content and function of respiratory complexes. Therefore, the content of mitochondrial complex IV increases under non-fermentative conditions until it equals the complex III content. Analysis by BNE ideally reveals almost no individual complexes III and IV, almost no small supercomplex (III_2IV_1) and almost exclusively the larger supercomplex (III_2IV_2). We have never observed a complex IV:III ratio larger than 1:1 in yeast mitochondria suggesting that no III_2IV_n-supercomplex containing more than two copies of complex IV is formed in yeast mitochondria [74]. A maximum of two copies of monomeric complex IV seems to be bound to the central complex III-dimer, as verified by 2-D electron microscopy [82]. The III_2IV_2 supercomplex therefore is better described as $IV_1III_2IV_1$ supercomplex. Dimers of complex IV can form in yeast if we assume that $IV_1III_2IV_1$ supercomplexes further associate into megacomplexes via their complex IV constituents (Fig. 2C).

3.4. Complex III–IV interface in the yeast respirasomes

Analyzing null mutants of a number of subunits of yeast complexes III and IV revealed that some subunits are not essential for supercomplex formation. These are the Rieske iron–sulfur protein Rip1p, Qcr6p, Qcr9p, and Qcr10p of complex III, and Cox8p, Cox12p, and Cox13p of complex IV. Loss of Qcr6p even promoted supercomplex formation. Including information obtained from a bacterial supercomplex [27] it could be concluded that specific transmembrane helices of cytochromes *b* and c_1 and of Qcr8p, forming a dent in the membrane part of complex III, are in physical contact with one of the mitochondrially encoded subunits of complex IV, Cox1, 2, or 3 [90].

Two phospholipids (cardiolipin L5) and phosphatidylethanolamine (L4) are bound in this dent formed by transmembrane helices of cytochromes *b* and c_1 and of Qcr8p [91]. In order to find out whether cardiolipin in general or the specific cardiolipin L5 at the interface between complexes III and IV is important for the assembly/stability of the respirasomes, we analyzed cardiolipin synthase deficient strains (Δ*crd1*) that are generally cardiolipin-deficient. We also analyzed mutant strains that cannot bind the specific cardiolipin L5, because specific lysines in the cardiolipin-binding transmembrane helix of cytochromes c_1 were replaced by neutral amino acids. General loss of cardiolipin caused instability of yeast respiratory supercomplexes under the conditions of BNE [74,90] which, however, could still be formed in the mitochondrial membrane and identified using the mild technique of CNE. Dowhan and coworkers could not identify supercomplexes in the cardiolipin-deficient strain by CNE. Therefore they suggested that cardiolipin is essential for supercomplex formation [92,93]. The question whether specifically cardiolipin L5 at the interface of complexes III and IV is important to stabilize the interaction of complexes is addressed by Wenz et al. in this volume [94]. Briefly: cardiolipin seems necessary to neutralize lysine charges in the transmembrane helix of cytochrome c_1 that participates in the interface of complexes III and IV. Charge neutralization is important for the interaction of complexes III and IV. One function of cardiolipin therefore is to stabilize supercomplexes. Supercomplexes in turn are necessary to keep cells and organisms in healthy condition.

3.5. Role of cardiolipin in health and disease

Loss of cardiolipin in yeast correlated with structural lability of the respiratory supercomplexes and with functional deficiency of the complex IV moiety that was found to be in an almost inactive but reversible resting state [90]. It was therefore interesting to investigate whether also milder defects in the cardiolipin biosynthesis pathway had similar effects. Reduced stability of yeast respirasomes was in fact observed in studies of Δ*taz1* mutants with defective cardiolipin-transacylase (taffazin) that contain modified cardiolipin with altered fatty acid chain length and changed degree of unsaturation on position C2 [95]. Similarly, studies of Barth syndrome patients with deficient cardiolipin-remodeling due to mutations in the taffazin gene, showed reduced stability of human supercomplexes. The reduced stability became evident from an enhanced release of complex IV from $I_1III_2IV_n$ supercomplexes during BNE [96]. This means that the altered cardiolipin affected specifically the stability of the complex III–IV interaction in yeast and human respirasomes. The devastating effects of Barth syndrome can be explained by a reduced amount of respiratory complexes which is regarded as a secondary effect of the reduced respirasome stability.

3.6. Assembly of respirasomes is required for stability of individual respiratory complexes

Human mitochondrial encephalomyopathies are often characterized by multiple deficiencies of two or more respiratory complexes. Sometimes, however, single complexes are specifically reduced, for example, complex I or complex IV. Since complex IV amounts can be normal in patients with specific deficiency of complex I, and vice versa, complexes I and IV seem not to require each other for stability in humans [28], similar to complexes III and IV in yeast that are stable in the absence or the other complex [24,25]. The situation was found to be quite different in mutants from *P. denitrificans* indicating for the first time that assembly in respirasomes is required to stabilize bacterial respiratory complex I [27]. The necessity of stably assembled human complex III for the stability of complex I was later demonstrated using muscle biopsies and cultured patient cells. Human complex I was almost completely lacking in the absence of assembled complex III [28,29]. Stabilization effects of respirasomes seem to vary in a species-specific way. In contrast to the dependence of complex I on complex III found in humans, down regulation of the nuclear-encoded subunits of complexes III and IV disrupted their respective complexes but not complex I in procyclic *Trypanosoma brucei* [97]. Mitochondrial complex I mutations in *Caenorhabditis elegans* produced cytochrome *c* oxidase deficiency [98] and cytochrome *c* oxidase was required for the assembly/stability of respiratory complex I in mouse fibroblasts [99].

3.7. Respirasomes are required for substrate channelling

Substrate channelling between bovine complexes I and III via ubiquinone could be demonstrated by the group of Lenaz using flux control analysis while no substrate channelling between complexes III and IV via cytochrome *c* was evident [30]. The chance to identify substrate channelling between complexes III and IV by flux control analysis seems not very high, since it depends on the binding of cytochrome *c* to supercomplexes rather than on supercomplex assembly itself. The authors agreed that complexes I and III form a tight "core supercomplex" but they also suggested that the mitochondrial respiratory chain is only partially organized in a supercomplex assembly. We cannot agree with the latter part of this conclusion. As pointed out before (3.1.), the possibility of differential solubilization of the mitochondrial complexes indicates that no detectable amounts of complex III and almost no complex IV exist as individual complexes or as supercomplexes with masses smaller than 3 MDa in the mitochondrial membrane. Therefore, we would like to emphasize that almost all complex I, III, and IV must be integral part of a large and fairly immobile protein network in the mitochondrial inner membrane of mammalia. This is currently visualized by structural models, as exemplified in Fig. 2, but more detailed studies are required.

3.8. Potential additional roles related to respirasome-associated proteins and complexes

Tim21, a specific subunit of the sorting active presequence translocase (Tim23 complex) of the inner membrane, has recently been postulated to physically interact with proton-pumping respiratory chain complexes and to stimulate preprotein insertion. Thus the presequence translocase may cooperate with the respiratory chain and promote membrane-potential-dependent protein sorting into the inner mitochondrial membrane [100,101]. Since respiratory chain complexes are around 10-times more abundant than the Tim23 complexes, Tim23 must be bound substoichiometrically. Further evidence points to physical interaction also of the ADP/ATP translocator with the Tim23/respiratory chain supercomplex [102]. However, no physical interaction with the ATP synthase or complex V was identified [103] which might be expected if ATP synthase and the translocators for phosphate and adenine nucleotides assemble into ATP synthasomes [62,63].

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 472, Molecular Bioenergetics, Project P11 (H.S.).

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