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# $Ca^{2+}$ as cofactor of the mitochondrial $H^+$-translocating $F_1F_O$-ATP(hydrol)ase

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Running title: $H^+$-translocation driven by ATP hydrolysis by the $Ca^{2+}$-activated $F_1F_O$-ATPase

## Abstract

The mitochondrial $F_1F_O$-ATPase in the presence of the natural cofactor $Mg^{2+}$ acts as the enzyme of life by synthesizing ATP, but it can also hydrolyze ATP to pump $H^+$. Interestingly, $Mg^{2+}$ can be replaced by $Ca^{2+}$, but only to sustain ATP hydrolysis and not ATP synthesis. When $Ca^{2+}$ inserts in $F_1$, the torque generation built by the chemomechanical coupling between $F_1$ and the rotating central stalk was reported as unable to drive the transmembrane $H^+$ flux within $F_O$. However, the failed $H^+$ translocation is not consistent with the oligomycin-sensitivity of the $Ca^{2+}$-dependent $F_1F_O$-ATP(hydrol)ase. New enzyme roles in mitochondrial energy transduction are suggested by recent advances. Accordingly, the structural $F_1F_O$-ATPase distortion driven by ATP hydrolysis sustained by $Ca^{2+}$ is consistent with the permeability transition pore signal propagation pathway. The $Ca^{2+}$-activated $F_1F_O$-ATPase, by forming the pore, may contribute to dissipate the transmembrane $H^+$ gradient created by the same enzyme complex.

Keywords: $Ca^{2+}$ cofactor; $F_1F_O$-ATPase; mitochondria; $H^+$ pump; oligomycin; permeability transition pore; bioenergetics.

## 1. Introduction

The mitochondrial $F_1F_O$-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 $F_O$ 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 $\alpha_3$, $\beta_3$, $\gamma$, $\delta$, and $\varepsilon$ subunits. An alternated arrangement of $\alpha$ and $\beta$ subunits forms a globular hexamer around the $\gamma$ 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 ($\Delta p$). *In vitro* the $\gamma$ 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 $\alpha/\beta$ 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 $\beta_E$ (empty), $\beta_{DP}$ (which hosts ADP) and $\beta_{TP}$ (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 $F_1F_O$-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 $F_1F_O$-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 $F_O$ 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

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enzyme section. All these subunits connect the soluble stator subunits with $\alpha_{TP}$ subunit of $F_1$ domain. In addition, the top of $\alpha_{TP}$, $\alpha_{DP}$, $\alpha_E$ and the $\beta_{DP}$ and $\beta_E$ 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 $F_1F_O$-ATPase sustains either ATP synthesis or hydrolysis [9]. In the "forward" mode the Mitchell's proton motive force $\Delta p$ created by mitochondrial respiration drives ATP formation from ADP and Pi. 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 $\Delta p$ drops [10]. Both ATP synthase and hydrolase activities are opposite $F_1F_O$-ATPase functions that depend on the bioenergetic state of mitochondria. The bi-functional ATP synthesis/hydrolysis mode coupled to $H^+$ translocation of $F_1F_O$-ATPase is a mechanism unique in biology sustained by the natural cofactor $Mg^{2+}$. The $F_1F_O$-ATPase can replace $Mg^{2+}$ by $Ca^{2+}$ losing the ATP synthesis function, but preserving the $F_1F_O$-ATP(hydrol)ase activity [11]. High $Ca^{2+}$ concentrations in mitochondria activate of $F_1F_O$-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 $F_O$ 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 $F_O$ 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 $F_1F_O$-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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evaluate the mitochondrial $F_1F_O$-ATPase activities, the mitochondrial preparations obtained as described by Nesci et al. [11], were added to the reaction system that contained 3 mM ATP and 2 mM $Ca^{2+}$ or $Mg^{2+}$ in 75 mM ethanolammine–HCl buffer, pH 8.8. The enzyme activity was spectrophotometrically detected and evaluated after subtraction of the non-specific ATP hydrolysis in the blank[16]. The sensitivity of the $F_1F_O$-ATPase activity, either sustained by $Mg^{2+}$ or by $Ca^{2+}$, to the specific $F_1F_O$ -ATPase inhibitor oligomycin witnessed the functional and structural coupling between the two sectors $F_1$ and $F_O$ [14].

### 2.2. Protein model

The structural details of the protein arrangement in the $F_1F_O$-ATPase subunits were obtained by the Chem3D program of ChemOffice Professional 19.1.1 software [17] using the deposited structures in PDB.

### 2.3. Calculations and statistics

In each set of experiments, the data represent the mean ± SD of the number of analyses carried out on at least three distinct mitochondrial preparations. The coupling index was calculated as the ratio between the total $F_1F_O$-ATPase activity and the oligomycin-sensitive $F_1F_O$-ATPase activity, being the latter obtained from the difference between the total $F_1F_O$-ATPase activity and the enzyme activity detected in the presence of 3mg/mL oligomycin, a dose which ensured maximal inhibition of the $F_1F_O$-ATPase [18].The differences between the enzyme activity data in differently treated mitochondria were evaluated by one way ANOVA followed by Student-Newman-Keuls' test when *F* values indicated significance ($P \leq 0.05$). The significance of the difference between the mean coupling index of the $Mg^{2+}$-dependent $F_1F_O$-ATPase and the $Ca^{2+}$-dependent $F_1F_O$-ATPase was evaluated by Student's *t* test ($P \leq 0.05$). Percentage data were *arcsin*-transformed before statistical analyses to ensure normality.

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## 3. Results and Discussion

The catalytic and non-catalytic subunits of the $F_1F_O$-ATPase show specific amino acid residues and secondary structure motifs required for the molecular interaction with adenine nucleotides and divalent cations. An eight amino acids sequence, *GXXXXGKT*, conserved in all ATPases [19] is the basic structural feature of the P-loop, known as a phosphate binding loop, on *α* and *β* subunits (Fig. 1B). The motif interacts with $Mg^{2+}$ and phosphate (Pi) groups of ATP by coordinating β-Pi and γ-Pi to exchange the terminal γ-Pi when the ATP is synthesized or hydrolysed. The positions and specific amino acid composition in the pig sequence are $_{158}$*GGAGVGKT*$_{165}$ and $_{169}$*GDRGTGKT*$_{176}$ in the *β* and *α* subunits, respectively. $T_{165}$ is the only residue that plays a key role in coordinating $Mg^{2+}$ in the *β* subunits of enzyme during ATP hydrolysis, while $T_{176}$ of *α* subunits could bind the cofactor. Similarly to $Mg^{2+}$, $Ca^{2+}$ can also bind to all the catalytic sites and probably also to the non-catalytic sites [4]. The relative affinities for divalent cations and ATP in the reverse reaction of ATP hydrolysis are modulated by mutagenesis of these specific residues of *β* subunits [20]. However, $Ca^{2+}$, which has higher steric hindrance than $Mg^{2+}$, can change the coordination geometry of the $Mg^{2+}$-binding site from the octahedral bipyramide which binds six ligands up to allow eight ligands when $Ca^{2+}$ is inserted in replacement of $Mg^{2+}$ [21]. Therefore, the rigid octahedral complex changes to a less rigid geometry with irregular bond distances and angles and variable coordination number. This flexible arrangement may explain the non-competitive $Ca^{2+}$ inhibition of the $Mg^{2+}$-activated $F_1F_O$-ATPase [14]. The $Ca^{2+}$-dependent $F_1F_O$-ATP(hydrol)ase is capable of sustaining torque generation of the rotor. The rotational motion was found to be similar to that induced by $Mg^{2+}$ in the $F_1$-ATPase [22]. $Ca^{2+}$ binding could have the functional consequence to prevent the building of the transmembrane $H^+$ gradient, as shown by ACMA fluorescence quenching [23]. However, these results cannot exclude that the rotation driven by ATP hydrolysis stimulated by $Ca^{2+}$ [22] is coupled to $H^+$ translocation. The $Mg^{2+}$-activated $F_1F_O$-ATPase can display $H^+$ flow across $F_O$ in the absence of adenine nucleotides bound to $F_1$. This uncoupled proton leakage, known as “proton slip”, is associated with a free-wheeling of the central stalk under non-physiological conditions [10]. In addition, the proton slip is abolished by $F_O$ inhibitors (*e.g.* oligomycin), but it is insensitive to $F_1$ inhibitors. Conversely, the $Ca^{2+}$-dependent $F_1F_O$-ATP(hydrol)ase activity was shown to inhibited by various $F_1$ inhibitors [14] and insensitive to other Ca-

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ATPase inhibitors [24]. Since the $F_1F_O$-ATPase in the presence of $Ca^{2+}$shows a four orders of magnitude lower enzyme activity than the $Mg^{2+}$-activated $F_1F_O$-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 $F_1F_O$-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 $\Delta p$ [11]. The loss of $F_1F_O$-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 $F_O$ [30]. However, the $Ca^{2+}$-dependent $F_1F_O$-ATP(hydrol)ase is inhibited by oligomycin [24]. 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 $H^+$ translocation [31]. In swine heart mitochondria oligomycin displays a higher inhibition efficiency on the $Ca^{2+}$-activated $F_1F_O$-ATPase than on the $Mg^{2+}$-activated $F_1F_O$-ATPase, as shown by the lower $IC_{50}$ value (Fig. 2B). The coupling index (the ratio between the total $F_1F_O$-ATPase activity and the oligomycin-sensitive $F_1F_O$-ATPase activity) is statistically similar, namely 94.7±1.8% and 91.6±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 $F_O$ 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 channel [32] and by the cryo-EM maps of the enzyme exposed to $Ca^{2+}$ [4]. The lack of apparent $H^+$ translocation with $Ca^{2+}$-dependent $F_1F_O$-ATP(hydrol)ase may be rather due to $H^+$ backflow through the open PTP [33]. Indeed, different $Ca^{2+}$ $F_1F_O$-ATPase states during ATP hydrolysis were not identified in the $Mg^{2+}$-activated $F_1F_O$-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 $F_O$ [4] (Fig. 3). In all likelihood, oligomycin inhibits ATP hydrolysis sustained by $Ca^{2+}$ in the first conformational stages of the $Ca^{2+}$-dependent $F_1F_O$-ATP(hydrol)ase when $F_1$ is still coupled to $F_O$. Indeed, oligomycin, venturicidin, and DCCD, which block $H^+$ translocation by binding to the *c*-ring, can reduce the calcein quenching rate [34], while small-molecules obtained from the oligomycin structure target

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the *c* subunits and inhibit the PTP [35]. 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 $Ca^{2+}$-activated $F_1F_O$-ATPase than the $Mg^{2+}$-activated $F_1F_O$-ATPase [11]. Moreover, in inside-out submitochondrial particles the oligomycin sensitive ATP hydrolysis was shown to be similarly affected by Δ*p* when the enzyme activity is sustained by $Ca^{2+}$ or by $Mg^{2+}$ [11]. Therefore, most likely, when the activating cation is $Ca^{2+}$, $H^+$ translocation may be masked by the high ionic conductance of the open PTP.

To sum up, the $H^+$-translocating $Ca^{2+}$-dependent $F_1F_O$-ATP(hydrol)ase is a (mono)functional mode of the mitochondrial F-type ATPase complex. The $F_1$ domain which hydrolyzes ATP in the presence of $Ca^{2+}$ drives the mechanical-power transmission which results in $F_O$ conductance to $H^+$. Consistently, the poor $H^+$-pumping activity of the $Ca^{2+}$-dependent $F_1F_O$-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 Δ*p* formation [33,36].

## 4. Conclusion

Since long-time $Ca^{2+}$ has been involved in the $F_1F_O$-ATPase modulation in heart mitochondria [37]. 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 $Ca^{2+}$-driven conformational changes of the $F_1F_O$-ATPase shoulder the idea that, among the multiple $Ca^{2+}$ actions in mitochondria, the $Ca^{2+}$ 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 $F_1F_O$-ATPase monomers in mammalian mitochondria (A). The enzyme subunits are drawn as ribbon representations obtained from modified PDB ID codes: 6TT7. $\Delta p$, 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 $F_1F_O$-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 $\beta_{TP}$ and $\alpha_{TP}$ 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 $F_1F_O$-ATPase. A) F-ATPase activities in the presence of $Ca^{2+}$ or $Mg^{2+}$ are shown as bar chart. B) Dose-response curve of oligomycin on the $F_1F_O$-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 µg/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 ± SD (vertical bars) from three experiments carried out on different mitochondrial preparations. * indicates significantly different values ($P \leq 0.05$).

Figure 3. $F_1F_O$-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 $F_1F_O$-ATPase conformation. Indeed, the transition of the $Ca^{2+}$-dependent $F_1F_O$-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-phophatidylserine plug out of the *c*-ring at the inner mitochondrial membrane side, while the $F_1F_O$ destabilization pulls out phospatidylserine at the matrix side.

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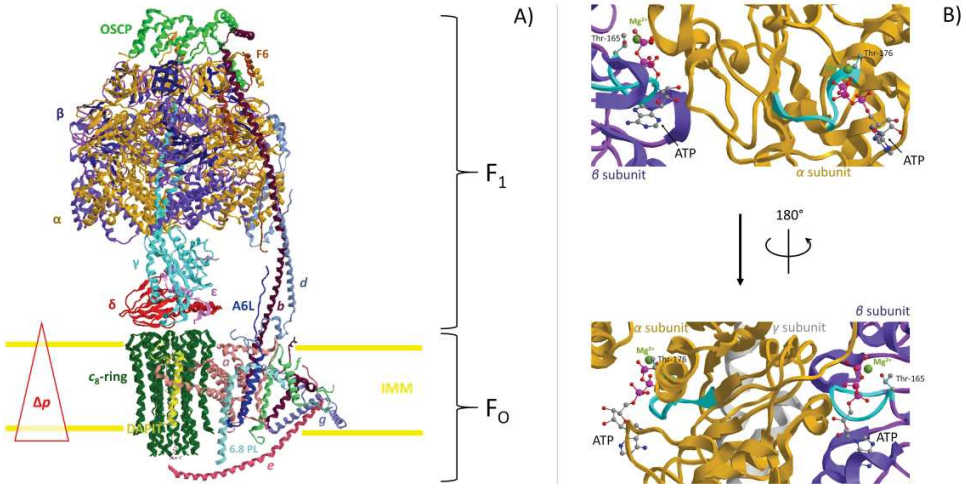


Figure 1. Representative structure of F1FO-ATPase monomers in mammalian mitochondria (A). The enzyme subunits are drawn as ribbon representations obtained from modified PDB ID codes: 6TT7. Δp, Mitchell's proton motive force, IMM, inner mitochondrial membrane. The letter colors are the same as those of the subunit to which belong. B) Catalytic binding site of F1FO-ATPase. The ATP substrate and Mg2+ cofactor (in ball and stick representation) are located in the β and α subunits, drawn as ribbon model (modified PDB ID code: 6J5J) in βTP and αTP conformation, respectively, which show the position of key amino acid residues that bind Mg2+. 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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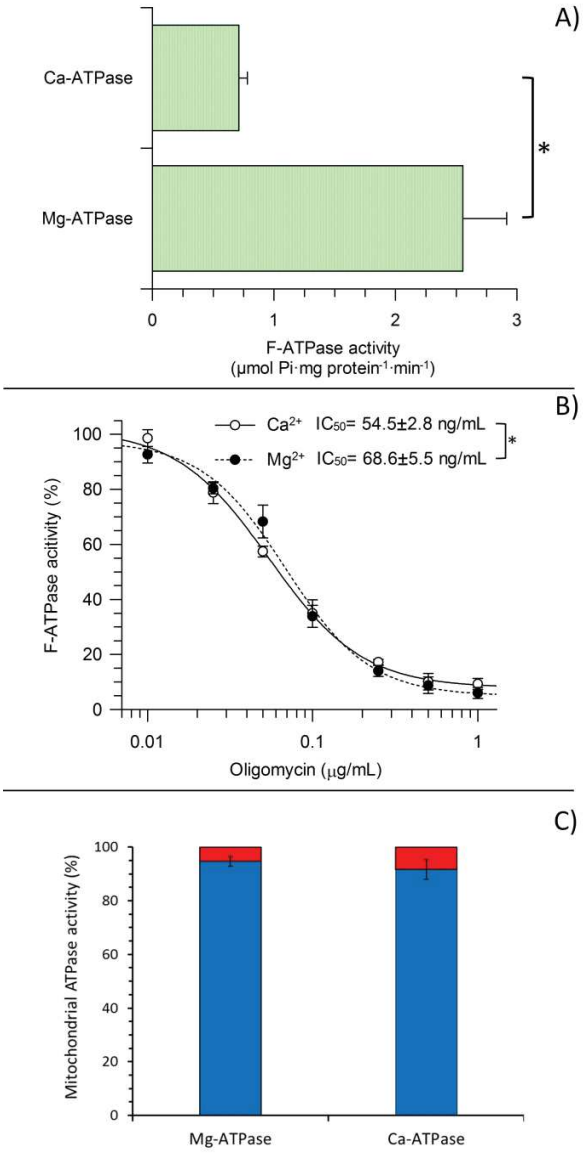


Figure 2. Effect of divalent cations on ATP hydrolysis by the mitochondrial F1FO-ATPase. A) F-ATPase activities in the presence of Ca2+ or Mg2+ are shown as bar chart. B) Dose-response curve of oligomycin on the F1FO-ATPase activated by Ca2+ or Mg2+ expressed as percentage of the enzyme activity in the absence of oligomycin. C) The oligomycin-sensitive ATPase activity (▉ blue) and the oligomycin-insensitive ATPase activity in presence of 3 µg/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 ± SD (vertical bars) from three experiments carried out on different mitochondrial preparations. * indicates significantly different values (P≤0.05).

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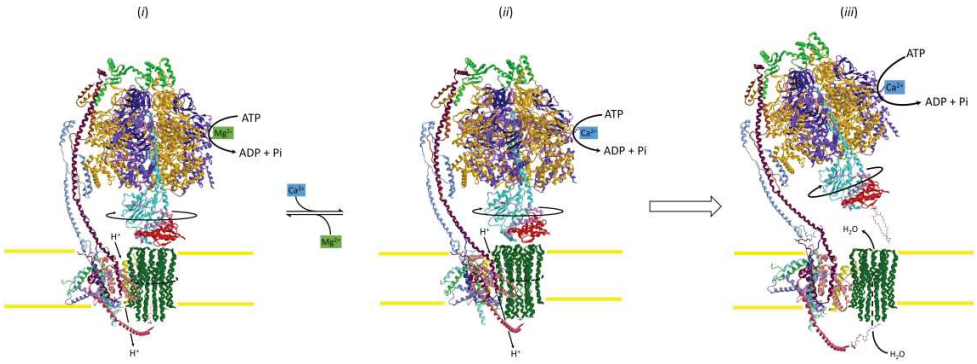


Figure 3. F1FO-ATPase activity raised by Mg2+ or Ca2+ as cofactors. ATP hydrolysis sustained by Mg2+ (i) or Ca2+ (ii) is coupled to H+ translocation. The different size of the two cofactors changes the F1FO-ATPase conformation. Indeed, the transition of the Ca2+-dependent F1FO-ATP(hydrol)ase from the 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-phophatidylserine plug out of the c-ring at the inner mitochondrial membrane side, while the F1FO destabilization pulls out phospatidylserine at the matrix side.

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