From the Ca^{2+}-activated F_1F_O-ATPase to the mitochondrial permeability transition pore: an overview

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Abstract

Based on recent advances on the Ca^{2+}-activated F_1F_O-ATPase features, a novel multistep mechanism involving the mitochondrial F_1F_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 F_1F_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 F_1F_O-ATPase dimer dissociation and MPTP opening.

Keywords: F_1F_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 $FADH_2$, 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 bongkrekic 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 F_1F_O-ATPase may mutually interact through cardiolipin which would somehow connect these proteins to form the ATP synthasome. Consistently, conformational changes triggered by Ca^{2+} 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 F_1F_O-ATPase -a splendid molecular machine- [17] has been proposed to form the pore structure [18,19].

2. The F_1F_O-ATPase: from an old to a new story as pore former

From its discovery around the middle of the 20th century, the F_1F_O-ATPase has undergone a sort of on-going evolution, stimulated by the increasing development of techniques and of knowledge, which lead to a continuous re-evaluation of the roles of this intriguing enzyme complex [17,20]. At present, we can say that new and up to now unsuspected roles for this ubiquitous enzyme are emerging in mitochondria. As widely known, in eukaryotic mitochondria the F_1F_O-ATPase constitutes the amazing molecular machine that exploits the electrochemical energy produced by the respiratory chain in the form of Mitchell's proton motive force (Δp) to produce ATP via a chemo-mechanical coupling mechanism [21]. Even if ATP synthesis represents the classical enzyme task, the catalytic mechanism is long known to work also in *reverse* to energize the IMM by ATP hydrolysis [20,22]. In practice, the direction of catalysis depends on Δp, being ATP synthesized to dissipate Δp and conversely ATP hydrolyzed to re-build Δp. The F_1F_O-ATPase structure is quite complex and can be roughly defined as an oligomer structurally composed by a hydrophilic F_1 catalytic domain and by a membrane-embedded F_O domain. These two domains are joined by a central and a peripheral stalk (Fig. 1). In turn the F_1 sector, which protrudes in the mitochondrial matrix, shows a $\alpha_3\beta_3\gamma\delta\varepsilon$ subunit composition and stoichiometry [23]. The three α subunits alternate with three β subunits to form the F_1 globular hexamer. The adenine nucleotide binding sites, namely three non-catalytic sites on α subunits and three catalytic sites on the β subunits, open at the interfaces between the α and β subunits of this spherical complex [24]. The membrane-embedded F_O sector is also formed by multiple proteins, namely the *a* subunit, the short amphipathic *b* subunit with the two transmembrane α-helices, *e*, *f*, *g*, A6L, DAPIT (diabetes-associated protein in insulin-sensitive tissue) subunits, a 6.8 kDa proteolipid and the c_n-ring, in which the subunit number is species-dependent [25,26]. The γ subunit extends from the center of the $(\alpha\beta)_3$ structure of F_1 to the F_O domain where it joins the δ and the ε subunits to form the foot of central stalk [27]. The core of F_O is formed by the *c*-ring, which is directly attached to the central stalk and constitutes the enzyme rotor, which transmits the rotational energy to F_1. Laterally, the *b*, *d*, F_6 and OSCP subunits form the peripheral stalk, which not only links the $(\alpha\beta)_3$-catalytic structure to the *a* subunit in the F_O domain, forming the integral enzyme stator, but also plays the role of resisting the torque generation of rotor [28]. Actually, the F_1F_O-ATPase/synthase is a rotary engine which matches rotation to catalysis. The clockwise rotation (seen from the intermembrane space) is driven by Δp which makes H^+ downhill translocate across the IMM through the *a* subunit/*c*-ring complex interface. This rotation transmitted from F_O to F_1 produces one ATP molecule per each β subunit, namely three ATP molecules are built in a 360° cycle. The opposite rotation, which pumps H^+, in the intermembrane space and re-constitutes Δp, is coupled to ATP hydrolysis. The nucleotide binding in the catalytic site requires the coordination of the essential cofactor Mg^{2+}, which contributes to ATP synthesis/hydrolysis and to the asymmetry of the three catalytic sites, which produces the differences in affinity for nucleotides [29]. Accordingly, each β subunit is asymmetric and during the rotation, by interacting with the γ subunit, undergoes three distinct conformational states β_E (always empty), β_{DP}, which contains bound MgADP and β_{TP} which binds MgATP [30].

Interestingly, in mitochondria, the F_1F_O-ATPases are assembled in supra-molecular dimeric complexes by the transmembrane F_O domain [31] which form extensive rows [32,33] distributed along the tightly curved ridges of the IMM *cristae* [34]. This localization exploits the higher H^+ density on the surface in the curved membrane regions [35] created by the respiratory complexes crowding at either side of the rows [36]. Structure, localization and function are tightly connected. Accordingly, the F_1F_O-ATPase structural arrangement and localization have relevant implications for the mechanism of mitochondrial energy transduction [34] and substantiate the F_1F_O-ATPase active role in membrane bending and *cristae* formation [35,37], thus contributing to mitochondrial

morphology. Indeed, the enzyme complex assembly locally produces an extreme membrane curvature in either concave (negative curvature) where the membrane invaginates or convex (positive curvature) at the edge of the *cristae*, as seen from the matrix [35].

The F_1F_O-ATPase energy-transduction mechanism of bioenergetics [22] and its modeling ability on mitochondria [38] turns into an energy-dissipating machinery when the mitochondrial Ca^{2+} concentration abruptly increases under pathological conditions [39]. In this case the F_1F_O-ATPase activated by Ca^{2+} instead of Mg^{2+} would form a channel which matches the conductance properties of the MPTP [40,41]. The pore opening leads to transient IMM depolarization and allows the diffusion of solutes, water enters and ATP is hydrolyzed by the F_1F_O-ATPase [3]. Disruption of mitochondrial homeostasis induces swelling and bursting of IMM, an event which has been linked to pathways leading to cell death [42,43]. The MPTP opening has been reported to be affected by a large variety of effectors and conditions, spanning from ion concentrations to physical changes. Accordingly, Ca^{2+}, Mg^{2+}, adenine nucleotides, P_i, H^+ and membrane potential have been claimed as F_1F_O-ATPase modulators and MPTP inducers/inhibitors [44]. On considering that the F_1F_O-ATPase is not only ruled by its own substrates/products, but also responds to post-translational modifications (PTMs) on different subunits [30], PTMs affecting the enzyme function or its super-complex organization may greatly impact MPTP modulation [44]. Furthermore, cyclosporin A (CsA) blocks the MPTP binding to CyPD, a protein which modulates the MPTP without being an essential component of its structure [45,46]. Additionally, the putative MPTP-CypD interaction involves OSCP subunit of F_1 sector [47], which connects the catalytic $(\alpha\beta)_3$ spherical complex to the peripheral stalk. However, since MPTP opening was recently reported to remain CsA-sensitive also in the absence of OSCP [48] or pH-dependent by protonation of the unique histidine in OSCP subunit [49], the whole mechanism need to be clarified.

On balance, the experimental evidence accumulated up to now points out that the MPTP-forming properties in eukaryotes [50] are apparently linked to the dual F_1F_O-ATPase function. Even if some points remain controversial and require to be elucidated, the enzyme of life that synthesizes ATP, activated by Mg^{2+}, apparently turns into the enzyme of death when hydrolyzes ATP in the presence of Ca^{2+}.

3. The hypotheses on the F_1F_O-ATPase involvement in the MPTP

From the beginning of the story, namely since cell death was associated with an abruptly increased mitochondrial permeability, it seemed quite obvious that the pore formation should involve mitochondrial membrane components whose conformational changes under certain conditions eventually made the inner membrane itself permeable to water and solutes. The most likely candidates were membrane-bound proteins, since the pore always supposed to be proteinaceous [4,44]. However, the MPTP constitution was never elucidated and it was also proposed to include a non-proteinaceous ion-conducting module [51]. Assumed that the F_1F_O-ATPase contributes to MPTP formation, as recent advances strongly hint, up to now two main mechanisms have been hypothesized: the channel forms within the *c*-ring of the F_O sector [52] or, alternatively, at the interface between the contact region of the dimer [19].

The "*c*-ring hypothesis" is sustained by intriguing hints on the channel conductance, obtained on mammalian pure *c* subunits reconstituted in lipid bilayers capable of generating spontaneous electrical oscillations activated by cGMP and inhibited by Ca^{2+} [53]. However, the current

generated through the *c* subunit pore was found to be cation-selective [54], while the MPTP is known to be induced by Ca^{2+} and non-selective for solutes with a molecular weight of up to 1,500 Da [55]. Otherwise, the dephosphorylation of a peptide related to *c* subunits promoted by Ca^{2+} and prevented by CsA emerged as MPTP inducer [56]. The formation of a voltage-sensitive channel in reconstituted *c* subunits or purified F_1F_O-ATPase in liposomes was hypothesized. The pore would be formed by CyPD and Ca^{2+}-dependent *c*-ring expansion and F_1 detachment [52]. This multi-conductance channel lacked cation selectivity, but was resistant to CsA, insensitive to Ca^{2+} and inhibited by the β subunit of F_1F_O-ATPase [52]. Experiments of overexpression or depletion of endogenous *c* subunits by specific siRNA downregulation and consequent MPTP inactivation strengthened the "*c*-ring channel" hypothesis [18]. A proper *c*-ring conformation is required for MPTP opening [57] and the highly conserved *c* subunit Gly zipper domain apparently plays a key role in the *c*-ring assembly linked to MPTP sensitivity [52,57]. Recent insights are provided by the Ca^{2+}-induced *de novo* water-permeable MPTP complex, which is inhibited by CsA, and apparently made up by *c* subunits associated with polyphosphate (polyPi) and polyhydroxybutyrate (PHB) [58]. The interaction of the *c* subunits with polyPi and PHB, by generating a charged polymer, provides an environment compatible with the hydrophobicity of these proteolipids. Indeed, the *c*-ring ion conduction gaps were offset by the electrophysiological properties of the non-proteinaceous Ca^{2+}-selective polyPi/PHB channel [51]. Otherwise, due to its structure, the *c*-ring cannot form a water-filled channel as suggested by the atomistic simulations of two *c*-rings of different lumen width [59]. The *c*-ring lumen contains lipids in bacteria [60], while in mitochondria it would be occluded by lipid molecules and forms a non-conducting channel [59]. Moreover, even in a potentially conducting state (*i.e.* hydrated state of the *c*-ring interior), molecular dynamics simulations demonstrated that the biophysical properties of such channel were not consistent with the high ionic conductance attributed to the MPTP [59]. However, atomic simulations on the *c*-ring do not take the polyPi/PHB model into account. Accordingly, due to the PHB amphipathic properties, lipids may localize in the *c*-ring hydrophobic core and even allow ion flux [58]. Even if the structural bases of the "*c*-ring channel" opening [52] necessarily require CyPD binding and inhibition by CsA only after Ca^{2+} addition, CyPD-null mice showed electrophysiological MPTP features indistinguishable from those of wild-type individuals in presence of high Ca^{2+} loads [61,62]. The possibility that the channel may be opened by the displacement of the two main F_1F_O-ATPase domains has also been considered. However, strip down F_1 from F_O sector generally occurs under drastic conditions, for instance in the presence of high urea concentrations, and leads to irreversible F_1F_O-ATPase denaturation, whereas the MPTP reversibly shifts between open/closed states. Moreover, the displacement of the two F_1F_O-ATPase sectors is not a likely mechanism to create a channel within F_O, because the γ, δ and ε subunits of F_1 cannot return to their native position within the hydrophobic sector. Additionally, it seems difficult to think that free β subunits can inhibit the MPTP formation because the catalytic subunits are linked into $(\alpha\beta)_3$ globular hexamer resistant to denaturation, yet the hexamer does not interact with the embedded membrane sector in wild-type F_1F_O-ATPase [63]. Finally, human cells in which the *c* subunit genes are disrupted preserve the typical MPTP properties [64]. These vestigial F_1F_O-ATPases in cells unable to synthesize the *c* subunits are also structurally devoid of *a* and A6L subunits and cannot translocate H^+, even if the CsA-sensitive MPTP formation is maintained [64]. On these bases it seems reasonable to conclude that in the absence of *c* subunits the MPTP could be formed and be sensitive to CypD, but it remains unclear if its conductance properties occurs through an unregulated MPTP pathway or not [65].

If the "*c*-ring hypothesis", even if intriguing, show some inconsistencies, other recent findings corroborate the involvement of the dimeric form of the F_1F_O-ATPase. Accordingly, the MPTP formation was observed after reconstitution into lipid bilayers of gel-purified F_1F_O-ATPase dimers associated with the detection of an indistinguishable channel current ascribable the MPTP electrophysiological equivalent mitochondrial mega-channel [19]. Cross-linking experiments from Bernardi's group indicate that the OSCP subunit of peripheral stalk interacts with CyPD [47] and benzodiazepine 423 (Bz-423), a MPTP inducer which overlaps the CyPD binding site [19]. Bz-423 inhibits the F_1F_O-ATPase activity similarly to the P_i-dependent CyPD which, by binding to the OSCP subunit, decreases the Mg^{2+}-dependent ATP hydrolysis in the absence of CsA [47]. Thus, CyPD modulates MPTP opening and CyPD binding to OSCP may propagate the conformational changes of the catalytic sites through the stator to enzyme membrane portions [66]. The high matrix Ca^{2+} concentration features MPTP activation. Therefore the "F_1F_O-ATPase peripheral stalk/dimer hypothesis" is fully consistent with the occupation of the catalytic site by Ca^{2+} in replacement of natural cofactor Mg^{2+} [44,66]. In the catalytic site the ${}_{\beta}Thr^{163}$ of the P-loop is directly linked to Mg^{2+}, while the ${}_{\beta}Arg^{189}$, ${}_{\beta}Glu^{192}$, ${}_{\beta}Asp^{256}$ residues are coordinated with Mg^{2+} by three water molecules respectively [67] (Fig. 2b). The metal binding pocket can be occupied by other divalent cations such as Ca^{2+} [68]. Interestingly, experiments on prokaryotes showed that the single mutation of ${}_{\beta}Thr^{159}Ser$ at the catalytic site equivalent to ${}_{\beta}Thr^{163}Ser$ in eukaryotes is the only aminoacid substitution which allows the normal F_1F_O-ATPase function if sustained by MgATP, but not by CaATP [69]. A recent paper [70] investigated the effects of the ${}_{\beta}Thr^{163}Ser$ mutation in human F_1F_O-ATPase. In comparison with wild type mitochondria, ATP hydrolysis driven by Mg^{2+} was stimulated, while the Ca^{2+}-dependent F_1F_O-ATPase activity was nearly completely inhibited. Moreover, the mutation in β subunit apparently decreased the MPTP sensitivity to Ca^{2+}, since higher Ca^{2+} levels were required to induce MPTP opening [70]. The ${}_{\beta}Thr^{163}$ is probably the only aminoacid which directly binds to the metal cation in the catalytic site. Consistently, while in presence of Mg^{2+} the mutation in the catalytic subunit apparently favors ADP release during hydrolysis, the larger Ca^{2+} would cause a spatial rearrangement that stiffens the F_1 sector and limits OSCP motility. Molecular dynamics simulations suggest that the mechanical energy of Ca^{2+} bound to β-subunits sites may be transmitted through a long connecting loop to the "crown region" of the OSCP subunit (Fig. 2a). According to this mechanism, the motion starting from the catalytic sites would be transferred through the lateral stalk to the membrane subunits where the MPTP opens [70]. Looking at the residues putatively involved in this mechanism, some concern might arise about the Ca^{2+} specific conformational changes transmission from the catalytic binding sites to OSCP, since only the N-terminus of the three α subunits interacts with OSCP at the N-terminal α-helical domain. Other interactions between the $(\alpha\beta)_3$ spherical domain and the peripheral stalk are established, connecting the α subunit N-terminal region to *b* and F_6 subunits respectively and the α subunit C-terminus to *d* subunit [38,71]. However, He et al, [48] recently provided experimental evidence that peripheral stalk subunits (*i.e.* OSCP and *b* subunit) are not involved in the MPTP formation. Consistently, the F_1 modification induced by Ca^{2+} cannot be transmitted to the membrane subunits when the stator is defective. In cells with F_1F_O-ATPase devoid of OSCP or if the peripheral stalk lacks the two transmembrane α-helices of *b* subunit, mitochondria retained a reduction of MPTP-dependent swelling rate responsive to CsA inhibition, thus suggesting that the binding site for CyPD is not provided by OSCP and in this vestigial F_1F_O-ATPase the channel size is affected [48]. Nevertheless, the inhibition of the MPTP opening by acidic pHs is sensitive to the protonation of the unique histidine in OSCP subunit (${}_{OSPC}His^{112}$ in humans) [49], thus leading Antoniel and colleagues suspect that more than one subunit of the peripheral stalk can transmit the full-conductance signal for MPTP opening. In addition, the overexpression of *e* subunit, which is

known to promote the F_1F_O-ATPase dimerization [72,73], limits the MPTP induction by Ca^{2+}. Briefly, even if there is still much work to be done to clarify the MPTP opening mechanism and some still unexplained contradictions emerge from literature data, experimental evidence gathered up to now indicates that MPTP opens when the F_1F_O-ATPase dimers dissociate and the *c*-ring maintains an adequate conformation [57].

4. A new conformational transmission model for MPTP opening

On considering the hypotheses so far proposed, some questions are still open. The MPTP, the F_1F_O-ATPase and Ca^{2+} depict an enigmatic triangle [74], in which Ca^{2+} apparently plays the leading role. From the available literature data and of some recent findings in our lab, we become increasingly convinced that Ca^{2+} by interacting with the F_1F_O-ATPase triggers subsequent conformational events which ultimately lead to form a pore in the inner mitochondrial membrane. In our opinion, there are many clues that lead to build an intriguing and quite realistic model. Accordingly, when the mitochondrial Ca^{2+} concentration increases, it replaces the natural cofactor Mg^{2+} in the catalytic site of the F_1F_O-ATPase [70]. As recently pointed out [75], the catalytic mechanism of ATP hydrolysis and H^+ translocation by the Ca^{2+} and Mg^{2+}-dependent F_1F_O complexes are apparently similar, in contrast with previous reports [76–78]. Interestingly, small molecules or cofactors (*e.g.* nitrite or NAD^+) were found to act differently on the enzyme when it is activated by Ca^{2+} or by Mg^{2+}, namely they inhibit the Ca^{2+}-dependent F_1F_O-ATPase without affecting the Mg^{2+}-dependent F_1F_O-ATPase. Therefore, the catalysis modulation may represent a molecular mechanism which is somehow involved in MPTP regulation [79,80]. As far as we are aware, the Ca^{2+}-activated F_1F_O-ATPase cannot synthesize ATP, but it is capable of ATP hydrolysis, probably by adapting the catalytic mechanism, which is compatible with the greater steric hindrance of Ca^{2+} with respect to Mg^{2+} when inserted in the β subunits [75]. So, in the presence of the larger Ca^{2+} radius, the coordination geometry of the cofactor-binding site of the enzyme would change from six-fold octahedral up to allow eight ligands, resulting into a less rigid geometry with irregular distances and angles [81]. The Mg^{2+} and Ca^{2+} stimulated enzyme activities, thus suggesting that the two cations similarly interact with the protein and the nucleotides [75,81]. Nevertheless, Ca^{2+} could bind to the same aminoacid residues as Mg^{2+} with even greater affinity (Fig. 2b,c). Moreover, ATP hydrolysis, which causes the mitochondrial ATP pool depletion associated with the MPTP [3], drives the torsional mechanism of the central stalk in the Ca^{2+}-activated F_1F_O-ATPase [82]. This torsion is coupled to H^+ pumping [75] through the IMM, even if H^+ translocation is unable to re-energize the IMM [44,75]. As a result, when the F_1F_O-complex works in the *reverse* mode (ATPase) driven by Ca^{2+} there is ATP dissipation without membrane polarization. So, it seems reasonable to think that the Ca^{2+}-activated F_1F_O-ATPase can start a multistep process resulting into MPTP formation and opening [83]. According to this model, the spatial rearrangement within F_1 would arise from Ca^{2+} binding to the catalytic sites, and, in the form of conformational change signal, would be transmitted to reach the hydrophobic F_O sector, where it would promote the dissociation of F_1F_O-ATPase dimers into monomers and determine the loss of the local curvature of *cristae*, thus making the MPTP open (Fig. 3). These events, namely the F_1F_O-ATPase activation by Ca^{2+} and the deformation of *cristae*, are both associated with mitochondrial dysfunction and cell death due to MPTP opening. The F_1F_O-ATPase dimerization in mammalian mitochondria could arise from the *a* and *e* subunits, while in yeast mitochondria also *i/j* and *k* subunits would participate in maintaining the dimer joined. These supernumerary membrane subunits in yeasts could be functional orthologs of the 6.8 proteolipid and DAPIT subunits in mammalian mitochondrial F_1F_O-ATPase, respectively. Interestingly, two

adjacent *a* subunits in mammalian form a "dimerization motif" in which the *a* subunit of each monomer has a strand planar structure that connects the two monomers. The *cristae* are bent by *e*, *g*, and the N-terminal portion of *b* subunits forming an unusual transmembrane domain [31] which, when the F_1F_O-ATPase dimerizes, induces the positive curvature of the membrane which produces the morphology of the *cristae*. Indeed, F_1F_O-ATPase monomers are *per se* sufficient to produce curvature in lipid bilayers (a 43° inclination is imposed by the membrane stator domain) and the detachment of dimers prevents the formation of the edge of the *cristae,* resulting in a zig-zag topology of the membrane [84]. Conversely, the positioning of dimers along rows in a ridge is a self-association of side-by-side union of multiple dimeric F_1F_O-ATPase super-complexes which does not require additional protein-protein interactions [35].

The stiffness of the peripheral stalk holds the $(\alpha\beta)_3$ globular hexamer in a stationary position with respect to the IMM, even if the stator has the adequate flexibility to allow the rotary catalytic cycle [85]. The dimer interface is formed at the basis of the peripheral stalk. Thus, any unusual flexibility induced in this structure can compromise the stability of the dimeric F_1F_O-ATPase supra-molecular assembly. However, MPTP persists in the absence of the peripheral stalk subunit [48]. Interestingly, vestigial F_1F_O-ATPase complexes, which lack the *c*-ring, the *a* and the A6L subunit or either the *b* subunit or the OSCP subunit, retain the MPTP features. All these vestigial enzymes were found abundantly associated with two forms of the intrinsic inhibitor protein (IF_1) [48,64]: a mature inactive IF_1 form (IF_1-M1 but not the IF_1-M2 isoform) and the import precursor IF_1, IF_1-P (a mature IF_1 form with import sequence). The same proteins were also associated with the monomeric F_1F_O-ATPase derived from ρ^0 cells [86]. It is still unknown whether IF_1-M1 and IF_1-P differ in the entry pathway to mitochondria or in ATP hydrolysis inhibition, but probably the specific association of IF_1-P with the F_1F_O-ATPase could prevent dimerization [86]. Moreover, the active IF_1 form (an antiparallel α-helical coiled coil dimeric structure) in the matrix can associate with five F_1 subunits in different conformations [87]. The active IF_1 is not essential for dimer formation [88,89], even if it can promote the F_1F_O-ATPase dimeric structure super-complexes [90]. The dimeric IF_1 role is the ATPase inhibition by binding with a ratchet-like mechanism to the α/β_{DP} site [91] proximal to the peripheral stalk [38]. However, some clues suggest that IF_1 may also play a role in MPTP opening [92]. The active form of IF_1 occurs at pH values below 6.5 [93], when the mitochondrial ATP hydrolysis to re-energize the IMM causes a disastrous ATP drop and cellular acidification. Interestingly, when pH lowers to 6.5, the protonation of histidine residue(s) near the region of dimer combination shifts the IF_1 equilibrium from tetramers (inactive IF_1 form) to dimers (active IF_1 form) so as to interact with F_1 sector [93]. The reversible histidine(s) protonation on the mitochondrial matrix side [94] as well as the $_{OSPC}His^{112}$ protonation [49] are known to inhibit the MPTP. As a matter of fact, when the matrix pH decreases below 7.0, the MPTP is in the closed conformation. However, dithyl pyrocarbonate (DPC) allows MPTP opening at pH 6.5 and maintains the Ca^{2+}-dependent channel sensitivity to CsA. Accordingly, DPC reacts with histidyl residues and prevents their reversible protonation [94]. Therefore, at acidic pHs IF_1 is in the active form and the MPTP is closed. Conversely, at pH values ≥ 7.0, the IF_1 dimers aggregate into a tetramer which occludes the inhibitory portion of F_1 sector [95]. At alkaline pHs, to avoid detachment from the F_1F_O-ATPase under non-inhibitory conditions, the C-terminus of IF_1 is kept anchored to F_O [96,97], while the N-terminal region of IF_1 is cross-linked to the *α* subunit [98]. In the presence of an incomplete structure of the peripheral stalk, the Ca^{2+}-induced conformational change within F_1 could be transmitted to the membrane subunits by the inactive form of IF_1 [99]. Accordingly, IF_1 by anchoring to the *α* subunit [98] and to a still unidentified membrane receptor of known molecular mass (approximately M_r 5400-6400 Da) [96] corresponding to one of the membrane subunits (A6L,

e, *f*, *g*, DAPIT subunits and 6.8 kDa proteolipid) [100], could create a low density bridge-like structure between the two monomers [32]. This connecting structure may be responsible for the conformational transmission and drive the conformational change from the Ca^{2+}-bound catalytic subunits to the membrane subunits [99]. This transmission would draw up the dimer stalks and cause the reduction or even the loss of the membrane convexity between the two matched F_O sectors [101]. Moreover, the MPTP inhibition at low osmotic strength is linked to changes in the IMM curvature [102]. Consistently, the pore formation between the two F_1F_O-ATPase monomers undoes the supra-molecular dimeric structure [57]. According to this model, the torque generation driven by ATP hydrolysis pushes the rotors of two adjacent F_1F_O-complexes towards opposite directions, allowing the respective monomer axis to contribute to reduce the distance between the two F_1 sectors from 15 to 10 nm [103]. The consequent curvature inversion of the *cristae*, associated with the spatial re-arrangement of the membrane subunits responsible for the "*bridge shape domain*", results in MPTP opening [104] (Fig. 4). Noteworthy, the rotation of the Ca^{2+}-activated F_1F_O-ATPase [82], driven by ATP hydrolysis, can only occur in the presence of a correct structure of the *c*-ring or even in the absence of the *c*-ring. Accordingly, a proper *c*-ring or vestigial F-ATPases unable to build the *c*-ring preserve the MPTP properties [57,64]. Once transmitted through conformational changes, such rotation may be indirectly responsible for the stalk-to-stalk distance modification involved in MPTP opening. The supra-molecular arrangement of the F_1F_O-ATPase complexes is consistent with the ultrastructure of the *cristae* and with the ideal mitochondrial bioenergetics. When the F_1F_O-ATPase dimeric and oligomeric forms were destabilized by mutated *e* subunits, the F_1F_O-ATPase activity was maintained, even if unexpectedly accompanied by IMM $\Delta\varphi$ reduction [105]. On the other hand the *e* subunit overexpression, which supports the dimeric structure, was found to limit MPTP formation and prevent IMM depolarization [57].

To sum up, the experimental evidence accumulated up to now strongly suggests that MPTP formation and opening result from a multi-step process in which conformational changes play a key role. The data accumulated up to now are well compatible with a conformational mechanism triggered by the Ca^{2+}-dependent F_1F_O ATP hydrolysis, which implies a spatial rearrangement within F_1. According to the depicted model, this conformational change would constitute a structural signal that, once transduced to the membrane subunits, promotes the monomerization of F_1F_O-ATPase super-complexes and the curvature inversion at the apex of the *cristae*. So, the features of the Ca^{2+}-activated F_1F_O-ATPase are fully consistent with the $\Delta\varphi$ loss and ATP hydrolysis, known as mitochondrial events which open the MPTP [3,83].

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6. References

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Captions to Figures

Figure 1. Subunit composition and structural arrangement of the F_1F_O-ATPase monomer. Protein subunits are drawn as ribbon representations (modified PDB ID codes: 5ARA and 6B2Z). Olive, *α* subunits; red, *β* subunits; blue, *γ* subunit; fuchsia, *δ* subunit; turquoise, *ε* subunit; orange, ring of *c* subunits; violet, *a* subunit; purple, A6L subunit; gold, *f* subunit; green, *b* subunit; pink, *d* subunit; sky-blue, F_6 subunit; grey, OSCP subunit. *e* and *g* subunit drawn in ball and stick mode, are blue and light blue, respectively. DAPIT and the 6.8 kDa proteolipid, still undefined membrane subunits, are not represented.

Figure 2. Detailed representation of the F_1F_O-ATPase subunits involved in catalysis. a) The OSCP subunit (grey) and the *β* subunit (red) with the "long connecting loop" and the "crown region" highlighted in olive and blue, respectively. The CaATP substrate in the catalytic binding site is depicted as ball and stick model (modified PDB ID code: 5ARA). The cofactors Mg^{2+} (yellow) (b) and Ca^{2+} (c) (turquoise) are depicted as inserted spheres in the β_{TP} site (modified PDB ID code:2JDI). ATP molecule and the side chains of Thr^{163}, Glu^{192}, Asp^{256}, Arg^{189} are drawn as ball and stick models.

Figure 3. Putative involvement of the Ca^{2+}-activated F_1F_O-ATPase complex(es) in MPTP formation. A) The dimeric form of the Mg^{2+}-activated F_1F_O-ATPase super-complex is associated with a highly convex membrane curvature which protrudes into the matrix; B) the dissociation of the F_1F_O-ATPase dimers, produced by the mechanical signal transduction from the Ca^{2+}-activated F_1 sector to the F_O sector (as detailed in the text), reduces the membrane curvature at the apex of the *cristae*. By this mechanism, the channel forms between two adjacent monomers. (Modified PDB ID codes: 5ARA and 6B2Z).

Figure 4. F_O dimerization and IMM curvature change as related events. The membrane dimeric domain is formed by the membrane-intrinsic α-helices of *b* subunit, the *a* subunit, the A6L subunit, the *f* subunit, the *e* subunit and the *g* subunit of each monomer. According to the model, the reduction of the concave IMM curvature dissociates the membrane-embedded F_O dimer and creates a pore at the monomer-monomer interface. Color subunits are the same as in Figure 1. (Modified PDB ID code: 6B2Z).

Figure 1
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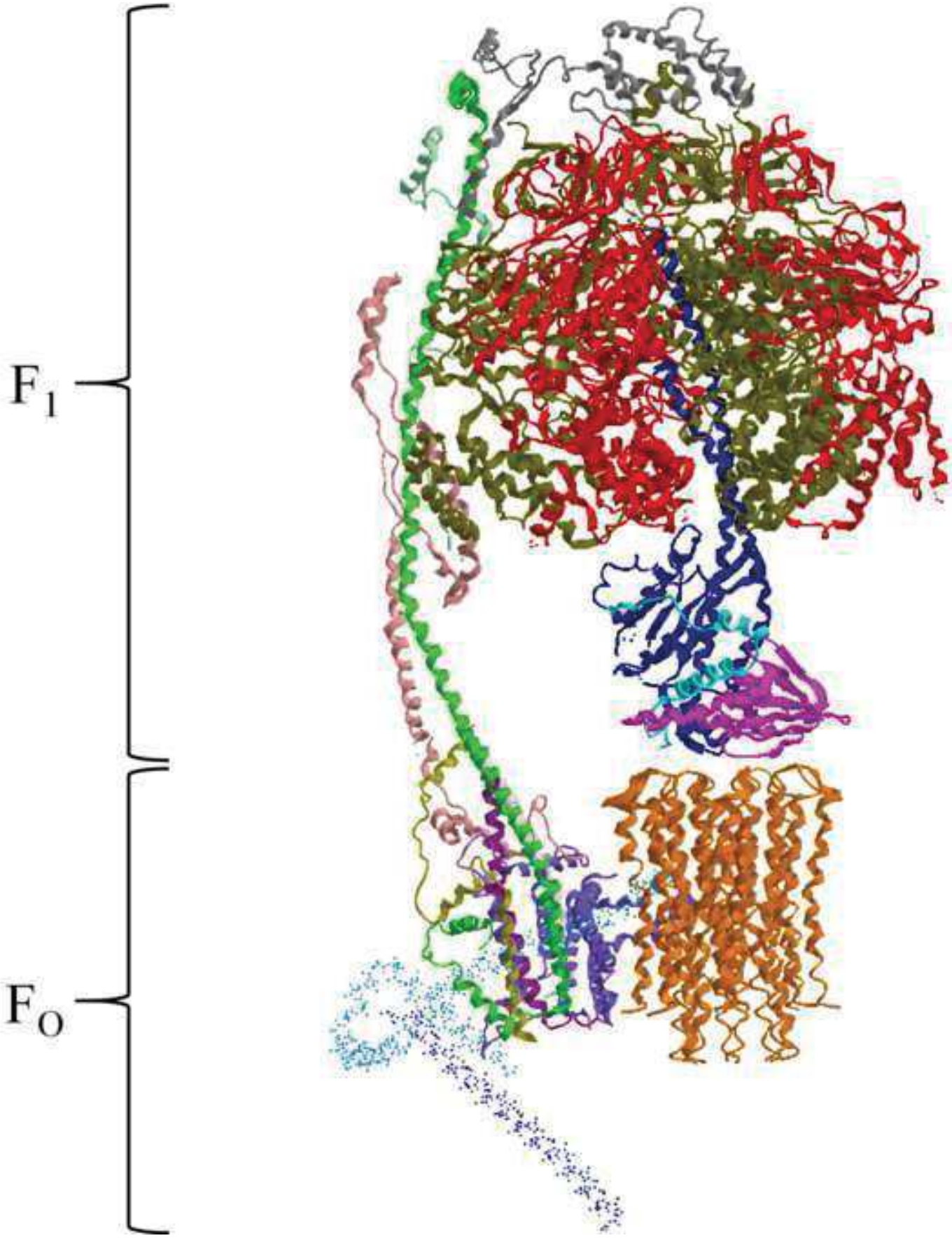

Figure 2
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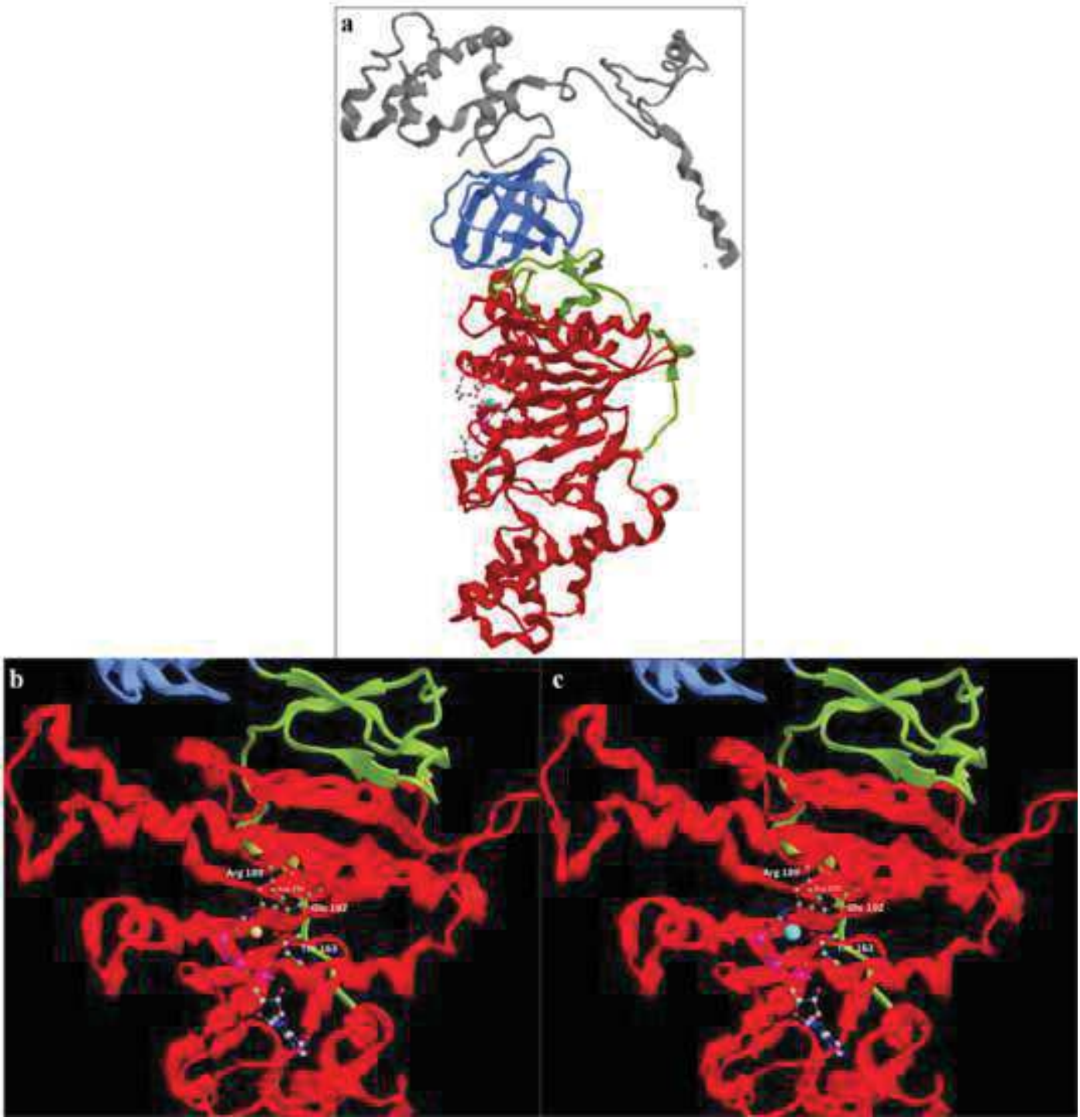

Figure 3

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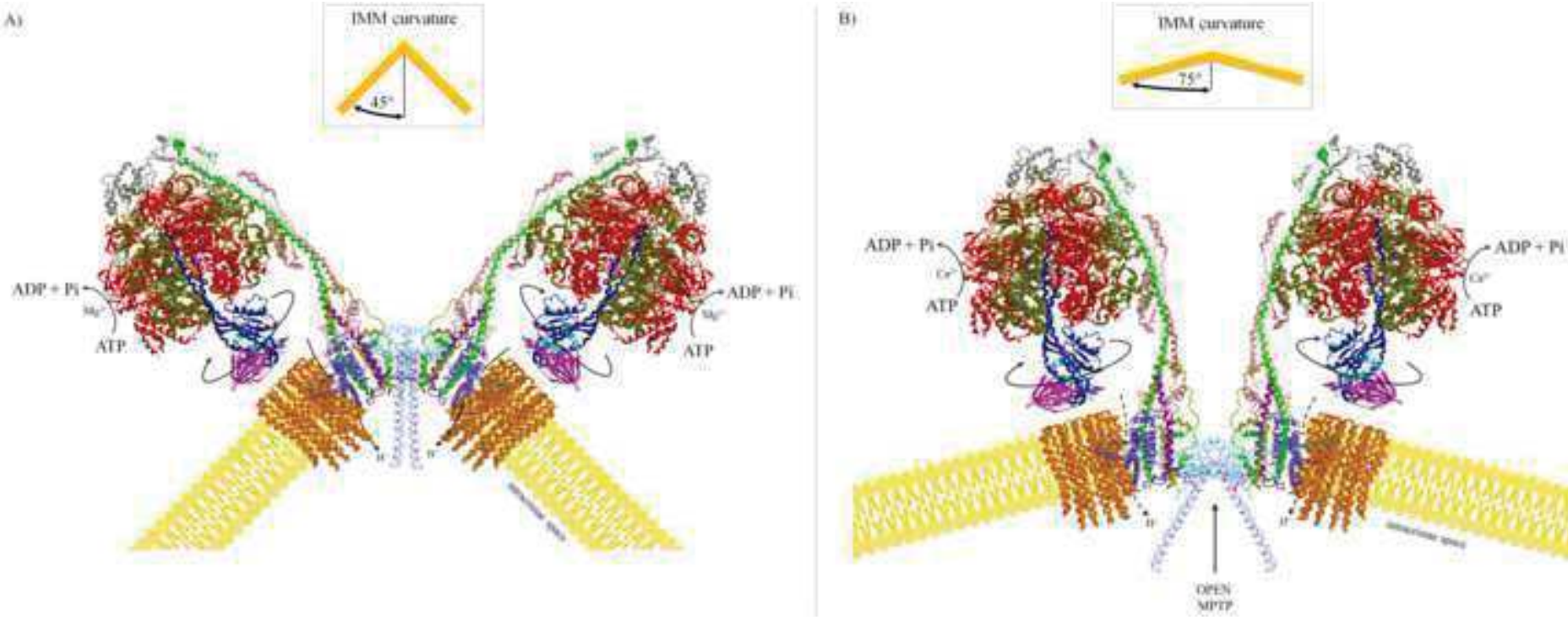

Figure 4

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