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From the Ca²⁺-activated F₁F₀-ATPase to the mitochondrial permeability transition pore: an overview

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Abstract

Based on recent advances on the Ca^{2+} -activated $\text{F}_1\text{F}_\text{O}$ -ATPase features, a novel multistep mechanism involving the mitochondrial $\text{F}_1\text{F}_\text{O}$ complex in the formation and opening of the still enigmatic mitochondrial permeability transition pore (MPTP), is proposed. MPTP opening makes the inner mitochondrial membrane (IMM) permeable to ions and solutes and, through cascade events, addresses cell fate to death. Since MPTP forms when matrix Ca^{2+} concentration rises and ATP is hydrolyzed by the $\text{F}_1\text{F}_\text{O}$ -ATPase, conformational changes, triggered by Ca^{2+} insertion in F_1 , may be transmitted to F_O and locally modify the IMM curvature. These events would cause $\text{F}_1\text{F}_\text{O}$ -ATPase dimer dissociation and MPTP opening.

Keywords: $\text{F}_1\text{F}_\text{O}$ -ATPase; calcium ion; mitochondrial permeability transition pore; conformational mechanism

1. Introduction

Oxidative phosphorylation, which features mitochondria in eukaryotes, is based on the cooperation and interplay between multiple enzyme complexes. Briefly, these complexes are dehydrogenases which transfer electrons according to the electrochemical gradient from reduced respiratory substrates, namely NADH and FADH₂, to the final acceptor molecular oxygen, and, by pumping protons in the intermembrane space, generate a H⁺ current through the inner mitochondrial membrane (IMM). Finally, the transmembrane electrochemical gradient of H⁺ ($\Delta\mu_{H^+}$) created by respiratory chain substrate oxidation drives ATP synthesis by the ATP synthase [1]. The formation of a large channel in the IMM, namely the so-called mitochondrial permeability transition pore (MPTP), dissipates the $\Delta\mu_{H^+}$ and, differently from the accepted bases of chemiosmotic hypothesis [2], eludes ATP production and causes loss of substrates and nucleotides from the mitochondrial matrix [3]. MPTP opening, by dramatically changing the IMM electrophysiological features, leads to mitochondrial dysfunction. The MPTP regulation and role in different forms of cell death, including autophagy, and in various pathologies have been the subject of intense and fruitful research, sustained by the hope to exploit this mitochondrial event to fight cancer, ischemic damage and neurodegeneration [4]. On the other hand, recent studies suggest that the MPTP may also play a relevant role in mitochondrial function, cell differentiation and development [5]. The MPTP structure has long remained a mystery, even if its identity was intensively searched for among known membrane components, above all membrane-bound proteins. At first, the voltage-dependent anion channel (VDAC) and the peripheral benzodiazepine receptor on the outer mitochondrial membrane (OMM) together with the IMM adenine nucleotide translocase (ANT) seemed the most likely candidates to take part in the enigmatic mechanism of MPTP formation [6]. In this putative mechanism, ANT was thought to constitute the MPTP fulcrum since the ANT inhibitors atractyloside (ATR) and bongkreikic acid (BGK) modulated the MPTP. In detail, BGK inhibited the MPTP by locking ANT in the M conformation (closed MPTP), while ATR maintained it in the C conformation (open MPTP) [7]. However, the ANT channel showed a similar conductance to that of the MPTP [8]. Subsequent findings pointed out that MPTP formation involved a supra-molecular complex, namely the assembly of different proteins [9]. Accordingly, differently localized proteins, namely hexokinase bound to the cytosolic surface of OMM, creatine kinase and nucleoside diphosphate kinase in the intermembrane space, and cyclophilin D (CypD) in the matrix apparently contributed to form the MPTP. An alternative model, in which the P_i carrier by interacting with ANT and CypD induced MPTP opening, was depicted [10]. However all the models proposed over 40 years of studies did not fully match the electrophysiological MPTP features [4] or were undermined by genetic deletion tests, which, one by one, excluded that any of these proteins are essential for MPTP formation [11–14]. ANT, the P_i carrier and the F₁F₀-ATPase may mutually interact through cardiolipin which would somehow connect these proteins to form the ATP synthasome. Consistently, conformational changes triggered by Ca²⁺ within the ATP synthasome may perturb the interface between these structures and produce the pore [15]. The ATP synthasome dynamics is ruled by the metabolic demand and is CypD-dependent [16]. Moreover, changes in the contact sites between the inner and outer mitochondrial membranes could intervene in MPTP opening [15]. At present, it seems likely that the MPTP may coincide with a conserved mitochondrial protein of key role in mitochondria. Recently, the F₁F₀-ATPase -a splendid molecular machine- [17] has been proposed to form the pore structure [18,19].

2. The F₁F₀-ATPase: from an old to a new story as pore former

1 From its discovery around the middle of the 20th century, the F₁F₀-ATPase has undergone a sort of
2 on-going evolution, stimulated by the increasing development of techniques and of knowledge,
3 which lead to a continuous re-evaluation of the roles of this intriguing enzyme complex [17,20]. At
4 present, we can say that new and up to now unsuspected roles for this ubiquitous enzyme are
5 emerging in mitochondria. As widely known, in eukaryotic mitochondria the F₁F₀-ATPase
6 constitutes the amazing molecular machine that exploits the electrochemical energy produced by the
7 respiratory chain in the form of Mitchell's proton motive force (Δp) to produce ATP via a chemo-
8 mechanical coupling mechanism [21]. Even if ATP synthesis represents the classical enzyme task,
9 the catalytic mechanism is long known to work also in *reverse* to energize the IMM by ATP
10 hydrolysis [20,22]. In practice, the direction of catalysis depends on Δp , being ATP synthesized to
11 dissipate Δp and conversely ATP hydrolyzed to re-build Δp . The F₁F₀-ATPase structure is quite
12 complex and can be roughly defined as an oligomer structurally composed by a hydrophilic F₁
13 catalytic domain and by a membrane-embedded F₀ domain. These two domains are joined by a
14 central and a peripheral stalk (Fig. 1). In turn the F₁ sector, which protrudes in the mitochondrial
15 matrix, shows a $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit composition and stoichiometry [23]. The three α subunits alternate
16 with three β subunits to form the F₁ globular hexamer. The adenine nucleotide binding sites, namely
17 three non-catalytic sites on α subunits and three catalytic sites on the β subunits, open at the
18 interfaces between the α and β subunits of this spherical complex [24]. The membrane-embedded
19 F₀ sector is also formed by multiple proteins, namely the *a* subunit, the short amphipathic *b* subunit
20 with the two transmembrane α -helices, *e*, *f*, *g*, A6L, DAPIT (diabetes-associated protein in insulin-
21 sensitive tissue) subunits, a 6.8 kDa proteolipid and the *c_n*-ring, in which the subunit number is
22 species-dependent [25,26]. The γ subunit extends from the center of the ($\alpha\beta$)₃ structure of F₁ to the
23 F₀ domain where it joins the δ and the ε subunits to form the foot of central stalk [27]. The core of
24 F₀ is formed by the *c*-ring, which is directly attached to the central stalk and constitutes the enzyme
25 rotor, which transmits the rotational energy to F₁. Laterally, the *b*, *d*, F₆ and OSCP subunits form
26 the peripheral stalk, which not only links the ($\alpha\beta$)₃-catalytic structure to the *a* subunit in the F₀
27 domain, forming the integral enzyme stator, but also plays the role of resisting the torque generation
28 of rotor [28]. Actually, the F₁F₀-ATPase/synthase is a rotary engine which matches rotation to
29 catalysis. The clockwise rotation (seen from the intermembrane space) is driven by Δp which makes
30 H⁺ downhill translocate across the IMM through the *a* subunit/*c*-ring complex interface. This
31 rotation transmitted from F₀ to F₁ produces one ATP molecule per each β subunit, namely three
32 ATP molecules are built in a 360° cycle. The opposite rotation, which pumps H⁺, in the
33 intermembrane space and re-constitutes Δp , is coupled to ATP hydrolysis. The nucleotide binding
34 in the catalytic site requires the coordination of the essential cofactor Mg²⁺, which contributes to
35 ATP synthesis/hydrolysis and to the asymmetry of the three catalytic sites, which produces the
36 differences in affinity for nucleotides [29]. Accordingly, each β subunit is asymmetric and during
37 the rotation, by interacting with the γ subunit, undergoes three distinct conformational states β_E
38 (always empty), β_{DP} , which contains bound MgADP and β_{TP} which binds MgATP [30].
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51 Interestingly, in mitochondria, the F₁F₀-ATPases are assembled in supra-molecular dimeric
52 complexes by the transmembrane F₀ domain [31] which form extensive rows [32,33] distributed
53 along the tightly curved ridges of the IMM *cristae* [34]. This localization exploits the higher H⁺
54 density on the surface in the curved membrane regions [35] created by the respiratory complexes
55 crowding at either side of the rows [36]. Structure, localization and function are tightly connected.
56 Accordingly, the F₁F₀-ATPase structural arrangement and localization have relevant implications
57 for the mechanism of mitochondrial energy transduction [34] and substantiate the F₁F₀-ATPase
58 active role in membrane bending and *cristae* formation [35,37], thus contributing to mitochondrial
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1 morphology. Indeed, the enzyme complex assembly locally produces an extreme membrane
2 curvature in either concave (negative curvature) where the membrane invaginates or convex
3 (positive curvature) at the edge of the *cristae*, as seen from the matrix [35].

4 The F₁F₀-ATPase energy-transduction mechanism of bioenergetics [22] and its modeling ability on
5 mitochondria [38] turns into an energy-dissipating machinery when the mitochondrial Ca²⁺
6 concentration abruptly increases under pathological conditions [39]. In this case the F₁F₀-ATPase
7 activated by Ca²⁺ instead of Mg²⁺ would form a channel which matches the conductance properties
8 of the MPTP [40,41]. The pore opening leads to transient IMM depolarization and allows the
9 diffusion of solutes, water enters and ATP is hydrolyzed by the F₁F₀-ATPase [3]. Disruption of
10 mitochondrial homeostasis induces swelling and bursting of IMM, an event which has been linked
11 to pathways leading to cell death [42,43]. The MPTP opening has been reported to be affected by a
12 large variety of effectors and conditions, spanning from ion concentrations to physical changes.
13 Accordingly, Ca²⁺, Mg²⁺, adenine nucleotides, P_i, H⁺ and membrane potential have been claimed as
14 F₁F₀-ATPase modulators and MPTP inducers/inhibitors [44]. On considering that the F₁F₀-ATPase
15 is not only ruled by its own substrates/products, but also responds to post-translational
16 modifications (PTMs) on different subunits [30], PTMs affecting the enzyme function or its super-
17 complex organization may greatly impact MPTP modulation [44]. Furthermore, cyclosporin A
18 (CsA) blocks the MPTP binding to CyPD, a protein which modulates the MPTP without being an
19 essential component of its structure [45,46]. Additionally, the putative MPTP-CypD interaction
20 involves OSCP subunit of F₁ sector [47], which connects the catalytic ($\alpha\beta$)₃ spherical complex to
21 the peripheral stalk. However, since MPTP opening was recently reported to remain CsA-sensitive
22 also in the absence of OSCP [48] or pH-dependent by protonation of the unique histidine in OSCP
23 subunit [49], the whole mechanism need to be clarified.

24 On balance, the experimental evidence accumulated up to now points out that the MPTP-forming
25 properties in eukaryotes [50] are apparently linked to the dual F₁F₀-ATPase function. Even if some
26 points remain controversial and require to be elucidated, the enzyme of life that synthesizes ATP,
27 activated by Mg²⁺, apparently turns into the enzyme of death when hydrolyzes ATP in the presence
28 of Ca²⁺.

3. The hypotheses on the F₁F₀-ATPase involvement in the MPTP

34 From the beginning of the story, namely since cell death was associated with an abruptly increased
35 mitochondrial permeability, it seemed quite obvious that the pore formation should involve
36 mitochondrial membrane components whose conformational changes under certain conditions
37 eventually made the inner membrane itself permeable to water and solutes. The most likely
38 candidates were membrane-bound proteins, since the pore always supposed to be proteinaceous
39 [4,44]. However, the MPTP constitution was never elucidated and it was also proposed to include a
40 non-proteinaceous ion-conducting module [51]. Assumed that the F₁F₀-ATPase contributes to
41 MPTP formation, as recent advances strongly hint, up to now two main mechanisms have been
42 hypothesized: the channel forms within the *c*-ring of the F₀ sector [52] or, alternatively, at the
43 interface between the contact region of the dimer [19].

44 The “*c*-ring hypothesis” is sustained by intriguing hints on the channel conductance, obtained on
45 mammalian pure *c* subunits reconstituted in lipid bilayers capable of generating spontaneous
46 electrical oscillations activated by cGMP and inhibited by Ca²⁺ [53]. However, the current
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1 generated through the *c* subunit pore was found to be cation-selective [54], while the MPTP is
2 known to be induced by Ca^{2+} and non-selective for solutes with a molecular weight of up to
3 1,500 Da [55]. Otherwise, the dephosphorylation of a peptide related to *c* subunits promoted by
4 Ca^{2+} and prevented by CsA emerged as MPTP inducer [56]. The formation of a voltage-sensitive
5 channel in reconstituted *c* subunits or purified F_1F_0 -ATPase in liposomes was hypothesized. The
6 pore would be formed by CyPD and Ca^{2+} -dependent *c*-ring expansion and F_1 detachment [52]. This
7 multi-conductance channel lacked cation selectivity, but was resistant to CsA, insensitive to Ca^{2+}
8 and inhibited by the β subunit of F_1F_0 -ATPase [52]. Experiments of overexpression or depletion of
9 endogenous *c* subunits by specific siRNA downregulation and consequent MPTP inactivation
10 strengthened the “*c*-ring channel” hypothesis [18]. A proper *c*-ring conformation is required for
11 MPTP opening [57] and the highly conserved *c* subunit Gly zipper domain apparently plays a key
12 role in the *c*-ring assembly linked to MPTP sensitivity [52,57]. Recent insights are provided by the
13 Ca^{2+} -induced *de novo* water-permeable MPTP complex, which is inhibited by CsA, and apparently
14 made up by *c* subunits associated with polyphosphate (polyPi) and polyhydroxybutyrate (PHB)
15 [58]. The interaction of the *c* subunits with polyPi and PHB, by generating a charged polymer,
16 provides an environment compatible with the hydrophobicity of these proteolipids. Indeed, the *c*-
17 ring ion conduction gaps were offset by the electrophysiological properties of the non-proteinaceous
18 Ca^{2+} -selective polyPi/PHB channel [51]. Otherwise, due to its structure, the *c*-ring cannot form a
19 water-filled channel as suggested by the atomistic simulations of two *c*-rings of different lumen
20 width [59]. The *c*-ring lumen contains lipids in bacteria [60], while in mitochondria it would be
21 occluded by lipid molecules and forms a non-conducting channel [59]. Moreover, even in a
22 potentially conducting state (*i.e.* hydrated state of the *c*-ring interior), molecular dynamics
23 simulations demonstrated that the biophysical properties of such channel were not consistent with
24 the high ionic conductance attributed to the MPTP [59]. However, atomic simulations on the *c*-ring
25 do not take the polyPi/PHB model into account. Accordingly, due to the PHB amphipathic
26 properties, lipids may localize in the *c*-ring hydrophobic core and even allow ion flux [58]. Even if
27 the structural bases of the “*c*-ring channel” opening [52] necessarily require CyPD binding and
28 inhibition by CsA only after Ca^{2+} addition, CyPD-null mice showed electrophysiological MPTP
29 features indistinguishable from those of wild-type individuals in presence of high Ca^{2+} loads
30 [61,62]. The possibility that the channel may be opened by the displacement of the two main F_1F_0 -
31 ATPase domains has also been considered. However, strip down F_1 from F_0 sector generally occurs
32 under drastic conditions, for instance in the presence of high urea concentrations, and leads to
33 irreversible F_1F_0 -ATPase denaturation, whereas the MPTP reversibly shifts between open/closed
34 states. Moreover, the displacement of the two F_1F_0 -ATPase sectors is not a likely mechanism to
35 create a channel within F_0 , because the γ , δ and ϵ subunits of F_1 cannot return to their native
36 position within the hydrophobic sector. Additionally, it seems difficult to think that free β subunits
37 can inhibit the MPTP formation because the catalytic subunits are linked into $(\alpha\beta)_3$ globular
38 hexamer resistant to denaturation, yet the hexamer does not interact with the embedded membrane
39 sector in wild-type F_1F_0 -ATPase [63]. Finally, human cells in which the *c* subunit genes are
40 disrupted preserve the typical MPTP properties [64]. These vestigial F_1F_0 -ATPases in cells unable
41 to synthesize the *c* subunits are also structurally devoid of *a* and A6L subunits and cannot
42 translocate H^+ , even if the CsA-sensitive MPTP formation is maintained [64]. On these bases it
43 seems reasonable to conclude that in the absence of *c* subunits the MPTP could be formed and be
44 sensitive to CypD, but it remains unclear if its conductance properties occurs through an
45 unregulated MPTP pathway or not [65].
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1 If the “*c*-ring hypothesis”, even if intriguing, show some inconsistencies, other recent findings
2 corroborate the involvement of the dimeric form of the F₁F₀-ATPase. Accordingly, the MPTP
3 formation was observed after reconstitution into lipid bilayers of gel-purified F₁F₀-ATPase dimers
4 associated with the detection of an indistinguishable channel current ascribable the MPTP
5 electrophysiological equivalent mitochondrial mega-channel [19]. Cross-linking experiments from
6 Bernardi’s group indicate that the OSCP subunit of peripheral stalk interacts with CyPD [47] and
7 benzodiazepine 423 (Bz-423), a MPTP inducer which overlaps the CyPD binding site [19]. Bz-423
8 inhibits the F₁F₀-ATPase activity similarly to the P_i-dependent CyPD which, by binding to the
9 OSCP subunit, decreases the Mg²⁺-dependent ATP hydrolysis in the absence of CsA [47]. Thus,
10 CyPD modulates MPTP opening and CyPD binding to OSCP may propagate the conformational
11 changes of the catalytic sites through the stator to enzyme membrane portions [66]. The high matrix
12 Ca²⁺ concentration features MPTP activation. Therefore the “F₁F₀-ATPase peripheral stalk/dimer
13 hypothesis” is fully consistent with the occupation of the catalytic site by Ca²⁺ in replacement of
14 natural cofactor Mg²⁺ [44,66]. In the catalytic site the βThr¹⁶³ of the P-loop is directly linked to
15 Mg²⁺, while the βArg¹⁸⁹, βGlu¹⁹², βAsp²⁵⁶ residues are coordinated with Mg²⁺ by three water
16 molecules respectively [67] (Fig. 2b). The metal binding pocket can be occupied by other divalent
17 cations such as Ca²⁺ [68]. Interestingly, experiments on prokaryotes showed that the single mutation
18 of βThr¹⁵⁹Ser at the catalytic site equivalent to βThr¹⁶³Ser in eukaryotes is the only aminoacid
19 substitution which allows the normal F₁F₀-ATPase function if sustained by MgATP, but not by
20 CaATP [69]. A recent paper [70] investigated the effects of the βThr¹⁶³Ser mutation in human F₁F₀-
21 ATPase. In comparison with wild type mitochondria, ATP hydrolysis driven by Mg²⁺ was
22 stimulated, while the Ca²⁺-dependent F₁F₀-ATPase activity was nearly completely inhibited.
23 Moreover, the mutation in β subunit apparently decreased the MPTP sensitivity to Ca²⁺, since
24 higher Ca²⁺ levels were required to induce MPTP opening [70]. The βThr¹⁶³ is probably the only
25 aminoacid which directly binds to the metal cation in the catalytic site. Consistently, while in
26 presence of Mg²⁺ the mutation in the catalytic subunit apparently favors ADP release during
27 hydrolysis, the larger Ca²⁺ would cause a spatial rearrangement that stiffens the F₁ sector and limits
28 OSCP motility. Molecular dynamics simulations suggest that the mechanical energy of Ca²⁺ bound
29 to β-subunits sites may be transmitted through a long connecting loop to the “crown region” of the
30 OSCP subunit (Fig. 2a). According to this mechanism, the motion starting from the catalytic sites
31 would be transferred through the lateral stalk to the membrane subunits where the MPTP opens
32 [70]. Looking at the residues putatively involved in this mechanism, some concern might arise
33 about the Ca²⁺ specific conformational changes transmission from the catalytic binding sites to
34 OSCP, since only the N-terminus of the three α subunits interacts with OSCP at the N-terminal α-
35 helical domain. Other interactions between the (αβ)₃ spherical domain and the peripheral stalk are
36 established, connecting the α subunit N-terminal region to *b* and F₆ subunits respectively and the α
37 subunit C-terminus to *d* subunit [38,71]. However, He et al, [48] recently provided experimental
38 evidence that peripheral stalk subunits (*i.e.* OSCP and *b* subunit) are not involved in the MPTP
39 formation. Consistently, the F₁ modification induced by Ca²⁺ cannot be transmitted to the
40 membrane subunits when the stator is defective. In cells with F₁F₀-ATPase devoid of OSCP or if
41 the peripheral stalk lacks the two transmembrane α-helices of *b* subunit, mitochondria retained a
42 reduction of MPTP-dependent swelling rate responsive to CsA inhibition, thus suggesting that the
43 binding site for CyPD is not provided by OSCP and in this vestigial F₁F₀-ATPase the channel size
44 is affected [48]. Nevertheless, the inhibition of the MPTP opening by acidic pHs is sensitive to the
45 protonation of the unique histidine in OSCP subunit (OSPCHis¹¹² in humans) [49], thus leading
46 Antoniel and colleagues suspect that more than one subunit of the peripheral stalk can transmit the
47 full-conductance signal for MPTP opening. In addition, the overexpression of *e* subunit, which is
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1 known to promote the F₁F₀-ATPase dimerization [72,73], limits the MPTP induction by Ca²⁺.
2 Briefly, even if there is still much work to be done to clarify the MPTP opening mechanism and
3 some still unexplained contradictions emerge from literature data, experimental evidence gathered
4 up to now indicates that MPTP opens when the F₁F₀-ATPase dimers dissociate and the *c*-ring
5 maintains an adequate conformation [57].
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9 4. A new conformational transmission model for MPTP opening

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11 On considering the hypotheses so far proposed, some questions are still open. The MPTP, the F₁F₀-
12 ATPase and Ca²⁺ depict an enigmatic triangle [74], in which Ca²⁺ apparently plays the leading role.
13 From the available literature data and of some recent findings in our lab, we become increasingly
14 convinced that Ca²⁺ by interacting with the F₁F₀-ATPase triggers subsequent conformational events
15 which ultimately lead to form a pore in the inner mitochondrial membrane. In our opinion, there are
16 many clues that lead to build an intriguing and quite realistic model. Accordingly, when the
17 mitochondrial Ca²⁺ concentration increases, it replaces the natural cofactor Mg²⁺ in the catalytic site
18 of the F₁F₀-ATPase [70]. As recently pointed out [75], the catalytic mechanism of ATP hydrolysis
19 and H⁺ translocation by the Ca²⁺ and Mg²⁺-dependent F₁F₀ complexes are apparently similar, in
20 contrast with previous reports [76–78]. Interestingly, small molecules or cofactors (*e.g.* nitrite or
21 NAD⁺) were found to act differently on the enzyme when it is activated by Ca²⁺ or by Mg²⁺, namely
22 they inhibit the Ca²⁺-dependent F₁F₀-ATPase without affecting the Mg²⁺-dependent F₁F₀-ATPase.
23 Therefore, the catalysis modulation may represent a molecular mechanism which is somehow
24 involved in MPTP regulation [79,80]. As far as we are aware, the Ca²⁺-activated F₁F₀-ATPase
25 cannot synthesize ATP, but it is capable of ATP hydrolysis, probably by adapting the catalytic
26 mechanism, which is compatible with the greater steric hindrance of Ca²⁺ with respect to Mg²⁺
27 when inserted in the β subunits [75]. So, in the presence of the larger Ca²⁺ radius, the coordination
28 geometry of the cofactor-binding site of the enzyme would change from six-fold octahedral up to
29 allow eight ligands, resulting into a less rigid geometry with irregular distances and angles [81]. The
30 Mg²⁺ and Ca²⁺ stimulated enzyme activities, thus suggesting that the two cations similarly interact
31 with the protein and the nucleotides [75,81]. Nevertheless, Ca²⁺ could bind to the same aminoacid
32 residues as Mg²⁺ with even greater affinity (Fig. 2b,c). Moreover, ATP hydrolysis, which causes the
33 mitochondrial ATP pool depletion associated with the MPTP [3], drives the torsional mechanism of
34 the central stalk in the Ca²⁺-activated F₁F₀-ATPase [82]. This torsion is coupled to H⁺ pumping
35 [75] through the IMM, even if H⁺ translocation is unable to re-energize the IMM [44,75]. As a
36 result, when the F₁F₀-complex works in the *reverse* mode (ATPase) driven by Ca²⁺ there is ATP
37 dissipation without membrane polarization. So, it seems reasonable to think that the Ca²⁺-activated
38 F₁F₀-ATPase can start a multistep process resulting into MPTP formation and opening [83].
39 According to this model, the spatial rearrangement within F₁ would arise from Ca²⁺ binding to the
40 catalytic sites, and, in the form of conformational change signal, would be transmitted to reach the
41 hydrophobic F₀ sector, where it would promote the dissociation of F₁F₀-ATPase dimers into
42 monomers and determine the loss of the local curvature of *cristae*, thus making the MPTP open
43 (Fig. 3). These events, namely the F₁F₀-ATPase activation by Ca²⁺ and the deformation of *cristae*,
44 are both associated with mitochondrial dysfunction and cell death due to MPTP opening. The F₁F₀-
45 ATPase dimerization in mammalian mitochondria could arise from the *a* and *e* subunits, while in
46 yeast mitochondria also *i/j* and *k* subunits would participate in maintaining the dimer joined. These
47 supernumerary membrane subunits in yeasts could be functional orthologs of the 6.8 proteolipid and
48 DAPIT subunits in mammalian mitochondrial F₁F₀-ATPase, respectively. Interestingly, two
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1 adjacent *a* subunits in mammalian form a “dimerization motif” in which the *a* subunit of each
2 monomer has a strand planar structure that connects the two monomers. The *cris*tae are bent by *e*, *g*,
3 and the N-terminal portion of *b* subunits forming an unusual transmembrane domain [31] which,
4 when the F₁F₀-ATPase dimerizes, induces the positive curvature of the membrane which produces
5 the morphology of the *cris*tae. Indeed, F₁F₀-ATPase monomers are *per se* sufficient to produce
6 curvature in lipid bilayers (a 43° inclination is imposed by the membrane stator domain) and the
7 detachment of dimers prevents the formation of the edge of the *cris*tae, resulting in a zig-zag
8 topology of the membrane [84]. Conversely, the positioning of dimers along rows in a ridge is a
9 self-association of side-by-side union of multiple dimeric F₁F₀-ATPase super-complexes which
10 does not require additional protein-protein interactions [35].
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14 The stiffness of the peripheral stalk holds the ($\alpha\beta$)₃ globular hexamer in a stationary position with
15 respect to the IMM, even if the stator has the adequate flexibility to allow the rotary catalytic cycle
16 [85]. The dimer interface is formed at the basis of the peripheral stalk. Thus, any unusual flexibility
17 induced in this structure can compromise the stability of the dimeric F₁F₀-ATPase supra-molecular
18 assembly. However, MPTP persists in the absence of the peripheral stalk subunit [48]. Interestingly,
19 vestigial F₁F₀-ATPase complexes, which lack the *c*-ring, the *a* and the A6L subunit or either the *b*
20 subunit or the OSCP subunit, retain the MPTP features. All these vestigial enzymes were found
21 abundantly associated with two forms of the intrinsic inhibitor protein (IF₁) [48,64]: a mature
22 inactive IF₁ form (IF₁-M1 but not the IF₁-M2 isoform) and the import precursor IF₁, IF₁-P (a mature
23 IF₁ form with import sequence). The same proteins were also associated with the monomeric F₁F₀-
24 ATPase derived from ρ^0 cells [86]. It is still unknown whether IF₁-M1 and IF₁-P differ in the entry
25 pathway to mitochondria or in ATP hydrolysis inhibition, but probably the specific association of
26 IF₁-P with the F₁F₀-ATPase could prevent dimerization [86]. Moreover, the active IF₁ form (an
27 antiparallel α -helical coiled coil dimeric structure) in the matrix can associate with five F₁ subunits
28 in different conformations [87]. The active IF₁ is not essential for dimer formation [88,89], even if it
29 can promote the F₁F₀-ATPase dimeric structure super-complexes [90]. The dimeric IF₁ role is the
30 ATPase inhibition by binding with a ratchet-like mechanism to the α/β_{DP} site [91] proximal to the
31 peripheral stalk [38]. However, some clues suggest that IF₁ may also play a role in MPTP opening
32 [92]. The active form of IF₁ occurs at pH values below 6.5 [93], when the mitochondrial ATP
33 hydrolysis to re-energize the IMM causes a disastrous ATP drop and cellular acidification.
34 Interestingly, when pH lowers to 6.5, the protonation of histidine residue(s) near the region of dimer
35 combination shifts the IF₁ equilibrium from tetramers (inactive IF₁ form) to dimers (active IF₁ form)
36 so as to interact with F₁ sector [93]. The reversible histidine(s) protonation on the mitochondrial
37 matrix side [94] as well as the OSCP^{His112} protonation [49] are known to inhibit the MPTP. As a
38 matter of fact, when the matrix pH decreases below 7.0, the MPTP is in the closed conformation.
39 However, dithyl pyrocarbonate (DPC) allows MPTP opening at pH 6.5 and maintains the Ca²⁺-
40 dependent channel sensitivity to CsA. Accordingly, DPC reacts with histidyl residues and prevents
41 their reversible protonation [94]. Therefore, at acidic pHs IF₁ is in the active form and the MPTP is
42 closed. Conversely, at pH values \geq 7.0, the IF₁ dimers aggregate into a tetramer which occludes the
43 inhibitory portion of F₁ sector [95]. At alkaline pHs, to avoid detachment from the F₁F₀-ATPase
44 under non-inhibitory conditions, the C-terminus of IF₁ is kept anchored to F₀ [96,97], while the N-
45 terminal region of IF₁ is cross-linked to the α subunit [98]. In the presence of an incomplete
46 structure of the peripheral stalk, the Ca²⁺-induced conformational change within F₁ could be
47 transmitted to the membrane subunits by the inactive form of IF₁ [99]. Accordingly, IF₁ by
48 anchoring to the α subunit [98] and to a still unidentified membrane receptor of known molecular
49 mass (approximately *M_r* 5400-6400 Da) [96] corresponding to one of the membrane subunits (A6L,
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1 *e*, *f*, *g*, DAPIT subunits and 6.8 kDa proteolipid) [100], could create a low density bridge-like
2 structure between the two monomers [32]. This connecting structure may be responsible for the
3 conformational transmission and drive the conformational change from the Ca²⁺-bound catalytic
4 subunits to the membrane subunits [99]. This transmission would draw up the dimer stalks and
5 cause the reduction or even the loss of the membrane convexity between the two matched F_O
6 sectors [101]. Moreover, the MPTP inhibition at low osmotic strength is linked to changes in the
7 IMM curvature [102]. Consistently, the pore formation between the two F₁F_O-ATPase monomers
8 undoes the supra-molecular dimeric structure [57]. According to this model, the torque generation
9 driven by ATP hydrolysis pushes the rotors of two adjacent F₁F_O-complexes towards opposite
10 directions, allowing the respective monomer axis to contribute to reduce the distance between the
11 two F₁ sectors from 15 to 10 nm [103]. The consequent curvature inversion of the *cristae*,
12 associated with the spatial re-arrangement of the membrane subunits responsible for the “*bridge*
13 *shape domain*”, results in MPTP opening [104] (Fig. 4). Noteworthy, the rotation of the Ca²⁺-
14 activated F₁F_O-ATPase [82], driven by ATP hydrolysis, can only occur in the presence of a correct
15 structure of the *c*-ring or even in the absence of the *c*-ring. Accordingly, a proper *c*-ring or vestigial
16 F-ATPases unable to build the *c*-ring preserve the MPTP properties [57,64]. Once transmitted
17 through conformational changes, such rotation may be indirectly responsible for the stalk-to-stalk
18 distance modification involved in MPTP opening. The supra-molecular arrangement of the F₁F_O-
19 ATPase complexes is consistent with the ultrastructure of the *cristae* and with the ideal
20 mitochondrial bioenergetics. When the F₁F_O-ATPase dimeric and oligomeric forms were
21 destabilized by mutated *e* subunits, the F₁F_O-ATPase activity was maintained, even if unexpectedly
22 accompanied by IMM Δφ reduction [105]. On the other hand the *e* subunit overexpression, which
23 supports the dimeric structure, was found to limit MPTP formation and prevent IMM depolarization
24 [57].

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33 To sum up, the experimental evidence accumulated up to now strongly suggests that MPTP
34 formation and opening result from a multi-step process in which conformational changes play a key
35 role. The data accumulated up to now are well compatible with a conformational mechanism
36 triggered by the Ca²⁺-dependent F₁F_O ATP hydrolysis, which implies a spatial rearrangement within
37 F₁. According to the depicted model, this conformational change would constitute a structural signal
38 that, once transduced to the membrane subunits, promotes the monomerization of F₁F_O-ATPase
39 super-complexes and the curvature inversion at the apex of the *cristae*. So, the features of the Ca²⁺-
40 activated F₁F_O-ATPase are fully consistent with the Δφ loss and ATP hydrolysis, known as
41 mitochondrial events which open the MPTP [3,83].
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52 6. References

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Captions to Figures

1
2 Figure 1. Subunit composition and structural arrangement of the F₁F₀-ATPase monomer. Protein
3 subunits are drawn as ribbon representations (modified PDB ID codes: 5ARA and 6B2Z). Olive, α
4 subunits; red, β subunits; blue, γ subunit; fuchsia, δ subunit; turquoise, ϵ subunit; orange, ring of c
5 subunits; violet, a subunit; purple, A6L subunit; gold, f subunit; green, b subunit; pink, d subunit;
6 sky-blue, F₆ subunit; grey, OSCP subunit. e and g subunit drawn in ball and stick mode, are blue
7 and light blue, respectively. DAPIT and the 6.8 kDa proteolipid, still undefined membrane subunits,
8 are not represented.
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16 Figure 2. Detailed representation of the F₁F₀-ATPase subunits involved in catalysis. a) The OSCP
17 subunit (grey) and the β subunit (red) with the “long connecting loop” and the “crown region”
18 highlighted in olive and blue, respectively. The CaATP substrate in the catalytic binding site is
19 depicted as ball and stick model (modified PDB ID code: 5ARA). The cofactors Mg²⁺ (yellow) (b)
20 and Ca²⁺ (c) (turquoise) are depicted as inserted spheres in the β_{TP} site (modified PDB ID
21 code:2JDI). ATP molecule and the side chains of Thr¹⁶³, Glu¹⁹², Asp²⁵⁶, Arg¹⁸⁹ are drawn as ball
22 and stick models.
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31 Figure 3. Putative involvement of the Ca²⁺-activated F₁F₀-ATPase complex(es) in MPTP formation.
32 A) The dimeric form of the Mg²⁺-activated F₁F₀-ATPase super-complex is associated with a highly
33 convex membrane curvature which protrudes into the matrix; B) the dissociation of the F₁F₀-
34 ATPase dimers, produced by the mechanical signal transduction from the Ca²⁺-activated F₁ sector
35 to the F₀ sector (as detailed in the text), reduces the membrane curvature at the apex of the *cristae*.
36 By this mechanism, the channel forms between two adjacent monomers. (Modified PDB ID codes:
37 5ARA and 6B2Z).
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46 Figure 4. F₀ dimerization and IMM curvature change as related events. The membrane dimeric
47 domain is formed by the membrane-intrinsic α -helices of b subunit, the a subunit, the A6L subunit,
48 the f subunit, the e subunit and the g subunit of each monomer. According to the model, the
49 reduction of the concave IMM curvature dissociates the membrane-embedded F₀ dimer and creates
50 a pore at the monomer-monomer interface. Color subunits are the same as in Figure 1. (Modified
51 PDB ID code: 6B2Z).
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Figure 1
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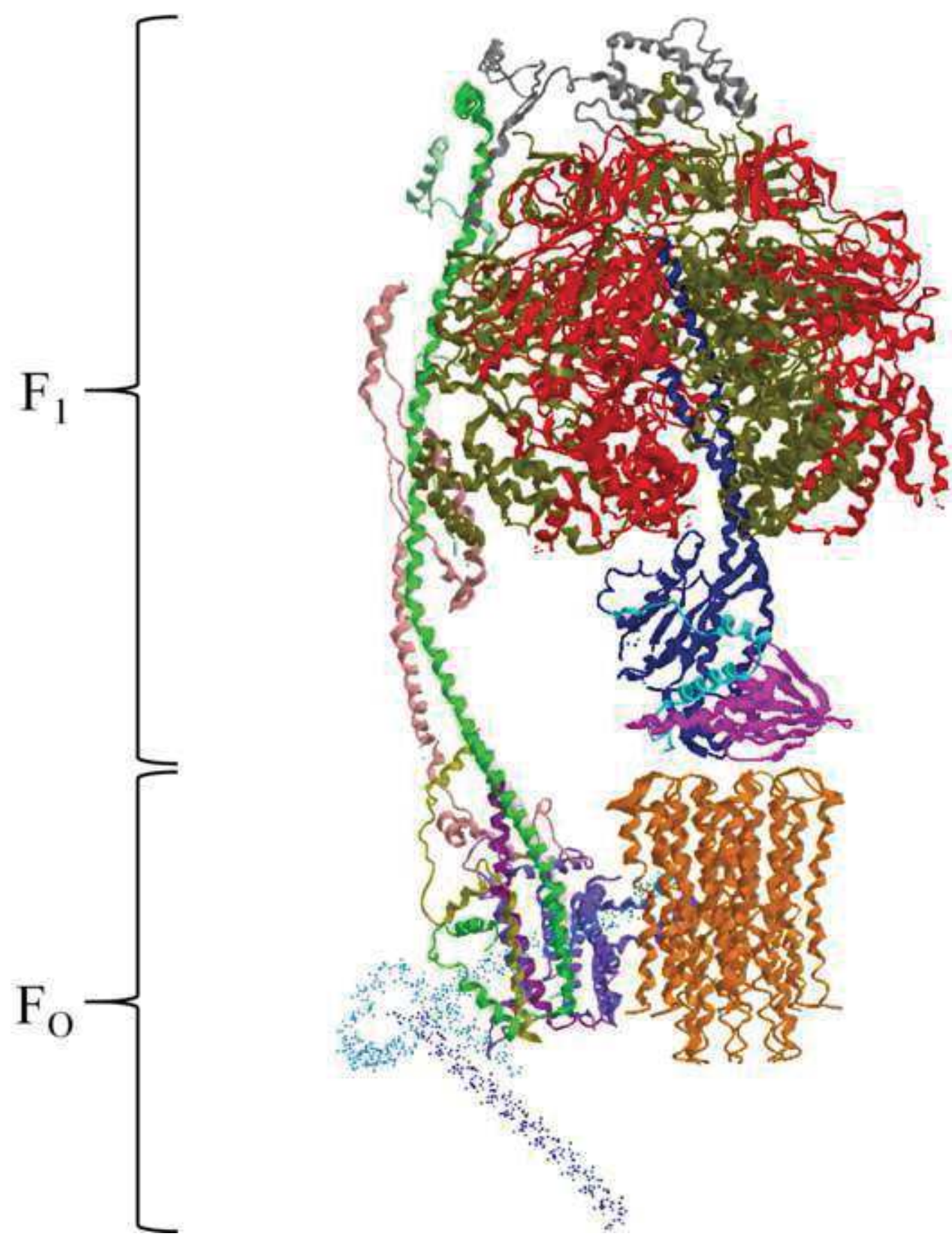


Figure 2
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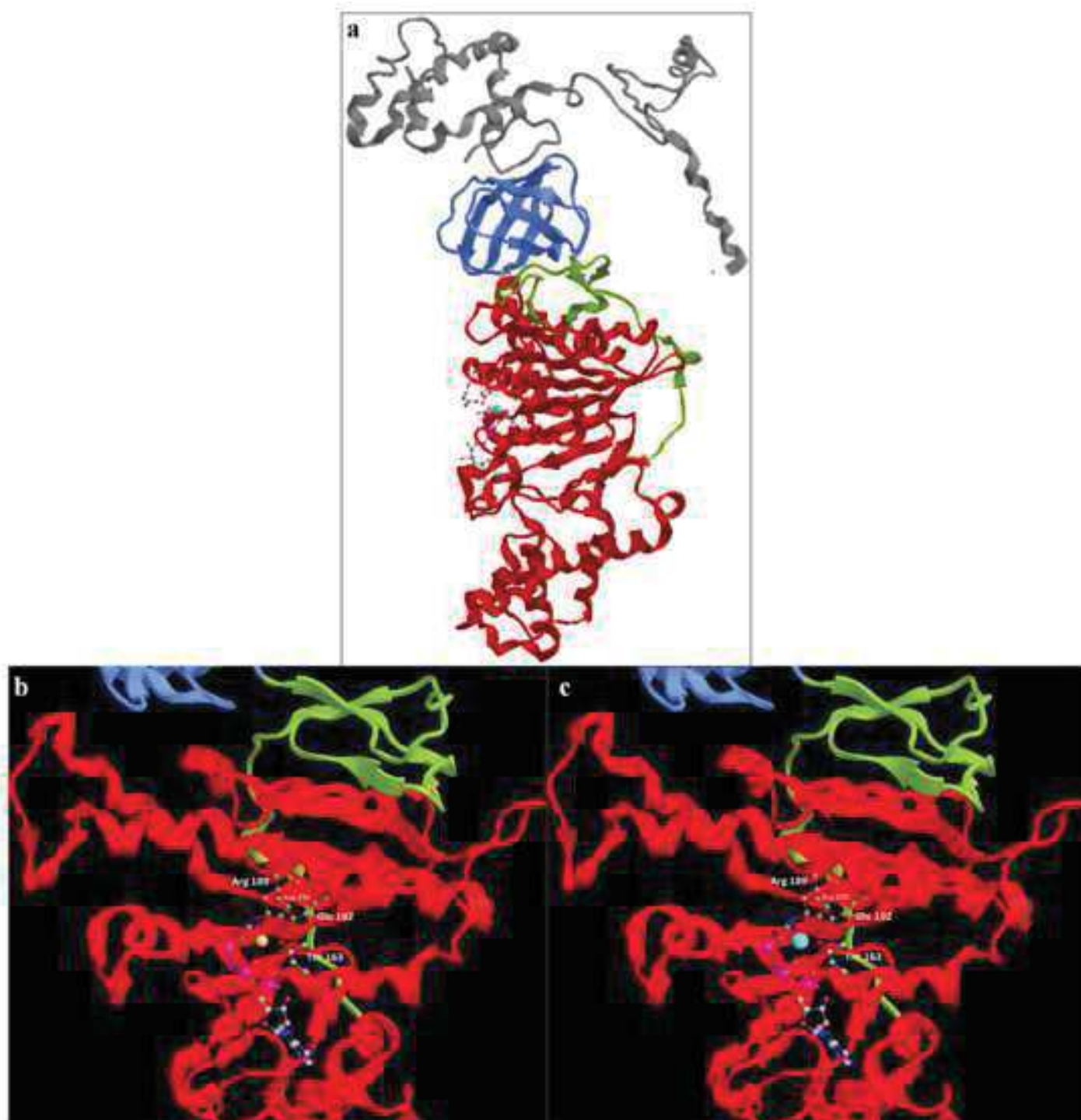


Figure 3
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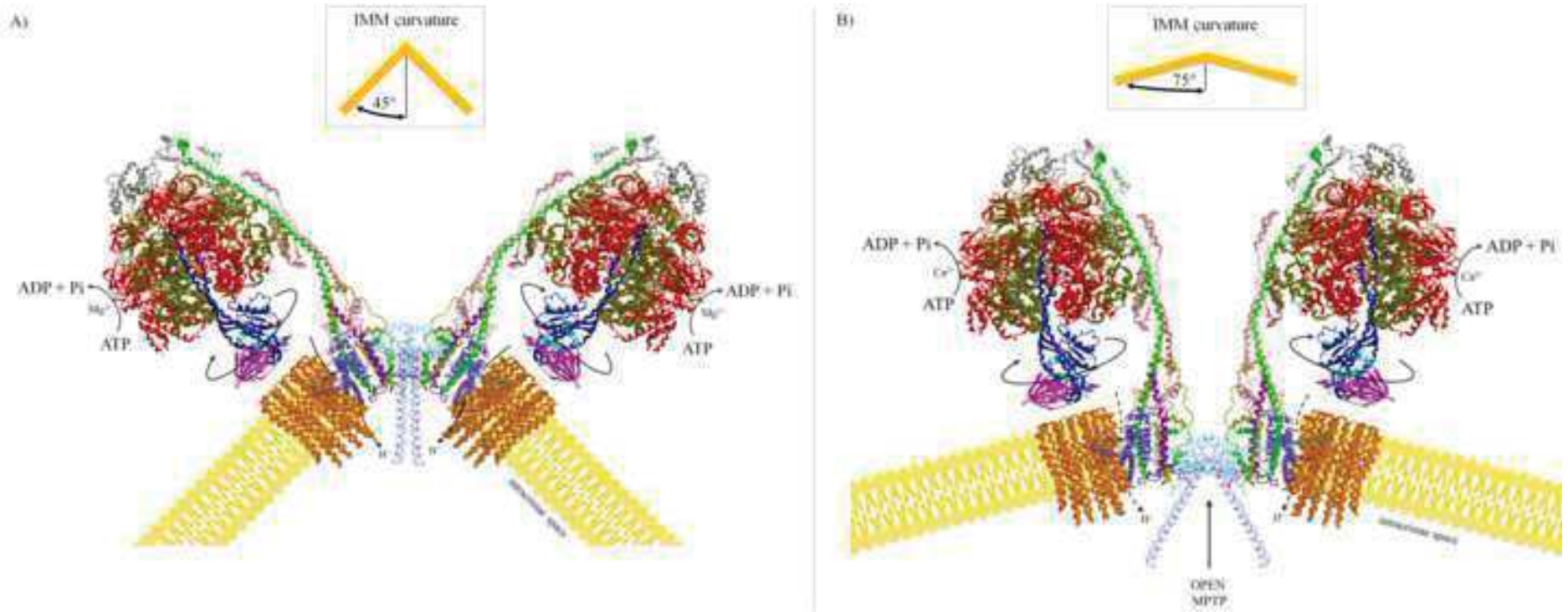


Figure 4
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