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Structures of mitochondrial oxidative phosphorylation supercomplexes and mechanisms for their stabilisation☆

Yuriy Chaban a,1, Egbert J. Boekema b, Natalya V. Dudkina b,* 2

a Institute of Structural and Molecular Biology, Malet street, Birkbeck College, London WC1E 7HX, UK
b Electron Microscopy Group, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747AG Groningen, The Netherlands

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1. Introduction

Mitochondria play a number of vital roles in the eukaryotic cell, among which the most important one is the production of ATP during oxidative phosphorylation (OXPHOS). The heavily folded inner membranes of mitochondria called cristae accommodate many copies of the respiratory chain components, or OXPHOS complexes (I–IV). Together with ATP synthase (complex V) they form the machinery for production of ATP, the energy currency of the cell. Complexes I–IV are multi-subunit enzymes that work in concert to create an electrochemical proton gradient across the mitochondrial inner membrane that is used by the F1F0 ATP synthase (complex V) to produce ATP via oxidative phosphorylation, although complex II is not directly able to pump protons. NADH or succinate, generated during glycolysis, fatty acid oxidation and in the citric acid cycle, form the fuel for the respiratory chain. During catalysis, there is electron transfer between the complexes mediated by two small components: lipid-soluble ubiquinone and water-soluble cytochrome c. They diffuse between the respiratory complexes I and III, and II and IV, respectively, and the latter takes them to form water from molecular oxygen (Fig. 1).

For understanding OXPHOS at the molecular level it was all-important that atomic structures of the individual complexes were solved. Complex I or NADH dehydrogenase is the largest enzyme of the electron transport chain (ETC) and has a characteristic L shape with an extensive part being embedded in the lipid bilayer and a smaller shoulder protruding into the mitochondrial matrix. The recent X-ray structure of the bacterial complex I suggests the coupling mechanism for NADH dehydrogenase [1]. Complex I binds NADH substrate to the distal end of the hydrophilic arm and transfers two electrons, one at a time, via FMN and seven iron–sulphur clusters to the bound ubiquinone at the interface between two arms [2]. Reduction of the ubiquinone induces conformational changes in the membrane arm resulting in translocation of four protons across the membrane via four channels [1].

Complex II or succinate dehydrogenase is the second independent entrance point of electrons to the respiratory chain. It oxidises succinate and transfers electrons through three iron–sulphur clusters to ubiquinone. Complex II is not a proton pump and it does not contribute directly to the proton gradient formation.

Complex III or cytochrome c oxidoreductase, which is known to exist in the membrane as a dimer, oxidises ubiquinone to ubiquinol and as a result it can pump two protons to the intermembrane space. The electrons from ubiquinol are passed to the carrier cytochrome c via cytochromes b and c1 of complex III.

The last enzyme of the mitochondrial electron transport chain is complex IV or cytochrome c oxidase. It accepts electrons from cytochrome c and delivers them to an oxygen molecule to convert it to two water molecules. Four protons are pumped to the intermembrane space during this process.
ATP synthase or complex V uses the energy stored in a proton gradient to turn ADP into ATP. It is a complex formed by 15–18 subunits with a total mass of 600 kDa [3]. The water-soluble F1-part consists of three α and three β catalytic subunits. The F1-part is connected to the membrane-embedded ring-like subunit c oligomer of the F0-part by a central and a peripheral stalk. The F0-part is composed of subunits α (Su 6), A6L (Su 8), e, f, g, the central stalk consists of the γ, δ and ε subunits and the peripheral stalk is made from subunits OSCP (Su 5), b, d, F6 (h) (the nomenclature of subunits in yeast is indicated in brackets, reviewed in [4]). The yeast enzyme has two specific additional subunits, i and k, which belong to the membrane part. Protons cross the membrane via the proton channel in the membrane part rotating the multi-subunit c ring and the central stalk. The α:β:γ trimer of F1-part is prevented from rotation by the peripheral stalk and catalyses ATP synthesis via the binding change mechanism [5].

2. Supramolecular organisation of the respiratory chain

The most important experimental strategy to characterise the organisation of the OXPHOS system has been blue-native polyacrylamide gel electrophoresis (BN-PAGE), first introduced by Hermann Schägger and colleagues [6,7]. BN-PAGE is based on the mild solubilisation of mitochondrial membranes with non-ionic detergents and is able to separate the largest stable protein complexes that can withstand solubilisation. This technique allowed the separation of respiratory supercomplexes on gels and marked the beginning of the study of the higher level of structural organisation of the OXPHOS system. Electron microscopy single particle analysis provided the first structural evidence for the respiratory supercomplexes [8,9]. In the last two decades, BN-PAGE and other biochemical techniques have triggered a considerable change in the view on the organisation of the electron transport chain in mitochondrial membranes. The idea of random diffusion of complexes in cristae was ruled out by a number of experiments. Flux-control measurements combined with inhibitor titration revealed that the yeast electron transport chain behaves as a single functional unit [10,11].

Respiratory supercomplexes have been found in organisms belonging to different kingdoms of eukaryotes (Table 1). Despite their phylogenetic distances from each other, all of them have in common that their OXPHOS systems have supramolecular organisation. Based on composition, all supercomplexes can be divided into four main groups. Complexes I, III2, and IV were found to assemble into I + III2, III2 + IV1–2 or I + III2 + IV1–4 supercomplexes. ATP synthases form dimers, which constitute oligomeric chains in cristae. Complex II is the only enzyme of the respiratory chain, which does not associate with the other respiratory complexes. Although Acín-Perez et al. reported complex II to be a part of the mouse respirasome, which is able to transfer electrons from succinate [12], this was not supported by any other studies. Unlike the other enzymes of the OXPHOS system, complex II is not only involved into respiration but also directly participates in the citric acid cycle. This could explain why complex II was not found to be part of the respiratory supercomplexes. Interestingly, a recent study revealed that the electron flow from the FAD substrate to complex III is independent from the electron pathway that originates from NAD+ and takes place within the I + III2 supercomplex ([13] Fig. 1). These two pathways use two different populations of quinone [13]. The abundance of the supercomplexes with different compositions varies from the organism to the organism. Thus, the supercomplex with composition I + III2 is the most abundant in plants ([14], Table 1), where 90–100% of complex IV is found in the monomeric form [15]. I + III2 + IV1–4 supercomplex is higher in abundance in mammals [7] and III2 + IV2 in fungi [7,16].

Structural studies of mitochondrial membranes revealed supramolecular assemblies of the OXPHOS system in situ. Rapid-freeze deep-etch electron microscopy provided the first demonstration of oligomeric rows of ATP synthase dimers in mitochondria of Paramecium in 1989 [17]. This work also suggested ordered linear arrays of the complex I in cristae [17]. The emergence of cryo-electron tomography (ET) has given rise to the study of supramolecular organisation of the respiratory

![Fig. 1. Plasticity model of the mitochondrial electron transport chain in which complexes I–IV are partly organised into supercomplexes, except for complex II, which feeds electrons via ubiquinone to complex III non-bound to complex I. Red arrows show electron pathways. In yellow, the complex I, marked as I; in red the complex II, marked as II; in green the complex III2, marked as III2; in purple the complex IV, marked as IV; in blue the dimeric ATP synthase, marked as V2; in violet the cytochrome c; Q, ubiquinol. The positions of the matrix (M), the intermembrane space (IMS) and cristae or inner membrane (IM) are indicated.](image-url)
chain in cristae membranes and intact mitochondria without using any fixatives at a resolution that allows the visualisation of individual macromolecules. A number of cryo-ET studies have demonstrated the existence of oligomeric chains of ATP synthases in the cristae membranes of various organisms [18–21]. Results of these experiments suggest different arrangements of the respiratory enzymes in tubular and lamellar types of mitochondria. Oligomeric rows of ATP synthases were found along cristae edges of lamellae, while complexes I–IV would be expected on flat surface of the cristae [20]. In contrast, the tubular cristae of the green alga Polyctomella were fully covered by oligomeric rows of ATP synthases [19], suggesting that the other OXPHOS enzymes might be arranged in between, but these could not be visualised due to the tight packing of ATP synthases. Interestingly, in tomographic reconstructions of mitochondria from some organisms as bovine, yeast, Arabidopsis etc. it is very hard to observe oligomeric chains of dimeric ATP synthases [20], Dudkina unpublished data), but Polyctomella is an exception to the rule [19]. A possible explanation is that the dense matrix of the organelles in most organisms obscures many of the internal features [20]. Another explanation could be the variable abundance of ATP synthase complexes among various organisms. Alternatively, ATP synthases could undergo rearrangement in membranes as the dimeric ATP synthases do not physically interact with each other and may drive the deformation of cristae membranes as suggested recently [21].

3. Significance of the OXPHOS supercomplexes

The structural study of the OXPHOS supercomplexes is of special importance for understanding the function of the respiratory chain complexes. Most mitochondrial diseases and disorders, including Parkinson’s and Alzheimer’s diseases [22,23], are associated with genes that encode for OXPHOS proteins. In addition, mitochondrial dysfunction appears to be a factor in cancer etiology. Mutations of the mitochondrial or nuclear DNA that affect components of the mitochondrial OXPHOS system result in reactive oxygen species (ROS) overproduction and promote tumour cell proliferation [24]. Furthermore, modifications in the levels of ROS have been linked to the resistance of cancer stem cells to chemotherapy [25]. Therefore, the OXPHOS system appears to be an attractive drug target in treatment of cancer and mitochondria-associated diseases. Understanding the supramolecular organisation of the OXPHOS system and its role in functioning of the respiratory chain is essential for the development of such treatment.

4. Respiratory supercomplexes

Complexes I, III2, and IV form three types of supercomplexes. One copy of complex I can associate with a dimeric complex III to form the I + III2 supercomplex, which is mainly found in plant mitochondria (Table 1). Electron microscopy revealed that the complex III2 is laterally attached to the membrane arm of complex I in its concave portion [26–28]. In some organisms complex III2 was found to associate with one or two copies of complex IV [16,28–30]. The III2 + IV2 supercomplex is the most stable in Saccharomyces cerevisiae, which lacks complex I. No free monomeric or dimeric forms of complex IV could be detected upon detergent solubilisation [16], indicating that all complex IV makes part of the supercomplex. The detailed EM analysis revealed that the complex III2 is flanked by both sides by monomeric complexes IV [16]. This study established that complex IV is not a functional dimer as previously proposed on the basis of crystallization experiments [31]. A pseudo-atomic 3D model showed that the monomeric cytochrome c oxidase complexes are attached to dimeric complex III at two alternate sides with their convex sides facing the complex III2 [16]. This is opposite to the side involved in the formation of complex IV dimers as described by X-ray crystallography. The recent cryo-EM map of the supercomplex is in agreement with the pseudo-atomic model, which was based on the negatively stained projections [32]. From the cryo data authors concluded that the distance between cytochrome c binding sites in complexes III and IV is about 6 nm [32], in contrast to the shorter 4 nm predicted from the 2D averages [16].

The largest respiratory chain supercomplex is formed by complexes I, III2, and up to 4 copies of complex IV [7]. It was called the respirasome because it creates the bulk of the proton motive force in the presence of cytochrome c and quinone in one unit. Recently, the structure of the bovine respirasome was determined by two different EM methods: cryo-ET of digitonin-solubilised respirasomes [33] and cryo-EM of amphipol-solubilised respirasomes [34]. Both methods gave compatible structures and demonstrated that the complex III2 sits in the arc of the membrane arm of complex I while complex IV is attached to the tip of NADH dehydrogenase. Docking of X-ray structures of complexes III2 and IV showed that the interaction sites of complexes III2 and IV differ from S. cerevisiae [31], but in both the yeast and bovine models, the complex IV dimerization interface is free. The differences between the organisation of the bovine and yeast respirasomes might be partly explained by necessity of complex III to interact with both complexes I and IV in mammalian mitochondria. However, even taking into account the interaction between complexes I and III2 in bovine there is no spatial restriction within the bovine respirasome for the complexes III2 and IV to interact in the same way as in S. cerevisiae. Another possible explanation would be a string-like association between I + III2 + IV supercomplexes via complexes IV, hypothesized from the respiratory chain fragments resolved on blue-native gels [35]. Early work on Paramecium hints to the possible supramolecular organisation of the complex I [17]. However, in the recent work based on cryo-ET authors exclude the existence of such strings [20].

4.1. Stabilising factors for the respirasome

In various organisms respirasomes appear to be very stable because they can be isolated as whole entities without significant degradation. In the last years a lot of effort has been placed into the search for factors that are responsible for gluing together the respirasome components. Cardiolipin is an anionic phospholipid formed by two phosphatidylglycerol groups bound by a glycerol. It is exclusively found in the mitochondrial inner membrane where one of its major roles is to specifically interact with proteins and modulate their functions (reviewed in [36]). The cryo-EM maps of the yeast and bovine respirasomes clearly showed that the complexes I, III2, and IV are at some distance within the supercomplex in the lipid bilayer and the spaces between complexes appear as a lower density material in the electron microscopy maps [32–34]. Thus, the space is most likely to be filled with lipids. It was shown previously that cardiolipin is required for stability of single complexes III and IV and formation of the stable III2 + IV2 supercomplex [37,38]. A content of about 50 molecules of cardiolipins per yeast supercomplex was determined by mass spectrometry analysis [32]. Due to larger distances between complexes, the bovine respirasome can potentially accommodate several times more cardiolipin molecules [34].

Sometimes lipids co-crystallize with membrane protein complexes. In the crystal structure of the bovine complex IV, one cardiolipin molecule was identified at subunit III [39], which is at the interface with complex III in the bovine respirasome EM structure [33], but not in the yeast respirasome [32]. However, photolabeling experiments suggested that the cardiolipin molecule was bound between subunits VIIa and Vlc [40], which are to some extent interacting with complex III in the yeast respirasome [16,32]. Several lipid molecules were found to be associated with the yeast complex III2. Cardiolipin is bound in a hydrophobic cleft at the interface between cytochromes b and c1 [41]. Cyt b and c1 are facing complex IV in the yeast supercomplex [16], but not in the bovine respirasome. However, a molecular dynamics simulation identified three additional binding sites for cardiolipins, which might be involved in interaction [42]. The authors suggested that the complexes first form an encounter supercomplex by interacting via cardiolipin binding sites. Following this, slow and small rearrangements in the relative orientations of the proteins may occur together with the release of the
excess of cardiolipins from the binding sites. By mass spectrometry it was found that eight and two molecules of cardiolipins associate with complexes III and IV, respectively [43].

Besides lipids, subunit VIIa of cytochrome c oxidase and proteins unrelated to the respiratory chain were shown to play a role in the stability of the respirasome. The cytochrome c oxidase subunit VIIa polypeptide 2-like (Cox7-a2l) was identified in the respirasome and III2 + IV supercomplex in mouse fibroblasts, but not in free complexes III or IV [13]. In the absence of Cox7-a2l, complex IV does not assemble into supercomplexes though the electron flux from substrates is not affected. The electrons will pass through a single pool of cyt c to a single pool of complex IV [13]. The cytochrome c oxidase subunit 7a-related protein (COX7RP) was also identified as a factor that promotes supercomplex assembly [44].

The abundant isoform of the ADP/ATP carrier Aac2, which is facilitating the exchange of ADP and ATP across the mitochondrial inner membrane, was found in association with the III2 + IV supercomplex [45,46] and it was shown that it requires cardiolipin for proper functioning [45]. Two novel small proteins Rcf1 (respiratory supercomplex factor 1) and Rcf2 (respiratory supercomplex factor 2) of 18 and 25 kDa separately associate with the respirasome and were demonstrated to be important for the stability of the yeast respirasome [47,48]. Rcf1 interacts with the Cox3 subunit of complex IV and is required for the proper assembly and functioning of the supercomplex [47,49]. Rcf1 was found to be in a close proximity to Aac2 [47]. Rcf2 can be specifically found in yeast, while Rcf1 has human homologs RCF1a and RCF1b [48]. Both proteins are found in association with complex IV and Rcf1 plays a crucial role in the stability of the supercomplex. High-resolution structures of the respirasomes will help to assign the positions of these novel components in the supercomplex.

A recent study of the relationship between respiration in mitochondria and cristae ultrastructure suggests that the cristae morphology plays a stabilising role for the respiratory supercomplexes in mice [50].

4.2. Respirasome functions

In the respiratory chain all enzymes are linked by the electron exchange. Ubiquinone physiologically interconnects complexes I and III by accepting electrons from the first and delivering them to the latter. It is known that most of the harmful mitochondrial superoxide originates from complexes I and III [51]. Complexes I and III are assembled within the respirasome such that the ubiquinone binding sites in the bovine respirasome are at a close distance of 13 nm [33,34]. Short movements of ubiquinone make sense not only to speed up the electron transfer process as a whole, but also to minimize loss of the electrons during transfer and the production of harmful oxygen radicals. Another crucial point in the OXPHOS electron pathway is the shuttling of cytochrome c between complexes III and IV. Recent data suggest that the distance that cytochrome c has to diffuse between the sites of complexes III and IV is 10 nm in the bovine respirasome [33,34], but less than 6 nm in the III2 + IV supercomplex in yeast [32]. Thus, the formation of the respirasome brings these sites closer to each other and is intended to make the transfer more efficient than if complexes III and IV were randomly distributed in the membrane. However, it is questionable if the distance between electron transport components is the main reason for the supercomplex formation. Taking into account that the diameter of cytochrome c is about 3.4 nm, Althoff et al. [34] raised the possibility of direct electron channelling of cytochrome c between complexes III and IV. However, a strong argument against this conclusion is that the cytochrome c binding sites of complexes III and IV are not as close to each other as they could be. Flux control experiments do not provide evidence for substrate channelling between complexes III and IV via cytochrome c [11], and a time-resolution spectrophotometric technique has shown that cytochrome c is not restricted in its diffusion [52]. Both data rather support the idea that the substrate channelling is not the main reason for the respirasome formation, although substrate channelling was found between bovine complexes I and III via ubiquinone [11].

A recent study of electron flux in the OXPHOS system points to separate pathways in the ETC depending on the presence of the assembly factor Cox7A2L. The complex IV can receive electrons from either the NADH substrate via the I + III2/I + III2 + IV supercomplex, from FAD via the complex II and the single complexes III2 and IV/III2 + IV supercomplex, or from both NADH and FAD ([13] Fig. 1). Such organisation of the respiratory chain may be essential for optimizing the simultaneous oxidation of multiple substrates [13]. Indeed, the ratio between electrons coming from NADH and FAD is higher when glucose is the main respiratory substrate and lower if fatty-acid oxidation is the main source of substrates [13]. Therefore, the dynamic supramolecular organisation of the ETC might be essential for efficient oxidation of available substrates.

Another intriguing question is how the presence of significant amounts of lipids between single complexes is consistent with the overall efficiency of the electron transfer. Are the lipids ultimately required for the stability of the supercomplex or is their presence a prerequisite for the ubiquinone exchange? Based on the finding that the electron flow from FAD to complex III is independent from the pathway that uses electrons from NAD+ within the I + III2/I + III2 + IV supercomplex [13], we propose that the limited space for lipids between complexes I and III could serve as a diffusion microdomain for the ubiquinone, confining it to the I + III2/I + III2 + IV supercomplex. A similar mechanism was suggested for the plastoquinone in chloroplasts [53].

Among other possible functions, the supercomplex can play a role in assembly of its components. Mutations in subunits of complex III or IV destabilize complex I in human and mouse cells [54,55] and complex I is necessary for the full assembly of the complex III in human cells [56,57]. Intriguingly, in the cytoplasmic membrane of the possible ancestor of eukaryotic mitochondria, Paracoccus denitrificans, the assembly of respiratory complexes I, III, and IV into the respirasome I + III4 + IV4 stabilizes the complex I [58].

Finally, the dynamic nature of the respiratory chain has to be taken into account. The supercomplexes can undergo rearrangement in response to the demand for ATP of particular cell types at a given time [59].

5. Dimeric ATP synthase

The first biochemical evidence for a dimeric organisation of the ATP synthase complex in yeast came from the BN-PAGE work by Arnold and colleagues [6]. Later dimers were found in bovine, Arabidopsis and several other organisms [7,14]. Interestingly, dimerisation of ATP synthases seems to be a characteristic feature of mitochondria, because no evidence has yet been obtained that supports the existence of dimers in bacterial membranes [60,61]. Structural information about the dimeric ATP synthases comes from either single particle EM of detergent-solubilised proteins or subtomogram averaging in situ. The latter method provides direct insight into supramolecular organisation of macromolecules in membranes but at lower resolution than single particle reconstructions. Low-resolution structures of dimeric ATP synthase were obtained from a wide range of eukaryotes, including the animal Bos taurus [9], the alga Polytomella sp. [8], the fungus S. cerevisiae [21,62], the protozoan Tetrahymena thermophila [63] and other organisms (Table 2). Remarkably, these organisms are phylogenetically very distant from each other but nonetheless share similar ultrastructures of the dimeric ATP synthase in their mitochondria (Fig. 2). In nearly all organisms two monomers associate via the membrane F0 parts. A special feature of the dimers is the unusual angle of 40-90° between the monomers, as observed in 2D projections where the dimers are seen from the side (Table 2, Fig. 2a, b, d). However, no such kink between monomers is visible in the EM map of the ATP synthase dimer from Tetrahymena (Table 2, Fig. 2c, e).
Table 2

<table>
<thead>
<tr>
<th>Organism</th>
<th>Angles (degrees) by subtomogram averaging in situ</th>
<th>Angles by single particle analysis of isolated supercomplexes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polytomella sp.</td>
<td>70 [19]</td>
<td>50 [67]</td>
</tr>
<tr>
<td>Bos taurus</td>
<td>55-90 [18]</td>
<td>40 [9,66]</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td>70 [18]</td>
<td>-</td>
</tr>
<tr>
<td>Tetrahymena thermophilus</td>
<td>-</td>
<td>0 [this work]</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>86 [21]</td>
<td>35, 90 [62, 66]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40 [65]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>55-142 [64]</td>
</tr>
<tr>
<td>Yarrowia lipolytica</td>
<td>80 [20]</td>
<td>-</td>
</tr>
<tr>
<td>Podaspera arentea</td>
<td>80 [20]</td>
<td>-</td>
</tr>
</tbody>
</table>

* Dashes in the table cells indicate that for the corresponding organism no structural information by the corresponding EM or ET method is available.

5.1. Angular distribution in dimers of ATP synthases

In ATP synthase dimers from different species the interacting monomers make a range of different angles that needs to be discussed. Obviously, detergent treatment during isolation may affect the angle. Several groups observed different angles between monomeric ATP synthases in S. cerevisiae using different amounts of detergent. 2D averaging of the dimers extracted with digitonin at a ratio of 5 g per g of protein revealed mainly two types of supercomplexes with angles of either 35° or 90° [62]. A similar study showed that the angles ranged from 35° to 142° for dimers extracted with a digitonin–protein ratio of 1.75 (w/v) [64]. However, in the latter work more than 50% of isolated dimers had angles of 83° or 93°. A third group extracted dimeric ATP synthases in presence of ADP with 2 g of digitonin per g of protein and reported typical angles of 36–48° for their preparation, although they could also find a small number of 90° and 140° dimers [65]. The recent subtomogram averages of the dimeric ATP synthases in yeast in situ did not reveal supercomplexes with small angles and reported only wide-angle dimers of 86° in membranes [21]. This value is more trustworthy because no detergent was used at all and the dimers were observed in their natural environment.

For the bovine supercomplex, the dimers with an angle of 40° were extracted with the low amount of digitonin 2.5 g per g of protein [9] and a small amount of 90° dimers were found at a ratio 25 g/g [66]. The first reported subtomogram averages of dimeric enzymes in bovine cristae fragments revealed a range of angles between 55 and 95° [18] and the same group later demonstrated that only the angle of 80° is found in membrane fragments [20]. Lower concentrations of detergent seem to increase the level of flexibility between monomers. However, a closer look at the EM averages suggests that the angular difference is likely not only due to flexibility between monomers [62,64,65]. The dimer-specific subunits may also play a role. The wide-angle dimer does not show the same interaction of two monomers as the small-angle dimer because the number of additional subunits located between two F_{0}-parts is different. The membrane part of the wide-angle dimer is significantly larger than that of the narrow-angle dimer and likely has a different composition. Therefore, the previously proposed different breakdown products of the oligomeric chain can still explain the angle difference [62]. The variability of angles between dimers in a row discovered by cryo-ET [21] could explain the variability in the dimer if it is formed by monomers of two neighbouring dimers. In some way, the tomography data must provide a more reliable estimate of angles because tomography can be applied without detergent solubilisation.

In contrast, the dimeric ATP synthase from *Polytomella* seems to be very rigid because both the subtomogram averages and the 2D averages of isolated supercomplexes demonstrated just one fixed angle of 70° [8,19]. A smaller angle of 50° between monomers was reported in a single particle 3D EM study of negatively stained *Polytomella* ATP synthase [67]. However, to establish whether the angle between monomers of ATP synthases in membrane in situ is fixed or can vary within a certain narrow range, it will be necessary to implement the three-dimensional classification of all previously reported for different organisms subtomogram averages. Therefore, this work needs further investigation and preferably on intact mitochondria, which have not been disturbed by mechanical forces during fragmentation.

5.2. Dimer specific subunits

Multiple studies suggest that dimeric ATP synthases are stabilised by protein–protein interactions. A lot of effort was put into the identification of proteins involved in monomer–monomer interfaces within dimeric ATP synthases from various organisms. The subunit compositions of dimers from baker’s yeast and green alga were extensively studied. Although the core-structures of monomeric ATP synthases in these organisms are conserved, their dimeric ATP synthases have unique dimer specific subunits that differ between species but nevertheless ensure the conservation of the angular association of monomers into dimers and oligomers.

5.2.1. Green algae

Dimeric ATP synthases of 1600 kDa from the closely related algae *Polytomella* and *Chlamydomonas* were investigated in detail. It was revealed that in both enzymes the main components of complex V are subunits α, β, γ, δ, ε, a, c and OSCP. The peripheral stalk subunits AGL, b, d, f, F6 and dimer specific subunits e and g, which are typical for the bovine enzyme, are absent in algal enzymes. Instead, they contain nine
specific subunits in the range of 9 to 66 kDa named ASA1 to ASA9 (for ATP Synthase Associated) [68,69], which seem to be shared by other members of Chlorophyceae [70]. The topology of these subunits was extensively studied by cross-linking and partial dissociation of the Polytomella supercomplex either in membranes [71] or in isolation [67]. Based on these approaches two different models were proposed [67,71] for the localisation of subunits ASA1–9. 2D EM projections and subtomogram averages of the dimeric ATP synthase from Polytomella showed the presence of a large, unusually shaped peripheral stalk [8,19], which is larger than that of the yeast or bovine enzymes and likely accommodates the unrelated ASA subunits. The major difference between two models is that the ASA1 subunit is proposed to constitute either the bulky peripheral stalk [71] or to belong to the membrane part of ATP synthase [67]. The model suggested by van Lis et al. [71] is supported by the fact that the ASA1 subunit is water soluble and large enough (66 kDa) to fill the peripheral stalk density [71]. ASA6 and ASA9 are mainly found in the dimeric form of the enzyme and were proposed to be at the dimer–dimer interface in the membrane [67,71]. Additional experiments are required to verify the models.

5.2.2. Yeast

ATP synthase from S. cerevisiae comprises three additional subunits e, g and k, associated with the F$_o$-part. They were found in the dimeric form of the enzyme and were assigned as dimer-specific [6]. Several studies suggested the indispensable role of the dimer-specific subunits in stabilization of the monomer–monomer interface in the dimer [6,72]. Further attempts to map the subunits at the monomer–monomer interface were based on chemical cross-linking. Such studies suggested that in addition to the e, g and k subunits, a number of other subunits of the monomeric enzyme might also be involved in dimerization. It was shown that subunit 4 (b in bovine) [73], 6 (a in bovine) [4,74] of the peripheral stalk together with e and g play a major role in the ATP synthase dimerization process. The subunit 6 (F6 in bovine) of the peripheral stalk [75] and the yeast-specific subunit i form homodimers [76], which were found to be associated with ATP synthase dimers even in the absence of subunits e and g, although subunit i is not essential for the dimer formation [72]. Tomography data convincingly show the oligomeric state of the ATP synthase complex, which is the association of dimers into rows, but are far too low in resolution to reveal concrete subunit interfaces and which of them are essential because it provides information about the 3D position of the monomers that cannot be deduced from the 2D averages (Fig. 2c, e). Although the 3D map does not reveal an angle between two monomers in the usual direction, it shows a displacement of the monomers by 45 ° in a direction orthogonal to the long axis through the F$_o$ parts (Fig. 3), as discussed below. Until now, all available structures of dimeric ATP synthases from various organisms demonstrated that the interaction between two monomeric enzymes was limited to their F$_o$-parts. Although there were structural hints in 2D EM averages to possible association via the peripheral stalk, which were supported by the cross-linking experiments (reviewed in [77]), they were not resolved in the 3D maps of yeast [65] and alga [19,67] due to the limited resolution. Our 3D map from Tetrahymena clearly shows that the monomers are linked not only via the membrane F$_o$-part, but are also connected via additional protein densities in the lower part of the peripheral stalks (Fig. 3f, h, j, green arrowheads). Two monomers interact with each other not in parallel, but in an antiparallel configuration in which the peripheral stalks are rotated outwards from the dimerisation axis by approximately 50° (Fig. 3a, e, i, asterisks). Remarkably, the c-rings of every monomer are not in close proximity, but are separated from each other by approximately 135 Å (Fig. 3k) and are linked via unassigned (protein) densities between them (Fig. 3f, h, i, blue arrows). Another density bridges two monomers at the intermembrane side of the dimer (Fig. 3k, dotted line). The dimeric ATP synthase in Tetrahymena has apparently undergone significant changes in comparison to the conventional supercomplexes from yeast and bovine. These changes have resulted in an unusual structure that requires additional protein densities to stabilise the unique construction of the protozoan enzyme. Proteomic analysis identified at least 13 novel proteins that constitute subunits apparently limited to the ciliate lineage [63]. A mitochondrially encoded protein, Ymf66 with eight predicted transmembrane domains could be a substitute for the subunit a of the F$_o$ sector [63]. Other subunits might also constitute unknown protein domains of the dimeric ATP synthase.

5.3. Functions of the dimeric ATP synthase

Oligomerisation of ATP synthases in cristae must have a special reason. The angular association of ATP synthases in membranes points to the possible role of complex V in a local bending of the cristae membrane. Indeed, tomography studies of cristae membranes from various organisms revealed ATP synthases in highly curved areas of cristae [18–20]. It is likely that the shape of the curved dimers causes the membrane curvature, rather than that a curved membrane induces the kink in the ATP synthase dimers. The observations that disrupted mitochondria retain the bent membranes [18–20] and that isolated dimers keep their shape [8,9,62] strongly points to their active role in shaping the cristae. Additionally, yeast mutants devoid of the dimer-specific e and g subunits and lacking ATP synthase dimers have an abnormal inner membrane ultrastructure. The mitochondrial membranes of these mutant strains exhibit unusual onion- [72,79] or balloon-shaped morphology [21]. This strongly indicates that the dimerization of ATP synthases is essential for cristae formation. One of the main consequences of the membrane bending is creating a large surface area to accommodate more OXPHOS complexes. Furthermore, it has been proposed that in lamella-type mitochondria protons are directed along flat membrane surfaces from the respiratory complexes towards ATP synthases at the ridges of cristae, thus optimizing local proton concentration for ATP synthesis [21].
There is another strong argument supporting the role of ATP synthases in the membrane curvature. ATP synthase dimers have so far only been reported for eukaryotic organisms, possibly reflecting their role in formation of mitochondrial cristae. Flat membranes in bacterial cells could have a direct link to the absence of dimerization process in prokaryotes. The subunits 4, e and g, which are essential for dimerization of ATP synthase in yeast mitochondria [6,21,73], are all encoded by nuclear DNA and might be a feature acquired during the evolution of eukaryotic organisms. It is not clear why the angular association of ATP synthase dimers is different in *Tetrahymena* and the impact of such unusual angular arrangement on morphology and efficiency of mitochondria has to be investigated.

It is interesting to note that in ATP synthase dimers the c-subunit rings of F₀ rotor parts would rotate into opposite directions with respect to the dimerization axis during proton translocation in ATP synthesis or hydrolysis. This could possibly play a role in stabilising the dimer by balancing the forces acting upon it due to the torque generated in individual ATPsynthase molecules. Additional interaction between monomers via connection of the peripheral stalks, which was observed in *Tetrahymena* and is likely present in *Polytomella* would strengthen the function of these stalks, whose primary role is to prevent the F₁-domains from rotation and which will require a greater amount of static force in dimers.

6. Future vision

In the last years, steadfast progress has been made in structural studies of the OXPHOS supercomplexes. Cryo-electron tomography experiments on mitochondrial cristae put an end to the debate about the existence of supercomplexes in mitochondria. Low-resolution structures of several supercomplexes became available, creating a framework for further functional and structural studies of the OXPHOS system. We see further progress in several directions. First of all, ongoing development of electron microscopy instrumentation and data processing in tomography and subtomogram averaging will permit more detailed studies of the respiratory chain and its physiological role in situ. Second, solving medium- and high-resolution structures of dimeric ATP synthases and respirasomes is within reach. Initial difficulties in creating the first 3D models of membrane macromolecules were overcome by the use of cryo-ET in combination with subtomogram averaging [33,80]. Finally, improvements in automated single particle image acquisition allow the collection of massive amounts of data necessary for the further refinement of structures. High-resolution studies will eventually help to identify stabilising factors for supercomplexes and particularly protein–protein interfaces in dimeric ATP synthases. Altogether, this will provide a basis for predicting mechanisms of cristae formation and modelling metabolic processes in mitochondria.

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