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Review

ATP synthase superassemblies in animals and plants: Two or more are better

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ABSTRACT

ATP synthases are part of the sophisticated cellular metabolic network and therefore multiple interactions have to be considered. As discussed in this review, ATP synthases form various supramolecular structures. These include dimers and homooligomeric species. But also interactions with other proteins, particularly those involved in energy conversion exist. The supramolecular assembly of the ATP synthase affects metabolism, organellar structure, diseases, ageing and vice versa. The most common approaches to isolate supercomplexes from native membranes by use of native electrophoresis or density gradients are introduced. On the one hand, isolated ATP synthase dimers and oligomers are employed for structural studies and elucidation of specific protein–protein interactions. On the other hand, native electrophoresis and other techniques serve as tool to trace changes of the supramolecular organisation depending on metabolic alterations. Upon analysing the structure, dimer-specific subunits can be identified as well as interactions with other proteins, for example, the adenine nucleotide translocator. In the organellar context, ATP synthase dimers and oligomers are involved in the formation of mitochondrial cristae. As a consequence, changes in the amount of such supercomplexes affect mitochondrial structure and function. Alterations in the cellular power plant have a strong impact on energy metabolism and ultimately play a significant role in pathophysiology. In plant systems, dimers of the ATP synthase have been also identified in chloroplasts. Similar to mammals, a correlation between metabolic changes and the amount of the chloroplast ATP synthase dimers exists. Therefore, this review focusses on the interplay between metabolism and supramolecular organisation of ATP synthase in different organisms.

1. Introduction

Mitochondria and chloroplasts serve as power plants of living cells. By generating the biological energy currency ATP, ATP synthases play a decisive role in this process [1,2]. At the beginning of the 1960s, the systematic study of isolated mitochondria by electron microscopy commenced. In the micrographs, the so-called “elementary particles” were among the first components unambiguously identified as important units of mitochondrial function [3]. These particles, similar to the structures shown in Fig. 1A, represent the ATP synthase [4]. They consist of a globular water-soluble subcomplex F_1, responsible for ATP synthesis (and hydrolysis) and a membrane embedded, proton translocating subcomplex F_o (see Fig. 1C). The letter “o” results from experiments in which ATP hydrolysis was inhibited by adding oligomycin ([5], see Fig. 1C). Since that time, a lot of knowledge accumulated, particularly about structure and function of F_1 (e.g. [6,7]). In contrast, the membrane integral part F_o is still experimentally demanding and a high-resolution structure of the complete ATP synthase (F_1F_o) is lacking to date. To overcome technical hurdles, pieces of information can be combined. On the one hand, single-particle electron microscopy provides an overview of the holoenzyme F_1F_o and shows the three dimensional outline [8]. On the other hand, a refinement is possible by docking high-resolution data of single subunits or small buildings blocks thereof into this model, e.g. from the membrane extrinsic region [9], from a complex of F_1 with F_o subunits [10] or from parts of the F_o complex [11,12]. Assembly of such jigsaw pieces provides an insight into a fascinating nano-machine, which unifies two mechanically connected biological motors: a chemical and an electrical (ion-driven), F_1 and F_o [13–16]. Besides this complex assembly, numerous subunits composing the individual ATP synthase, sophisticated enzyme complexes have to be considered in the complex cellular context. In recent years, the perception of the cell is changing from randomly colliding enzymes to a well-structured network [17]. This is also relevant to the ATP synthase. Based on its function during oxidative phosphorylation (or photophosphorylation), protein–protein interactions with other energy converting proteins are conceivable. Moreover, electron microscopy reveals that ATP synthases are arranged vicinal to each other (see Fig. 1D and [18,19]).

Abbreviations: ANT, adenine nucleotide translocator; ASA, ATP synthase associated; BHM, bovine heart mitochondria; BN, blue-native; CBBG-250, Coomassie Brilliant Blue G-250; CF_1F_o, chloroplast ATP synthase; (C)F_o, membrane integral part of the (chloroplast) ATP synthase; CN, colourless native/clear native; DCCD, *N,N′*-dicyclohexylcarbodiimide; F_1, hydrophilic part of the ATP synthase; OxPhos, oxidative phosphorylation

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We will discuss the supramolecular organisation of ATP synthases (and other supercomplexes) and how it differs from mammals to plants. Differences might arise in terms of structure and function. Moreover it has to be revealed if the supramolecular organisation is depending on the metabolic state and vice versa. Additionally, the influence of the supramolecular organisation on development, ageing, and cristae membranes has to be considered.

2. Composition and function of the ATP synthase

To synthesise ATP, an electrochemical proton gradient induces an intramolecular rotation of hydrophobic subunits in the membrane integral part of the ATP synthase [1,14,20,21]. The rotor subunits III (in chloroplasts) or *c* (mitochondria or bacteria) are organised as a cylinder-shaped oligomer III_x/c_x ([22–24], see Fig. 1B). A special feature of the subunits III/*c* is their high hydrophobicity, they are designated as proteolipids [25]. *N*,*N*′-dicyclohexylcarbodiimide (DCCD) binds specifically to the subunits III/*c* and inhibits proton translocation ([26,27], see Fig. 1C). When balancing the transformation of the electrochemical proton gradient into chemical energy, the following calculation is used: a full turn of the oligomer III_x/c_x through 360° results in synthesis of three ATP molecules, whereby the number of protons is equivalent to the number of subunits. The quantity of protons per ATP, the so-called coupling ratio, is an important value to judge the energetic efficiency of ATP synthases and all upstream processes (respiratory chain or photosynthesis) [28]. For mitochondria, P. Mitchell and J. Moyle at first communicated H^+/ATP ratios of 2 [29]. Subsequent studies reported values between 2 and 3.8 for mitochondria, for chloroplasts between 2 and 5 [30]. To date, the structurally determined values are 3.3 and 2.7 for yeast and bovine heart mitochondria, respectively [31,32]. For spinach chloroplasts the H^+/ATP ratio is significantly higher, 4.7 [33–39].

The interest in the membrane intrinsic part of the ATP-synthase is enhanced by the research of the pharmaceutical industry, which expects a new approach to develop novel drugs [40]. One focus is directed towards neuronal ceroid-lipofuscinoses, the Batten disease [41,42]. An important characteristic of this disease is the accumulation of lipopigment, which consists largely of subunit *c* of the mitochondrial ATP synthase [43]. Subunit *c* of persons with Batten disease is trimethylated [44]. The other important application concerns tuberculosis, where mycobacteria are the pathogens which have infected approx. a third of the world's population. Diarylquinolines can inactivate selectively the ATP synthases of mycobacteria [45]. The target protein of the treatment is the mycobacterial subunit *c* [46,47].

While the III_x/c_x oligomer forms the membrane embedded portion of the H^+-driven rotor, subunit γ is the main rotor component in F_1. In relation to this rotor, the subunits forming the peripheral stalk of the ATP synthase function as a stationary element (stator). In the current model of the ATP synthase, the stator is required to store energy [2,48]. As another important function, the stator subunits participate in the assembly of dimers/oligomers.

3. Supramolecular organisation of energy converting membrane proteins

In contrast to aqueous systems employed for biochemical in vitro studies, the concentration of macromolecules in the interior of biological cells is very high. The protein concentration averages about 200–300 g/L [49,50]. Such crowded environment establishes a basis for manifold protein–protein interactions. Some of the interactions emerge randomly, but additionally scaffolding for well-organised protein networks is put up. By arranging enzymes into stoichiometric complexes, specific metabolic pathways (e.g. protein, glycogen, or lipid biosynthesis) can be organised in a manner allowing the direct transfer of intermediates to the next step of processing (substrate channelling). This kind of organisation as "quinary" structures [51–53] allows the cell to increase the efficiency of enzymatic reactions.

Very high local protein concentrations occur in biological membranes [54] but also at its surface [55]. Moreover, the membrane proteins have a preferential orientation in the plane and across the lipid bilayer. As a consequence, interactions of membrane proteins and formation of protein complexes are facilitated [56,57]. Also lipids are involved in the process of organisation and can form lattices [58]. Therefore, a supramolecular array emerges in the membrane, as found for receptors [59] or aquaporins [60]. The highest ratios of membrane proteins compared to lipids occur in energy-transducing membranes. As a result of this crowding, a large number of protein–protein interactions are expected. Currently, supramolecular protein complexes are being isolated and characterised from inner mitochondrial membranes [38,61–67] and thylakoid membranes [68–70].

3.1. Isolation of supercomplexes

A gentle isolation procedure is the most important prerequisite for the in-depth analysis of supramolecular structures. This aspect has to be considered just from beginning the preparation of mitochondria (e.g. [38,71]). In this context, especially the solubilisation of the native membrane influences strongly the success of obtaining intact supercomplexes (see Fig. 2). Very mild solubilisation is achieved by non-ionic detergents. Particularly digitonin, n-dodecyl-β-D-maltoside (DDM) or Triton X-100 are very useful agents for this task. Digitonin is isolated from foxglove (*Digitalis purpurea*) [72]. In addition to its solubilisation properties [73,74], an important feature of digitonin is the interaction with cholesterol [75–77]. In contrast to the natural compound digitonin, DDM and Triton X-100 are produced by chemical synthesis and are available in larger scale [78]. DDM proved its worth for deciphering the structure of membrane proteins. A multitude of common detergents are useful for solubilisation, purification and crystallisation of membrane proteins [79–81]. But about 25% of all procedures leading to crystals applied DDM [82]. In contrast to DDM, which is frequently employed to obtain intact individual complexes of the respiratory chain, Triton X-100 has the advantage of stabilising supramolecular structure. A prerequisite for this stabilisation is to apply Triton X-100 at low detergent to protein ratios [83,84].

Even with sophisticated biochemical equipment isolation and structural characterisation of membrane proteins pose a challenge. Especially in the case of multimeric protein complexes, the long-term stability is an important issue [80]. This leads to restrictions regarding the purification procedure. In contrast to X-ray crystallography with its specific requirements in respect to the sample purity and homogeneity, single-particle electron microscopy studies can be conducted subsequent to detergent-based solubilisation and crude purification [85,86].

One important tool for identification and separation of supercomplexes is blue-native electrophoresis (BN-PAGE, see Fig. 3). This technique, pioneered by H. Schägger, employs the net-negatively charged dye Coomassie Brilliant Blue G-250 to allow/improve the migration of proteins in a gel matrix driven by an electric field [87]. Moreover, also fragile protein-protein interactions are maintained allowing the electrophoresis of protein complexes and supercomplexes in native state. However, particularly in the case of supramolecular structures of the ATP synthase, the omission of the dye can be necessary. Even for monomeric ATP synthase, this dye leads to partial dissociation into the subcomplexes F_1 and F_o [88] and only ATP synthases of some species are unaffected by this treatment [89]. For applications with fragile complexes, clear or colourless native PAGE (CN-PAGE) [90,91] or the variant high resolution CN-PAGE [92] are the methods of choice (see Fig. 2). In native gels, protein complexes and supercomplexes persist structurally and functionally intact [93–95]. This condition allows detection of enzyme activities directly in the gel (see Fig. 3) [96–98]. Moreover, a scale up of native PAGE paves the way to isolate intact membrane protein complexes by

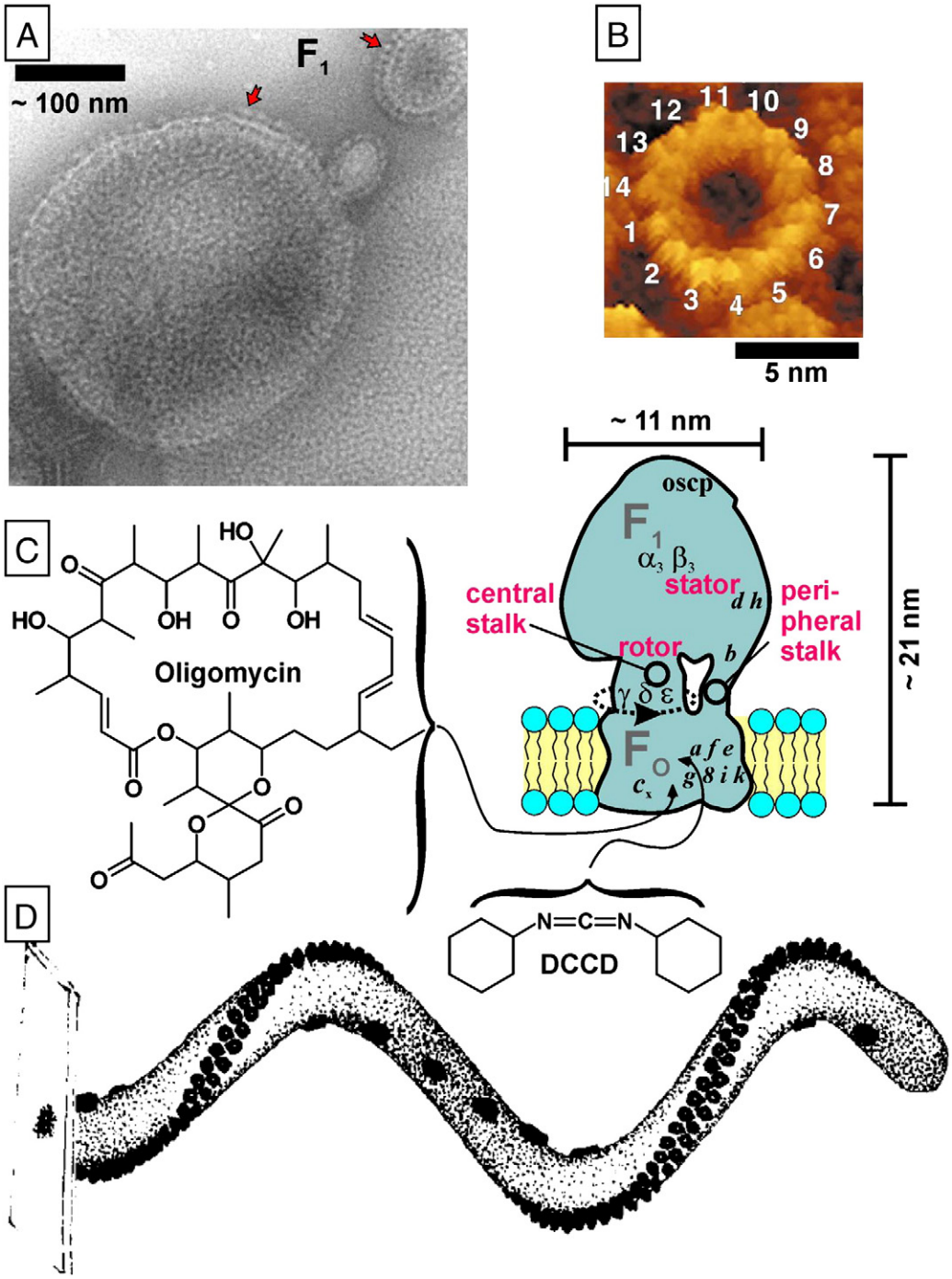

Fig. 1. ATP synthases and their supramolecular structures. A) Electron micrograph of chloroplast ATP synthase containing vesicles. Highly pure CF_1F_o from spinach chloroplasts, according to [249], was reconstituted at high protein to lipid ratio [94] into soy lecithin liposomes. This sample was stained negatively with 1% uranyl acetate and micrographs were recorded with a CCD camera at a Tecnai Spirit electron microscope. B) Atomic force micrograph of the spinach chloroplast oligomer III_{14}, taken from [33]. Reprinted by permission from Macmillan Publishers Ltd: NATURE 405, 418–419, copyright 2000. C) Schematic view of the ATP synthase and interaction sites of the inhibitors DCCD and oligomycin (structure of oligomycin B according to [250]), which block proton translocation. Different parts of the enzyme complex are assigned. D) Arrangement of ATP synthases into supramolecular structures. Helically linked F_1F_o complexes are depicted on tubular cristae structures. This classic model was taken from [19]. ©The Rockefeller University Press. The Journal of Cell Biology, 1989, 108: 2233–2240. doi:10.1083/jcb.108.6.2233.

elution [34,88,93,95,99,100]. Therefore, one path from biological membranes to supercomplexes in buffer solution is to solubilise the membranes by mild detergents, separate the proteins via native electrophoresis and subsequently elute them from the gel (see Fig. 2). By conducting this procedure, a protein complex is obtained in solution which provides the opportunity for a variety of functional and structural studies [99]. This approach has been applied to respiratory chain supercomplexes of bovine heart [93,101], to chloroplast ATP synthase monomers [34] but also to mitochondrial ATP synthase dimers from potato [102]. An alternative way of isolating respiratory chain supercomplexes is density gradient centrifugation, as used for *Arabidopsis thaliana*, yeast, *Zea mays*, and potato [102–105]. Here, the solubilised protein complexes are loaded on a preformed sucrose or glycerol gradient and centrifuged to separate the various species. While the loading capacity is superior to preparative gels, the resolution is significantly lower. Fractions from density gradients served as samples for the structural characterisation of the ATP synthase dimer from bovine heart, the alga *Polytomella*, and

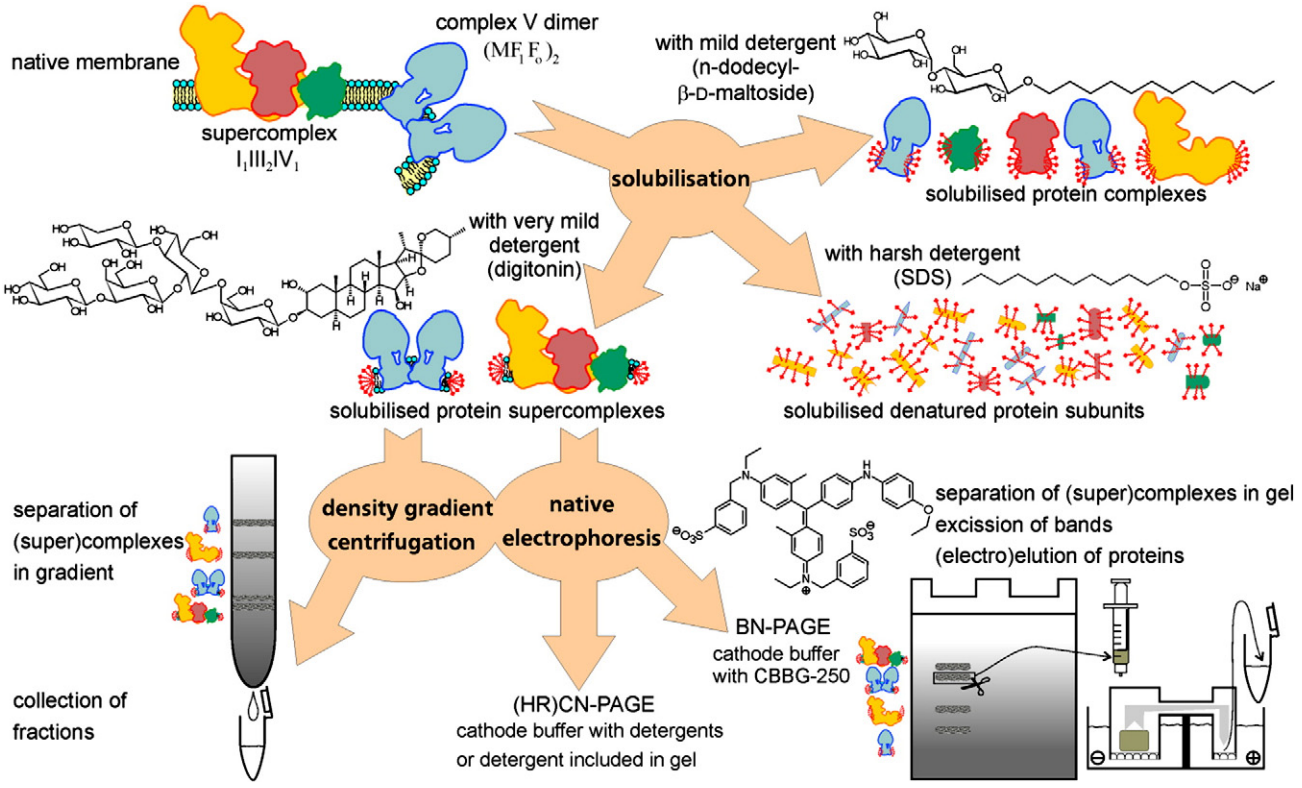

Fig. 2. Isolation of supramolecular structures of ATP synthases and respiratory chain supercomplexes. The choice of detergent for solubilisation determines the success of obtaining intact supercomplexes. One of the mildest detergents is digitonin (structure drawn according to [251]). Most frequently, density gradient centrifugation and (preparative) native electrophoresis are employed as purification steps subsequent to the solubilisation. Samples from density gradients can be directly employed for functional and structural studies. Native gels are applicable for in-gel activity tests, but in depth study require elution of the supercomplexes from the gel matrix.

yeast [106–109]. In contrast to preparative native PAGE and density gradient centrifugation, the otherwise frequently employed chromatography is something of a rarity in context with membrane protein supercomplexes. One recent exception is the use of size exclusion chromatography for the separation of different oligomeric form of the ATP synthase [110].

3.2. Identification of supercomplexes of the ATP synthase

The first hints for a supramolecular organisation of ATP synthase were provided by electron microscopy. Even in early studies of mitochondria often a regular arrangement of ATP synthase particles was described (e.g. [111–115]). Remarkably, also the row-like organisation [115] and the preferred occurrence of the particles on the convex side of curved tubuli [114] were recognised in these micrographs. A detailed model of the arrangement of ATP synthases in mitochondrial cristae membranes resulted from studies with rapid-freeze deep-etch electron microscopy. Mitochondria of the unicellular ciliate protozoon *Paramecium multimicronucleatum* were examined by this technique and demonstrated that F_1 complexes are arranged as a double row of particles (see [18,19] and Fig. 1D).

To date, native electrophoresis techniques are dominant in detecting supercomplexes due to their simplicity and cost-efficacy. In the first gel-based report of ATP synthase dimers, Triton X-100 was employed for the solubilisation of yeast mitochondria [84]. But in case of Triton X-100 the detergent to protein ratio has to be chosen carefully. While 2.4 g/g is required for quantitative solubilisation, isolation of the dimer succeeded at about 0.6 g/g but not at 2.4 g/g [84,116]. Upon employing digitonin, the detergent to protein ratio can be handled more flexible. In a wide range of 1.5–8.0 not only the dimer is preserved, but also the solubilisation efficacy is very high [116].

Schägger and Pfeiffer [116] also presented a second organism with dimers of the mitochondrial ATP synthase: cow. Meanwhile, the dimers have been identified via native electrophoresis in mitochondria from a large variety of organisms. The range of organisms covers the ciliate *Tetrahymena* [117], green plants [102,118–120], algae [121,122] diverse fungi (*Podospora anserina*, *Neurospora crassa*, *Yarrowia lipolytica*) [123–128], but also rat [129–134] and human cell cultures [135,136]. Recently, more and more organisms are added to this list, e.g. *Drosophila* [137]. Our own research now includes the fish *Nothobranchius furzeri* and here the ATP synthase dimers are also detected [138]. By BN-PAGE and particularly CN-PAGE not only dimeric species of the ATP synthase can be identified, but also higher oligomers, as described in yeast [139] and mammals (rat and bovine) [91,129]. In the archaeon *Methanothermobacter thermautotrophicus* a different kind of organisation was reported: homomeric supercomplexes of the ATP synthase stalk subcomplex [140].

In addition to native electrophoresis as screening technique to identify supramolecular assemblies of the ATP synthase, recently the structural characterisation again grows in relevance to reveal more details than the first electron microscopy studies listed at the beginning of this section. In 2005 two studies were published with single particle analysis of electron micrographs. The structure of dimeric ATP synthase from bovine heart mitochondria displayed connections between the hydrophobic as well as between hydrophilic domains of ATP synthase monomers as dimerisation interfaces [108]. In contrast, in dimers from the algae *Polytomella* sp. the ATP synthase had only contact sites in the hydrophobic domain [106]. Another significant difference is the angle between the monomers, which was approximately 40° for the bovine dimer and approximately 70° for

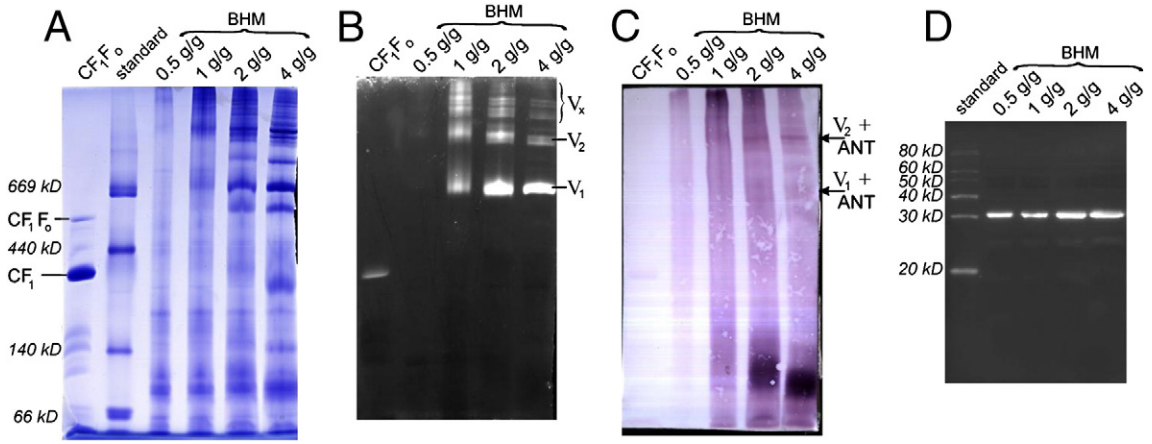

Fig. 3. Detection of supramolecular structures of ATP synthases and analysis of their activity. ATP synthase from spinach chloroplasts [249,252] treated to destabilise CF_1F_o to generate a large proportion of CF_1 and digitonin solubilised bovine heart mitochondria (BHM) with detergent to protein ratios from 0.5 to 4 g digitonin per g protein, according to [92] served as samples. In A–C, 5 μg CF_1F_o or 30 μg solubilised bovine heart mitochondria were applied to blue-native gels (T = 3.5–12%, [253]). For molecular mass calibration, native high molecular weight marker (66–669 kDa) from GE Healthcare (lane "standard") was applied. A) Gel stained with Coomassie blue R-250. B) Gel incubated in a lead(II)nitrate-containing buffer to detect ATP hydrolysis activity according to [96]. Several ATP synthase species were indicated: monomer (V_1), dimer (V_2), and oligomers (V_x). C) Gel blotted onto a PVDF membrane. The membrane was incubated with a primary antibody against adenine nucleotide translocator (ANT; sc-9299 from Santa Cruz Biotechnology) and an alkaline phosphatase conjugated secondary antibody. Bands were visualised by incubation in BCIP/NBT solution. Arrows indicate supercomplexes of ANT with ATP synthase monomers and dimers. D) The specificity of the ANT antibody was verified by Western blotting of a denaturing gel. 15 μg solubilised BHM were loaded on a SDS gel (T = 14%). For molecular mass calibration, MagicMark XP from Invitrogen (lane "standard") was applied. SDS gel blotted onto a PVDF membrane. The membrane was incubated with the ANT primary antibody and a horseradish peroxidase conjugated secondary antibody. Bands were visualised by incubation in West Dura Substrate (Pierce). Only one band is detected, located at the molecular mass of the ANT.

Polytomella. A subsequent study revealed, that two categories of dimers occur in *Polytomella* and in yeast: wide-angle dimers (70–90°) and small-angle dimers (35–40°) [107]. The classification as true and pseudo dimers lead to controversies which continue on [141,142]. Also the recently published single-particle analysis of the dimer from yeast mitochondria, demonstrating an angle of ~45°, will resume the discussion [109].

In addition to the study of isolated ATP synthase dimers, these structures were also examined in their native environment, the inner mitochondrial membrane. With atomic force microscopy rows of ATP synthase dimers were revealed in yeast mitochondrial membranes [143]. Advances in the electron microscopy techniques allowed to refine the models derived from the studies before 1995. By cryo electron tomography of rat liver and bovine heart sub-mitochondrial particles, dimeric ATP synthase was found to be arranged in ~1 μm long rows [144]. The dimers were proposed to enforce a strong local curvature on the membrane, which could act as a proton trap [144]. For yeast mitochondria, a combination of negative stain electron microscopy with cryo electron tomography studies was conducted and resulted in a model of zipper-like dimer ribbons [145]. Whereas the angle between ATP synthases varied from 30 to 55° in yeast mitochondria [145], in mammalian mitochondria angles of 70° and more occur [144]. For the yeast ATP synthase dimer, a dependency of the angle from the state of the sample was observed. The solubilised dimers exhibited larger angles than membrane bound ATP synthases, indicating an increased flexibility of the F_o–F_o hinge due to the presence of detergent [145]. Recently, by cryo electron tomography a 3D reconstruction of intact mitochondria of the algae *Polytomella* could be generated, which show oligomeric ATP synthase at 5.7 nm resolution [146]. In this reconstruction the angle between ATP synthase monomers was 70°, very similar to the mammalian dimers. Additionally, the 3D tomography data indicate a vicinity of the peripheral stalks [146]. Therefore, besides the membrane integral part F_o also extramembrane structures seem to be crucial for the stability of ATP synthase dimers. This aspect includes not only mitochondrial ATP synthase di-/oligomers, but also the dimer of the chloroplast enzyme. In the chloroplast ATP synthase dimer, vanadate-ions as an analogue of phosphate, which bind to the F_1 part, lead to dissociation of the dimer [147].

3.3. Subunit interactions in ATP synthase di-/oligomers

One main aspect of supramolecular structures of the ATP synthase is which subunits stabilise dimers and oligomers, i.e. which portions of the ATP synthase are the major contact sites.

3.3.1. Subunits e, g, k, and i

In the first electrophoresis-based report of ATP synthase dimers, the subunits *e*, *g*, and *k* were identified to be important for dimerisation [84]. Additionally also subunit *i* is required [148]. In yeast mitochondria, these four proteins belong to the membrane intrinsic F_o part of the ATP synthase. Subunit *e* is known to form homodimers [149] and has been structurally resolved in detail by NMR [150]. The C-terminal coiled-coil region of subunit *e* functions to stabilise the ATP synthase dimer [151]. Another important domain for the dimerisation process is the GxxxG motif in the transmembrane segment of subunit *e* [152]. The same motif is also found in subunit *g* and stabilises ATP synthase supercomplexes [153]. Subunit *e* together with *g* bind to monomeric yeast ATP synthases to initiate the assembly of ATP synthase dimers [154]. Upon changing the amounts of subunit *e* relative to *g*, the morphology of mitochondrial cristae is altered in yeast [155]. This finding points the way to decipher potential functions of supramolecular ATP synthase assemblies: Dimerisation and oligomerisation plays an important role in the formation of mitochondrial cristae. Recent studies demonstrate a variety of factors in context with the shaping of the mitochondrial morphology. One aspect is that formation of cristae depends on the antagonism of subunits *e*/*g* and the protein Fcj1 [110]. In addition to the subunits *e* and *g* also the small subunits *k* and *i* are involved during stepwise assembly of ATP synthase dimers. Subunit *i* organises the incorporation of new subunits, while subunit *k* tightly binds to the mature dimer and stabilises this complex [156].

3.3.2. The inhibitor protein IF_1

Another component whose rule is discussed in context of ATP synthase dimers is IF_1. IF_1 was first discovered in bovine heart [157] and is an inhibitor of the ATP hydrolysis activity of mitochondrial ATP synthases [158]. The IF_1 protein of mammals has homologues in yeast and plant mitochondria [159]. But also in *Paracoccus denitrificans* and related α-proteobacteria similar proteins have been identified [160].

The binding and release of IF_1 to ATP synthases is regulated by ATP and the membrane potential [161]. By utilising the membrane potential, IF_1 conserves the ATP supply of the cell [162]. Onto identification of ATP synthase dimers by BN-PAGE, a role of IF_1 during dimerisation was proposed [163]. But the exact function kept nebulous, because only two years later, the dependence of the dimerisation on the presence of IF_1 was challenged [164]. As affirmation, another group demonstrated in the same year that formation of dimers of the mitochondrial ATP synthase in yeast does not require the IF_1 homologue Inh1 [165]. This discussion was continued by a novel finding of IF_1 promoting the dimerisation of F_1F_o [130].

3.3.3. The ASA subunit of algae

In the green algae *Chlamydomonas reinhardtii*, the mitochondrial ATP synthase dimer was identified in 2003 independently by two groups [121,122]. This green alga as well as its colourless close relative *Polytomella* sp. have eminent peculiarities concerning the subunit composition of the ATP synthase [166]. The Chlamydomonadales algae have lost typical ATP synthase subunits as *b*, *d*, *f*, A6L, and F_6 but also subunits discussed above in context with dimerisation: *e*, *g* and IF_1 [167]. This loss is compensated by the gain of new subunits named "ATP Synthase-Associated" proteins ASA 1–9 [167–169]. A potential role of the ASA subunits is the stabilisation of dimeric ATP synthase, because another feature of the algae mitochondrial ATP synthase is its outstanding stability [121,168–170]. In the recent model of this ATP synthase dimer, the ASA subunits occupy important positions in the contact sites of the two monomers [142]. The modification of the ATP synthase also lead to an exceptional resistance towards the inhibitor oligomycin [171].

3.3.4. Role of the peripheral stalk

In mitochondrial ATP synthases, the peripheral stalk has a significantly different subunit composition compared to homologues from chloroplast or bacteria [172–174]. While bacterial ATP synthases possess a dimer of *b*-subunits as peripheral stalk, in mitochondria only one subunit *b* (subunit 4 in yeast) is present. Therefore, the identification of two *b*-subunits in close vicinity is surprising and indicates a role of this subunit in supporting ATP synthase-ATP synthase interactions [175,176]. This subunit is located near to subunit *g*, which suggests a concerted involvement of the peripheral stalk together with the subunits *e*, *g*, *i*, and *k* during dimerisation [177]. Even in yeast mutants, where the dimerisation subunit *e* is lacking, a systematic association between ATP synthases occurs and two *b*-subunits come in molecular proximity [178]. Particularly the membrane integral domain is fundamental for this process [179]. This domain consists of two transmembrane segments which were connected by an intermembrane space loop. Not only the α-helical parts but also the loop is important for the stability of supramolecular species of the mitochondrial ATP synthase [180]. Besides subunit *b* the *h*-subunit is the second component of the peripheral stalk in mitochondrial ATP synthases [181,182]. Both, *b* and *h* are involved in the dimerisation of the ATP synthase, whereby the interactions of the peripheral stalk subunits are presumably independent of subunits *e* and *g* [183]. In addition to the structural role, the subunits of the peripheral stalk also modulate the activity of the ATP synthase. In this context, the peptidyl prolyl *cis-trans* isomerase cyclophilin D is of particular importance [184]. Cyclophilin D binds to the ATP synthase and decreases hydrolysis and synthesis activity [185]. As binding site, the subunits OSCP, *b*, and *d* have been identified [186]. Upon displacement of cyclophilin D by addition of cyclosporine A, the ATP synthase is activated and a switch in the oligomeric assembly is suggested [186]. Also in algae, the peripheral stalk of the mitochondrial ATP synthase is suggested to play a main role in stabilisation of dimeric ATP synthases. Here, some ASA subunits substitute the peripheral stalk subunits *b*, *d*, *f*, A6L, and F_6 and may support the dimerisation [167,168]. In the chloroplast ATP synthase of algae, the subunits of the peripheral stalk are also proposed to be important components during dimerisation [147].

3.4. Involvement of further subunits

While the participation of peripheral stalk subunits during dimerisation seems to be reasonable, some new observations are irritating: they suggest an involvement of subunits directly responsible for the rotary mechanism. One component is subunit γ, the central rotating entity. During catalysis, whether ATP synthesis or hydrolysis, γ induces conformational changes and is a basic component of the mechanism [187–189]. The N- and C-termini of subunit γ are also relevant during the assembly process, for the formation of ATP synthase monomers and dimers [190]. In the membrane integral F_o section, the transport of protons is coupled to a rotational movement. While the *c*/III-oligomer serves as proton-driven rotor in the recent mechanistic model, subunit *a* provides the access and release channels for the protons [20,191,192]. Similar to the *b*-subunit of the peripheral stalk, subunit *a* can be connected to *a* from another ATP synthase complex, showing the proximity and a significant monomer–monomer interface in dimeric ATP synthase [193]. When an ATP synthase dimer is being assembled, the effect of subunits *e*/*g* and *a* on dimerisation is additive [194]. A further look into the sequence of events during dimer assembly demonstrates that primarily the dimers are formed and later the IF_1 protein is incorporated [194].

4. Supramolecular assembly of the ATP synthase and the organellar context

4.1. Interaction of ATP synthases with other proteins/protein complexes

Besides interactions of ATP synthases among themselves they are part of a sophisticated network of interactions with other proteins. A controversially discussed component is the so-called factor B, which is suggested to be an additional subunit of the ATP synthase [195]. This protein restores the energy coupling activity of the ATP synthase complexes in submitochondrial particles depleted of their factor B [195]. Meanwhile, the structure of factor B is known in detail [196]. A putative function of this protein with about 175 amino acids could be an optimisation of ATP synthesis under proton-limited conditions by binding of factor B tetramers to ATP synthase dimers [197]. As a result of this interaction, factor B is involved in the oligomerisation of ATP synthases and may be crucial for the cristae morphology [198].

Protein–protein interactions play a key role in coordinating ATP production and oxygen consumption in mitochondria. To adapt the energy metabolism to varying environmental conditions the flux of substrates has to be regulated. Important controlling steps are the cytochrome *c* oxidase (complex IV of the respiratory chain) and transport proteins (i.e. phosphate/proton exchange) [199]. To date it is nebulous whether this regulation occurs kinetically [199] or by direct contact of protein complexes [200].

The electrochemical proton gradient is the driving force for the ATP synthase, but ATP formation also requires a sufficient supply with the substrates ADP and phosphate. Both substrates are transported across the mitochondrial membranes by specific carriers: the adenine nucleotide translocator (ANT) and the phosphate carrier. The ANT is present in high amount in mitochondria and can be found as common contaminant in preparations of other mitochondrial proteins. Therefore, unambiguous data is needed to demonstrate specific protein–protein contact with the ANT. In the mid of the 1970s indications of a close-by localisation of the ANT and the F_1 subcomplex occurred [201]. In bovine heart preparations, the translocator is detected in the same density gradient fraction as the ATP synthase [202]. But in face of these hints, several years passed by until specific interactions between ATP synthases and transport proteins became increasingly clear.

Upon isolation of mitochondria from rat heart, cristae-like vesicles can be obtained by extensive subfractionation which allows extraction of the so-called ATP synthasome [203]. This structure is a supercomplex of an ATP synthase monomer with the ANT and the phosphate carrier. Similar to the ATP synthase dimers and oligomers, which require adequate handling to prevent dissociation, also for the ATP synthasome specific treatment is essential. The choice of detergent influences strongly the successful isolation of the ATP synthasome. A screening revealed that this supercomplex can be obtained with tridecyl-maltoside but not with DDM [203]. In the ATP synthasome both carriers (as heterodimer) are located adjacent to subunit *c*, as revealed by single-particle analysis [204]. The ATP synthasome is not a special feature of rat, similar structures are also reported in bovine heart [205,206]. We employed antibodies to study the interaction network of bovine heart ATP synthase in Western blots of native gels. Our results affirm the presence of the adenine nucleotide translocator in a complex with this ATP synthase (see Fig. 3). Surprisingly, the signal of the ANT antibody is very weak in complexes with monomers of the ATP synthase as obvious in Fig. 3. But at the position of dimers/oligomers the antibody labelling is much more pronounced, which is in accordance with recent indications [206].

Besides mammals, ATP synthasome structures have been identified also in evolutionary quite distinct organisms. *Leishmania*, which live as intracellular parasites of insects and invertebrates, likewise possess a complex of ATP synthase and the adenine nucleotide translocator [207]. Such ATP synthasomes allow an optimisation of ATP synthesis by carrying substrates near to the catalytic centre of F_1. Furthermore, the just formed ATP will be removed from the ATP synthase to minimise interference of the ATP synthesis reaction or to prevent unwanted ATP hydrolysis. A study with rat liver mitochondria demonstrated that the ATP synthasome is part of an even larger complex which includes the succinate dehydrogenase and an ABC transporter [208]. Therefore, secondary transporters are also included in the sophisticated interaction network of the ATP synthase [209,210]. To demonstrate interactions of the ATP synthase with the succinate dehydrogenase in living organisms, fluorescent fusion proteins have been expressed in *Bacillus subtilis*. This study revealed a co-localisation of both enzyme complexes in discrete membrane domains [211], which confirms the result for rat liver. Besides ATP, phosphocreatine molecules are main carriers of energy under physiological conditions. A connection of the ATP synthesis to the production of this second energy carrier is proposed by the formation of a mitochondrial interactosome [212]. This interactosome is suggested to consist of the ATP synthasome, the creatine kinase of the mitochondrial intermembrane space, the voltage-dependent anion channel VDAC and tubulin [212]. Such an organisation allows an efficient regulation of energy fluxes and encloses this system in the filamentous network of the cell.

Similar to the optimisation of the metabolite transport by forming a complex with carriers, also an improvement of proton transfer towards the ATP synthase enhances the efficiency of this enzyme complex. One way is to arrange the proton translocating complexes of the respiratory chain near to the ATP synthase. Another way is a more efficient proton migration along membrane surfaces [213,214]. Up to now, no stable supercomplex of respiratory chain complexes with the ATP synthase has been isolated, but several lines of evidence point to interactions of the ATP synthase with the cytochrome *c* oxidase (complex IV). In 1986, it was demonstrated that the incorporation of subunit 9 of yeast (homologous to subunit *c*) into the mitochondrial ATP synthase affects the assembly of complex IV [215]. Upon modifying the yeast ATP synthase by a mutated subunit γ with the redox regulatory region of chloroplasts, the activity of the cytochrome *c* oxidase drops by 90% [216]. A correct proton transfer requires both complexes in intact form. This is confirmed by the finding that the biogenesis of complex IV is only completed successfully when the ATP synthase is fully assembled and able to transport protons [217]. With this regulatory mechanism the quantity of proton donors and acceptors can be matched to the bioenergetic requirements.

In yeast, the dimerisation subunit *g* of the ATP synthase is crucial for the interactions with complex IV. Upon deletion of subunit *g*, not only ATP production decreases but also the activity of the cytochrome *c* oxidase. In addition to *g*, also subunit *e* affects complex IV. For maximum cytochrome *c* oxidase activity, both *e* and *g* have to be present in the ATP synthase complex [218]. As a consequence of the supramolecular organisation of the respiratory chain, alterations in the ATP synthase can affect the function/assembly of complex IV containing supercomplexes. In addition to a decreasing complex IV activity, the composition of the III_x–IV_y supercomplexes changes once both subunits *e* and *g* were deleted. Therefore, yeast mutants without *e* and *g* exhibit more individual cytochrome bc_1 complex (or complex III dimers) and less III_x–IV_y supercomplexes [218]. But the interaction of the ATP synthase with complex III is not a direct contact. This was determined with differential scanning calorimetry and electron paramagnetic resonance, which indicated only interactions of the ATP synthase with the cytochrome *c* oxidase but not with the cytochrome bc_1 complex [219]. Interactions of the ATP synthase with complex IV are not limited to mitochondria. In the electron transfer chain of aerobic bacteria, some enzyme complexes are very similar to the cytochrome *c* oxidase. In cyanobacteria, a mutual regulation of the activity of the ATP synthase with the cytochrome *b*-563/*c*-554 complex occurs [220]. Alkaliphilic bacteria require a 1:1 stoichiometry of the ATP synthase with a cytochrome caa_3 complex to synthesise ATP under conditions of very low proton motive force [221].

4.2. Formation of cristae and supramolecular organisation of ATP synthases

Even in the early electron micrographs of mitochondria it is clearly recognisable that ATP synthases are arranged near to tubular membrane structures. In the model of cristae membranes derived from rapid-freeze deep-etch electron microscopy (see Fig. 1 and [18,19]) ATP synthase double rows (dimers) can be found in strongly curved regions. A direct link between morphology and dimerisation was identified upon growing yeast cells deficient in non-essential subunits of the ATP synthase. A deletion of either subunits *e* or *g* leads to an altered cristae morphology with numerous digitations and onion-like structures [139]. The data suggests that the dimerisation is involved in the control of the genesis of the inner mitochondrial membrane [139]. When subunit γ was modified by fusion to a fluorescent protein (DsRed) known as an in vivo crosslinker, yeast cells expressing this fusion protein as a subunit of the mitochondrial ATP synthase grew slower compared to control cells [222]. The cause for this impaired growth is the elimination of the cristae structure which is attended by the occurrence of artificial ATP synthase tetramers [223]. In contrast to native ATP synthase oligomers, the assembly of these tetramers was mediated through subunits not normally associated with dimerisation, demonstrating the impact of correct supramolecular organisation to cells in vivo indispensable for normal mitochondrial function. In addition to the subunits *e* and *g* also IF_1 is important for formation of cristae: IF_1 overexpression increases the formation of dimeric ATP synthase complexes and the density of mitochondrial cristae is increased [162].

The dimerisation subunits of the ATP synthase are key players in organising the mitochondrial cristae structure but they are not the only components [224,225]. Mitophilin is a critical organiser of the mitochondrial cristae morphology in HeLa cells [226] and *Caenorhabditis elegans* [227]. In yeast, Fcj1 is a homolog of the mitophilin protein and here the antagonism of Fcj1 and the ATP synthase subunits *e*/*g* modulates the oligomeric state of the ATP synthase and controls membrane curvature of cristae [110]. As proposed, Fcj1 interferes with the formation of higher oligomers and thereby favours negative membrane bending, i.e. convex structures [110]. In contrast, ATP oligomers induce a positive curvature to the inner mitochondrial membrane [144]. Therefore, one important pathway to regulate the cristae morphology is the ATP synthase-mitophilin way [224]. The

second mechanism involves dynamin-like GTPases and prohibitins [228,229]. This system seems to basically rely on the size or the proteolytic processing of the dynamin-like GTPase Opa1 (in human) or Mgm1p (in yeast) [228,230]. Also this second way is connected to the ATP synthase. Mgm1p serves as an upstream regulator of the dimerisation subunit *e* (Tim11p) which affects protein stability, ATP synthase assembly, and cristae morphology [231]. While the stability of respiratory chain supercomplexes is significantly affected by cardiolipin, previous data indicated that the lack of this anionic phospholipid had little effect on the stability of dimeric ATP synthase [232]. In contrast, recent data suggest that also cardiolipin promotes the oligomerisation of ATP synthases [137].

4.3. Activity of ATP synthase dimers and metabolic control of dimerisation

One important question in context with dimerisation of ATP synthases is: are there any differences in the activity between monomeric and dimeric/oligomeric species of the ATP synthase? A putative role of the dimerisation could be the stabilisation of the interaction within each ATP synthase by compensating rotational torque [143]. In the case of the respiratory chain complexes, the assembly to supercomplexes has been proven to boost the catalytic activity (of the complexes I and III_2 by complex IV [93] and of complex I by III_2 and IV [134]). To study the performance of the ATP synthase, native gels can be directly employed to perform *in-gel* activity tests which allow a comparison of different ATP synthase species in one sample (see Fig. 3). Even if these tests only concern the hydrolysis activity of the enzyme, they are a valuable tool in many studies. When bovine heart mitochondria were incubated in three different buffer systems, the monomer/dimer ratio, judged by the Coomassie dye staining intensity, was constant [164]. But in all buffers the ATPase activity of the dimer was almost zero whereas the monomer was active and produced pronounced white lead phosphate bands in this assay [164]. In contrast, in yeast mitochondria the ATP synthase dimer displayed the most intense activity compared to the monomer [139]. An important aspect for this activity test is the kind of gel used for the assay. While blue native gels are mild, the milder variant is CN-PAGE [91,233]. The application of CN-PAGE not only facilitates the detection of higher oligomers of the ATP synthase but also avoids the dye which affects the stability of the enzyme complex [88,89,94] and leads to low *in-gel* hydrolysis activities [96,234]. Moreover, when CN- instead of BN-PAGE is used, the hydrolysis activity can be inhibited specifically by adding oligomycin [91,232]. This variant of the assay allows discrimination between intact ATP synthases with F_o fully coupled to F_1 and individual F_1 sections which lost their sensitivity towards the inhibitor. Upon deploying CN-PAGE, the ATPase activities of monomers, dimers and oligomers in yeast and mammals were similar [91,129,232]. During the studies of the *in-gel* ATPase activity more factors were identified to be crucial. Also the detergent employed during solubilisation has an influence on the activity. As shown for bovine heart, Triton X-100 extracts have a lower activity than digitonin-solubilised ATP synthase dimers [235]. This may also explain why the Triton X-100 solubilised bovine heart dimer had almost no ATPase activity in the study mentioned above [164]. In addition, the assay temperature strongly affects the ATPase hydrolysis test. While at 20 °C the activities of monomers and dimers are similar, an increase of the temperature reveals significant differences [236]. At 30 and 37 °C dimers in heavy bovine heart mitochondria and mitoplasts had greater specific activity than monomers, but not dimers in submitochondrial particles [236]. This points to a role of dimerisation in the regulation of the nanomotor function of the ATP synthase [141]. A regulatory role is confirmed by studies of phosphorylation of monomer vs. dimer. By an anti-phosphotyrosine antibody the F_1 γ subunit of bovine heart mitochondrial ATP synthase was detected to be phosphorylated in the monomer but not in the dimer [237]. In yeast, phosphorylation of a serine residue of the dimerisation subunit *g* (see above) was found to be critical for the dimerisation [238]. Exchange of this serine by a phosphomimetic residue inhibited dimerisation, whereas exchange by alanine enhanced the level of dimerisation [238]. Experiments in yeast mutants demonstrated a link between phosphorylation, dimerisation and activity. A phosphomimetic mutation of a threonine residue in the β subunit (involved in formation/maintenance of dimers) leads to a decreased ATPase activity [239].

Since the main function of the ATP synthase is the production instead of hydrolysis of ATP, the most interesting measurements are those in synthesis direction. Such experiments were performed with yeast ATP synthase reconstituted into vesicles and the ATP synthesis rate and ATP yield was compared as a function of the ATP synthase concentration. A conclusion of this study was that monomeric ATP synthase catalyses high rates of proton transport driven ATP synthesis and that dimerisation, if it occurs, does not influence the ATP synthesis [240]. This observation is in line with the result that deletion of the dimer subunits *e* and *g* has no influence of the catalytic activity of the ATP synthase [84]. Maybe one ambiguity of such measurements is the microenvironment. The ATP synthase resides at the apex of cristae which should increase the local pH gradient allowing ATP synthesis even under proton-limited conditions [144]. This is in accordance to the finding that the mitochondrial membrane potential is dependent on the oligomeric state of ATP synthases [241]. In this study, a role for the supramolecular structures of the ATP synthase in organising microdomains not only of the ATP synthase but also of other supercomplexes within the inner membrane was proposed, which should optimise metabolite channelling [241].

In contrast to the counterpart in yeast or mammals, the mitochondrial ATP synthase dimer of green algae is particularly stable [121,122,168,169]. Therefore, the comparison of the activity monomer vs. dimer requires a specific treatment to monomerise the dimer. One approach is incubation of the dimeric enzyme with taurodeoxycholate [170]. The study of different features of both ATP synthase species indicated a significantly higher stability of the dimer and moreover demonstrated that the dimer is the active form of algae mitochondrial ATP synthase [170]. Since algae not only contain mitochondria but also chloroplasts, this circumstance enables a direct comparison of two types of ATP synthases in the same organism. When *C. reinhardtii* is cultivated at different growth conditions, the proteomic pattern of many thylakoid membrane proteins changes [122]. In addition, the various growth parameters varied had a significant influence on the metabolic state of the green algae. But in face of these changes, the composition of the chloroplast ATP synthase, particularly the number of subunits in the cylinder-shaped oligomer III_x, remained constant [242]. Likewise, the dimer of the mitochondrial ATP synthase displayed no alterations in subunit composition [243]. In contrast, the supramolecular organisation of the chloroplast ATP synthase varies. Whereas the mitochondrial ATP synthase dimer of algae is very stable, the chloroplast homologue is more susceptible towards environmental effects [147]. One important factor is phosphate, which serves as substrate for the synthesis of ATP. Phosphate binds to the catalytic centre of the ATP synthase at a nucleotide-free catalytic site [244]. As a consequence, conformational changes occur. When single F_1 subcomplexes were studied in hydrolysis direction, phosphate release drives the last 40° of the 120° step in the catalytic mechanism [245]. In case of the chloroplast ATP synthase dimer of algae, this dimer dissociates into monomers upon incubation with phosphate and vanadate (as transition-state analogue of phosphate) but not by incubation with molybdate [147]. In contrast, the mitochondrial dimer is not affected by either incubation. This suggests a distinct dimerisation mechanism for mitochondrial and chloroplast ATP synthase and indicates that the contact sites between monomers may differ [147]. A main contact site for the chloroplast ATP synthase dimer might be the peripheral stalk. To study the regulation by cell physiological processes quantitatively, stable isotope labelling of living *C. reinhardtii* cells and blue-native

PAGE have been employed. By comparing to photoautotrophic growth, an increased assembly of chloroplast ATP synthase dimers on the expense of preexisting monomers during photomixotrophic growth was observed, demonstrating a metabolic control of the dimerisation process for the chloroplast enzyme [243].

Switching back to the mitochondrial ATP synthase again, the interplay between metabolism and dimerisation reveals upon analysing changes of the (supramolecular) protein organisation dependent on age or pathophysiological processes. In rat brain and liver, the abundance of the intact ATP synthase decreases during ageing (1.5–2 fold) [131,132,246,247], also in skeletal muscle [248] as well as in the fungus *P. anserina* and in human cells [136,247]. One important indicator of specific mitochondrial diseases is the presence of unbound F_1 part in increased amount [234]. Such F_1 subcomplexes hydrolyse ATP and shorten energy supply of the cell. During ageing, the abundance of unbound F_1 increases, as demonstrated for rat brain [131,132]. Surprisingly, the proportion of the ATP synthase monomer in comparison to the oligomers in rat brain also changes during ageing [38,131,132]. The findings establish the proton ATP synthase firmly as one of the prime mitochondrial targets of age-related changes. However significant details, namely the correlation of supramolecular organisation, ATP synthase activity and the energy status of the cell, are still nebulous and require intensified efforts of elucidation.

Demonstrated but not understood in molecular functional detail, protein–protein interactions and homo/heteromeric assembly of supercomplexes, metabolomes and interactomes are of crucial importance for metabolism.

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