




ATP synthase F_0F_1 structure, function, and structure-based drug design

Alexey V. Vlasov^{1,2} · Stepan D. Osipov¹ · Nikolay A. Bondarev¹ · Vladimir N. Uversky^{1,3} · Valentin I. Borshchevskiy^{1,4,5} · Mikhail F. Yanyushin⁶ · Ilya V. Manukhov¹ · Andrey V. Rogachev^{1,2} · Anastasiia D. Vlasova¹ · Nikolay S. Ilyinsky¹ · Alexandr I. Kuklin^{1,2} · Norbert A. Dencher^{1,7} · Valentin I. Gordeliy^{1,4,5,8} 

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Abstract

ATP synthases are unique rotatory molecular machines that supply biochemical reactions with adenosine triphosphate (ATP)—the universal “currency”, which cells use for synthesis of vital molecules and sustaining life. ATP synthases of F-type (F_0F_1) are found embedded in bacterial cellular membrane, in thylakoid membranes of chloroplasts, and in mitochondrial inner membranes in eukaryotes. The main functions of ATP synthases are control of the ATP synthesis and transmembrane potential. Although the key subunits of the enzyme remain highly conserved, subunit composition and structural organization of ATP synthases and their assemblies are significantly different. In addition, there are hypotheses that the enzyme might be involved in the formation of the mitochondrial permeability transition pore and play a role in regulation of the cell death processes. Dysfunctions of this enzyme lead to numerous severe disorders with high fatality levels. In our review, we focus on F_0F_1 -structure-based approach towards development of new therapies by using F_0F_1 structural features inherited by the representatives of this enzyme family from different taxonomy groups. We analyzed and systematized the most relevant information about the structural organization of F_0F_1 to discuss how this approach might help in the development of new therapies targeting ATP synthases and design tools for cellular bioenergetics control.

Keywords F_0F_1 ATP synthase · Membrane proteins · Intrinsically disordered proteins (IDP) · Small-molecule cofactors · Isoprenoid quinones · Structure-based drug design

Introduction

Cellular bioenergetics is essentially based on the catalysis of the energy storage molecules using conformational changes of protein complexes involved in the oxidative phosphorylation (OXPHOS) or photophosphorylation processes. The

actual synthesis of these small molecules, which are vital for most organisms, takes place at the interfaces between different subunits of these protein complexes.

ATP synthase is a key enzyme in the OXPHOS and photophosphorylation processes. It produces adenosine triphosphate (ATP)—a small energy storage molecule powering

✉ Valentin I. Gordeliy
g.valentin@fz-juelich.de

¹ Research Center for Molecular Mechanisms of Aging and Age-Related Diseases, Moscow Institute of Physics and Technology, 141700 Dolgoprudny, Russia

² Joint Institute for Nuclear Research, 141980 Dubna, Russia

³ Department of Molecular Medicine and Byrd Alzheimer's Research Institute, Morsani College of Medicine, University of South Florida, Tampa, FL, USA

⁴ Institute of Biological Information Processing (IBI-7: Structural Biochemistry), Forschungszentrum Jülich, 52425 Jülich, Germany

⁵ JuStruct: Jülich Center for Structural Biology, Forschungszentrum Jülich, 52428 Jülich, Germany

⁶ Institute of Basic Biological Problems, Russian Academy of Sciences, 142290 Pushchino, Moscow region, Russia

⁷ Physical Biochemistry, Department Chemistry, Technische Universität Darmstadt, Alarich-Weiss-Straße 4, 64287 Darmstadt, Germany

⁸ Institut de Biologie Structurale Jean-Pierre Ebel, Université Grenoble Alpes-Commissariat à l'Energie Atomique et aux Energies Alternatives-CNRS, 38027 Grenoble, France

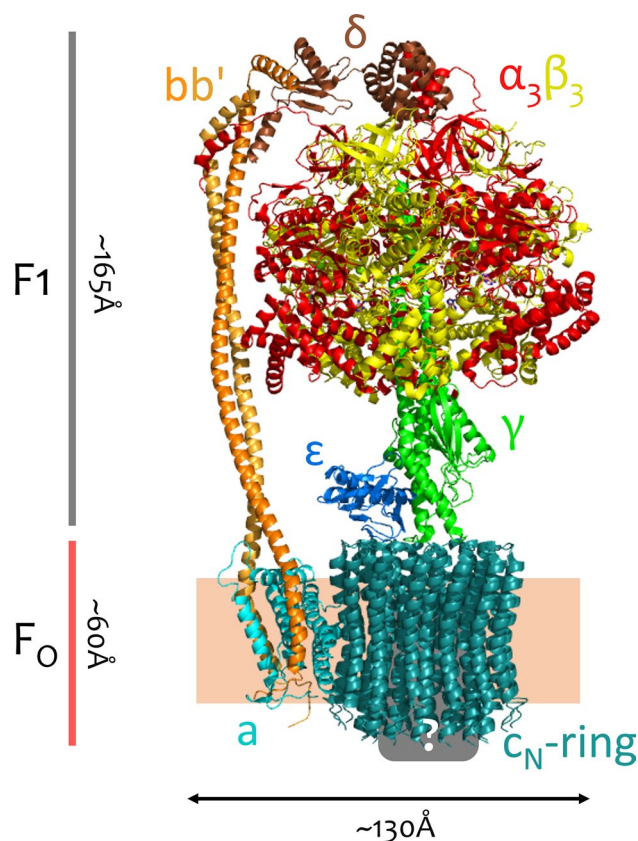


Fig. 1 An overall view of the cF_0F_1 ATP synthase (most of the bF_0F_1 comprise similar subunits). F_0 part consists of $abb'c_N$ subunits, F_1 part consists of $\alpha_3\beta_3\gamma\epsilon\delta$ subunits, bb' is a peripheral stalk. Inside the c_N -ring pore, there are molecules that interact with its inner surface. The PDB structure 6FKF of cF_0F_1 from the spinach chloroplasts is shown [3]. The figure is reprinted from [4]

vital biochemical reactions in living cells. F-type ATP synthase consists of F_1 and F_0 multisubunit complexes (water-soluble and membrane integral, respectively), which are connected by central and peripheral stalks. The central stalk rotates with the c-ring as a rigid body, triggering conformational changes and rotation of the catalytic part $\alpha_3\beta_3$ (Fig. 1). ATP is synthesised at the interaction interface of the α/β catalytic subunits of the ATP synthase by chemomechanical coupling via the rotation of the c-ring subunit complex in the membrane [1, 2].

ATP synthase is extremely wide-spread and is present in almost all organisms from bacteria and archaea to eukaryotes. F-type ATP synthase was found in the bacterial cellular membranes, thylakoid membranes of chloroplasts, and cristae of mitochondria in eukaryotes. Various types of structural oligomeric organization of ATP synthases were found in different species. Bacterial ATP synthases (bF_0F_1) are considered to be monomers [5–7]. Chloroplast ATP synthases (cF_0F_1) are also found to be mostly monomers [3, 8]. However, some evidence showed

dimerization of cF_0F_1 , which is probably connected with the regulation processes of ATP synthesis in chloroplasts [9]. Mitochondrial F-ATP synthases (mtF_0F_1) are arranged as dimers, tetramers, and, in some cases, hexamers [10], thereby determining the topology of mitochondrial cristae in eukaryotes [5]. Intriguingly, slight differences in the peripheral subunit composition may lead to the significantly different assemblies of ATP synthases among organisms, even if the assembly of the mtF_0F_1 dimer (type of dimer) remains the same [10, 11].

A vital importance of ATP synthase for cellular bioenergetics implies that dysfunctions of this enzyme result in a number of severe diseases with high fatality. Severe metabolic disorders, such as malfunctioning of tissues and organs and diabetes, are only a part of the problems that can be caused by improper functioning of ATP synthase [12, 13]. In addition, there is a hypothesis that ATP synthase might be involved in the formation of the mitochondrial permeability transition pore (mPTP) [14–16], also known as the mitochondrial mega-channel (MMC) [17–22], which represents a non-selective mega-channel in the inner mitochondrial membranes. The permeability transition phenomenon of acute swelling and uncoupling of mitochondria when exposed to high calcium concentrations and phosphate, oxidative stress, or other conditions leads to the dissipation of the proton motive force (PMF, promoting movement of protons across membranes downhill the electrochemical potential) and, consequently, to the cell death.

The membrane rotary part of the ATP synthases—c-ring and, in particular, its inner pore—might play a crucial role in proper functioning of the whole enzyme. Furthermore, a hypothesis suggesting that the c-ring itself might act as a mega-channel in the mPTP was proposed [14]. In our previous reports [4, 23], we hypothesised that the isoprenoid quinones might be present inside the inner pore of the c-rings in almost all organisms, especially in bacterial and chloroplast ATP synthases. They might form a complex dynamic interaction interface with the inner α -helices of the c-ring, and this interface is extremely important in stabilization of the c-ring, preventing ion leakage, and influencing the rate of the ATP synthesis.

The gaps in the knowledge of the molecular mechanism of the ATP synthase action can be filled mainly via structural studies of the enzyme. However, obtaining high-resolution structures of the protein complex is not sufficient. To clearly understand molecular mechanisms of stabilization of the ATP synthase during two synchronized rotation processes ($\alpha_3\beta_3$ catalytic part and the c-ring in the membrane), it is necessary to track the conformational changes or obtain a series of high-resolution structures at different stages of the enzyme action [7]. Nevertheless, static high-resolution structures of ATP synthases are also extremely useful for the development of new therapies of various diseases by

F₀F₁-structure-based drug design. One of the successful examples of using F₀F₁-structure-based approach was understanding the molecular mechanisms of a novel anti-tuberculosis (TB) drug bedaquiline (BDQ), based on the high-resolution structures of a c-ring [24] and the complete bF₀F₁ complex [25] from mycobacteria.

In this review, we highlight recent advances in structural biology of ATP synthases, systematize high-resolution structural data, and describe F₀F₁-structure-based approach to design new therapies. We demonstrate the similarities in structural organization of various ATP synthases found in the representatives of different phylogenetic groups. We discuss how to use these structural features for developing ligands capable of binding to the corresponding F₀F₁ and other possible ways of modulating F₀F₁ functioning. We emphasise such an approach involves information on high-resolution structural data of different families of F₀F₁.

Similarity and diversity of ATP synthases

In different organisms, ATP synthases demonstrate unexpectedly high diversity of their subunit composition. It is intriguing that almost all bF₀F₁ and cF₀F₁ have minimal differences in the subunit composition of the enzyme ($\alpha_3\beta_3\gamma\epsilon\delta\text{bb}'\text{can}$ —9 types of subunits, except Mycobacteria, which lack the δ -subunit—it is an extension of the b-subunit of a peripheral stalk [25]). Furthermore, all these enzymes are mostly monomers, although subtomographic studies of chloroplasts showed that the population of the cF₀F₁ represents a distribution of oligomeric forms containing 85% of monomers, 12% of “pairs”/dimers, and 3% of higher oligomers of cF₀F₁ [8]. On the other hand, cryo-EM studies of spinach cF₀F₁ revealed that the enzyme is characterized by a monomeric structure that can be resolved to about 3 Å [3]. However, the sample preparation included reconstitution of the ATP synthase into nanodiscs with the scaffold protein MSP2N2. This means that the maximum diameter of these nanodiscs was about 145 Å [26], which is able to fit only a monomeric form of the cF₀F₁ (Fig. 1).

In contrast to bacteria, eukaryotes have significantly different subunit composition of ATP synthases, which are incorporated into the inner membranes of their mitochondria. Mitochondria themselves are thought to descend from alphaproteobacteria (a class of bacteria in the Proteobacteria phylum), and the bacterial intracellular membranes that were used to harness energy are thought to become the mitochondrial cristae [27]. These alphaproteobacteria had a monomeric ATP synthase, which further should have been modified to create dimers in the mitochondrial cristae. Therefore, the machinery, which determines the topology of the cristae [28], should have occurred simultaneously with the advent of the ATP synthase dimers. The transition from

the monomeric bF₀F₁ to the mtF₀F₁ dimers added functionality to the enzyme. In fact, the determination of the cristae topology and, therefore, the morphology of the mitochondria in different species was the direct consequence of that transition [29].

mtF₀F₁

Interestingly, the core subunits of ATP synthases ($\alpha_3\beta_3\gamma\epsilon$, a-subunit and the membrane part of a c-ring) are present with slight modifications in different groups of the Eukaryota superkingdom. At the same time the peripheral subunits are significantly different, so that mtF₀F₁ has four types of dimers (Fig. 2).

Currently Eukaryota superkingdom contains seventeen groups of different rank according to the NCBI taxonomy classification [30]. The structures of complete ATP synthases are available for representatives of only four of them: Opisthokonta, Sar and Discoba clades, and Viridiplantae. Interesting fact is that mtF₀F₁ dimers have differences in their subunit composition and consequently are characterized by different shape only between representatives of different groups [5].

The catalytic activity of purified and isolated ATP synthase was demonstrated only for limited cases [3, 25, 31, 32]. The rest of the papers reported the high-resolution structures of F₀F₁ do not report about validation of ATP synthesis activity.

Inside each group, mtF₀F₁ dimers belong to the same type and show minimal differences according to available information. For example, Opisthokonta clade representatives have V-shape mtF₀F₁ dimers of type I, which produce ~86° of membrane curvature (Fig. 3A, E). High-resolution structures are available for mtF₀F₁ from yeast [33] and porcine heart [34]. The mtF₀F₁ tetramer was described as the largest assembly for this group (Fig. 3A). On the other hand, the Viridiplantae representatives have V-shape mtF₀F₁ dimers of type II, which results in the less membrane curvature ~55°–56° (Fig. 3B, F) than that of the I type. Viridiplantae also have chloroplasts with the monomeric cF₀F₁, which can assemble to oligomers under specific conditions [9, 35, 36]. High-resolution structures are available for the mtF₀F₁ dimer from unicellular alga *Polytomella* sp. [37] and for the cF₀F₁ monomer from spinach leaves [3]. Dimeric form is the highest oligomeric state of the ATP synthases from this group (Fig. 3B). Sar clade representatives have mtF₀F₁ dimers of type III, which almost do not curve the membrane and have a U-shape structure (Fig. 3C, G). In this group, the representatives of Ciliophora phylum (Alveolata clade) have tetramers of mtF₀F₁ (two U-shape dimers), which determine the cristae morphology, as it was shown by solving the high-resolution structure of the mtF₀F₁ tetramer from *Tetrahymena thermophila* [11]. Interestingly, the representatives of

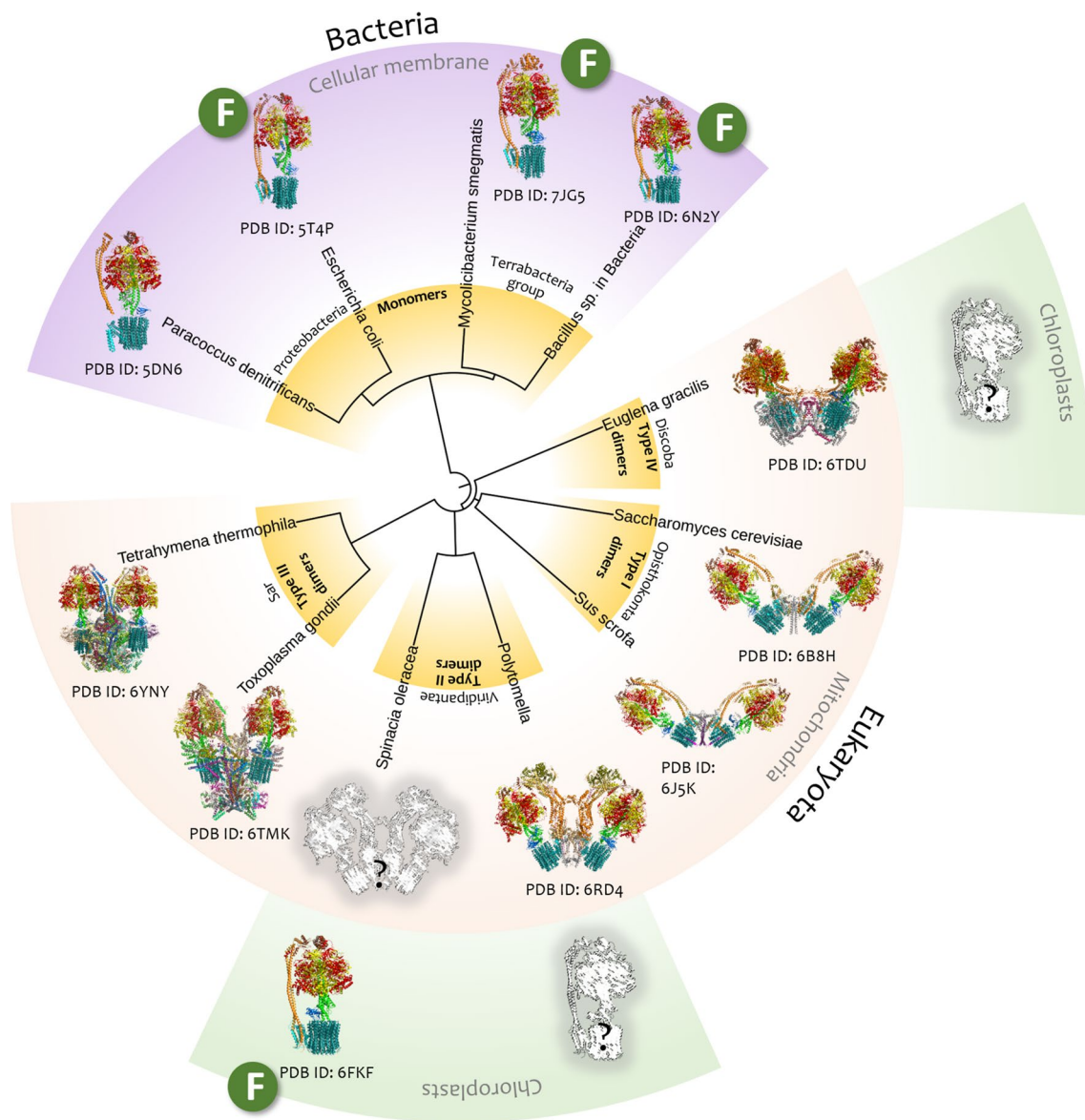


Fig. 2 Phylogenetic tree of species with available structural data on F-type ATP synthases. The phylogenetic tree includes the species from two superkingdoms (Bacteria and Eukaryota), and eukaryotes from Viridiplantae, Opisthokonta, Sar, and Discoba clades (the representatives of the superkingdom Archaea have A-type ATP synthases and are not discussed in this review). The type of ATP synthase oligomeric state is determined for cellular membrane bF_OF₁ in the case

of bacteria and for mtF_OF₁ in the case of eukaryotes. Some eukaryotes which have chloroplasts, and consequently cF_OF₁, are highlighted green. The letter “F” means the reported functional activity of purified and isolated F_OF₁. Structures of ATP synthases are marked with the corresponding PDB IDs. Unknown structures of ATP synthases are painted as a faded grey sketch with a question mark. The figure was slightly modified from ref. [4]

the Apicomplexa phylum (Alveolata clade) have six ATP synthases assembling in hexamers that form bipentagonal pyramids with icosahedric symmetry, which determines the cristae morphology (high-resolution cryo-EM structure is available for the mtF_OF₁ hexamer from *Toxoplasma gondii* [10]).

This is a new form of mtF_OF₁ organization in comparison with other known species, where the mtF_OF₁ complexes have a tendency to arrange into rows. Finally, representatives

of Discoba clade have V-shape mtF_OF₁ dimers of type IV, producing approximately the same membrane curvature as that generated by the type II of mtF_OF₁ dimers (~55°–56°) (Fig. 3D, H). These organisms also have chloroplasts with monomeric cF_OF₁. High-resolution structure is available for mtF_OF₁ dimer from *Euglena gracilis* [40], and no structural data are currently available for the cF_OF₁ from any representative of the Discoba clade. Considering subunit composition of ATP synthases, in bacteria and chloroplasts, bF_OF₁ and

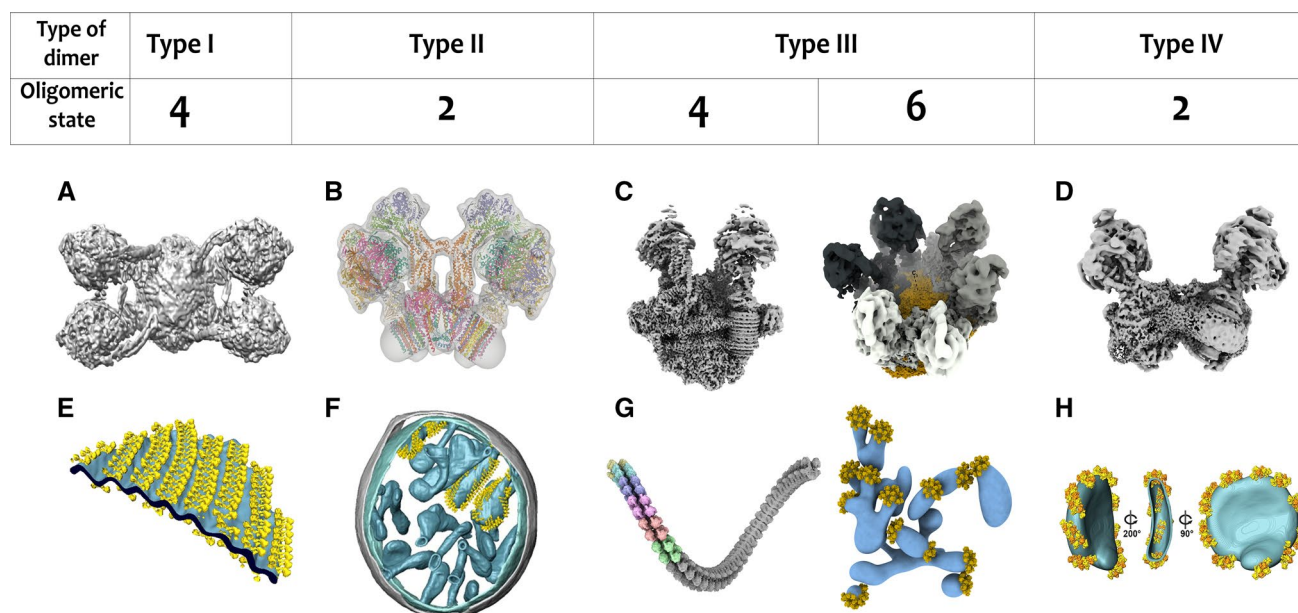


Fig. 3 Four types of mtF₀F₁ dimers. **A–D** Show cryo-EM structures of mtF₀F₁ and **E, F**—their arrangement in 3D volume cristae. **A, E**—the tetramers of mtF₀F₁ from animals & fungi (dimers of type I). **B, F**—the dimers of mtF₀F₁ from unicellular green algae (type II). **C, G**—the tetramers of mtF₀F₁ from ciliates and hexamers of mtF₀F₁

from Apicomplexa (dimers of type III). **D, H**—the dimers of mtF₀F₁ from *Euglena* and trypanosomes (dimers of type IV). **E, F** Were reprinted from ref. [38]. **C, G** were reprinted from refs. [10, 11]. **H** Was reprinted from ref. [39]

Table 1 Subunit structure and oligomeric state of different F-type ATP synthases according to the available structural data. The table is modified from ref. [4]

Super-kingdom	Bacteria	Eukaryota				
			Opisthokonta	Viridiplantae	Sar	Discoba
Organelles	cellular membranes	*chloroplasts		chloroplasts*		chloroplasts*
		thylakoid membranes	mitochondria			
Oligomeric state of F ₀ F ₁	monomers		dimers			
			Type I	Type II	Type III	Type IV
Subunit composition of F ₀ F ₁	α ₃ , β ₃ , γ, δ, ε, a, b, b', c _n	α ₃ , β ₃ , γ, δ, ε, a, b, b', c _n	α ₃ , β ₃ , γ, δ, ε, a, b, c _n , d, e, f, g, h, i (i/j or 6.8PL), k (DAPIT), l, 8, OSCP, F6	α ₃ , β ₃ , γ, δ, ε, c _n , a, OSCP, ASA(1 - 10)	α ₃ , β ₃ , γ, δ, ε, a, b, c _n , d, f, i/j, k, 8, OSCP, ATPPT(1 - 13)	α ₃ , β ₃ , γ, δ, ε, a, b, c _n , d, f, i/j, k, 8, p18, OSCP, ATPEG(1-8), ATPTB(1, 3, 4, 6, 12)

cF₀F₁ are similar and consist of 9 or 8 types of subunits. In mitochondria of eukaryotes, the mtF₀F₁ form dimers of type I, II, III, and IV, and have 19, 18, 27, and 28 different types of subunits, respectively (Table 1) according to the high-resolution structures of the corresponding mtF₀F₁ complexes.

The interesting fact is that there might be different mtF₀F₁ assemblies from the same type of the mtF₀F₁ dimer defining different morphology of the mitochondria cristae. These assemblies are occasionally defined by slight changes in the peripheral subunit machinery. This fact is also important in terms of the roles of different cofactors

and small-molecule ligands that participate in the assembly of oligomeric mtF_0F_1 complexes, especially the F_0 parts of ATP synthases (see details in the “[Small-molecule cofactors of the c-ring](#)”).

Interestingly, representatives of the Viridiplantae kingdom and the Discoba clade have both mitochondria and chloroplasts. Therefore, they carry two different ATP synthase species (mtF_0F_1 and cF_0F_1) in their proteomes. It might be very intriguing to learn how the ATP regulation is organized in these species in terms of the coordination of the biochemical processes as well as the structures of ATP synthases. However, no structural information about mtF_0F_1 and cF_0F_1 from one organism is currently available. Although there is a high-resolution structure of the cF_0F_1 from thylakoid membranes of spinach, there is no structural data about its mtF_0F_1 . A vice versa situation is observed for alga (*Polytomella* sp.), where a high-resolution structure of the mtF_0F_1 dimer is available [37] but there is no structural information about the corresponding cF_0F_1 . Therefore, the capability of the spinach mtF_0F_1 to form dimers of type II is only a hypothesis that needs to be directly confirmed by the high-resolution structural data.

cF_0F_1

Although the chloroplast ATP synthases were primarily found as monomers, in some cases they were actually reported as dimers [8, 35, 36], and biochemical evidence of their cF_0F_1 dimerization were shown. The existence of different cF_0F_1 (e.g. via F_1 – F_1 interface) dimerization mechanisms is claimed, which might depend on the phosphate concentration. Structural data obtained by small-angle X-ray scattering for cF_0F_1 from spinach chloroplasts were reported [41]. However, the lack of high-resolution structural data of cF_0F_1 dimer does not allow understanding whether such dimers are functional or non-functional protein complexes.

bF_0F_1

Concerning bacterial ATP synthases it is necessary to mention that although there is no significant differences between different clades in the form-factors of their enzymes, representatives included in one clade show structural differences between their bF_0F_1 . For example, bF_0F_1 from *Mycobacterium smegmatis* [25] and *Bacillus* sp. [32] belonging respectively to the Actinobacteria and Firmicutes phyla of the Terrabacteria group clade have 8 and 9 types of subunits and are characterized by different intersubunit interaction interfaces (less contacts within b/a interface, 35 a.a. extension of α -subunits, etc.) [25].

In addition, it would be interesting to investigate structures of ATP synthases from various bacterial and archaeal phyla from the point of view of their diversity and evolution.

For example bF_0F_1 from *Chloroflexus aurantiacus*, a representative of Chloroflexi phylum, contains four copies of b-subunit per complex instead of usual two and the connection between F_0 and F_1 moieties by the peripheral stalk of b- and δ -subunits is designed differently [42, 43]. Structural comparison of proton and sodium ATP synthases seems very perspective, as well as comparative structural analysis of bacterial and archaeal ATP synthases, seem very promising approaches.

Therefore, while highlighting the structural features of the bF_0F_1 , one should pay closer attention to the phylum than to the clades for the corresponding organisms.

High-resolution structural studies

Since the first high-resolution structure (2.8 Å) of the F_1 part of mtF_0F_1 from the bovine mitochondria was solved by X-ray diffraction (XRD) crystallography technique [44] many attempts were made to get the high-resolution structure of the whole complex. However, crystallization of the whole F_0F_1 ATP synthase is still a challenge. As a result, since 1999, there were only six successful attempts, which resulted in XRD high-resolution structures of ATP synthase from different organisms [45–50] (Table 2). These structures generated data crucial for understanding the molecular mechanisms of action of the important enzyme.

Firstly, the mechanism of the transmission of c-ring rotation to the conformational changes in the $\alpha_3\beta_3$ domain powering ATP synthesis became clear. For example, the structure of monomeric yeast mtF_0F_1 showed ten c-subunits in the c-ring and close contacts between the c-ring and $\gamma\delta$ subunits [45]. This allowed the authors to suggest an ensemble rotation of $c\gamma\delta$ as a rigid body. In ref. [46], the structure of Mg-ADP-inhibited state of the yeast ATP synthase was described. The contacts between the c-ring/ $\gamma\delta$ were found to be formed by electrostatic forces, and Glu59 in the active center of the c-ring appeared not to be involved in the hydrogen bonding between c-subunits, therefore indicating that this residue does not determine a c-ring stoichiometry.

The paradigm of structural organization of ATP synthases changed when the c-rings with a stoichiometry that is not multiple of three were discovered. Besides observing this in the first structures of yeast mtF_0F_1 , for spinach chloroplast c-ring it was also shown that it has 14 c-subunits instead of 12 as assumed before [51], and a c-ring with eight subunits was later discovered in the bovine mtF_0F_1 [47].

The tilt between the F_1 central stalk and the c-ring [48] provided understanding of a flexibility of ATP synthase during its functioning. The work reported in ref. [6] demonstrated twelve c-subunits in bacterial ATP synthase

Table 2 The structures of H⁺ F-ATP synthases containing at least the c-ring in addition to the F₁ part of the complex The table is modified from ref. [4]

No	c _N	Origin	Type	Year	Method	Res. (Å)	PDB ID(s), ref.
1	10	<i>S. cerevisiae</i>	H ⁺	1999	XRD	3.0–3.9	1QO1; 2XOK [45]
2	10	<i>S. cerevisiae</i>	H ⁺	2010	XRD	3.4	2WPD [46]
3	8	<i>Bos taurus</i>	H ⁺	2010	XRD	3.5	2XND [47]
3	10	<i>S. cerevisiae</i>	H ⁺	2012	XRD	6.5	3ZRY [48]
4	10	<i>S. cerevisiae</i>	H ⁺	2012	Cryo-EM	37.0	4B2Q [29]
5	8	<i>Bos taurus</i>	H ⁺	2015	Cryo-EM	6.4–7.4	5FIL; 5ARI; 5ARH; 5FIK; 5FIJ; 5ARA; 5ARE [52]
6	12	<i>Paracoccus denitrificans</i>	H ⁺	2015	XRD	4.0	5DN6 [6]
7	10	<i>Polytomella</i>	H ⁺	2015	Cryo-EM	7.0	wwPDB: EMD-2852 [53]
7	10	<i>E. coli</i>	H ⁺	2016	Cryo-EM	6.9–8.5	5T4P; 5T4O; 5T4Q [31]
8	10	<i>Pichia angusta</i>	H ⁺	2016	Cryo-EM	7.0–7.9	5LQX; 5LQY; 5LQZ [54]
9	10	<i>Yarrowia lipolytica</i>	H ⁺	2016	XRD/Cryo-EM	3.5/7.7	5FL7/wwPDB: EMD-8151 [50]
10	10	<i>S. cerevisiae</i>	H ⁺	2017	Cryo-EM	3.6	6B8H; 6B2Z [33]
11	10	<i>S. cerevisiae</i>	H ⁺	2018	Cryo-EM	3.6	6CP6 [55]
12	14	<i>Spinacia oleracea</i>	H ⁺	2018	Cryo-EM	3.15–4.3	6FKI; 6FKF; 6FKH [3]
13	10	<i>Polytomella</i>	H ⁺	2019	Cryo-EM	2.9–3.2	6RDV; 6RDE; 6RE4; 6RE1; 6RE7; 6REU; 6RDP; 6REA; 6RDS; 6RDY; 6RED; 6RDI; 6RDB; 6RDG; 6RDM; 6RER; 6RD9; 6RE2; 6RE3; 6RE5; 6RE6; 6RE0; 6RDX; 6RDW; 6RET; 6RES; 6REB; 6REC; 6RDC; 6RDI; 6RDH; 6REP; 6RDK; 6REF; 6REE; 6RDZ; 6RDR; 6RDL; 6RDU; 6RDT; 6RE9; 6RE8; 6RDO [37]
14	10	<i>Bacillus PS3 (recombinant)</i>	H ⁺	2019	Cryo-EM	3.0–3.2	6N30; 6N2Z; 6N2Y [32]
15	10	<i>Euglena gracilis</i>	H ⁺	2019	Cryo-EM	2.8–4.3	6TDU; 6TE0; 6TDY; 6TDZ [40]
16	8	<i>Sus scrofa</i>	H ⁺	2019	Cryo-EM	3.3–6.2	6J5K; 6J5J; 6J5I [34]
17	10	<i>E. coli</i>	H ⁺	2020	Cryo-EM	3.1–3.4	6OQR; 6OQS; 6OQT; 6OQU; 6PQV; 6WNN; 6OQV; 6OQW; 6WNR; 6VWK [7]
18	10	<i>Tetrahymena thermophila</i>	H ⁺	2020	Cryo-EM	2.5–3.1	6YNV; 6YNW; 6YNX; 6YNY; 6YNZ; 6Y00 [11]
19	9	<i>Mycobacterium smegmatis</i>	H ⁺	2020	Cryo-EM	3.4–3.7	7JG5; 7JG6; 7JG7; 7JG8; 7JG9; 7JGA; 7JGB; 7JGC [25]
20	10	<i>Toxoplasma gondii</i>	H ⁺	2021	Cryo-EM	2.8–4.8	6TMH; 6TMG; 6TMJ; 6TMI; 6TML; 6TMK [10]

“c_N” column lists the stoichiometry of the c-ring. The resolution is shown as a range in case if more than one PDB structure has been published

from *Paracoccus denitrificans*, described the structure of peripheral stalk bb' subunits, and a-subunit in F₀ part of bF₀F₁. This was one of the first studies, where a close interaction interface of subunit a with the c-ring was shown, and a mechanism of proton transfer through the a/c-ring interface via two half-channels formed by the a-subunit was suggested. This provided a better understanding of the molecular mechanisms of converting proton gradient electrochemical energy to ATP synthesis. The studies of α₃β₃ active center pointed to a native regulation by Mg-ATP and Mg-ADP inhibitors of ATP synthases [50].

The first Cryo-EM structure of F₀F₁ (resolution 20 Å) was obtained in 2003 [56]. Due to the significant progress in cryo-EM technique, more high-resolution structural data of ATP synthases became available. The resolution of cryo-EM-based structures of the enzyme was improved from 20 Å in 2003 to 2.5 Å in 2020, and an increasing number of structures of the complete F₀F₁ was collected during the recent 5 years [3, 7, 10, 11, 29, 31, 32, 34, 37,

40, 50, 52–55, 57]. These studies revealed the structural and functional organization of ATP synthases and their assemblies, helped understanding the chemo-mechanical coupling and transmembrane proton transport mechanisms, and revealed how organisation of the mtF₀F₁ influences the cristae topology and defines a morphology of mitochondria.

The fact that mtF₀F₁ can assemble into dimers, which determine the topology of cristae in mitochondria of mammals, plants, and fungi was demonstrated in refs. [8, 58–60]. However, for a long time, the mechanisms determining the form of ensembles of mtF₀F₁ dimers remained hidden. The understanding that structural elements of F₀ are responsible for dimerization and larger assemblies was developed by subsequent studies [29]. The structural organization of the a-subunit was established [52] for the bovine mtF₀F₁, and the following studies [53] discovered that four α-helices of the a-subunit arrange parallel to the membrane plane. This arrangement creates the invaginations in F₀ called

half-channels which establish proton transport via a/c-ring interaction interface.

Revealing the existence of the different conformational states of ATP synthases allowed modelling the whole rotational cycle of the enzyme [31, 53]. The participation of F_O subunits in the ATP synthase functioning revealed that ATP8 subunit provides an additional brace between the peripheral stalk and the a-subunit in yeast mtF_OF_1 , and flexibility of the peripheral stalk bdh subunits allows a-subunit to adjust lateral movements during the activity of F_1 part [54]. The involvement of F_O subunits in the ATP synthase dimerization process revealed that the f-subunit establishes F_O - F_O contacts, whereas e- and g-subunits determine a membrane curvature of $\sim 100^\circ$ forming so-called V-shape dimer of yeast mtF_OF_1 [50]. Further improvement of the yeast mtF_OF_1 V-shape dimer model showed [57] that subunits f,8,b determine the base of a peripheral stalk with d-subunit, which acts as a clip. Subunits a, i/j are also responsible for the dimerization and the b-, e-, g-, and k-subunits define a membrane curvature for the mtF_OF_1 V-shape dimer.

High-resolution structures of entire ATP synthases and their parts opened a possibility of designing small-molecule cofactors capable of binding to ATP synthases and modulating or inhibiting their activity. For example, the detailed structure of the F_O part of the yeast mtF_OF_1 allows suggesting that the ATP synthase inhibitor oligomycin binds to the c-ring residing in the lipid surrounding [55]. More details of functioning of cF_OF_1 from spinach chloroplasts were summarized in ref. [3], and the adaptation mechanism of chloroplast ATP synthase redox potential to the day-night cycle was uncovered.

Structural studies of mtF_OF_1 from green algae *Polytomella* sp. demonstrated flexibility of the a-subunit during rotation of the c_{10} -ring and discrete ATP synthesis [37]. In addition, ten subunits composing a rigid peripheral stalk established dimerization contacts between two enzymes and participated in the formation of an mtF_OF_1 dimer of type II (see the “[Similarity and diversity of ATP synthases](#)”). This type of mtF_OF_1 dimers was demonstrated to have an ion (presumably Zn^{2+}) coordinated by the His-248 and His-252 of aH5 of a-subunit, and it possibly synchronizes protonation of the c-ring active center with its rotation.

Another type of dimeric organization (type IV) of mtF_OF_1 was reported in ref. [5]. The work showed a number of peripheral subunits, associated small-molecule cofactors, such as cardiolipins and other phospholipids, and unusual structural organization of a c-ring, where the extension of the c-subunits form a β -barrel that have contacts with ATPEG1-subunit [40].

Very recently, high-resolution structures of ATP synthases revealed more complex assemblies of these enzymes for dimeric types I, III, and IV (corresponding oligomerization states are 4, 4 and 6, respectively). The structure of the

mtF_OF_1 tetramer from mammalian mitochondria revealed that subunits b, k, g, and inhibitory factor 1 (IF1) are responsible for connecting two V-shape mtF_OF_1 dimers and form a H-shape tetramer [34]. The authors also suggested that a 6.8PL subunit is inside the c-ring pore. The authors proposed three different mechanisms of mtF_OF_1 inhibition: by IF1, by Mg-ADP and by e-subunit establishing contacts with the 6.8PL subunit.

The structure of the mtF_OF_1 tetramer from ciliates was recently resolved [11]. This mtF_OF_1 forms U-shaped dimers of a type III. The peripheral subunit machinery establishing dimerization was determined including a subunit ATPPT2 which occurred to be a single copy in the mtF_OF_1 dimer. Two U-shape dimers create a tetramer that determines an angle of membrane curvature of $\sim 11^\circ$. Interestingly, small-molecule cofactors, such as cardiolipins, NAD, and CoQ-8, were found associated with the subunits in F_O and their function remains unknown.

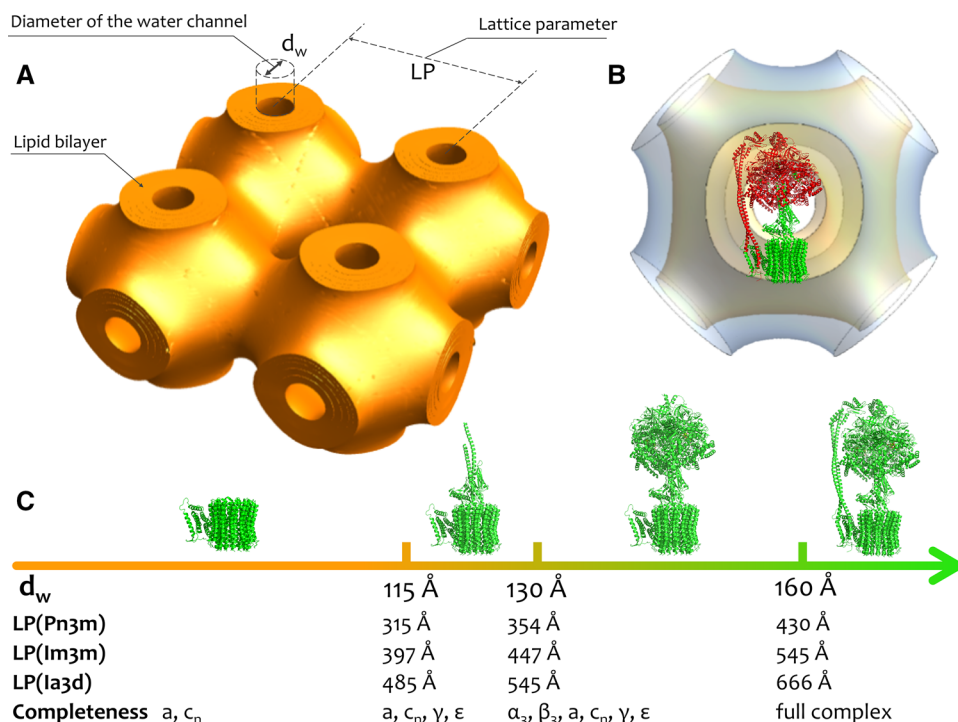
The structure of the mtF_OF_1 (V-shape dimer of a type IV) from apicomplexan parasite revealed hexameric cyclic assembly of the enzyme that forms pentagonal pyramids [10]. The subunit ATPTG11 was found responsible for assembling the hexamers, and its knock-out resulted in the disruption of the pentagonal nets of the mtF_OF_1 hexamers and altered cristae morphology.

Besides mtF_OF_1 , bacterial ATP synthases were also studied by cryo-EM method. The structure of recombinant bF_OF_1 helped clarify the mechanisms of enzyme inhibition by the e-subunit and the proton pathway through the membrane [32].

It is worth noting that successful crystallization trials of ATP synthases were done by vapour diffusion (VD) method, which however, has limitations, such as leading to rapid kinetics of molecules in solution and destabilization of membrane proteins. Therefore, the VD method is commonly used for crystallization of water-soluble proteins and is not optimal for membrane protein complexes [61]. This might also explain the fact of the existence of only partial crystallization of ATP synthases (separate domains or subunits) by VD method even when using the whole protein complex as a starting crystallization material [62].

Crystallization in lipid mesophases (in meso method) is a promising technique to obtain crystals of complete membrane protein complexes, such as F_OF_1 because it uses membrane-mimicking systems which provide more native conditions for membrane proteins than VD method does [63, 64]. However, there are limitations on size of membrane systems when using in meso-crystallization method. A lipid cubic phase (LCP) forms two non-intersecting nets of water channels. The size of the channel diameter is limited by the physico-chemical properties of a matrix lipid that is used to prepare LCP and the composition of a precipitate, where the LCP with the membrane protein is embedded (Fig. 4A,

Fig. 4 **A** Schematic representation of a lipid cubic phase (LCP) of Im3m type of symmetry. The lattice parameter (LP) and the diameter of water channels (d_w) are shown. **B** A schematic reconstruction of one ATP synthase molecule in the LCP. Subunits colored in red do not fit in the unit cell of the LCP. **C** Required d_w and LP and completeness of the ATP synthase complex that can be reconstituted in corresponding LCPs of different types of symmetries are shown. The PDB model 6FKF of ATP synthase from spinach chloroplasts was used for illustration [3]. The figure was taken from ref. [4]



B). F₁ part of ATP synthase is approximately 160 Å, which is about 3–4 times larger than the average diameters of the water channel of common LCPs (Fig. 4C) [65].

It might be also useful to swell lipid mesophases by using charged glycerophospholipids (1,2-Distearoyl-sn-glycero-3-phosphoglycerol) (DSPG) [66]. However, this method works well only if a low-salt precipitant (not higher than 150 mM NaCl of ionic strength) is used. Precipitants with high salinity will shrink the LCP and decrease the size of water channel diameters.

Cryo-EM techniques are commonly working successfully with proteins larger than 100 kDa, which is a suitable case for studying ATP synthases ($M_w > 500$ kDa), and they have recently allowed improving a resolution up to 2.5–3.0 Å in the case of the F₁ part. Unfortunately, the resolution of the F₀ part hardly reaches 3.0–3.1 Å. The lack of the structural information makes it difficult to clearly understand the molecular mechanisms of intersubunit interactions, which play key roles in functioning of the enzyme.

A possible way for obtaining a high-resolution structure of ATP synthase uses complementary XRD and cryo-EM techniques. In this approach, XRD can allow obtaining high-resolution structures of separate membrane subunits, which can be crystallized in meso, and the cryo-EM could provide high-resolution structures of F₁ part of ATP synthase. Then the mosaic model can be constructed as it was done in ref. [50] (see Table 2).

Intrinsically disordered regions in ATP synthases

High-resolution structural studies of ATP synthases is still a challenge, despite great recent advances that were achieved with new developments of X-ray crystallography or cryo-EM studies of membrane proteins. There are several reasons for that in the case of complex structural organization and transmembrane nature of such proteins. Another important factor that is commonly hindering the attempts of the crystallographic analysis of proteins is the presence of intrinsically disordered regions [67]. Among well-known examples of disordered proteins recalcitrant to crystallization is a myelin basic protein (MBP) [68]. In fact, due to the exhaustive series of attempts to crystallize MBP for X-ray diffraction, where 4600 different crystallization conditions failed to induce crystallization of MBP, this protein was included to the category of “uncrystallizable” proteins [69]. Even proteins with solved crystal structures can possess noticeable levels of intrinsic disorder, since very significant fraction of proteins in PDB contains regions with missing electron density, which are potentially intrinsically disordered [70, 71]. In line with these considerations, analysis of the PDB structure 6FKF of cF₀F₁ from the spinach chloroplasts revealed that each subunit of this machine contains variable regions of missing electron density: residues 1–7 and 504–507 in chain α; residues 1–17 and 495–498 in chain β; residues 1–42 and 364 in chain γ; residues 1–70 and 250–257 in chain δ; residues 132–134 in chain ε; residues 1–21 and 245–247 in chain a; residues 1–22 and 183–184 in chain b; residues

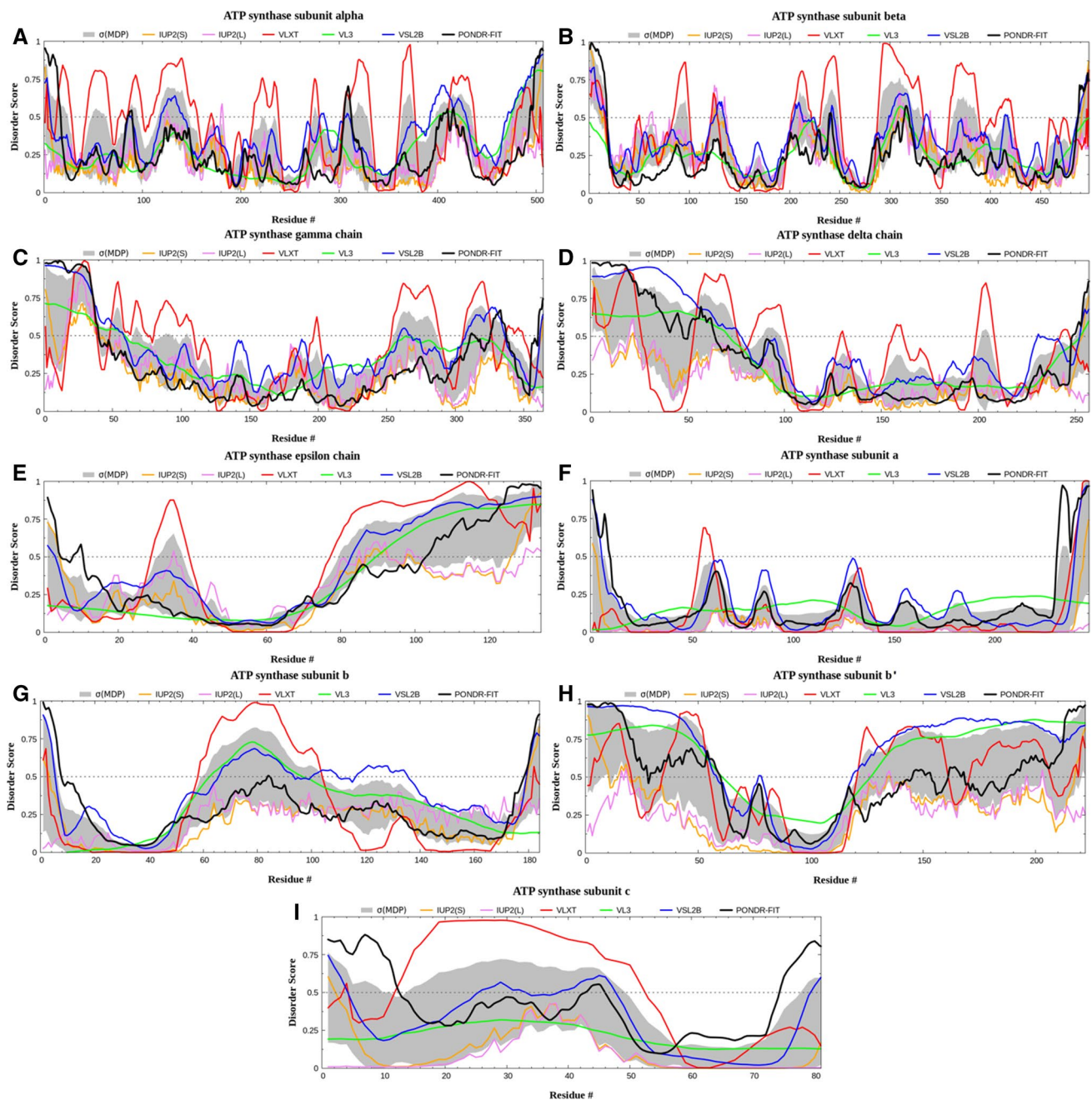


Fig. 5 Per-residue intrinsic disorder predisposition of the subunits of cFOF₁ from the spinach chloroplasts. Intrinsic disorder profiles were generated based on the outputs of six commonly used disorder predictors: PONDR® VLXT [72], PONDR® VL3 [73], PONDR® VLS2 [74], PONDR® FIT [75], and IUPred2 (Short) and IUPred2 (Long) [76]. The outputs of the evaluation of the per-residue disorder propensity by these tools are represented as real numbers between 1 (ideal

prediction of disorder) and 0 (ideal prediction of order). A threshold of 0.5 is used to identify disordered residues and regions in query proteins. Residues with the disorder scores (DS) $0.25 \leq DS < 0.5$ are considered as moderately disordered, whereas residues with $0.15 \leq DS < 0.25$ are flexible. The figure clearly shows that all subunits contain noticeable levels of intrinsic disorder, with some of them being rather disordered

1–77 and 221–222 in chain b'; and residues 1–2 in chain c. Therefore, very significant parts of each cFOF₁ subunit are expected to be disordered even within the assembled complex. Figure 5 further illustrates this concept by showing per-residue disorder profiles generated for each cFOF₁ subunit.

The corresponding intrinsically disordered regions of ATP synthases might help to understand the mechanisms of regulation of the enzymes. For example, intrinsically disordered proteins (IDPs) were found to regulate activity of ATP synthases from bacteria (*E. coli*, *Paracoccus*

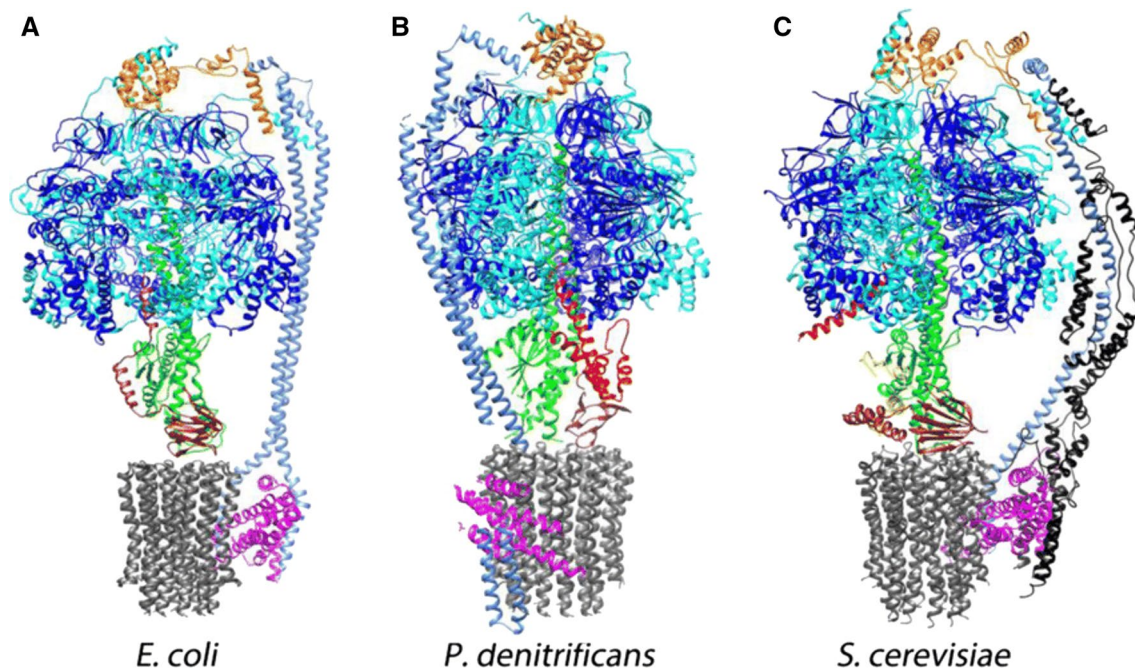


Fig. 6 The structures of inhibited ATP synthases by intrinsically disordered regions of their subunits (colored in red). **A** bF₀F₁ from *E. coli* inhibited by ϵ -subunit, **B** bF₀F₁ from *Paracoccus denitrificans*

inhibited by ζ -subunit, **C** mtF₀F₁ from *S. cerevisiae* inhibited by IF₁ subunit. The figure is from [77]

denitrificans) (Fig. 6A, B) as well as from mitochondria of eukaryotes (*S. cerevisiae*) (Fig. 6C) [77]. The activity regulation of chloroplast ATP synthases occurs via a redox switch on its γ -subunit [78]. Figure 5C shows the presence of intrinsically disordered regions in γ -subunit (residues 1–42 and 364), however, the structural changes of the redox switch are in another region of γ -subunit (residues 238–282) [78]. Therefore, additional experiments are required to reveal how the mechanisms of activity regulation of ATP synthases can be investigated in terms of IDPs.

However, even when high-resolution structures of query proteins are not available, there is always a possibility to try different organisms belonging to the same phylogenetic group as the organism of interest (e.g. pathogen), if one can properly account for the structural features of their F₀F₁, which are described in this review. There are a number of hints on which type of structural features might be used to affect or modulate the activity of ATP synthases. In the case of mitochondrial [11] and chloroplast [23] ATP synthases, the presence of isoprenoid quinones as the F₀ cofactors might be a strong indicator that different derivatives of ubiquinones (UQ) (mitochondria), plastoquinones (PQ) (chloroplasts) and menaquinones (MK) (bacteria) might influence the functioning of ATP synthases and can become the tools of cellular bioenergetic control. We discuss here a generalized approach based on the relevant structural information on the ATP synthases, which might be helpful

in the further studies, development of new therapies, and structure-based drug design.

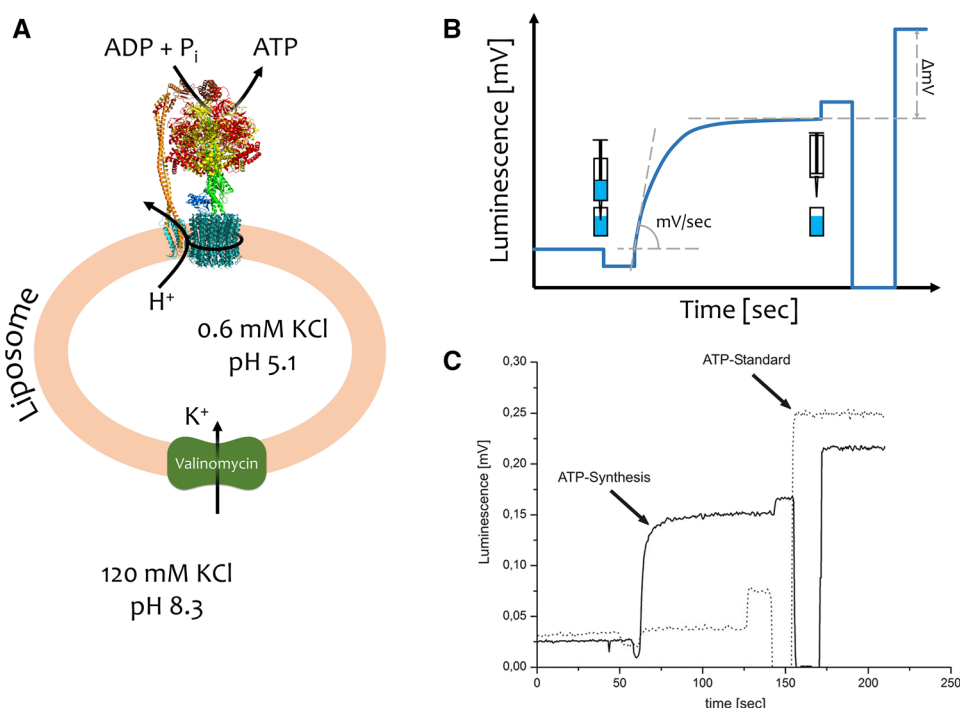
Validation of ATP synthase functionality

Structural information about ATP synthase is commonly validated via the functional activity assays of the investigated enzyme. Typically, functional studies are done by incorporating ATP synthases into liposomes and then checking the activity by the luciferin-luciferase assay [79–82].

A typical protocol includes preparation of the unilamellar vesicles (ULVs) of phosphatidyl choline lipids (PC) and reconstitution of the ATP synthase there. Here, ULVs are the spherical vesicles bounded by a single lipid bilayer. ATP synthase is initially solubilized in detergent (DDM, tPCC- α -M, digitonin, etc.). Thereafter, Bio-Beads or Amberlite XAD-2 resin is used to remove detergent and trigger incorporation of the enzyme into ULV bilayers (Fig. 7A). Detailed description of the protocol can be found in ref. [62].

The luciferin-luciferase assay is based on the capability of luciferase to hydrolyse ATP to AMP and luciferin to oxyluciferin emitting light during this chemical reaction, which can be detected by spectroscopy (Fig. 7B, C). Based on these data, the efficiency of ATP synthesis (ATP/sec/enzyme) is calculated in comparison with the ATP standard added (Fig. 7B, C).

Fig. 7 Quantitative analysis of ATP synthase activity by ATP synthesis luciferin-luciferase test. **A** Schematic model of a proteoliposome with reconstituted ATP synthases is shown with the imposed ΔpH and $\Delta\mu(\text{K}^+)$ gradients to energize ATP synthesis. **B** Schematic and **C** experimental data of the luminescence signal. **C** is from ref. [62] and the figure is from ref. [4]



The method for reconstitution of F_0F_1 into liposomes, energisation of the liposome membrane by an acid base transition and measurement of ATP synthesis (using radioactive phosphate as well as luciferin/luciferase) was developed for optimisation of the isolation of the F_0F_1 from chloroplasts. It resulted in isolated cF_0F_1 with all subunits showing activities of about half of the activity observed in thylakoid membranes. Reconstitution and ATP synthesis activity of the cF_0F_1 was reported first in ref. [83].

Reconstitution in liposomes with full ATP synthesis activity of a mtF_0F_1 driven by an acid–base transition was reported first in ref. [84]. H^+/ATP ratio of proton transport-coupled ATP synthesis have been first described in ref. [85]. In addition, mtF_0F_1 reconstituted into liposomes together with a photosensitive membrane protein bacteriorhodopsin showed light-induced ΔpH -driven ATP synthesis [86].

In earlier work the luciferin/luciferase was used to determine the amount of ATP synthesized in an acid base transition using a separate assay. This allows one to optimize the conditions for energisation with an acid base transition and, in the separate assay, the optimal conditions for the detection of ATP with luciferin/luciferase can be used [83].

These experiments revealed that different ATP synthases have different rates of ATP synthesis and even the same ATP synthase can be characterized by noticeable difference in its ATP synthesis rate. For example, cF_0F_1 from *Spinacia Oleracea* has a measured efficiency ranging from 40 to 200 $\text{ATP}/\text{F}_0\text{F}_1/\text{sec}$ in similar functional studies conducted in different groups [62, 87, 88].

Bacterial F_0F_1 was reported to be reconstituted into liposomes and its ATP synthesis activity was measured firstly by Fischer et al. in 1994 [79]. Bacterial ATP synthases were reported to have lower ATP synthesis rate, e.g. $\sim 11\text{--}15$ $\text{ATP}/\text{sec}/\text{enzyme}$ for bF_0F_1 from *Ilyobacter tartaricus* [89].

Surprisingly, functional activity measurements were reported only for several F_0F_1 from Fig. 2 and only for monomers of F_0F_1 . It could be interesting to measure the functional activity of dimeric F_0F_1 and compare it with one of monomeric F_0F_1 .

ATP-dependent luciferases from firefly or others are used for in vivo functional studies of ATP synthases [90, 91], for purified mitochondria or chloroplasts [92, 93], and for two-hybrid systems to evaluate the interaction of cofactors with ATP synthases [94]. A perspective approach might be the usage of combined sensory systems of eukaryotic (ATP-dependent) and bacterial (FMN—NADH-dependent) luciferases [95, 96] for functional studies of respiratory chain proteins and ATP synthases both in vivo and in vitro [97], as well as ATP-dependent processes such as refolding of thermoinactivated proteins [98].

Vinkler et al. [93] investigated the ATP synthesizing system of chloroplasts with electric field pulses and for ATP detection with the luciferin/luciferase included in the reaction medium. However, the results described in this work mainly seem to be artefacts resulting from electrode reactions and from the effect of the temperature jump generated by the voltage pulse in combination with the adenylate kinase omnipresent in chloroplasts.

Mitochondrial permeability transition pore (mPTP)

One of the most intriguing phenomena in mitochondria is the existence of the mitochondrial permeability transition pore (mPTP), also known as the mitochondrial mega-channel (MMC). Although this phenomenon has been intensively discussed during the last several decades, structural information about its molecular mechanisms is still missing. Importance of the mPTP is defined by the fact that activation of this pore triggers cell death (apoptosis and necrosis). PTP is defined as a Ca²⁺-dependent non-selective channel

permeable to ions and solutes with a molecular mass below 1500 Da. Although its maximal conductivity reaches 1.5 nS, for a number of substates the lower conductivity is observed [15].

In mammals, the PTP is positively modulated by binding of cyclophilin D (CyPD) and inhibited by cyclosporin A (CsA). Phenomenologically, the mPTP activation is characterized by high ion conductivity, rapid membrane depolarization, and cellular death. According to the recent discussions, mPTP is an assembly that might be either adenine nucleotide translocase (ANT) or/and ATP synthase [15, 99]. This model is based on the observations that the purified ANT, as well as purified ATP synthase were reconstituted

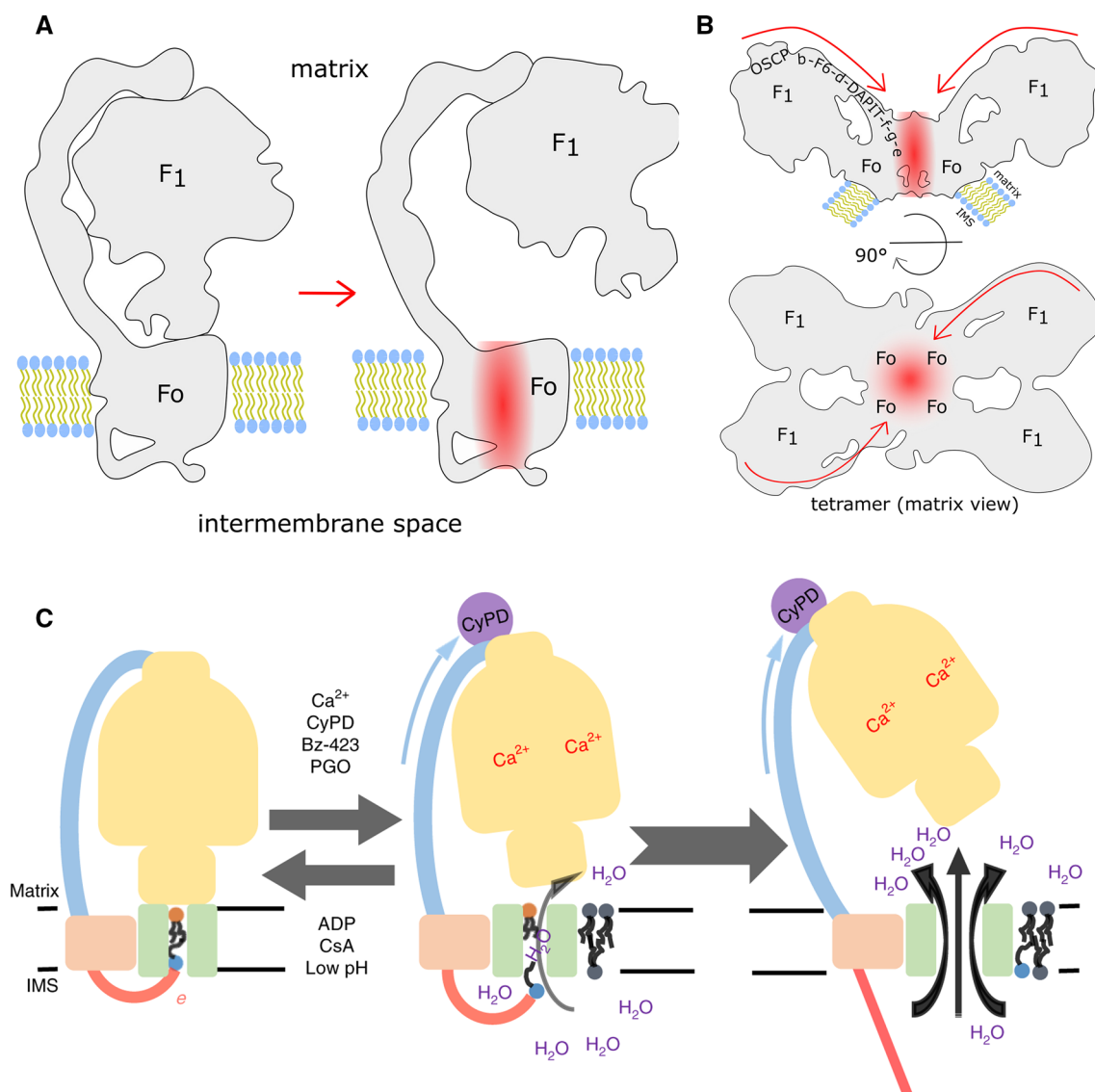


Fig. 8 Different hypotheses of ATP synthase involvement in mPTP phenomenon. **A** The c-ring hypothesis. **B** ATP synthase oligomer hypothesis suggesting that mPTP might occur between of F₀ parts of ATP synthase monomers. **C** A "death finger" hypothesis suggest-

ing that conformational changes induced by Ca²⁺ and CyPD lead to removal of e-subunit and of a lipid plug by conformational changes via b-subunit. **A**, **B** were adapted from ref. [15]; **C** from ref. [113]

into liposomes and showed electrophysiological characteristics similar to mPTP measured by patch clamp technique (conductivity of 300–600 pS for ANT [100] and 1–1.3 nS for ATP synthase [101], respectively). However, the mPTP phenomenon was shown to persist after the knockdown of ANT [102] and the c-subunit of ATP synthase expression [103].

Below we briefly describe the dominating hypotheses of the pore formation via ATP synthase and discuss how the oligomeric assemblies of the enzyme might form a huge non-selective channel in the mitochondrial inner membranes.

The c-ring hypothesis

The c-ring hypothesis suggests that the c-rings under the physiological conditions are capable of non-selective conductivity (Fig. 8A). The most notable evidence was described in ref. [104], demonstrating that the purified c-rings show biophysical characteristics similar to mPTP, but are not sensitive to Ca^{2+} and are not regulated by CsA. Therefore, a hypothesis was proposed that the regulatory elements are located in the F_1 part, and the pore itself consists of c-rings. F_1 participates in the inhibition of mPTP, and during the formation of a pore, F_1 dissociates from F_0 . It was shown by fluorescence microscopy applying the bipartite tetracycline display (FlAsh) method, which indicated that the structures of open and closed pores are different.

The theory of the mPTP formation via c-rings was often criticized [105–107]. Briefly, the group of Walker [107] showed that the channel inside the c-ring is hydrophobic and has the shape of a sand clock, narrowing from 14.2 to 10.4 Å in the middle. Based on these structural data, they suggested that the formation of a pore with conductivity up to 1.5 nS from c-rings is not possible. Along the same vein, Bernardi [105] claimed that (1) dissociation of F_1 from F_0 occurs under extremely specific conditions (2 M urea), and when returning to physiological conditions, reverse assembly occurs; (2) the assumption that the β -subunit can close the F_0 channel is quite controversial, since normally the β -subunit has no affinity for c-rings, and the presence of a free β -subunit itself is rather unlikely; (3) the induction of Ca^{2+} mPTP in intact/complete ATP synthase can be suppressed by the addition of CsA, and it is not clear how this can happen if F_1 has already completely dissociated from F_0 .

In addition, based on the atomic modeling [106] it was shown that (1) the hydrated c-ring does not exhibit the properties of mPTP; (2) under physiological conditions, the c-ring lumen part is not hydrated and is plugged with lipids. All this was confirmed for the cases of the c_{10} -ring from *Saccharomyces cerevisiae*, and for the c_{13} -ring from *B. pseudofirmus*. The models without two c-subunit monomers also showed that the c-ring remained clogged with lipid molecules. Therefore, the formation of a pore by the

ATP synthase c-rings in their physiological conditions is still questionable.

The oligomer hypothesis

Another hypothesis suggests the formation of a pore between two monomers of the ATP synthase dimer or between four monomers of the ATP synthase tetramer (Fig. 8B). This model [101] is based on the observations that dimers, but not monomers of the ATP synthase, after blue native gel purification and reconstitution into the lipid bilayers in the presence of Ca^{2+} , can open a channel with currents that were typical of the mitochondrial mega channel. The channel opening was inhibited by the ATP synthase inhibitor AMP-PNP and $\text{Mg}^{2+}/\text{ADP}$. Based on the additional considerations [108–110], it was suggested that the pore is formed at the dimeric interface.

The main refutation of this hypothesis was presented in [111], where the authors showed that mPTP requires the dissociation of ATP synthase dimers. Furthermore, the authors of other study [112] showed that the ATP synthase monomer was sufficient for the formation of a pore and criticized previous publications [101, 110], where the oligomeric state of the ATP synthase reconstituted in liposomes was not verified unambiguously.

In the beginning of 2020, Walker et al. claimed [16] that based on the knockout data of various ATP synthase subunits, it can be argued that mPTP is not necessarily associated with the ATP synthase dimer.

In conclusion [16], it seems that the final answer on the possibility of the mPTP formation via the ATP synthase dimer will be obtained via determining the high-resolution structure of the ATP synthase dimer. In 2020, the dimer structure was obtained by the J. Walker group using Cryo-EM technique [107], and it was shown that the c-rings cannot form a pore under native conditions. However, there was no statement about the model of the pore between the dimeric F_0 parts of ATP synthase.

The “death-finger” hypothesis

Despite the evidence against the hypothesis of pore formation via c-rings, research towards this hypothesis was continued, and new evidence supporting this model was recently obtained [112–114]. As a result, currently, the modified model of the mPTP formation via c-rings is suggested. This modified model of the mPTP formation via a c-ring is as follows. High Ca^{2+} levels lead to the displacement of Mg^{2+} from the pocket in the β -subunit and causes conformational changes in F_1 , which eventually lead to the separation of the e-subunit from the c-ring and the stretching of the lipid plug by conformational changes via the b-subunit (Fig. 8C). At

the initial stage, these changes are reversible, but eventually they lead to the separation of the F₁ part and the increase of the pores of c-rings to 2–3 nm [113]. It is quite possible that this increasing in the diameter of the c-ring pores occurs similarly to the formation of non-specific pores by amyloidogenic proteins [114]. Next (or simultaneously) this leads to the destruction of the oligomeric interaction interfaces and the dissociation of the dimers and tetramers of ATP synthase [111]. It is assumed [112] that the formation of mPTP occurs at the level of the ATP synthase monomer, and the decay of the oligomers is a consequence, but not the cause of this phenomenon. In this study, not only patch clamp measurements were performed with ATP synthase reconstituted in small unilamellar vesicles (SUVs), which showed that ATP synthase forms a voltage-gated and Ca²⁺—dependent channel, but also the monomeric state of ATP synthase was confirmed by the Cryo-EM.

Thus, there are different hypotheses on the nature of mPTP and additional experiments are required to reveal a real mechanism of the transition.

C-ring

An intriguing fact is the diversity of the molecular mechanisms utilized for stabilization of ATP synthases in their rotary part—c-ring. Different organisms have different contexts of interaction interfaces of their c-subunits. In fact, their ATP synthases can be found in completely different surroundings, show different subunit stoichiometry of the c-ring (Fig. 9), possess variability of other subunits of the ATP synthase that are interacting with the c-ring, and sometimes even contain the extended polypeptide chain of the c₁-subunit [40].

The stoichiometry of c-rings from different organisms is different and ranges from 8 to 17 subunits (Fig. 9). In the case of eukaryotes, the most common numbers of mitochondrial c-subunits are 8 and 10. Bacteria show more diverged c-ring stoichiometry, which is ranging from 9 to 17, with high variability being sometimes observed within the closely related organisms (Fig. 9). Some bacterial species have a hybrid F/V type Na⁺ c-ring with 9_F/1_V

Fig. 9 Phylogenetic tree showing the c-ring stoichiometry of different organisms. The histogram illustrates the number of the c-subunits. The stoichiometry of eukaryotic H⁺ mitochondrial c-rings is colored red and H⁺ chloroplast c-rings—green; bacterial H⁺ c-rings—purple and Na⁺ c-rings—orange. *Acetobacterium woodii* is colored pink due to a hybrid F/V type Na⁺ c-ring with a stoichiometry of 9_F/1_V. Red and green question marks indicate unknown stoichiometry of mtF₀F₁ and cF₀F₁ c-rings for the corresponding species, respectively. The figure was reprinted from ref. [4] with minor modifications

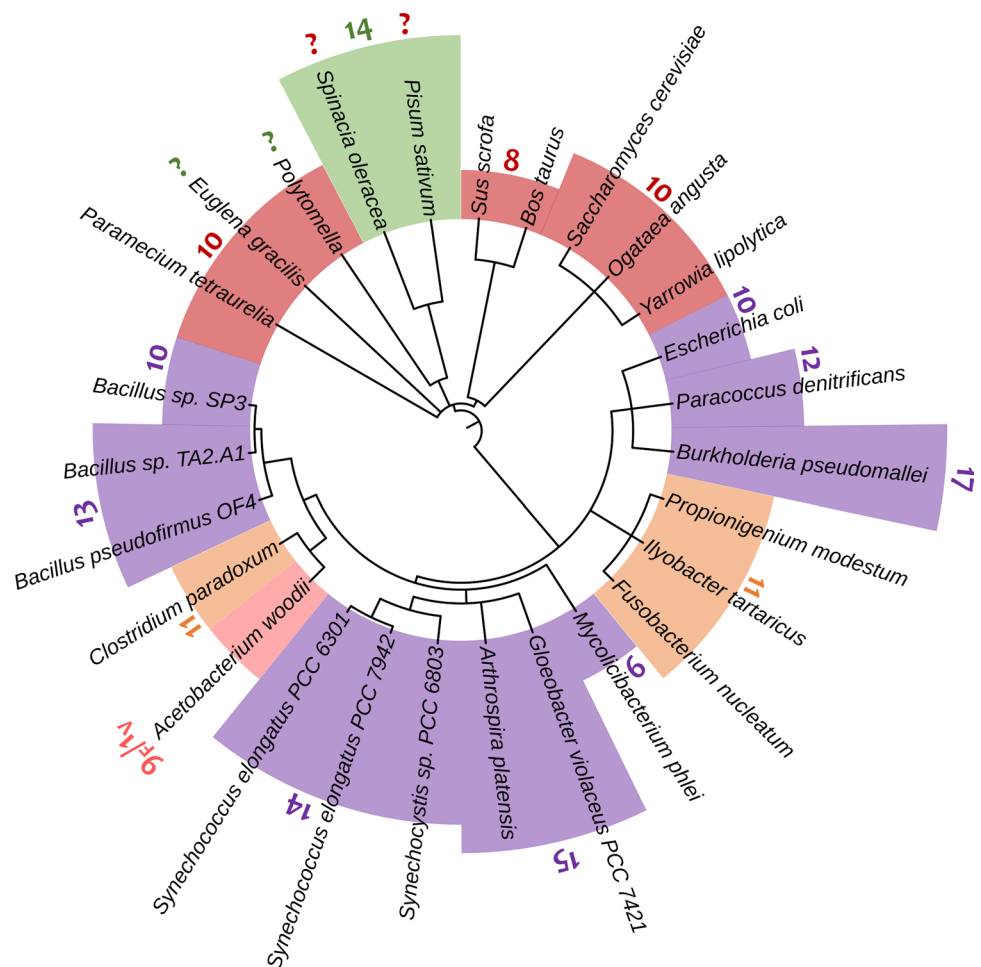
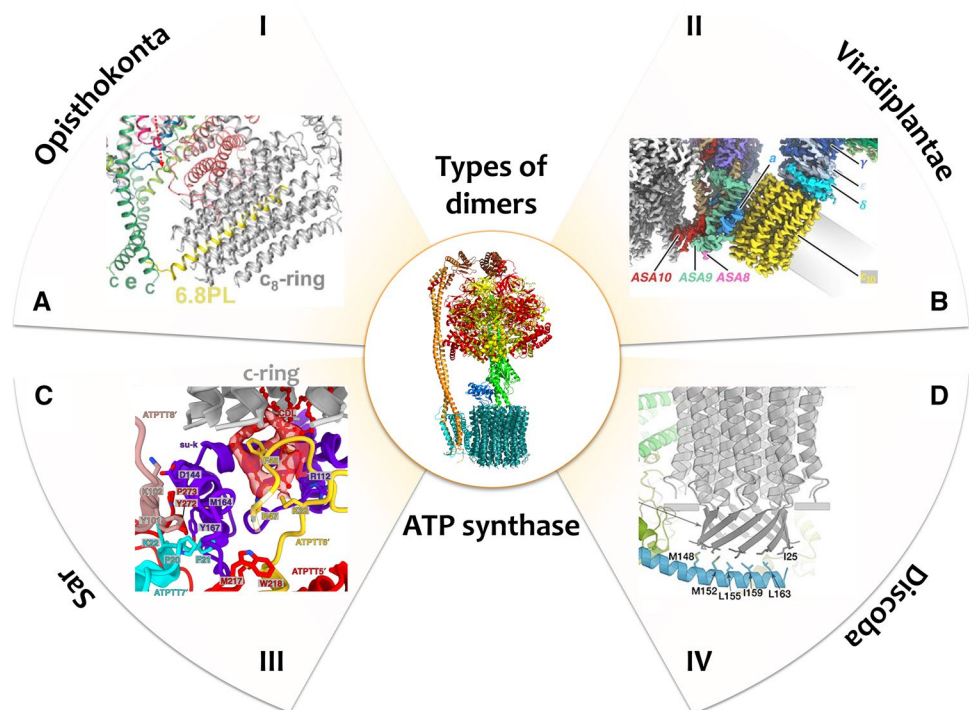


Fig. 10 Interaction interfaces for different types of mtF_0F_1 dimers with a focus on the c-ring. **A** mtF_0F_1 dimer of type I has a PL6.8 subunit inside the inner pore of the c-ring. **B** mtF_0F_1 dimer of type II has a large peripheral stalk stabilizing the c-ring. **C** mtF_0F_1 dimer of type III has an ATPIT6 subunit with a cardiolipin inside the inner pore of the c-ring. **D** mtF_0F_1 dimer of type IV has an extended polypeptide chain at the N-terminus, which forms a β -barrel establishing sliding contacts with ATPEG-1 subunit. The figure was reprinted from ref. [4] with modifications



stoichiometry, though the form factor of this c-ring is similar to a c-ring with 11_F protomers [115].

One of the important details here is that the c-ring might be involved in the mPTP formation. However, as we have discussed, determination of the exact molecular mechanisms of this phenomenon is still a big challenge. Based on the diversity of the stoichiometries of c-rings in different organisms (Fig. 9) and different context of interaction interfaces between a c-ring and surrounding subunits of ATP synthase (Fig. 10), it seems that the different types of mtF_0F_1 dimers will undergo different pathways of disassembly, therefore presenting diversified mechanisms of the processes of mPTP formation in eukaryotes. There are also interesting questions on the existence in bacteria or chloroplasts of the processes similar to mPTP in mitochondria and molecular mechanisms that could underlie these processes.

C-rings differ not only by the number of c_1 -subunit copies, but also by the secondary structure of the polypeptide chains. Typically, in most known cases, the c_1 -subunit consists of two α -helices connected by a conserved loop, which establishes contacts between a c-ring and a central stalk of an ATP synthase. Two transmembrane α -helices create a so-called hairpin, so that the N- and C-termini of the c_1 -subunit are on the same side and are very close to the membrane surface. However, the representatives from the Discoba clade have an extension at the N-terminus of the c_1 polypeptide chain, which forms a β -barrel serving as an interface, which

connects the c-ring with the ATPEG-1 subunit via sliding contacts (Fig. 10D).

Small-molecule cofactors of the c-ring

The question on what compounds can be found inside the inner pore of the c-rings of ATP synthases from different organisms was risen 2 decades ago while investigating the c_{11} -ring of bF_0F_1 from *Ilyobacter tartaricus* [116] and the c_{14} -ring of cF_0F_1 from spinach chloroplasts [51, 117]. Some authors claimed the lipidic nature of the so-called “plug” inside the central pore of c-rings and showed AFM data [116] or results of computer modelling [118, 119], whereas other authors claimed the proteinaceous nature of the “plug” [117]. Nowadays, due to the existence of the high-resolution structural information for ATP synthases from different species one can observe that the nature of the “plug” of the c-rings from mtF_0F_1 can be either lipid or protein or protein/lipid. In case of mtF_0F_1 dimers of type I, it was shown that a subunit PL6.8 is inserted inside the inner pore of the c_8 -ring of mtF_0F_1 from the porcine heart [34]. In case of mtF_0F_1 dimers of type III, a cardiolipin was found inside the pore of the c_{10} -ring [11]. The other types of mtF_0F_1 dimers (II and IV) might have a lipid “plug” preventing ion leakage through the corresponding c-rings [37, 40].

Concerning the chloroplast ATP synthases the purified samples of the c-ring from the ATP synthase of spinach chloroplasts were firstly published in 1987 [120]. Later, the

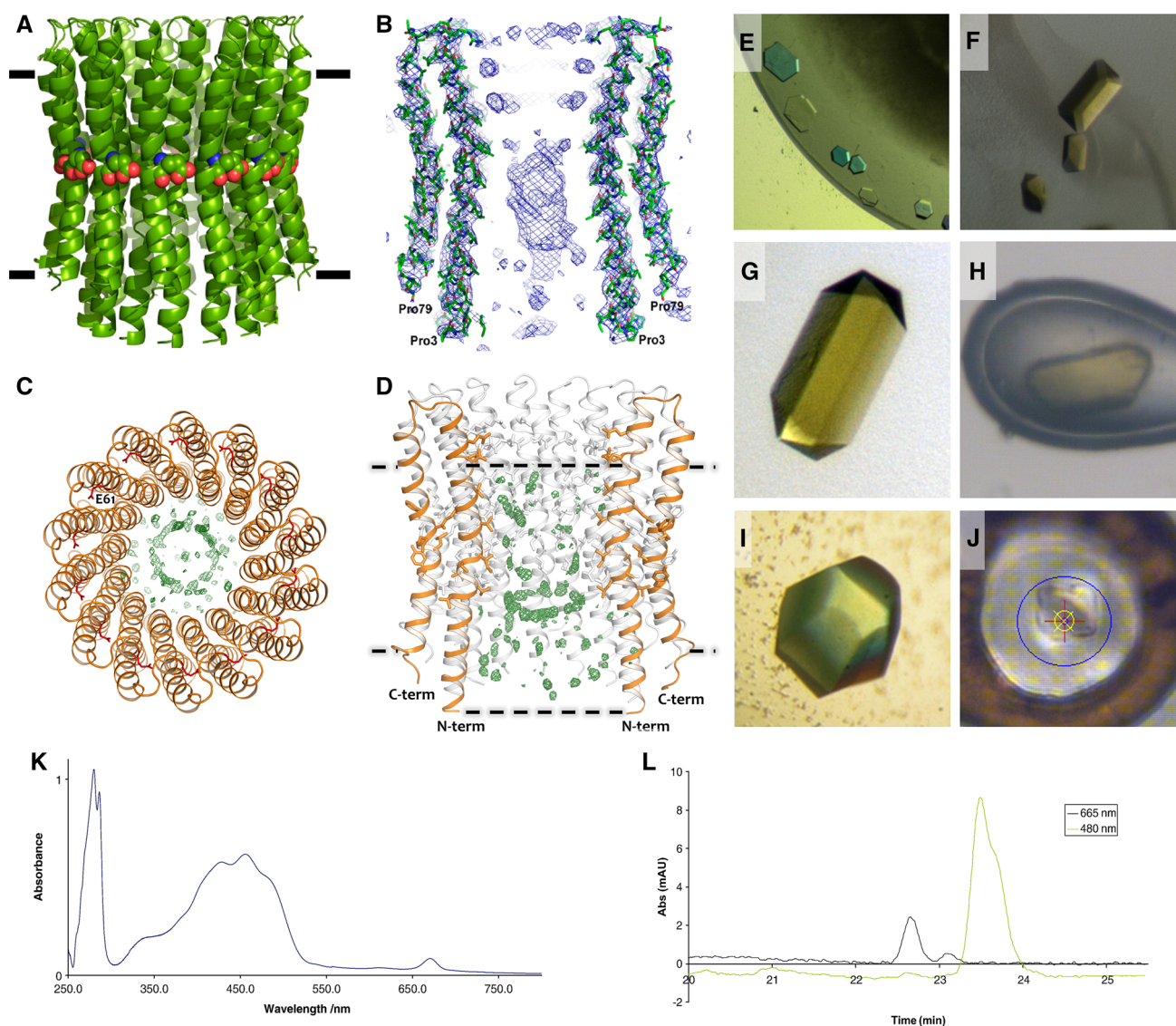


Fig. 11 **A, B** [121], **C, D** [23] Crystallographic data of the c₁₄-ring from spinach chloroplasts demonstrate additional electron densities inside the inner pore of the c-ring. **E–J** A gallery of the crystals of the c₁₄-ring from spinach chloroplasts [23, 62, 87]. **K** UV–Vis spec-

tra of dissolved c₁₄-ring crystals and **L** the reversed-phase HPLC diagram pointing to beta-carotene and chlorophyll *a* at 480 and 665 nm, respectively [87]. The figure was reprinted from ref. [4] with modifications

similar samples were reported to contain small-molecule supplements despite the harsh conditions of sample purification (1% w/v N-lauryl sarcosine (NLS), 65 °C) [87]. The structural evidence of the possible presence of small-molecule supplements inside the c-ring inner pore was presented in refs. [23, 121] based on the analysis of high-resolution crystallographic data (Fig. 11A–D). Another evidence of the presence of small molecules, such as beta-carotene or chlorophyll *a*, is also indicated by the corresponding color of the c-ring crystals [62, 87] (Fig. 11E–J), studies with UV–Vis spectroscopy (Fig. 11K) and reversed-phase HPLC (Fig. 11L) [87].

Additional experimental evidence of possible presence of small molecules in c-rings was reported in our previous work [23] (Fig. 12), where the purified samples of the c₁₄-ring from spinach chloroplasts were investigated by UV–Vis differential spectroscopy before and after a qualitative reaction with NaBH₄. The difference spectra between an oxidative and a reduced states of the samples showed the similar fingerprint as for a quinonic compound—trimethyl benzoquinone (TMBQ), which is a polar moiety of a plastoquinone-9 (PQ-9) (Fig. 12A, D, E).

In line with these observations, additional examples of the interaction of different isoprenoid quinones with ATP synthase were presented [11]. The authors demonstrated a

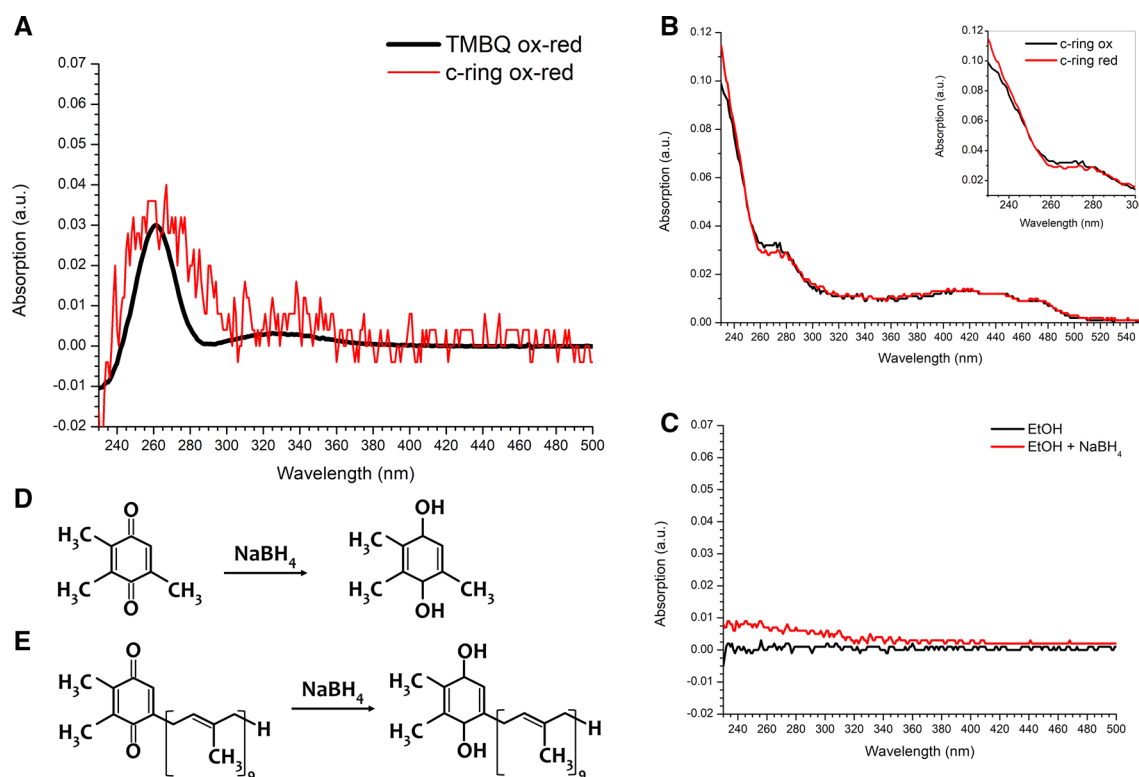


Fig. 12 Differential UV-Vis spectroscopy of the purified c_{14} -ring from spinach chloroplasts dissolved in EtOH. **A** “Ox-redox” differential spectra of the purified c-ring and TMBQ (colored in red and black, respectively). **B** UV-Vis spectra of the c-ring samples before (ox) and after addition of NaBH_4 (redox) (black and red lines, respec-

tively). **C** A control experiment with pure EtOH before and after addition of NaBH_4 (black and red lines, respectively). Schemes of reducing **D** TMBQ to TMBQH₂ and **E** PQ₉ to PQ₉H₂. The figure was reprinted from ref. [4]

Table 3 Distances (D_{in}) between polar/apolar interfaces inside c-rings from different organisms and the length of a hydrophobic chain of corresponding isoprenoid quinones (L_Q). The table is reprinted from ref. [4]

No	Origin, PDB ID(s)	Type of quinone	L_Q , Å	D_{in} , Å	D_{in}/L_Q
1	<i>Spinacia oleracea</i> , (6TQJ)	PQ-9	45.3	45.8 ± 0.3	1.01
2	<i>Saccharomyces cerevisiae</i> , (5BPS, 4F4S)	UQ-6	30.6	47.8 ± 0.3	1.56
3	<i>Bacillus pseudofirmus</i> , (4CBK, 2X2V)	MK-7	35.5	35.4 ± 1.6	1.00
4	<i>Mycobacterium phlei</i> , (4V1H)	MK-9	45.3	47.8 ± 0.7	1.08
5	<i>Arthrospira platensis</i> , (2XQU)	PQ-7 (?)	35.5	38.2 ± 1.5	1.08

direct interaction of UQ-8 with subunits in F_0 part of a tetrameric mtF_0F_1 from *Tetrahymena thermophila*.

Initially, hypothesis that isoprenoid quinones might be cofactors of ATP synthases from different organisms was proposed in [4, 23]. The authors showed the presence of additional positive electron densities inside the c-ring from spinach chloroplasts and found similar densities in the available high-resolution structures of c-rings from bacteria and mitochondria of eukaryotes. This hypothesis gains stronger evidence if one calculates the distances between the polar/apolar interfaces inside the c-rings from different organisms and compares them with the corresponding isoprenoid quinones that are natural components of their membranes (Table 3) [4].

Since 2019, a boom of cryo-EM studies on the ATP synthases resulted in the emergence of high-resolution structures of mtF_0F_1 from different species, where the electron densities observed inside the pore of the corresponding c-rings were annotated as a cardiolipin molecule [11] (mtF_0F_1 dimer of type III), or α -helix of PL6.8 subunit [34] (mtF_0F_1 dimer of type I). Intriguingly, if there are isoprenoid quinones inside the c-rings of mtF_0F_1 , there should be complex interaction interfaces between a lipidic mixture or a protein-lipid mixture and the inner surface of the c-ring (Fig. 13B) [4].

In case of bacterial and chloroplast ATP synthases the densities were not annotated in the literature due to low resolution of the corresponding structures. In addition to what is

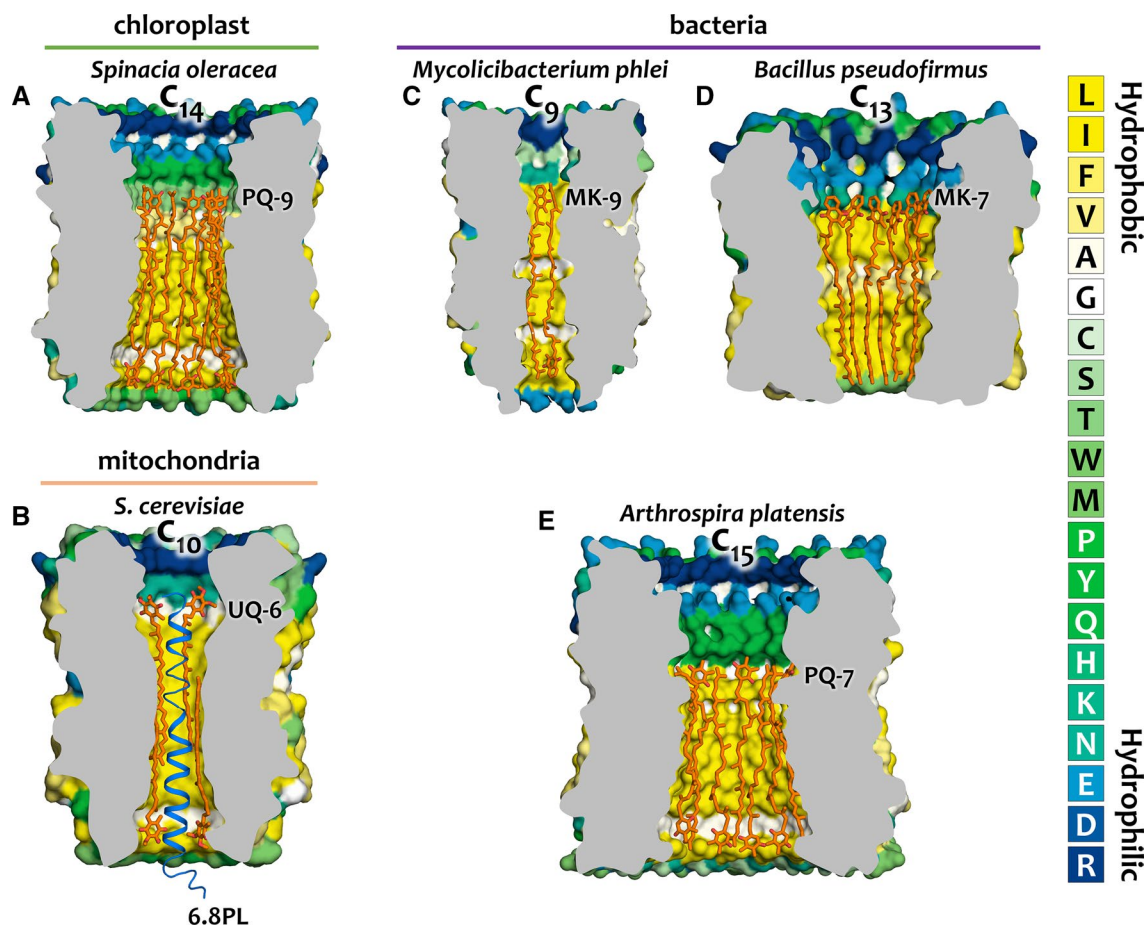


Fig. 13 Schematic picture of possible interaction interfaces of c-rings from different organisms. Isoprenoid quinones are shown in orange. **A** The c₁₄-ring from *Spinacia oleracea* (6TQJ) [23] and PQ-9 molecules. **B** The c₁₀-ring from *Saccharomyces cerevisiae* (4F4S) [122] and the possible UQ-6/6.8PL subunit interface. **C** The c₉-ring from

Mycobacterium phlei (4V1H) [123] and MK-9 molecules. **D** The c₁₃-ring from *Bacillus pseudofirmus* (4CBK) [123] and MK-7 molecules. **E** The c₁₅-ring from *Arthrospira platensis* (2XQU) [124] and PQ-7 molecules. The figure was reprinted from ref. [4]

known, there is no large peripheral machinery in bFOF₁ and cFOF₁ therefore it is highly likely that the plug inside corresponding c-rings might consist of isoprenoid quinones or at least a mixture of lipids and quinone molecules (Fig. 13A, C, D, E) [4].

ATP synthases as potential therapeutic targets

Recent advances in the structural biology of ATP synthases directly demonstrated that representatives of different taxonomy groups carry unique structural features of this enzyme, thereby representing potential targets for drug design and gene therapies, or in more general context, open new possibilities towards the development of promising tools for cellular bioenergetics control. For example, it was already shown [10, 11, 29, 125] that a

specific architecture of the mitochondria cristae is formed by different mtF₀F₁ oligomers (see Fig. 3), and their disassembly to mtF₀F₁ monomers leads to the loss of cristae shape and swelling.

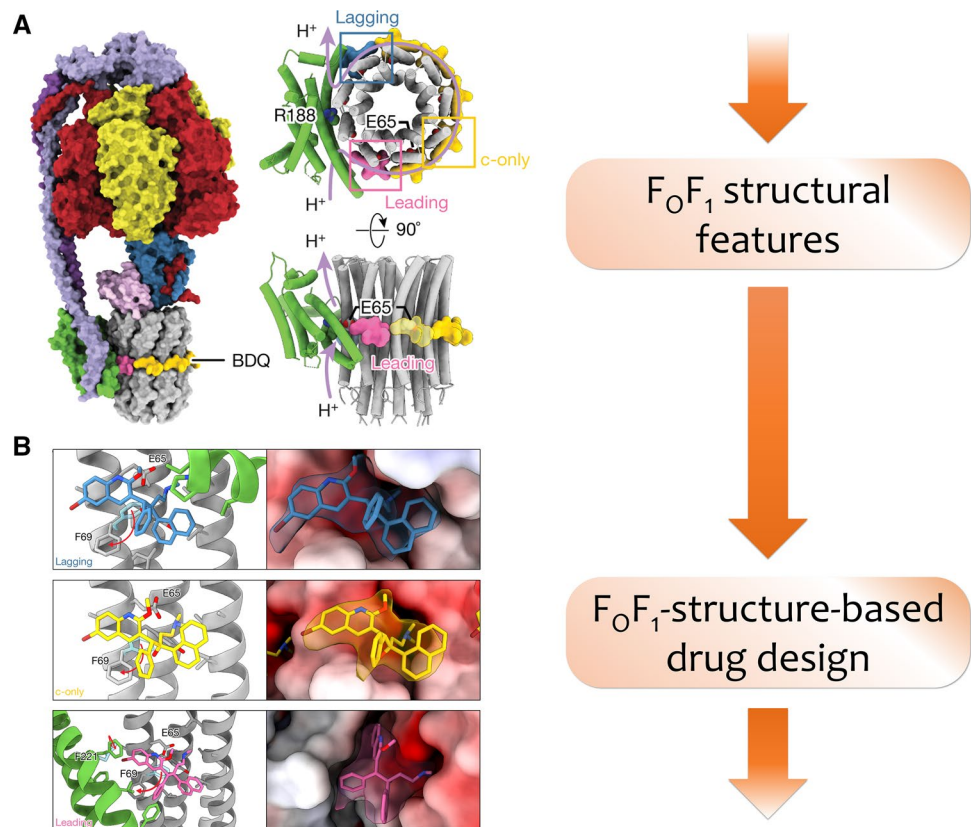
World Health Organization (WHO) presented a list of the diseases caused by the different pathogens that require new antibiotics for treatment (Table 4) [126]. We added to this list a taxonomy classification of the corresponding pathogens to highlight, which structural features of their ATP synthases might be used for therapies.

A successful case of drug design based on the structural features of ATP synthases is bedaquiline (BDQ), a drug developed against tuberculosis (Fig. 14). The pathogen *Mycobacterium tuberculosis* carries the same structural features of bF₀F₁ as *Mycobacterium smegmatis*, for which the high-resolution ATP synthase structure was solved by the cryo-EM and demonstrated an inhibition of the enzyme by BDQ via binding of this molecule to the c₉-ring active

Table 4 WHO priority list for research and development of new antibiotics for antibiotic-resistant bacteria [126] and corresponding taxonomy classification of the pathogens

Diseases	Pathogen	Taxonomy group	Available high-res F_0F_1 from the same group (PDB IDs)
<i>Bacterial pathogens</i>			
Tuberculosis	<i>Mycobacterium tuberculosis</i>	Bacteria; terrabacteria group; actinobacteria	<i>Mycobacterium smegmatis</i> (7JG5; 7JG6; 7JG7; 7JG8; 7JG9; 7JGA; 7JGB; 7JGC)
Pneumonia; bloodstream infections; meningitis; wound and surgical site infections, including necrotizing fasciitis; urinary tract infections	<i>Acinetobacter baumannii</i>	Bacteria; proteobacteria; gammaproteobacteria	<i>E. coli</i> (6OQR; 6OQS; 6OQT; 6OQU; 6PQV; 6WNNQ; 6OQV; 6OQW; 6WNR; 6VWK)
Pneumonia; septic shock; urinary tract infection; gastrointestinal infection; skin and soft tissue infections	<i>Pseudomonas aeruginosa</i>		
Plague; salmonellosis	<i>Enterobacteriaceae</i>		
Salmonellosis	<i>Salmonella sp.</i>		
Pneumonia; meningitis; epiglottitis	<i>Haemophilus influenzae</i>		
Shigellosis	<i>Shigella sp.</i>		
Enterococcal infections [127]	<i>Enterococcus faecium</i>	Bacteria; terrabacteria group; firmicutes	<i>Bacillus PS3 (recombinant)</i> 6N30; 6N2Z; 6N2Y
Abscesses; sinusitis; food poisoning	<i>Staphylococcus aureus</i>		
Meningitis; otitis media; sinusitis; community-acquired; pneumonia	<i>Streptococcus pneumoniae</i>		
Gastritis; duodenitis; stomach cancer	<i>Helicobacter pylori</i>	Bacteria; proteobacteria; delta/epsilon subdivisions; epsilonproteobacteria	N/A
Campylobacteriosis	<i>Campylobacter sp.</i>		
Gonorrhea	<i>Neisseria gonorrhoeae</i>	Bacteria; proteobacteria; betaproteobacteria	N/A
<i>Eukaryotic pathogens</i>			
African trypanosomiasis	<i>Trypanosoma brucei</i>	Eukaryota; discoba	<i>Euglena gracilis</i> (6TDU; 6TE0; 6TDY; 6TDZ)
Visceral leishmaniasis	<i>Leishmania donovani</i>		
Babesiosis or piroplasmosis	<i>Babesia spp.</i>	Eukaryota; Sar	<i>Tetrahymena thermophila</i> (6YNN; 6YNNV; 6YNNW; 6YNNX; 6YNY; 6YNZ; 6YOO)
Toxoplasmosis	<i>Toxoplasma gondii</i>		<i>Toxoplasma gondii</i> (6TMH; 6TMG; 6TMI; 6TML; 6TMK)
Cryptosporidiosis	<i>Cryptosporidium spp.</i>		
Malaria	<i>Plasmodium spp.</i>		
Balantidiasis	<i>Balantidium coli</i>		
Giardiasis	<i>Giardia lamblia</i>	Eukaryota; metamonada	N/A
Trichomoniasis	<i>Trichomonas vaginalis</i>		
Amoebiasis	<i>Entamoeba histolytica</i>	Eukaryota; amoebzoa	N/A

Fig. 14 **A** Structural features carried by bF₀F₁ from mycobacteria. In this case the active centers of the c₈-ring are found to have a special binding pocket [24, 25]. **B** F₀F₁-structure-based drug design demonstrates the molecular mechanism of inhibiting the bF₀F₁ by anti-tuberculosis drug BDQ. The figure was modified from [25]



centers of the bF₀F₁ from mycobacteria [25]. In 2015, the molecular mechanism of BDQ binding was elucidated by X-ray crystallographic analysis of a crystal of the c₉-ring [24]. Nowadays, the studies of BDQ efficiency showed high culture conversion rate (65–100%) and a satisfactory treatment outcomes [128].

Among human pathogens, a significant part is represented by protists. Their ATP synthases can become a potential target for new antibiotics and inhibitors. Since the mtF₀F₁ from different species within the same group are structurally similar, it makes them prospective targets for ligands and small-molecule drugs or other therapies targeting ATP synthases. To date, high-resolution structures are available only for four groups of eukaryotes (see Fig. 2).

Existing structure of mtF₀F₁ from *Euglena gracilis* [40] reveals structural features of the mtF₀F₁ (type IV dimer) belonging to the Discoba clade. This clade also includes pathogens from the genus *Leishmania* [129] that cause leishmaniasis. In addition to *Leishmania*, this group includes *Trypanosoma brucei* [130], the causative agent of African Trypanosomiasis. WHO classifies both of these diseases as so-called “forgotten” diseases. Although the diseases from this list (Table 4) do not attract public attention, as they are dangerous mainly for residents of developing countries, nevertheless, they take about half a million lives annually.

One more example of the potential targeting is the mtF₀F₁ dimers type III from *Tetrahymena thermophila* [11] and *Toxoplasma gondii* [10] belonging to the Ciliophora and Apicomplexa phylum, respectively, from the Alveolata clade of the Sar clade. As it was mentioned in the “[Similarity and diversity of ATP synthases](#)”, the high-resolution structures showed similar U-shape form-factors of the mtF₀F₁ dimers from these organisms. However, these dimers mtF₀F₁ showed completely different macroscale organization in mitochondria cristae. In the case of Ciliophora phylum, the tubular mitochondria cristae topology is formed by a tetrameric array of mtF₀F₁ (see Fig. 3C, G the left side). In the case of Apicomplexa phylum, the mitochondrial cristae are formed by the pentagonal bypiramids formed by the mtF₀F₁ hexamers (see Fig. 3C, G the right side). Though different oligomeric organization of mtF₀F₁ is present in representatives of the Sar clade, they carry similar structural features in their dimeric organization, except for the peripheral distant subunits in the F₀ part of the enzymes. The Sar clade includes a large number of pathogens, such as *Babesia* spp. (Apicomplexa phylum) [131], *Toxoplasma gondii* (Ciliophora phylum) [132], *Balantidium coli* (Ciliophora phylum) [133], and *Cryptosporidium* spp. (Apicomplexa phylum) [134]. In addition, the causative agent of malaria *Plasmodium* spp. (Apicomplexa phylum) [135] also belongs to Alveolata clade from the Sar clade.

However, there are pathogens belonging to the groups, for which there is still no mtF_0F_1 structural data (representatives of Amoebozoa and Metamonada clades). For example, the pathogen *Entamoeba histolytica* [136, 137], belonging to Amoebozoa clade, causes amoebiasis (also belongs to the forgotten diseases as well as trypanosomiasis and leishmaniasis). The Metamonada clade includes pathogens, such as *Giardia lamblia* [138], which causes giardiasis, and *Trichomonas vaginalis* [139], which causes trichomoniasis, a sexually transmitted disease. Obtaining high-resolution structures of the corresponding ATP synthases could help in the development of new drugs against these diseases.

Outlook

Despite recent advances in the structural biology of ATP synthases, these enzymes are still a focus of modern research due to their vital importance for living organisms, structural complexity, and diverse subunit composition in distant lineages. The main divergence of the ATP synthases generally follows the NCBI taxonomy [30]. For example, Bacteria superkingdom and chloroplasts carry ATP synthase monomers. Although in some cases, the cF_0F_1 dimers were reported, there is neither structural data showing the

presence of cF_0F_1 functional dimers nor biochemical evidence of the subunit machinery establishing these dimers.

ATP synthases from the Eukaryota superkingdom representatives reside within the mitochondria and chloroplasts. In mitochondria, ATP synthases tend to assemble into arrays, which determine the topology of their cristae. The next level of divergence of mtF_0F_1 subunit machinery occurs at the level of clades of Eukaryota superkingdom. For example, the representatives of Opisthokonta, Sar and Discoba clades, and Viridiplantae kingdom have significantly different types of dimeric assemblies (V- and U-shape dimers of four types). Before 2021, one could make the conclusion that a type of a dimer exactly defines the morphology of mitochondria. However, a recent study [10] showed that *Toxoplasma gondii*—a representative of Apicomplexa phylum from Alveolata clade belonging to Sar clade—has completely different assembly of mtF_0F_1 arrays (hexamers of mtF_0F_1 which form pentagonal bipyramids, with each hexamer consisting of three mtF_0F_1 dimers of type III) than *Tetrahymena thermophile*—a representative of a Ciliophora phylum, which also belongs to Alveolata clade (its mtF_0F_1 dimers of type III form arrays of tetramers creating tubular cristae) [11]. Unexpectedly, the difference in the organization of the mtF_0F_1 assembly is established by minimal changes in the peripheral subunit machinery of dimers of the enzyme. In particular, the

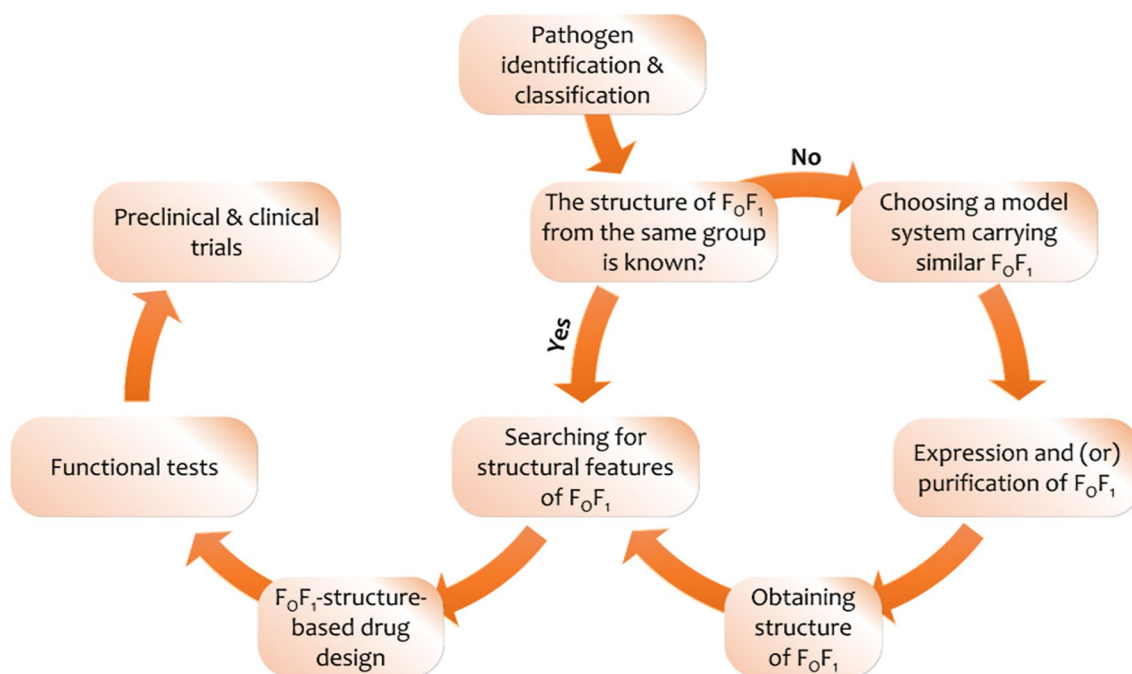


Fig. 15 Promising F_0F_1 -structure-based drug-design approach. If a target pathogen belongs to a phylogenetic group for which a corresponding high-resolution structure of F_0F_1 is unavailable, one might choose a model system carrying the same structural features of F_0F_1 for expression or purification of intact F_0F_1 from it. When the

high-resolution structure of the F_0F_1 is obtained one can search for structural features that can be utilized for F_0F_1 -structure-based drug-design with following standard procedures of functional testing, pre-clinical and clinical trials

hexameric net of the mtF_0F_1 is established by a single subunit ATPG11, making it an attractive target for therapies and other various applications.

The structural features of bacterial ATP synthases can also be utilized for the development of drugs. One of the promising cases is a development of the anti-tuberculosis drug BDQ, where the ATP synthase-structure-based approach revealed the molecular mechanisms of inhibiting the enzyme [24, 25], which might help improving the drug efficiency in the future.

We summarize here a possible systematic strategy for the development of new therapies based on the F_0F_1 -structure approach (Fig. 15). Briefly, the approach starts from the classification of pathogens (see Table 4). If a pathogen belongs to a phylogenetic group, for which a high-resolution structure of the corresponding F_0F_1 is available for one of the representatives, one can use it for searching for the specific structural features carried by the ATP synthase of these organisms. Usually, model systems chosen for studies by means of structural biology are non-pathogenic. However, they might accurately reflect the behavior of the corresponding pathogens belonging to the same phylogenetic group.

If a pathogen belongs to a phylogenetic group, for which there are no representatives with available high-resolution structure of F_0F_1 , then one has to choose an appropriate model system from the same phylogenetic group, preferentially at the phylum level, but at least at level of clades.

Then, one has to obtain a recombinant or intact ATP synthase, depending on the model system that was chosen, and after that obtain a high-resolution structure of F_0F_1 by means of structural biology according to modern protocols of cryo-EM studies (see Table 2) and probably using protein crystallization and X-ray diffraction as a complementary method to refine the high-resolution structure especially in a membrane F_0 region, where commonly the resolution of cryo-EM technique is lower than for the water-soluble part F_1 .

Finally, when the high-resolution structure of corresponding F_0F_1 is obtained one can search for unique structural features belonging to the exact phylogenetic group but missing in other groups. Typically, a host organism and an infecting pathogen belong to the distant lineages and therefore carry significant differences in the F_0F_1 organization. These structural differences become the target for small-molecule ligands that can partially or completely inhibit pathogenic F_0F_1 without affecting the activity of the host enzyme. Further functional tests, preclinical and clinical trials following standard procedures are finalizing the development of new F_0F_1 targeting drugs against the pathogenic infections.

The useful support on the way to new therapies targeted to F_0F_1 may also come from the state-of-the-art machine learning method such as AlphaFold [140, 141] and RoseTTAFold [142]. Despite revolutionary advances for protein

structure predictions, the use for drug development is still elusive [143]. However, when structural information for parts of F_0F_1 remains unavailable, predicted models may be a source of therapeutic ideas which can later be validated in functional tests.

The scheme in Fig. 15 is not the only way of how the approach using structural information on the F_0F_1 from different organisms can be used. F_0F_1 -structure-based approach is much wider and might open new perspectives for the gene therapies, drug design, and development of tools for the control of cellular bioenergetics. One of the intriguing perspectives is the possible role of mtF_0F_1 in the mPTP phenomenon, which is, however, still unknown in detail. The possibility of using mPTP as a target for therapies is actively discussed in the literature [144]. A complementary use of the F_0F_1 -structure-based approach might significantly help in the development of the mPTP-based therapies.

Regarding bacterial and chloroplast ATP synthases, the question of the small-molecule compounds inside the inner pore of their c-rings is still not yet settled. The possible presence of isoprenoid quinones inside the c-rings of corresponding F_0F_1 [23] is intriguing both in terms of understanding the mechanisms of the enzyme functioning and development of the powerful small-molecule ligands that can modulate the bF_0F_1 and cF_0F_1 efficiencies.

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Authors' contributions AVV designed, conceived and wrote the manuscript. SDO strongly contributed to the section “ATP synthases as potential therapeutic targets”, edited the text of the manuscript. NAB strongly contributed to the section “Mitochondrial permeability transition pore (mPTP)”. VNU strongly contributed to the section “Intrinsically disordered regions in ATP synthases”, contributed to all sections and edited the text of the manuscript. VIB contributed to the section “Outlook”, edited the text of the manuscript. MFY contributed to the section “Similarity and diversity of ATP synthases”. IVM contributed to the section “Validation of ATP synthase functionality”, edited the text of the manuscript. AVR organized funding acquisition, edited the text of the manuscript. ADV contributed to the section “Small-molecule cofactors of the c-ring”, edited the text of the manuscript. NSI contributed to the section “Intrinsically disordered regions in ATP synthases”, edited the text of the manuscript. AIK contributed to the section “High-resolution structural studies”. NAD contributed to all sections and edited the text of the manuscript. VIG supervised the

project, strongly contributed to all sections and edited the text of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Declarations

Conflict of interests The authors declare that they have no conflict of interest.

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Consent for publication Not applicable.

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