Mitochondria not only play a fundamental role in heart physiology but are also key effectors of dysfunction and death. This dual role assumes a new meaning after recent advances on the nature and regulation of the permeability transition pore, an inner membrane channel whose opening requires matrix Ca\(^{2+}\) and is modulated by many effectors including reactive oxygen species, matrix cyclophilin D, Pi (inorganic phosphate), and matrix pH. The recent demonstration that the F-ATP synthase can reversibly undergo a Ca\(^{2+}\)-dependent transition to form a channel that mediates the permeability transition opens new perspectives to the field. These findings demand a reassessment of the modifications of F-ATP synthase that take place in the heart under pathological conditions and of their potential role in determining the transition of F-ATP synthase from and energy-conserving into an energy-dissipating device. (Circ Res. 2015;116:1850-1862. DOI: 10.1161/CIRCRESAHA.115.306557.)

Key Words: Ca(2+)-Mg(2+)-ATPase ■ mitochondria ■ permeability transition pore

Besides their central role in ATP synthesis and oxidative metabolism of nutrients, mitochondria contribute to intracellular Ca\(^{2+}\) homeostasis and generation of reactive oxygen species (ROS) and are determinant for both cell survival and cell death. Important for any cell type, these functions are crucial for cardiac myocytes. Mitochondrial processes are highly compartmentalized because of the existence of 2 limiting membranes and to the selective localization of proteins, nucleotides, and coenzymes in the intermembrane and matrix spaces.

The outer mitochondrial membrane (OMM) is the interface between mitochondria and other cellular components and represents a physical barrier preventing the release of mitochondrial proteins involved in apoptosis, such as cytochrome c. OMM proteins are involved in apoptosis,\(^1\) intracellular signaling, tethering to the endoplasmic reticulum/sarcoplasmic reticulum,\(^2\) autophagy,\(^3,4\) and mitophagy.\(^5\) OMM permeability to metabolites and small peptides (<5 kDa) is regulated by the voltage-dependent anion channel and other transporters including the translocator protein of 18 kDa.\(^6\)

The inner mitochondrial membrane (IMM), whose permeability to solutes is controlled by highly specific transporters and tightly regulated channels, is the site of coupling between substrate oxidation and ATP synthesis in the process of oxidative phosphorylation. Mitochondria operate a sequence of energy conversion processes through which the exergonic flow of electrons along the respiratory complexes supports the endergonic pumping of protons from the matrix to the intermembrane space. The resulting proton motive force (\(\Delta p\)) drives the rotation of the Fo sector of ATP synthase leading to the synthesis of ATP in the F, sector. The 2 components of \(\Delta p\), namely \(\Delta \psi_m\) and \(\Delta pH\), are also used for the uptake of ADP and inorganic phosphate (Pi), respectively, and for the release of ATP into the cytosol in exchange for ADP. In addition, \(\Delta p\)
is also used by mitochondria for their biogenesis and for the maintenance of ion homeostasis, which is crucial for Ca\textsuperscript{2+}.\textsuperscript{7,8} In particular, the mitochondrial Ca\textsuperscript{2+} uniporter\textsuperscript{9,10} catalyzes Ca\textsuperscript{2+} uptake down the Ca\textsuperscript{2+} electrochemical gradient, which is largely driven by the inside-negative ΔΨ m, whereas release from the matrix to the intermembrane space, which is catalyzed by the mitochondrial Na\textsuperscript+/Ca\textsuperscript{2+} exchanger, ultimately depends on ΔpH. Indeed, the exchange is functionally coupled with the mitochondrial Na\textsuperscript+/H\textsuperscript{+} exchanger, so that mitochondrial Ca\textsuperscript{2+} release is paralleled by H\textsuperscript{+} reuptake.\textsuperscript{11}

A rise in intramitochondrial [Ca\textsuperscript{2+}] from a resting value of ≤0.1 to ≤1 μmol/L activates key enzymes of oxidative metabolism, such as pyruvate, isocitric, and oxoglutaric dehydrogenases.\textsuperscript{12} Therefore, the increase in ATP demand dictated by cytosolic Ca\textsuperscript{2+}-activated processes, especially contractile activity, is matched by increased ATP synthesis.\textsuperscript{13} Higher values of matrix [Ca\textsuperscript{2+}] could reflect the ability of mitochondria to buffer undesired elevation of cytosolic [Ca\textsuperscript{2+}]. but may also promote cell injury after opening of the permeability transition pore (PTP), an IMM channel, as discussed in detail in the following sections. More controversial is the contribution of mitochondrial Ca\textsuperscript{2+} homeostasis to cytosolic Ca\textsuperscript{2+} oscillations, especially contractile activity.27 A limited contribution of mitochondrial Ca\textsuperscript{2+} to cytosolic Ca\textsuperscript{2+} under physiological conditions, whereas they might shape cellular [Ca\textsuperscript{2+}] dynamics on prolonged elevations of cytosolic [Ca\textsuperscript{2+}].\textsuperscript{27} A limited contribution of mitochondrial Ca\textsuperscript{2+} homeostasis to cardiac function seems to be supported by the lack of a specific cardiac phenotype in mitochondrial Ca\textsuperscript{2+} uniporter knockout mice.\textsuperscript{28} This experimental model also seems to rule out an involvement of mitochondrial Ca\textsuperscript{2+} overload in ischemia/reperfusion (I/R) injury.

Mitochondria Are Double-Edged Swords

Coupling between the proton gradient and the F-ATP synthase, which is essential for life, can become a life-threatening process under conditions of cell injury, especially when oxygen availability is curtailed. In the absence of oxygen electron flow stops, the mitochondrial membrane potential collapses and mitochondrial ATP synthesis can no longer occur. If cytosolic ATP is available, it is taken up by mitochondria and hydrolyzed by F-ATP synthase, with regeneration of the proton gradient if the IMM permeability is low.\textsuperscript{29} If the IMM permeability increases after opening of the PTP, ATP hydrolysis is maximal and ATP depletion precipitates rigor contracture. This also explains the increased rate of glycolysis, the massive accumulation of lactate, and the drop in intracellular pH determined by anoxia or any defect of the respiratory chain. These concepts support the inhibition of ATP hydrolysis as an effective strategy for myocardial protection against I/R injury that is elicited by both self-defense mechanisms and pharmacological treatments.\textsuperscript{30} Indeed, the rigor contracture was prevented by exposure to the F-ATP synthase inhibitor oligomycin, emphasizing the power of F-ATP synthase as an ATP consumer.\textsuperscript{31,32}

Decreased flow of electrons along the respiratory chain promotes the partial reduction of oxygen, increasing ROS production. Complete reduction of dioxygen to H\textsubscript{2}O requires 4 electrons, a process occurring sequentially because oxygen accepts 1 electron at a time. Consequently, oxygen reduction inevitably implies the formation of partially reduced intermediates. The large majority of oxygen delivered to mitochondria is fully reduced to water at the level of complex IV. In this terminal step of the mitochondrial respiratory chain, oxygen is reduced sequentially, yet reaction intermediates remain bound to complex IV so that only the final product, that is, water, is released. However, electrons flowing through the respiratory chain can be donated to oxygen at other sites where reduction is

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>CsA</td>
<td>cyclosporin A</td>
</tr>
<tr>
<td>CyP</td>
<td>cyclophilin</td>
</tr>
<tr>
<td>IF</td>
<td>inhibitor factor 1</td>
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<tr>
<td>IMM</td>
<td>inner mitochondrial membrane</td>
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<tr>
<td>I/R</td>
<td>ischemia/reperfusion</td>
</tr>
<tr>
<td>OMM</td>
<td>outer mitochondrial membrane</td>
</tr>
<tr>
<td>OSCP</td>
<td>oligomycin sensitivity conferring protein</td>
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<tr>
<td>PTMs</td>
<td>post-translational modifications</td>
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<tr>
<td>PTP</td>
<td>permeability transition pore</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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not complete resulting in ROS formation.\textsuperscript{33} Significant amounts of ROS are also produced within mitochondria at sites other than the IMM such as monoamine oxidase in the OMM and p66\textsuperscript{Shc} in the intermembrane space.\textsuperscript{34} In brain mitochondria the high rate of H\textsubscript{2}O\textsubscript{2} formation from the respiratory chain elicited by the complex III inhibitor antimycin A, is still much lower than that originating from activity of monoamine oxidase.\textsuperscript{35,36}

ROS generated within mitochondria may serve a function in signaling processes that are crucial for the optimal response to physiological and pathological stimuli.\textsuperscript{37-39} Most of the effects elicited by ROS can be explained by post-translational modifications (PTMs) of proteins, especially at the level of Cys residues\textsuperscript{40} that might affect also protein (de)phosphorylation by modulating protein kinases\textsuperscript{41} and phosphatases.\textsuperscript{42} However, ROS can become detrimental, in a transition that has been dubbed mitochondrial stress.\textsuperscript{43,44} There is little doubt that formation of ROS can determine mitochondrial dysfunction and accelerate evolution of cell injury toward necrosis and apoptosis\textsuperscript{45,46}; and that ROS formation is an essential element favoring onset of the permeability transition.\textsuperscript{37

**Mitochondrial PTP**

The PTP of mammalian mitochondria is an IMM channel with a radius of \( \approx 1.4 \) nm\textsuperscript{48} and a conductance ranging between 0.9 and 1.5 nS in symmetrical 150 mmol/L KCl.\textsuperscript{49,50} It frequently enters a half-conductance substate that may be because of its dimeric composition,\textsuperscript{51-53} which will be further discussed below (see also a recent review by Szabo and Zoratti\textsuperscript{54}). Occurrence of mitochondrial permeabilization and its inhibition by adenine nucleotides had been appreciated since the early 1950s,\textsuperscript{55,56} but the potential relevance of this event to physiological and pathological stimuli\textsuperscript{37-39} was partially inhibited by small interfering RNAs resulting in apoptosis\textsuperscript{45,46}, and that ROS formation is an essential element favoring onset of the permeability transition.\textsuperscript{37}

Our recent identification of the F-ATP synthase complex as the likely channel-forming component of the PTP\textsuperscript{55} was made possible by 2 sets of critical observations. The first was that CyPD interacts with the F-ATP synthase, as shown by blue-native gel analysis and communoprecipitation.\textsuperscript{72} Binding occurred at the lateral stalk of the complex, was favored by Pi (which promotes the permeability transition in mammalian mitochondria), and was counteracted by CsA with matching effects on the catalytic activity. Indeed, Pi-dependent CyPD binding decreased the catalytic activity of F-ATP synthase by \( \approx 30\% \), and the enzyme was fully reactivated by CsA-induced detachment of CyPD.\textsuperscript{73} It is of note that the interactions with the PTP are affected by PTM of CyPD including phosphorylation by GSK3\( \beta \) (glycogen synthase kinase),\textsuperscript{76,79} acetylation,\textsuperscript{80} and S-nitrosylation.\textsuperscript{81}

The second insight was the identification of subunit oligomycin sensitivity conferring protein (OSCP) as the binding partner of CyPD,\textsuperscript{51} and the observation by Glick and coworkers\textsuperscript{82} that OSCP is also the binding site of the F-ATP synthase inhibitor Bz-423. After the demonstration that Bz-423 is a PTP inducer,\textsuperscript{53} we could show that purified dimers of F-ATP synthase form channels activated by Ca\textsuperscript{2+}, Bz-423, and oxidative stress and inhibited by Mg\textsuperscript{2+}/ADP in preparations from bovine hearts,\textsuperscript{51} Saccharomyces cerevisiae\textsuperscript{52} and Drosophila melanogaster.\textsuperscript{53} Channel formation by F-ATP synthase was also observed by Alavian et al,\textsuperscript{83} and a role for the enzyme is also supported by a study where translation of the c subunit was partially inhibited by small interfering RNAs resulting in PTP inactivation.\textsuperscript{84}

The mechanism through which the F-ATP synthase forms the PTP is the subject of intense investigation.\textsuperscript{47} We have proposed that the channel forms from dimers of F-ATP synthase after a conformational change that would follow replacement of Mg\textsuperscript{2+} with Ca\textsuperscript{2+} at the catalytic site, which is consistent with earlier literature on the F-ATP synthase. Indeed, when Ca\textsuperscript{2+} replaces Mg\textsuperscript{2+} ATP hydrolysis is not coupled to formation of a H\textsuperscript{+} gradient,\textsuperscript{85,86} suggesting that a conformational change takes place that leads to the apparent uncoupling of chemical catalysis from H\textsuperscript{+} transport. We suspect that the conformational change actually leads to PTP opening, and thus that the lack of measurable H\textsuperscript{+} translocation is because of H\textsuperscript{+} backflow through the channel. In mammalian mitochondria, binding of CyPD to OSCP would cause a conformational change affecting the accessibility of Me\textsuperscript{2+} to the catalytic binding sites, resulting in onset of the permeability transition if matrix [Ca\textsuperscript{2+}] is sufficiently high. The conformational change would be independently favored by ROS-dependent thiol oxidation and counteracted by thiol reduction, making replacement of Mg\textsuperscript{2+} with Ca\textsuperscript{2+} possible also in mitochondria lacking CyPD. Once the conformational change has occurred, permeation would take place at the interface between dimers, consistent with the effect of genetic ablation of the e and g subunits in yeast.\textsuperscript{52} The F-ATP synthase would then return to its basal coupled state.
when the catalytic site is reoccupied by Mg\(^{2+}\), consistent with the full reversibility of the permeability transition in populations of isolated mitochondria\(^{87}\) as well as in individual organelles\(^{20,21}\) and in intact cells.\(^{22}\) If the opening is long-lasting, however, a set of events takes place (ATP depletion, loss of substrates and pyridine nucleotides, matrix swelling causing IMM remodeling and eventually OMM rupture, release of cytochrome c, and other proapoptotic factors) that critically contributes to cell death.

**Regulation of the PTP**

\(\text{Ca}^{2+}\)-dependent channel formation under condition of oxidative stress (thiol oxidation or cross-linking) and its inhibition by Mg\(^{2+}\), adenine nucleotides and acidic pH seem to be general features of F-ATP syntheses, which have to date been observed in mitochondria from *Bos taurus*, *S cerevisiae*, and *D melanogaster*.\(^{51-53}\) However, marked differences exist in channel conductance, sensitivity to Pi, presence of a mitochondrial CyP, and inhibition by CsA. In bilayer experiments with purified dimers of the F-ATP synthase, channel conductance was 53 pS in Drosophila, 300 pS in yeast, and 500 pS in mammals, suggesting structural differences in the channel-forming subunit(s) of the enzyme.\(^{51-53}\) PTP opening is strongly favored by Pi in mammalian mitochondria (in spite of the Pi-dependent decrease of free matrix [Ca\(^{2+}\)]),\(^{88}\) whereas it is inhibited by Pi in yeast\(^{52,89,90}\) and Drosophila.\(^{59,91}\) Mammalian and *S cerevisiae* mitochondria possess matrix CyPs (CyPD and CPR3, respectively), yet the PTP is inhibited by CsA only in mammalian mitochondria. However, Drosophila does not possess a mitochondrial CyP, yet the expression of human CyPD in Drosophila *S. R*\(^{2+}\) cells sensitizes the PTP to Ca\(^{2+}\) in a process that is insensitive to CsA.\(^{53}\) These studies suggest that regulatory interactions between CyPD and the F-ATP synthase only emerged in mammals. This interaction may also contribute to explain the unique inducing effects of Pi in mammalian mitochondria, which could (in part at least) be a consequence of increased binding of CyPD to the F-ATP synthase.\(^{77}\)

Studies largely performed in the 1990s defined key mechanisms of PTP modulation and critical residues involved in its response to pathophysiological effectors. Pore opening requires the permissive presence of matrix Ca\(^{2+}\), which acts through a site that can bind other Me\(^{2+}\) ions such as Mg\(^{2+}\), Sr\(^{2+}\), and Mn\(^{2+}\), resulting in PTP inhibition.\(^{92}\) A second, external binding site for Me\(^{2+}\) has been defined, whose occupancy results in PTP inhibition even when Ca\(^{2+}\) is bound.\(^{92}\) Pore opening is strongly promoted by oxidation of pyridine nucleotides and of matrix dihithiols at discrete sites.\(^{93,94}\) These PTP-regulating diithiol-disulfide interconversions can be blocked by 1-chloro-2,4-dinitrobenzene and by monofunctional thiol reagents like N-ethylmaleimide and monobromobimane.\(^{95,96}\) A second class of regulatory thiols has been identified based on the effects of the impermeant oxidant copper-\(\alpha\)-phenanethroline, which promotes PTP opening in a process that, unlike that mediated by matrix Cys residues, cannot be inhibited by monobromobimane.\(^{94}\) This site is located on the outer surface of the IMM, because mitoplasts displayed an identical inducing effect of copper-\(\alpha\)-phenanethroline as intact mitochondria.\(^{92}\) The permeability transition is strongly affected by matrix pH. In de-energized mitochondria the pH optimum for PTP opening is 7.4, whereas the open probability decreases sharply below pH 7.4 through reversible protonation of His residues that can be prevented by diethylpyrocarbonate.\(^{98,99}\) The relevant His residues are not located on CyPD because PTP modulation by matrix pH is indistinguishable in mitochondria from CyPD-null and wild type animals.\(^{77}\) It should be kept in mind that in energized mitochondria an acidic pH can promote rather than inhibit PTP opening because of an increased rate of Pi uptake, an effect that could worsen PTP-dependent tissue damage in ischemic and posts ischemic acidosis.\(^{100}\) Finally, the PTP is affected by the transmembrane voltage, in the sense that depolarization increases the probability of pore opening\(^{95,98,101}\) in a process where Arg residues may play a critical role.\(^{102-104}\)

Given that F-ATP synthase forms channels that are indistinguishable from the bona fide PTP, it may be useful to briefly present the structural features of the heart F-ATP synthase before discussing potential points of regulation and residues where the effectors described above may exert their effects.

**Structure of Heart F-ATP Synthase**

The mitochondrial F\(_{10}\)F\(_{1}\) ATP synthase (F-ATP synthase or complex V of the oxidative phosphorylation system) is a well-conserved multisubunit complex located in the IMM\(^{105,106}\) that is responsible for the majority (>90%) of the aerobic ATP production in the heart. This large complex is a molecular motor and normally catalyzes the synthesis of ATP from ADP and Pi using the transmembrane proton-motive force generated by respiration. As mentioned in the first paragraph of the text, however, the complex is an ancestral ATPase that can act as an ATP-consuming device when the proton gradient is dissipated.\(^{107}\)

Heart mitochondria of mammals contain high amounts of F-ATP synthase, which has been estimated to be 0.38 to 0.45 nmol/mg protein\(^{108}\) and have been widely used as enzyme source for structural and functional studies. The complex is composed by a soluble catalytic F\(_{1}\) subcomplex protruding in the mitochondrial matrix and by a membrane-embedded F\(_{0}\) subcomplex through which the protons flow (Figure 1, where the complex is shown in its physiological dimeric form). These subcomplexes are connected by central and peripheral stalks. The whole complex has a molecular mass that varies between 540 and 585 kDa depending on the source.\(^{11}\) Numerous atomic structures have revealed that F\(_{1}\) comprises 3 copies of each of the nucleotide-binding subunits \(\alpha\) and \(\beta\), which alternate around the central \(\gamma\)-helical coiled coil of the \(\gamma\) subunit.\(^{112-116}\) Together with the \(\alpha\) subunits \(\delta\) and \(\varepsilon\), subunit \(\gamma\) forms the central stalk, which is connected to the F\(_{1}\) c-ring structure formed by 8 copies of subunit c (Figure 1) whose central region is probably occupied by phospholipids.\(^{117,118}\) The remaining F\(_{0}\) part consists of the subunits abdefg(A6L)F6, 2 subunits of which a and A6L are encoded by mitochondrial DNA. The remaining F\(_{0}\) subunits are encoded by nuclear genes, and complex assembly is a stepwise process assisted by several factors, which are still being actively investigated.\(^{119}\)

The structure and arrangement of the membrane subunits have not been defined yet except for subunit a, which associates with the c-ring peripherally, possibly forming an
angle of ≈70° relative to the c-ring helices, as recently resolved in the green alga *Polytomella*. Subunit α provides 2 half transmembrane water channels for H+ to access the con- 
drives not noted as β catalytic sites through at least 3 major functional states de-
≈ takes each of the 3 100 revolutions/s. Rotation of subunit α activity of IF1 to block the ATP-driven rotation of human F1 has been recently visualized. Moreover, dimers seem to be stabilized by binding of the matrix metalloprotein Factor B (or subunit s of F-ATP synthase), which gets in contact with the e and g subunits as well as with the ADP/ATP carrier. The existence of a second interface (the oligomerization interface) through e/e and g/g interactions originally proposed in yeast is still debated because the distance between dimers seems variable in electron cryomicrography of mitochondria from different sources, which would make direct protein contacts difficult. An interesting aspect of the dimeric/oligomeric structure is that the stabilizing contribution of the different subunits seems to be additive. Indeed, mutants lacking ≥1 of the above-mentioned subunits, such as ρ0 cells (which lack mitochondrial DNA) and therefore do not synthesize human cells totally or partially depleted of e and g subunits, still form lower amounts of F-ATP synthase dimers and oligomers as detected by native gel electrophoresis.

Electron cryomicroscopy of mitochondria from different sources clearly revealed that F-ATP synthase is organized in dimers, which are essential to maintain a high local curvature of the IMM and normal cristae morphology, and form long rows of oligomers at the cristae edges of mitochondria. An additional proposed role of F-ATP synthase dimers/oligomers is higher catalytic efficiency. Biochemical and genetic studies provided evidence that, for dimer formation, preferential interactions occur between F0 subunits α, h, e, and g, forcing the peripheral stalks (which are turned away from each other) and the 2 F1 heads at fixed angles of >70°. In mammals, the dimers have been reported to be further stabilized by interaction with IF1 favoring ATP synthesis in cells overexpressing IF1, but this proposal has been criticized based on biochemical studies in isolated ox heart membranes, as well as on the lack of effect on dimer stability of IF1, silencing in human osteosarcoma cells. Moreover, dimers seem to be stabilized by binding of the matrix metalloprotein Factor B (or subunit s of F-ATP synthase), which gets in contact with the e and g subunits as well as with the ADP/ATP carrier. The existence of a second interface (the oligomerization interface) through e/e and g/g interactions originally proposed in yeast is still debated because the distance between dimers seems variable in electron cryomicrography of mitochondria from different sources, which would make direct protein contacts difficult. An interesting aspect of the dimeric/oligomeric structure is that the stabilizing contribution of the different subunits seems to be additive. Indeed, mutants lacking ≥1 of the above-mentioned subunits, such as ρ0 cells (which lack mitochondrial DNA) and therefore do not synthesize human cells totally or partially depleted of e and g subunits, still form lower amounts of F-ATP synthase dimers and oligomers as detected by native gel electrophoresis.

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Figure 1. Structure of bovine F-ATP synthase. Left monomer, The F1 and F0 sectors are highlighted. Right monomer, The F1 and F0 subunits are shown. In the F1 sector, the front α and β subunits have been removed to reveal the F1-rotor (central stalk). The F, α and β subunits are colored in red and yellow, respectively. The F1 rotor γ, δ, ε, and ρ subunits are colored in shades of blue, the peripheral stalk subunits b, d, F6, and oligomycin sensitivity conferring protein (OSCP) in shades of green, and the c-ring in purple. The remaining F0 subunits a, e, f, g, A6L, whose structure has not been defined yet, are located in the F0 subcomplex colored in light blue. The image (lateral view) has been built starting from the yeast dimer molecular model (PDB ID 4b2q) and superimposing the cryo-electron microscopy map of bovine F-ATP synthase (EMD ID EMD-2901). The fit of molecular models to cryo-electron microscopy map was performed using the program ADP_EM. The molecular model for bovine F-ATP synthase was obtained by superimposing the 3-dimensional structure of the bovine F1-c-ring complex (PDB ID 4b2q) which contains the bovine subunits. Iterative magic fit. The lateral stalk was taken from the yeast dimer (PDB ID 4b2q) which contains the bovine subunits.

Figure 2. Interactors of F-ATP synthase and permeability transition pore formation. Reversible binding of cyclophilin D (CyPD) and IF1 to F-ATP synthase is shown, together with the putative region of channel formation (broken arrow). Factors promoting binding/release of interactors are also indicated. CsA indicates cyclosporin A.
Catalysis requires the binding of the nucleotide as a complex with Mg\textsuperscript{2+}, which plays a key role in shaping the high affinity catalytic sites located in the \( \beta \) subunits, where Mg\textsuperscript{2+} is hexa-coordinated by \( \beta \)Thr\textsubscript{160} (bovine numbering), by the oxygen atoms \( \beta \)O\textsubscript{2} and \( \gamma \)O\textsubscript{2} of ATP, and by 3 ordered water molecules (hydrogen bonded to \( \beta \)Asp\textsubscript{156}, \( \beta \)Glu\textsubscript{156}, and \( \beta \)Asp\textsubscript{126} (Figure 3). Mg\textsuperscript{2+} can be replaced by other divalent cations, including Ca\textsuperscript{2+}, and the ionic radius is the chief determinant of the ability to support catalysis.\textsuperscript{149} However, Ca\textsuperscript{2+} ions support \( \gamma \) subunit rotation and ATP hydrolysis, but not H\textsuperscript{+} translocation and ATP synthesis, as documented both in bacteria\textsuperscript{150} and in mammals,\textsuperscript{26} strongly suggesting that the catalytic site has a different conformation state when it is occupied by Ca\textsuperscript{2+}.

### F-ATP Synthase and PTP Formation in the Heart

The mechanism of PTP formation from F-ATP synthase remains unsolved and is the matter of considerable debate.\textsuperscript{47} It is clear, however, that all PTP effectors will eventually have to be assigned to specific sites of the F-ATP synthase. What follows is therefore a discussion of potential targets of well-characterized pore effectors in heart F-ATP synthase and of PTM that may affect or cause the transition to the PTP.

The permeability transition has been detected in \( \rho^0 \) cells lacking subunit a and A6L,\textsuperscript{150} whereas it was markedly inhibited in yeast mutants lacking subunits e and g,\textsuperscript{35} suggesting a different contribution of the F\textsubscript{O} subunits in forming the PTP. Based on the observation that PTP has been conserved from yeast to Drosophila and mammals,\textsuperscript{151} it seems reasonable to suggest that the conserved subunits b, e, and g of the F\textsubscript{O} subcomplex, which mediate the dimer/oligomer formation, may also favor PTP formation in the presence of Ca\textsuperscript{2+} ions and thiol oxidants, possibly also through other subunits.

In the heart Ca\textsuperscript{2+} ions are also regulatory elements of F-ATP synthase activity, which must match ATP utilization for muscle contraction, whose rate and force can vary in vivo up to 5- to 10-fold.\textsuperscript{123} Indeed, it is now well established that F-ATP synthase does not respond simply to the bioenergetic parameters, that is, to the levels of ATP, ADP, Pi and proton motive force, and reversible and rapid stimulation of F-ATP synthase by physiological intramitochondrial Ca\textsuperscript{2+} levels has been clearly demonstrated in isolated pig heart mitochondria\textsuperscript{14} and cultured rat cardiomyocytes.\textsuperscript{112} These changes were parallel to stimulation of the Ca\textsuperscript{2+}-sensitive dehydrogenases of the Krebs cycle and rapid enough (100 ms) to potentially support steep changes in myocardial workload.\textsuperscript{44} Intriguingly, the ability to upregulate the F-ATP synthase is lost in hypertension and hyperthyroidism,\textsuperscript{123} although its relationship to the pathogenesis of these disease states is not clear. Although a direct allosteric mechanisms mediated by Ca\textsuperscript{2+} ions was ruled out already in the 1990s,\textsuperscript{125} how Ca\textsuperscript{2+} modulates the F-ATP synthase activity has not been established yet.

In cardiomyocytes a Ca\textsuperscript{2+}-dependent interaction of F\textsubscript{0} with the S100A1 protein, which is expressed predominantly in cardiac muscle, has been reported to lead to increased ATP production.\textsuperscript{153} The F\textsubscript{0} subunit e has been hypothesized to contain a putative Ca\textsuperscript{2+}-dependent activating region exposed at the cytosolic site of F\textsubscript{0} sector, potentially ensuring the rapid decoding of increases of cytosolic [Ca\textsuperscript{2+}].\textsuperscript{154} Indeed, residues 34 to 56 of e subunit are highly homologous to the Ca\textsuperscript{2+}-dependent troponyosin-binding region for troponin T, and their interaction with a specific antibody mediates an increase of F-ATP synthase activity. Because of exposure of the Ca\textsuperscript{2+}-binding site to the mitochondrial intermembrane space, subunit e may be also involved in the Ca\textsuperscript{2+}-dependent inhibition of PTP opening.\textsuperscript{82} Subunit e is important for F-ATP synthase dimer formation,\textsuperscript{157,158} suggesting that Ca\textsuperscript{2+} inhibition could target the dimer structure and function (Figure 3).

Another conserved Ca\textsuperscript{2+} binding site has been described in the N terminus of F\textsubscript{1} subunit c,\textsuperscript{155,156} which could also represent a candidate for PTP inhibition by Ca\textsuperscript{2+} in the intermembrane space. Ca\textsuperscript{2+} binding to subunit c purified from neuronal plasma membrane and reconstituted in artificial membranes was able to block nonvalent cation currents,\textsuperscript{156} and Ca\textsuperscript{2+} binding to subunit c inhibited H\textsuperscript{+} translocation in bacteria and chloroplasts,\textsuperscript{157} suggesting that Ca\textsuperscript{2+} could alter the c-ring conformation. However, although the Ca\textsuperscript{2+}-binding capacity of subunit c from bacteria and chloroplasts is well established, that of the mammalian subunit c is debated.\textsuperscript{157} These data emphasize the complexity of the Ca\textsuperscript{2+}-dependent modulation of F-ATP synthase (Figure 3), and the need for further studies to clarify how different activating signals may be integrated in the normal heart and in the transition of F-ATP synthase to PTP formation.

We have already mentioned that when the proton motive force declines, F-ATP synthase reverses to a proton-pumping ATPase, and that this event plays a major role in I/R injury of the heart. It is worth mentioning that this condition may also apply to mitochondrial diseases.\textsuperscript{159,158} Interestingly, we observed that the sense of rotation of F-ATP synthase strongly affects the threshold of Ca\textsuperscript{2+} required for PTP opening in intact mitochondria.\textsuperscript{51,55} Because continuous ATP hydrolysis markedly reduces the Ca\textsuperscript{2+} sensitivity of PTP, the inverse rotation of F-ATP synthase during ischemia might contribute to maintain a closed pore. A tentative explanation is that rotation during ATP hydrolysis might destabilize the dimers by pulling the stators apart and vice versa, as proposed Buzhynskyy et al.,\textsuperscript{159} who demonstrated the existence of 2 classes of dimers characterized by different stalk-to-stalk distance in native IMM.

It is well established that heart mitochondria of both slow- and fast-beating species possess sufficiently high levels of IF\textsubscript{1} relative to the F-ATP synthase to completely inhibit ATPase activity with an effect equivalent to the action of oligomycin.\textsuperscript{125} Consistently, both in vitro and in vivo experimental models demonstrated that during ischemia the ATPase activity is (at least partially) inhibited by the reversible binding of IF\textsubscript{1}, thus mitigating ATP depletion. Indeed, in ischemic hearts of dog,\textsuperscript{160} goat,\textsuperscript{161} and rabbit,\textsuperscript{162} the loss of ATPase activity correlates with an increased IF\textsubscript{1} content. Downregulation of F-ATP synthase activity is also observed in anoxic cardiomyocytes, although the IF\textsubscript{1} contents were not measured. In HeLa and C2C12 cells transient IF\textsubscript{1} overexpression preserves ATP and protects from cell death induced by oxygen and glucose deprivation, a finding that would enlist IF\textsubscript{1} in prosurvival proteins.\textsuperscript{142} Nevertheless, the response to cell injury seems to depend on the cell type, as IF\textsubscript{1} content varies between species, tissues, and even between cell types within a given tissue, such as in central nervous system where neurons contain much more IF\textsubscript{1}.
by short ischemic periods before long-lasting ischemia and reperfusion, a phenomenon called ischemic preconditioning. During ischemic,163,165 as well pharmacological preconditioning,170,171 IF1 seems to mediate a peculiar, long-lasting ATPase inhibition in dog, goat, and rat hearts (until 30–120 minutes of reperfusion depending on the species or the experimental model), although contradictory results have been published, which could be explained by the different analytic methods used.172 As preconditioning results in sparing of ATP, lowering of membrane potential and Ca2+ accumulation, it may seem that IF1 favors conditions that inhibit PTP formation during reperfusion. Moreover, based on the observation that the lack of IF1 in Luft’s disease is associated with uncoupled mitochondria,173 long-lasting IF1 binding during ischemia might maintain tight coupling supporting oxidative phosphorylation during reperfusion. Clarification of the molecular events involving IF1 in preconditioning holds great promise for understanding preconditioning and for devising pharmacological strategies for cardioprotection.

PTMs of F-ATP Synthase and PTP Formation

Until recently, there were few reports about the PTMs of F-ATP synthase, which have been associated with specific biological function/processes in a limited number of cases. Considerable information has been obtained about oxidative modifications of F-ATP synthase. Experiments with isolated

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**Figure 3.** Map of Mg2+/Ca2+ binding sites and Ca2+-dependent interactions in bovine F-ATP synthase. Left monomer, The subunits involved in Ca2+-dependent interactions are highlighted, that is, subunits α and β, which interact with the matrix protein S100A153 (not shown in the picture), and the F0 region containing subunit e, which may interact with a hypothetical tropomyosin-like protein localized in the intermembrane space.154 Ca2+-regulatory sites located in the c-ring are also shown.155,156 Right monomer, The residues (T163, R189, E192, and D256) of β subunit interacting with the catalytic metal ions are mapped onto the 3-dimensional structure of the bovine F1–c-ring complex.

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**Figure 4.** Map of Cys residues in bovine heart F-ATP synthase. Position of Cys residues on the specified subunits (red dots) is mapped onto the 3-dimensional structure of the bovine F1–c-ring complex and of the bovine lateral stalk. OSCP indicates oligomycin sensitivity conferring protein.

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**Figure 5.** Posttranslational modifications of F-ATP synthase in the normal and failing heart. Left monomer, The residues involved in post-translational modifications (PTMs) in normal heart are shown, that is, complete trimethylation of cK43117; oxidation of W12, W53 of subunit d191; phosphorylation of αS184, αS419, γY52195, and γS121,196 dS30,192 Right monomer, Residues involved in PTMs associated with preconditioning or heart failure are shown, that is, phosphorylation of γS56, γT57, γT212, γS213; nitration of dY114 (numbering according to PDB ID 4b2q) and hydroxylation of dK71.182 Subunit α is also highlighted, being the target of tyrosine nitration.186
mitochondria from bovine heart demonstrated that F-ATP synthase contains a set of thiols located in F₆₅ (Figure 4), whose environment senses membrane energization and whose oxidation results in complete and reversible mitochondrial uncoupling. However, because uncoupling was not inhibited by oligomycin, the authors proposed that the permeability pathway is located on the intermembrane side of the oligomycin binding site. This position would suggest involvement of the unique Cys residue of subunit c, which is located near the Glu₄₅ residue essential for proton translocation. It is tempting to speculate that oxidation of this Cys residue may favor PTP formation in another part of F₆₅. This does not exclude the involvement of species-specific Cys residues located in other subunits, such as subunit b in bovine and human heart (Figure 4), whose modification in the bovine enzyme also affects catalytic function. In animal F-ATP synthase, factor B contains 2 vicinal Cys residues, whose oxidation leads to mitochondrial uncoupling. However, factor B is not present in yeast and D melanogaster, where the PTP is also modulated by SH reagents, thus suggesting that factor B is probably not involved in PTP formation. We showed that isolated F₆₅ from bovine heart F-ATP synthase is selectively inactivated by hydrogen peroxide through redox-active iron–protein adducts probably generating highly ROS in proximity of the catalytic nucleotide binding sites. Because of their conservation it is tempting to hypothesize that also these sites may be candidates for ROS-mediated PTP modulation.

Information on oxidative modifications of F-ATP synthase subunits has widely increased with the development of mass spectrometry-based methods, which demonstrated that F-ATP synthase is susceptible to oxidative/nitrosative stress associated with heart failure, central nervous system disorders, calorific restriction, and aging. Specifically, the α subunit is S-nitrosylated in response to GSNO (S-nitrosoglutathione) treatment of a mouse heart membrane fraction. Moreover, in the same subunit Tyr nitration was increased after I/R of mouse heart (Figure 5), suggesting that α subunit is actively involved in various oxidative modifications. Consistently, the formation of an intersubunit disulfide bridge between γC251 and γC78 has been observed in canine dysynchronous heart failure, which reverted after cardiac resynchronization therapy, and disulfide formation was proposed to be involved in PTP activation. However, these residues are distant in the assembled complex (Figure 4) suggesting that the disulfide may only form in a misfolded/aggregated enzyme. Interestingly, in mitochondria from aging cardiomyocytes, which are more prone to undergo the permeability transition during reperfusion in spite of an impaired ability to accumulate Ca²⁺ ions during ischemia and reperfusion, quantitative proteomics revealed increased Cys oxidation at several subunits, including OSCP and d, which might potentially affect CyPD binding to the peripheral stalk and PTP formation. Moreover, a pharmacoproteomic approach demonstrated that myocardial infarction in dogs caused several nitric oxide–related chemical modifications of d subunit, that is, nitration of Y114 and hydroxylation of K71 (numbering according to PDB ID 4b2q), followed by its myristoylation. These PTMs were abolished by the cardioprotective drug valsartan, which also induced phosphorylation of S29 (numbering according to PDB ID 4b2q) of the same subunit (Figure 5). Intriguingly, δPKC (protein kinase C), which plays key roles in I/R, is a modulator of F-ATP synthase in cardiomyocytes through direct binding to subunit d, which requires cardiolipin. Subunit d also contains 3 Trp residues, which have been classified as hot spots for oxidation in human heart being found in normoxic conditions (Figure 5). These data suggest subunit d as actively involved in various oxidative modifications, whose implication in PTP formation remains to be elucidated.

The most extensively characterized PTMs concern Lys43 of the c subunit, which is completely trimethylated in all vertebrates and in the majority of invertebrates (Figure 4). These residues are located in the loops exposed to the matrix and possibly provide sites for the specific binding of cardiolipin. In the normal heart, several phosphorylated residues have also been reported, and these are mainly located in the F₁ sector where they may be functionally relevant. The modified residues include αS184, αS419, γS52, and γS121 (Figure 5). The β subunit was found to be modified by preconditioning of rabbit myocytes with adenosine, and contained at least 5 phosphorylated residues, 3 of which (βS56, βT57, and βT212 or βS213) are accessible and therefore potentially regulated (Figure 5).

**Conclusions**

Identification of the F-ATP synthase as the most likely channel-forming element of the PTP has been a turning point for our understanding of mitochondrial pathophysiology. Conservation of the channel-forming ability across species points to a conserved function, and species-specific differences in conductance and regulation suggest an evolutionary role that still needs to be addressed. We are aware that a coherent picture explaining the mechanisms through which the energy-conserving enzyme turns into an energy-dissipating device is not possible at present, but we hope that this review will help address the many open mechanistic issues and inspire the molecular analysis of appropriate F-ATP synthase mutants.

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**Disclosures**

None.

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