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Ca²⁺ as cofactor of the mitochondrial H⁺-translocating F₁F₀-ATP(hydrol)ase

Salvatore Nesci, Alessandra Pagliarani

Department of Veterinary Medical Sciences, University of Bologna, Ozzano Emilia Via Tolara di
Sopra 50, 40064 Bologna, Italy.

Corresponding author: salvatore.nesci@unibo.it

Running title: H⁺-translocation driven by ATP hydrolysis by the Ca²⁺-activated F₁F₀-ATPase

Abstract

The mitochondrial F₁F₀-ATPase in the presence of the natural cofactor Mg²⁺ acts as the enzyme of life by synthesizing ATP, but it can also hydrolyze ATP to pump H⁺. Interestingly, Mg²⁺ can be replaced by Ca²⁺, but only to sustain ATP hydrolysis and not ATP synthesis. When Ca²⁺ inserts in F₁, the torque generation built by the chemomechanical coupling between F₁ and the rotating central stalk was reported as unable to drive the transmembrane H⁺ flux within F₀. However, the failed H⁺ translocation is not consistent with the oligomycin-sensitivity of the Ca²⁺-dependent F₁F₀-ATP(hydrol)ase. New enzyme roles in mitochondrial energy transduction are suggested by recent advances. Accordingly, the structural F₁F₀-ATPase distortion driven by ATP hydrolysis sustained by Ca²⁺ is consistent with the permeability transition pore signal propagation pathway. The Ca²⁺-activated F₁F₀-ATPase, by forming the pore, may contribute to dissipate the transmembrane H⁺ gradient created by the same enzyme complex.

Keywords: Ca²⁺ cofactor; F₁F₀-ATPase; mitochondria; H⁺ pump; oligomycin; permeability transition pore; bioenergetics.

1. Introduction

The mitochondrial F_1F_0 -ATPase is a multisubunit complex arranged in dimers or oligomers and placed at the edge of the *cristae* of the inner mitochondrial membrane (IMM) ¹. The monomer is formed by two domains, named F_1 and F_0 functionally and structurally linked to a stator (lateral stalk) and a rotor (central stalk). The F_1 portion, namely the hydrophilic domain that protrudes in the mitochondrial matrix, has a conspicuous lollipop shape formed by α_3 , β_3 , γ , δ , and ϵ subunits. An alternated arrangement of α and β subunits forms a globular hexamer around the γ subunit (Fig. 1A). The structure functions as a reversible rotary molecular motor which can build or hydrolyze ATP depending on the rotation direction, which in turn is driven by the transmembrane proton-motive force (Δp). *In vitro* the γ subunit of F_1 -ATPase was shown to rotate within the surrounding $\alpha_3\beta_3$ subunits, synthesizing or hydrolysing ATP in three separate catalytic sites on the α/β subunit interface. The catalytic sites are alternated with the non-catalytic sites, which can only bind adenine nucleotides ². During the kinetic reactions, the three non-equivalent conformation β_E (empty), β_{DP} (which hosts ADP) and β_{TP} (contains ATP or ADP) of the catalytic sites, with increasing affinity for ATP, change their conformation and binding properties every 120° with the rotation of the rotor ³. In addition, the F_1F_0 -ATPase catalytic and non-catalytic sites in their different conformations can also bind metal divalent cations ¹. In mammals, the membrane-embedded domain is composed by the a subunit, the transmembrane α -helices of b subunit, the c_n subunits (n = eight in mammals) which arranged as a cylindric palisade form the c -ring, A6L subunit, and the supernumerary subunits e , f , g , DAPIT (Diabetes-Associated Protein in Insulin-sensitive Tissue), 6.8 KDa proteolipid (PL) (Fig. 1A) ⁴. The H^+ translocation sector arises from a/c -ring interactions by forming two asymmetric half-channels with unexpected horizontal membrane-intrinsic α -helices in the a subunit. These two half-channels are mutually offset, while the H^+ binding sites are located on the C-terminal α -helix of each c subunit ⁵. In the mammalian F_1F_0 -ATPase the a and A6L membrane subunits are encoded by the mitochondrial DNA. The central stalk within the F_1 domain contains the γ subunit, which joined to the δ and ϵ subunits, forms a sort of foot which interacts with the loop region of c -ring. The lateral or peripheral stalk joins the two F_0 and F_1 enzyme domains (Fig. 1A). The b subunit spans the complete length of the lateral stalk and interacts with OSCP, F6 and d subunits which belong to the soluble

enzyme section. All these subunits connect the soluble stator subunits with α_{TP} subunit of F_1 domain. In addition, the top of α_{TP} , α_{DP} , α_E and the β_{DP} and β_E are only linked with OSCP. Some subunits of the lateral stalk, namely the membrane embedded portion of b , f and A6L subunits ⁶ and the supernumerary subunits, are transmembrane subunits ⁴. The lateral stalk shows a spectacular flexibility that plays the role of resisting the torque generation of the rotor by coupling F_1 catalysis to H^+ translocation ^{7,8}.

The H^+ -translocating F_1F_0 -ATPase sustains either ATP synthesis or hydrolysis ⁹. In the “forward” mode the Mitchell’s proton motive force Δp created by mitochondrial respiration drives ATP formation from ADP and P_i . In the so-called “reverse” mode, the phosphorylation potential generated by ATP breakdown is exploited by the enzyme complex to pump H^+ and energize the IMM when the Δp drops ¹⁰. Both ATP synthase and hydrolase activities are opposite F_1F_0 -ATPase functions that depend on the bioenergetic state of mitochondria. The bi-functional ATP synthesis/hydrolysis mode coupled to H^+ translocation of F_1F_0 -ATPase is a mechanism unique in biology sustained by the natural cofactor Mg^{2+} . The F_1F_0 -ATPase can replace Mg^{2+} by Ca^{2+} losing the ATP synthesis function, but preserving the F_1F_0 -ATP(hydrol)ase activity ¹¹. High Ca^{2+} concentrations in mitochondria activate of F_1F_0 -ATPase by direct Ca^{2+} binding to the $\alpha_3\beta_3$ globular hexamer that dissociates ATP hydrolysis from H^+ pumping. In this case F_1 was reported to become uncoupled from F_0 domain ¹². However, this assumption becomes questionable on considering the recent cryo-EM enzyme structure and structure activity relationship data on the effect of small molecules ¹³ and specific F_1 and F_0 domain inhibitors ^{11,14}. So, in search for a different interpretation of the findings up to now obtained, experimental and literature data ¹⁵ were combined to draw a pattern of the mechanism involved.

2. Materials and Methods

2.1. Preparation of the mitochondrial fractions and F_1F_0 -ATPase activity assays

Swine hearts (*Sus scrofa domesticus*) were collected at a local abattoir and transported to the lab. From homogenized heart tissue and then subjected to differential centrifugation ¹¹ the mitochondrial preparations, obtained in a divalent cation-free medium, were characterized as described in ¹⁴. To

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3 evaluate the mitochondrial F_1F_0 -ATPase activities, the mitochondrial preparations obtained as
4 described by Nesci et al.¹¹, were added to the reaction system that contained 3 mM ATP and 2 mM
5 Ca^{2+} or Mg^{2+} in 75 mM ethanolamine-HCl buffer, pH 8.8. The enzyme activity was
6 spectrophotometrically detected and evaluated after subtraction of the non-specific ATP hydrolysis
7 in the blank¹⁶. The sensitivity of the F_1F_0 -ATPase activity, either sustained by Mg^{2+} or by Ca^{2+} , to the
8 specific F_1F_0 -ATPase inhibitor oligomycin witnessed the functional and structural coupling between
9 the two sectors F_1 and F_0 ¹⁴.
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21 2.2. Protein model

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23 The structural details of the protein arrangement in the F_1F_0 -ATPase subunits were obtained by the
24 Chem3D program of ChemOffice Professional 19.1.1 software¹⁷ using the deposited structures in
25 PDB.
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33 2.3. Calculations and statistics

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35 In each set of experiments, the data represent the mean \pm SD of the number of analyses carried out
36 on at least three distinct mitochondrial preparations. The coupling index was calculated as the ratio
37 between the total F_1F_0 -ATPase activity and the oligomycin-sensitive F_1F_0 -ATPase activity, being the
38 latter obtained from the difference between the total F_1F_0 -ATPase activity and the enzyme activity
39 detected in the presence of 3mg/mL oligomycin, a dose which ensured maximal inhibition of the
40 F_1F_0 -ATPase¹⁸. The differences between the enzyme activity data in differently treated mitochondria
41 were evaluated by one way ANOVA followed by Student-Newman-Keuls' test when F values
42 indicated significance ($P \leq 0.05$). The significance of the difference between the mean coupling index
43 of the Mg^{2+} -dependent F_1F_0 -ATPase and the Ca^{2+} -dependent F_1F_0 -ATPase was evaluated by
44 Student's t test ($P \leq 0.05$). Percentage data were *arcsin*-transformed before statistical analyses to
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3 3. Results and Discussion
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6 The catalytic and non-catalytic subunits of the F_1F_O -ATPase show specific amino acid residues and
7 secondary structure motifs required for the molecular interaction with adenine nucleotides and
8 divalent cations. An eight amino acids sequence, *GXXXXGKT*, conserved in all ATPases ¹⁹ is the
9 basic structural feature of the P-loop, known as a phosphate binding loop, on α and β subunits (Fig.
10 1B). The motif interacts with Mg^{2+} and phosphate (Pi) groups of ATP by coordinating β -Pi and γ -Pi
11 to exchange the terminal γ -Pi when the ATP is synthesized or hydrolysed. The positions and specific
12 amino acid composition in the pig sequence are ₁₅₈*GGAGVGKT*₁₆₅ and ₁₆₉*GDRGTGKT*₁₇₆ in the β
13 and α subunits, respectively. *T*₁₆₅ is the only residue that plays a key role in coordinating Mg^{2+} in the
14 β subunits of enzyme during ATP hydrolysis, while *T*₁₇₆ of α subunits could bind the cofactor. Similarly
15 to Mg^{2+} , Ca^{2+} can also bind to all the catalytic sites and probably also to the non-catalytic sites ⁴. The
16 relative affinities for divalent cations and ATP in the reverse reaction of ATP hydrolysis are
17 modulated by mutagenesis of these specific residues of β subunits ²⁰. However, Ca^{2+} , which has
18 higher steric hindrance than Mg^{2+} , can change the coordination geometry of the Mg^{2+} -binding site
19 from the octahedral bipyramide which binds six ligands up to allow eight ligands when Ca^{2+} is
20 inserted in replacement of Mg^{2+} ²¹. Therefore, the rigid octahedral complex changes to a less rigid
21 geometry with irregular bond distances and angles and variable coordination number. This flexible
22 arrangement may explain the non-competitive Ca^{2+} inhibition of the Mg^{2+} -activated F_1F_O -ATPase ¹⁴.
23 The Ca^{2+} -dependent F_1F_O -ATP(hydrol)ase is capable of sustaining torque generation of the rotor.
24 The rotational motion was found to be similar to that induced by Mg^{2+} in the F_1 -ATPase ²². Ca^{2+}
25 binding could have the functional consequence to prevent the building of the transmembrane H^+
26 gradient, as shown by ACMA fluorescence quenching ²³. However, these results cannot exclude that
27 the rotation driven by ATP hydrolysis stimulated by Ca^{2+} ²² is coupled to H^+ translocation. The Mg^{2+} -
28 activated F_1F_O -ATPase can display H^+ flow across F_O in the absence of adenine nucleotides bound
29 to F_1 . This uncoupled proton leakage, known as “proton slip”, is associated with a free-wheeling of
30 the central stalk under non-physiological conditions ¹⁰. In addition, the proton slip is abolished by F_O
31 inhibitors (e.g. oligomycin), but it is insensitive to F_1 inhibitors. Conversely, the Ca^{2+} -dependent F_1F_O -
32 ATP(hydrol)ase activity was shown to inhibited by various F_1 inhibitors ¹⁴ and insensitive to other Ca-

ATPase inhibitors²⁴. Since the F_1F_0 -ATPase in the presence of Ca^{2+} shows a four orders of magnitude lower enzyme activity than the Mg^{2+} -activated F_1F_0 -ATPase (Fig. 2A), ATP hydrolysis sustained by Ca^{2+} may be unable to support a significant H^+ pumping to energize the membrane. Moreover, the Ca^{2+} -activated F_1F_0 -ATPase is now generally recognized to play an important role in the permeability transition pore (PTP) formation and opening^{25–29}, which can dissipate the Δp ¹¹. The loss of F_1F_0 -ATPase structural-functional integrity emerges as the most likely event involved in the decreased oligomycin sensitivity when the F_1 catalysis is not coupled to H^+ transport by F_0 ³⁰. However, the Ca^{2+} -dependent F_1F_0 -ATP(hydrol)ase is inhibited by oligomycin²⁴. A similar behaviour was described in pea stem mitochondria, where, since the Ca-ATPase activity was fully sensitive to oligomycin, ATP hydrolysis could be coupled to H^+ translocation³¹. In swine heart mitochondria oligomycin displays a higher inhibition efficiency on the Ca^{2+} -activated F_1F_0 -ATPase than on the Mg^{2+} -activated F_1F_0 -ATPase, as shown by the lower IC_{50} value (Fig. 2B). The coupling index (the ratio between the total F_1F_0 -ATPase activity and the oligomycin-sensitive F_1F_0 -ATPase activity) is statistically similar, namely $94.7 \pm 1.8\%$ and $91.6 \pm 3.7\%$ in presence of Mg^{2+} and Ca^{2+} , respectively. Therefore oligomycin blocks H^+ translocation coupled to ATP hydrolysis irrespective of the divalent cation (Fig. 2C). Consistently, these data suggest that mechanochemical coupling of Ca^{2+} -dependent F_1 -ATP(hydrol)ase works as a rotary chemical motor to drive H^+ translocation in the F_0 domain^{11,16}. The fact that the H^+ -pumping activity driven by Ca^{2+} may not energize IMM is not surprising, being supported by the new “bent-pull” model of the c-ring gated channel³² and by the cryo-EM maps of the enzyme exposed to Ca^{2+} ⁴. The lack of apparent H^+ translocation with Ca^{2+} -dependent F_1F_0 -ATP(hydrol)ase may be rather due to H^+ backflow through the open PTP³³. Indeed, different Ca^{2+} F_1F_0 -ATPase states during ATP hydrolysis were not identified in the Mg^{2+} -activated F_1F_0 -ATPase. Moreover recent data show that the PTP opens when the Ca^{2+} -enzyme in disassembled conformation has the peripheral stalk twisted and the F_1 detached from F_0 ⁴ (Fig. 3). In all likelihood, oligomycin inhibits ATP hydrolysis sustained by Ca^{2+} in the first conformational stages of the Ca^{2+} -dependent F_1F_0 -ATP(hydrol)ase when F_1 is still coupled to F_0 . Indeed, oligomycin, venturicidin, and DCCD, which block H^+ translocation by binding to the c-ring, can reduce the calcein quenching rate³⁴, while small-molecules obtained from the oligomycin structure target

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the *c* subunits and inhibit the PTP³⁵. Previous experiments in our lab showed that DCCD, which specifically blocks H⁺ translocation by covalently binding to the *c* subunit carboxylic groups which constitute H⁺ binding sites and inhibits ATP hydrolysis, more promptly reacts and binds to the Ca²⁺-activated F₁F₀-ATPase than the Mg²⁺-activated F₁F₀-ATPase¹¹. Moreover, in inside-out submitochondrial particles the oligomycin sensitive ATP hydrolysis was shown to be similarly affected by Δp when the enzyme activity is sustained by Ca²⁺ or by Mg²⁺¹¹. Therefore, most likely, when the activating cation is Ca²⁺, H⁺ translocation may be masked by the high ionic conductance of the open PTP.

To sum up, the H⁺-translocating Ca²⁺-dependent F₁F₀-ATP(hydrol)ase is a (mono)functional mode of the mitochondrial F-type ATPase complex. The F₁ domain which hydrolyzes ATP in the presence of Ca²⁺ drives the mechanical-power transmission which results in F₀ conductance to H⁺. Consistently, the poor H⁺-pumping activity of the Ca²⁺-dependent F₁F₀-ATP(hydrol)ase fails to energize the IMM, mainly because the same enzyme activity is a key PTP constituent, and most likely the PTP opening prevents and masks Δp formation^{33,36}.

4. Conclusion

Since long-time Ca²⁺ has been involved in the F₁F₀-ATPase modulation in heart mitochondria³⁷. Most knowledge comes from *in vitro* experiments, mainly due to difficulties in the detection of individual action mechanisms *in vivo*³⁸. The structural data which cast light on the Ca²⁺-driven conformational changes of the F₁F₀-ATPase shoulder the idea that, among the multiple Ca²⁺ actions in mitochondria, the Ca²⁺ intervention in the PTP is one of the most relevant mitochondrial roles of this multitasking cation in physiology and pathology.

Conflicts of interest

None.

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Figure 1. Representative structure of F_1F_0 -ATPase monomers in mammalian mitochondria (A). The enzyme subunits are drawn as ribbon representations obtained from modified PDB ID codes: 6TT7. Δp , Mitchell's proton motive force, IMM, inner mitochondrial membrane. The letter colors are the same as those of the subunit to which belong. B) Catalytic binding site of F_1F_0 -ATPase. The ATP substrate and Mg^{2+} cofactor (in ball and stick representation) are located in the β and α subunits, drawn as ribbon model (modified PDB ID code: 6J5J) in β_{TP} and α_{TP} conformation, respectively, which show the position of key amino acid residues that bind Mg^{2+} . The P-loop is in light blue in both subunits. The binding sites are viewed from the γ subunit (upper panel) and between the observer and the γ subunit (lower panel).

Figure 2. Effect of divalent cations on ATP hydrolysis by the mitochondrial F_1F_0 -ATPase. A) F_1F_0 -ATPase activities in the presence of Ca^{2+} or Mg^{2+} are shown as bar chart. B) Dose-response curve of oligomycin on the F_1F_0 -ATPase activated by Ca^{2+} or Mg^{2+} expressed as percentage of the enzyme activity in the absence of oligomycin. C) The oligomycin-sensitive ATPase activity (■) and the oligomycin-insensitive ATPase activity in presence of 3 $\mu g/ml$ of oligomycin (■) are expressed as percentages of the total mitochondrial ATPase activity sustained by Ca^{2+} or Mg^{2+} , respectively. Data expressed as column chart represent the mean \pm SD (vertical bars) from three experiments carried out on different mitochondrial preparations. * indicates significantly different values ($P \leq 0.05$).

Figure 3. F_1F_0 -ATPase activity raised by Mg^{2+} or Ca^{2+} as cofactors. ATP hydrolysis sustained by Mg^{2+} (i) or Ca^{2+} (ii) is coupled to H^+ translocation. The different size of the two cofactors changes the F_1F_0 -ATPase conformation. Indeed, the transition of the Ca^{2+} -dependent F_1F_0 -ATP(hydrol)ase from the assembled (ii) to the disassembled state (iii) could induce the loss of H^+ -translocation. Consequently, the PTP opens when a retracted e subunit pulls the lyso-phosphatidylserine plug out of the c-ring at the inner mitochondrial membrane side, while the F_1F_0 destabilization pulls out phosphatidylserine at the matrix side.

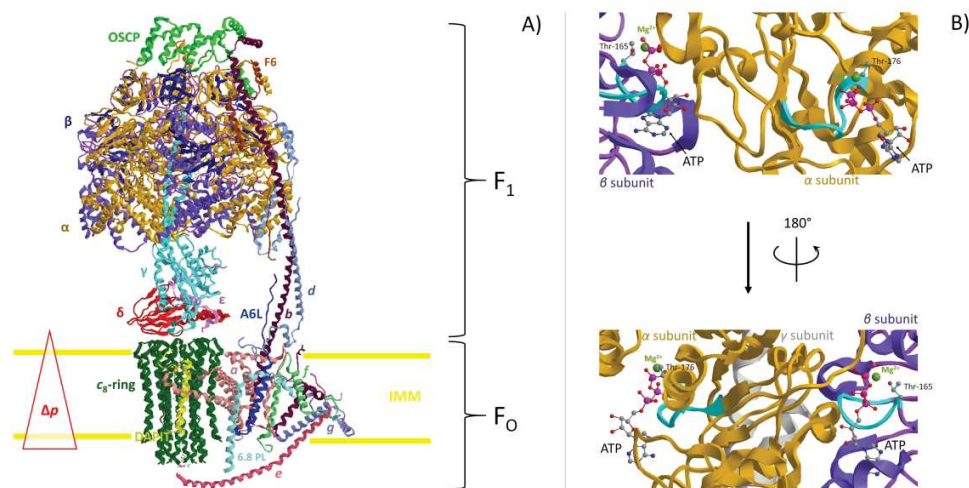


Figure 1. Representative structure of F1FO-ATPase monomers in mammalian mitochondria (A). The enzyme subunits are drawn as ribbon representations obtained from modified PDB ID codes: 6TT7. Δp , Mitchell's proton motive force, IMM, inner mitochondrial membrane. The letter colors are the same as those of the subunit to which belong. B) Catalytic binding site of F1FO-ATPase. The ATP substrate and Mg^{2+} cofactor (in ball and stick representation) are located in the β and α subunits, drawn as ribbon model (modified PDB ID code: 6J5J) in β TP and α TP conformation, respectively, which show the position of key amino acid residues that bind Mg^{2+} . The P-loop is in light blue in both subunits. The binding sites are viewed from the γ subunit (upper panel) and between the observer and the γ subunit (lower panel).

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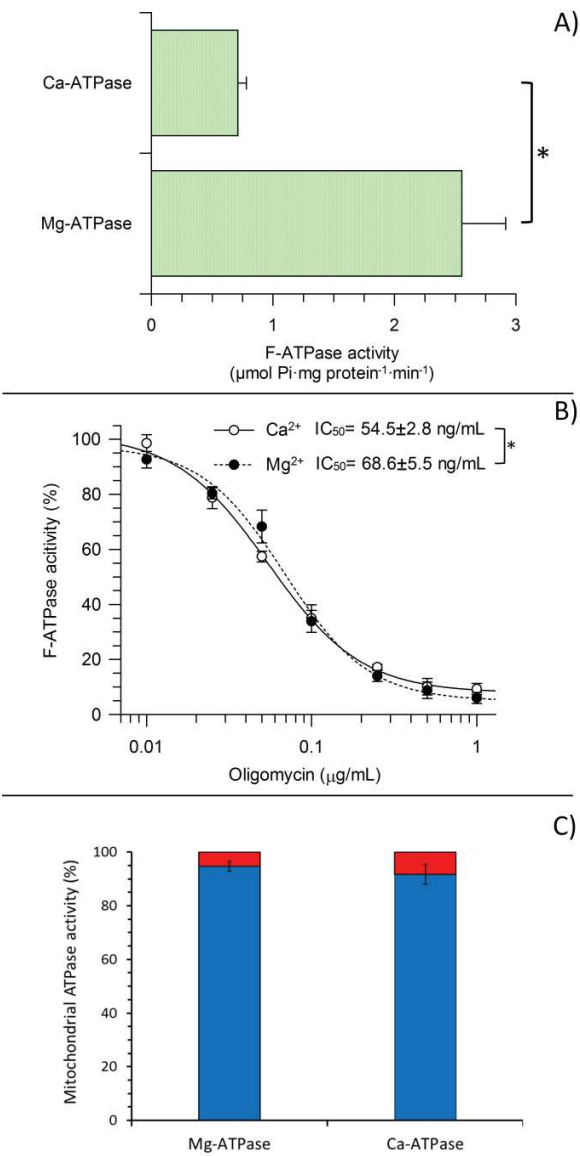


Figure 2. Effect of divalent cations on ATP hydrolysis by the mitochondrial F1FO-ATPase. A) F-ATPase activities in the presence of Ca²⁺ or Mg²⁺ are shown as bar chart. B) Dose-response curve of oligomycin on the F1FO-ATPase activated by Ca²⁺ or Mg²⁺ expressed as percentage of the enzyme activity in the absence of oligomycin. C) The oligomycin-sensitive ATPase activity (■ blue) and the oligomycin-insensitive ATPase activity in presence of 3 $\mu\text{g/ml}$ of oligomycin (■ red) are expressed as percentages of the total mitochondrial ATPase activity sustained by Ca²⁺ or Mg²⁺, respectively. Data expressed as column chart represent the mean \pm SD (vertical bars) from three experiments carried out on different mitochondrial preparations. * indicates significantly different values ($P \leq 0.05$).

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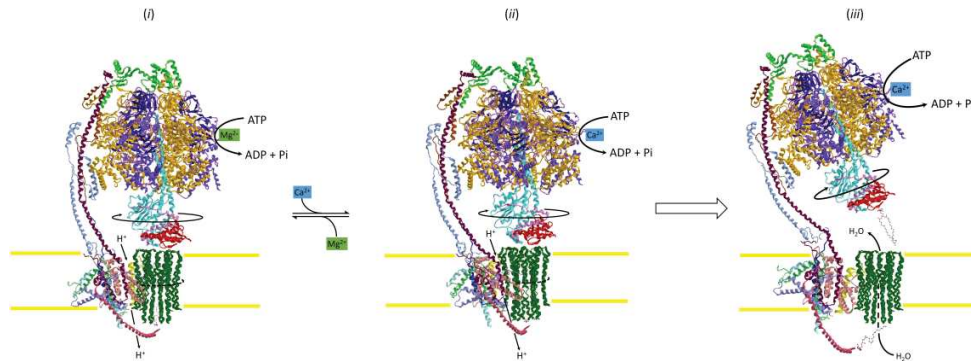


Figure 3. F1FO-ATPase activity raised by Mg²⁺ or Ca²⁺ as cofactors. ATP hydrolysis sustained by Mg²⁺ (i) or Ca²⁺ (ii) is coupled to H⁺ translocation. The different size of the two cofactors changes the F1FO-ATPase conformation. Indeed, the transition of the Ca²⁺-dependent F1FO-ATP(hydrol)ase from the assembled (ii) to the disassembled state (iii) could induce the loss of H⁺-translocation. Consequently, the PTP opens when a retracted e subunit pulls the lyso-phosphatidylserine plug out of the c-ring at the inner mitochondrial membrane side, while the F1FO destabilization pulls out phosphatidylserine at the matrix side.

381x143mm (600 x 600 DPI)