From ATP to PTP and back. A dual function for the mitochondrial ATP synthase

Paolo Bernardi, MD,
Department of Biomedical Sciences, University of Padova, Italy

Fabio Di Lisa, MD,
Department of Biomedical Sciences, University of Padova, Italy

Federico Fogolari, PhD, and
Department of Medical and Biological Sciences, University of Udine, Italy

Giovanna Lippe, PhD
Department of Food Science, University of Udine, Italy

Abstract

Mitochondria play a fundamental role in heart physiology, but are also key effectors of dysfunction and death. This dual role assumes a new meaning following 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 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.

Keywords

Mitochondria; heart pathophysiology; Ca^{2+}; permeability transition pore; F-ATP synthase

Introduction

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 due to the existence of 2 limiting membranes and to the quite 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 ER/SR,[2] autophagy[3,4] and mitophagy.[5] OMM permeability to metabolites and small peptides (< 5kDa) is regulated by the voltage-dependent anion channel (VDAC) and other transporters including the translocator protein of 18 kDa (TSPO).[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 (Δp) drives the rotation of the F_o sector of ATP synthase leading to the synthesis of ATP in the F_1 sector. The two components of Δp, namely $\Delta\psi_m$ and ΔpH, are also utilized for the uptake of ADP and Pi, respectively, and for the release of ATP into the cytosol in exchange for ADP. In addition, Δp is also utilized by mitochondria for their biogenesis and for the maintenance of ion homeostasis, which is crucial for Ca^{2+}.[7,8] In particular, the mitochondrial Ca^{2+} uniporter (MCU)[9,10] catalyzes Ca^{2+} uptake down the Ca^{2+} electrochemical gradient, which is largely driven by the inside-negative $\Delta\psi_m$, whereas release from the matrix to the intermembrane space, which is catalyzed by the mitochondrial Na^+/Ca^{2+} exchanger, ultimately depends on ΔpH. Indeed, the exchanger is functionally coupled with the mitochondrial Na^+/H^+ exchanger, so that mitochondrial Ca^{2+} release is paralleled by H^+ reuptake.[11]

A rise in intramitochondrial $[Ca^{2+}]$ from a resting value of about 0.1 μM to 1 μM activates key enzymes of oxidative metabolism, such as pyruvic, isocitric, and oxoglutaric dehydrogenases.[12] Therefore, the increase in ATP demand dictated by cytosolic Ca^{2+}-activated processes, especially contractile activity, is matched by increased ATP synthesis.[13] Higher values of matrix $[Ca^{2+}]$ could reflect the ability of mitochondria to buffer undesired elevation of cytosolic $[Ca^{2+}]$, 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^{2+} homeostasis to cytosolic Ca^{2+} oscillations related to myocardial contractile activity. Based upon kinetic analysis of mitochondrial Ca^{2+} uptake and release *in vitro*, beat-to-beat variations of matrix $[Ca^{2+}]$ should not occur because MCU exhibits high transport capacity but low affinity, so that even for values of cytosolic $[Ca^{2+}]$ measured at peak systole ($\approx$2 μM) mitochondrial Ca^{2+} uptake should be negligible. However, due to the close proximity between mitochondria and SR, MCU might be exposed to local domains of $[Ca^{2+}]$ higher than those occurring in the bulk cytosolic space.[14] As far as Ca^{2+} release is concerned, the Na^+/Ca^{2+} exchanger may be too slow to cope with very fast cytosolic oscillations, such as those

occurring in a beating mouse heart.[7] Nevertheless, clear evidence of beat-to-beat changes of mitochondrial [Ca^{2+}] in intact cardiomyocytes has been provided by different laboratories.[15–17] This issue is still the matter of debate,[18] and besides methodological questions a major point concerns the high energetic cost (or low convenience) that would be determined by beat-to-beat variations in mitochondrial [Ca^{2+}]. An adequate pathway for Ca^{2+} release could be provided by transient openings of the PTP,[19] which have been documented both in isolated mitochondria and *in situ*.[20–23] The advantage of an unselective channel of high conductance would be to provide charge compensation within the channel itself, allowing mitochondrial Ca^{2+} release at zero potential, i.e. even for small Ca^{2+} gradients.[7] Support for this idea comes from the finding that treatment of cardiomyocytes with CsA (which desensitizes the PTP) increased the uptake of $^{45}Ca^{2+}$ in mitochondria,[24] and that mitochondrial [Ca^{2+}] is elevated in hearts with genetic ablation of mitochondrial cyclophilin (CyP) D, the mitochondrial target of CsA.[25] The reader is referred to a recent review for a general discussion of this hypothesis[26] while the links between PTP and cardiac disease will be discussed further below.

Based upon a recent quantitative analysis, mitochondria do not appear to represent a significant dynamic buffer of cytosolic Ca^{2+} under physiological conditions while they might shape cellular [Ca^{2+}] dynamics upon prolonged elevations of cytosolic [Ca^{2+}].[27] A limited contribution of mitochondrial Ca^{2+} homeostasis to cardiac function appears to be supported by the lack of a specific cardiac phenotype in MCU knockout mice.[28] This experimental model seems also to rule out an involvement of mitochondrial Ca^{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.[29] If the IMM permeability increases following 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.[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.[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_2O requires four electrons, a process occurring sequentially since oxygen accepts one 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, i.e. water, is released. However, electrons flowing through the respiratory chain can be donated to oxygen at other sites where reduction is not complete resulting in ROS formation.[33] Very significant amounts of ROS are also produced within mitochondria at sites other than the IMM such as monoamine oxidase in the OMM and p66[Shc] in the intermembrane space.[34] In brain mitochondria the high rate of H_2O_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.[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.[37–39] Most of the effects elicited by ROS can be explained by post-translational modifications of proteins, especially at the level of Cys residues[40] that might impact also on protein (de)phosphorylation by modulating protein kinases[41] and phosphatases.[42] On the other hand, ROS can become detrimental, in a transition that has been dubbed mitohormesis.[43,44] There is little doubt that formation of ROS can determine mitochondrial dysfunction, and accelerate evolution of cell injury towards necrosis and/or apoptosis;[45,46] and that ROS formation is an essential element favoring onset of the permeability transition.[47]

The mitochondrial permeability transition pore

The PTP of mammalian mitochondria is an IMM channel with a radius of about 1.4 nm[48] and a conductance ranging between 0.9 and 1.5 nS in symmetrical 150 mM KCl.[49,50] It frequently enters a half-conductance substate that may be due to its dimeric composition,[51–53] which will be further discussed below (see also a recent review by Szabò and Zoratti).[54] Occurrence of mitochondrial permeabilization and its inhibition by adenine nucleotides had been appreciated since the early 1950s,[55,56] but the potential relevance of this event to heart pathophysiology was first proposed by Haworth and Hunter,[57–60] who coined the term permeability transition and provided the unique insight that it could be due to reversible opening of a proteinaceous pore of the IMM. The nature of the PTP has remained a mystery for 60 years.[61] Theories about pore formation by the IMM adenine nucleotide translocator and Pi carrier, the OMM VDAC and TSPO and by a variety of additional proteins did not stand the test of genetic inactivation of each of these putative pore component(s) and will not be covered here, but for discussion the reader is referred to a recent review where the importance of the pore as a target for cardioprotection is also addressed.[47]

A turning point of research on the PTP was the discovery that the pore can be inhibited by the immunosuppressant drug CsA[62,63] through its binding to CyPD,[64] a matrix protein that like all CyPs has peptidyl-prolyl *cis-trans* isomerase activity that assists in protein folding.[65,66] Through the use of CsA the role of the PTP in cardiac pathophysiology could be established in cellular models,[67] in hearts subjected to ischemia-reperfusion injury[68] and in a pilot study in infarcted patients.[69] It should be stressed that CsA is not a blocker of the PTP, nor an inhibitor in the strict sense of the word. Indeed, at higher Ca^{2+} loads and/or higher concentrations of inducers the permeability transition can still occur in the presence

of CsA,[70] and a more appropriate term to describe the effects of CsA on the pore is desensitization.[71] This key point was firmly established by the characterization of mitochondria from CyPD-null mice, which like CsA-treated wild-type mitochondria underwent PTP opening when the matrix Ca^{2+} load was increased[72–75] and possessed a channel with electrophysiological features identical to those found in wild-type mice.[76]

Our recent identification of the F-ATP synthase complex as the likely channel-forming component of the PTP[51] was made possible by two sets of critical observations. The first was that CyPD interacts with the F-ATP synthase, as shown by blue-native gel analysis and co-immunoprecipitation.[77] 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 about 30%, and the enzyme was fully reactivated by CsA-induced detachment of CyPD.[77] It is of note that the interactions with the PTP are affected by posttranslational modifications (PTM) of CyPD including phosphorylation by GSK3β,[78,79] acetylation[80] and S-nitrosylation[81].

The second insight was the identification of subunit OSCP as the binding partner of CyPD,[51] and the observation by Glick and coworkers that OSCP is also the binding site of the F-ATP synthase inhibitor Bz-423.[82] Following the demonstration that Bz-423 is a PTP inducer,[51] we could show that purified dimers of F-ATP synthase form channels activated by Ca^{2+}, Bz-423 and oxidative stress, and inhibited by Mg^{2+}/ADP in preparations from bovine hearts,[51] *S. cerevisiae*[52] and *D. melanogaster*.[53] Channel formation by F-ATP synthase was also observed by Alavian et al.,[83] and a role for the enzyme is also supported by a study where translation of the c subunit was partially inhibited by siRNAs resulting in PTP inactivation.[84]

The mechanism through which the F-ATP synthase forms the PTP is the subject of intense investigation.[47] We have proposed that the channel forms from dimers of F-ATP synthase after a conformational change that would follow replacement of Mg^{2+} with Ca^{2+} at the catalytic site, which is consistent with earlier literature on the F-ATP synthase. Indeed, when Ca^{2+} replaces Mg^{2+} ATP hydrolysis is not coupled to formation of a H^+ gradient,[85,86] suggesting that a conformational change takes place that leads to the apparent uncoupling of chemical catalysis from H^+ transport. We suspect that the conformational change actually leads to PTP opening, and thus that the lack of measurable H^+ translocation is due to H^+ backflow through the channel. In mammalian mitochondria, binding of CyPD to OSCP would cause a conformational change affecting the accessibility of Me^{2+} to the catalytic binding sites, resulting in onset of the permeability transition if matrix $[Ca^{2+}]$ is sufficiently high. The conformational change would be independently favored by ROS-dependent thiol oxidation and counteracted by thiol reduction, making replacement of Mg^{2+} with Ca^{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.[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

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 appear to be general features of F-ATP synthases, which have so far been detected in mitochondria from *B. taurus*, *S. cerevisiae* and *D. melanogaster*.[51–53] On the other hand, marked differences exist in channel unit 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] while it is inhibited by Pi in yeast[52,89,90] and Drosophila.[53,91] Mammalian and *S. cerevisiae* mitochondria possess matrix CyPs (CyPD and CPR3, respectively) yet the PTP is inhibited by CsA only in mammalian mitochondria. On the other hand, Drosophila does not possess a mitochondrial CyP, yet expression of human CyPD in Drosophila S_2R^+ 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 carried out 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 like 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 dithiols at discrete sites.[93,94] These PTP-regulating dithiol-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-*o*-phenanthroline, which promotes PTP opening in a process that, unlike that mediated by matrix Cys residues, cannot be inhibited by monobromobimane.[94,97] This site is located on the outer surface of the IMM, since mitoplasts displayed an identical inducing effect of copper-*o*-phenanthroline as intact mitochondria.[98] The permeability transition is strongly affected by matrix pH. In deenergized mitochondria the pH optimum for PTP opening is 7.4, while the open probability decreases sharply below pH 7.4 through reversible protonation of His residues that can be prevented by diethylpyrocarbonate.[99,100] 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.[101] It should be kept in mind that in energized mitochondria an acidic pH can promote rather than inhibit PTP opening due to an increased rate of Pi uptake, an effect that could worsen PTP-

dependent tissue damage in ischemic and postischemic acidosis.[102] Finally, the PTP is affected by the transmembrane voltage, in the sense that depolarization increases the probability of pore opening[95,103,104] in a process where Arg residues may play a critical role.[105–107]

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_OF_1 ATP synthase (F-ATP synthase or complex V of the oxidative phosphorylation system) is a well conserved multi-subunit complex located in the IMM[108,109] that is responsible for the majority (over 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 Introduction, however, the complex is an ancestral ATPase that can act as an ATP-consuming device when the proton gradient is dissipated.[110]

Heart mitochondria of mammals contain high amounts of F-ATP synthase, which has been estimated to be 0.38–0.45 nmol/mg protein,[111] 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_O 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.[112] Numerous atomic structures have revealed that F_1 comprises three copies of each of the nucleotide-binding subunits α and β, which alternate around the central α-helical coiled coil of the γ subunit.[113–117] Together with the F_1 subunits δ and ε, subunit γ forms the central stalk, which is connected to the F_O c-ring structure formed by 8 copies of subunit c (Figure 1) whose central region is probably occupied by phospholipids.[118,119] The remaining F_O part consists of the subunits abdefg(A6L)F6, two subunits of which - a and A6L - are encoded by mitochondrial DNA. The remaining F_O and F_1 subunits are encoded by nuclear genes, and complex assembly is a stepwise process assisted by several factors, which are still being actively investigated.[120]

The structure and arrangement of the membrane subunits has not been defined yet except for subunit a, which associates with the c-ring peripherally, possibly forming an angle of approximately 70° relative to the c-ring helices, as recently resolved in the green alga Polytomella.[121] Subunit a provides two half transmembrane water channels for H^+ to access the conserved carboxylic residues present in each c subunit. Indeed, proton translocation into the mitochondrial matrix through these (half) channels is concurrent with the clockwise rotation of the F_O c-ring (when viewed from the matrix side), which drives γ subunit rotation within the $\alpha_3\beta_3$ F_1 subcomplex at about 100 revolutions per second. Rotation of subunit γ takes each of the 3 catalytic sites through at least 3 major functional states denoted as βE,

βDP and βTP, thereby supporting the synthesis of 3 ATP molecules for each 360° rotation.[109,122,123] When the enzyme works in the direction of ATP hydrolysis, the counterclockwise rotation of the γ subunit and of the c-ring cause proton traslocation into the intermembrane space. This activity is selectively inhibited by reversible binding of the endogenous inhibitor factor 1 (IF_1) to F_1 with a 1:1 stoichiometry (Figure 2), resulting in full inhibition.[124,125] Accordingly, the ability of IF_1 to block the ATP-driven rotation of human F_1 has been recently visualized[126]. The peripheral stalk, comprising the F_O subunits oligomycin sensitivity conferring protein (OSCP), b, d and F6, is important to prevent the co-rotation of α and β subunits with γ and therefore the loss of enzyme efficiency.[127] As already mentioned, this subcomplex is also the target of CyPD, which binds to the OSCP subunit (Figure 2), possibly sharing a common binding site with the F-ATP synthase inhibitor Bz-423.[128,129] CyPD binding to OSCP is favored by Pi and counteracted by CsA;[77] CyPD sensitizes the PTP to Ca^{2+}, promoting the transition of the F-ATP synthase to a channel that may form at the interface between monomers[51–53,61] (Figure 2). Interestingly, OSCP has also been recently recognized as the binding site of 17β-estradiol, whose binding promotes an intrinsically uncoupled state of ATP synthase,[130] as well as of regulatory proteins, such as Sirtuin3,[131] highlighting its ability to sense metabolic signals.

Electron cryomicroscopy of mitochondria from different sources[132] clearly revealed that F-ATP synthase is organized in dimers, which are essential to maintain a high local curvature of the IMM[133] and normal cristae morphology,[134] 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.[133,135] Biochemical and genetic studies provided evidence that, for dimer formation, preferential interactions occur between F_O subunits a,[136] b,[137] e,[138,139] and g,[140] forcing the peripheral stalks (which are turned away from each other) and the two F_1 heads at fixed angles of $> 70°$.[133,141] In mammals the dimers have been reported to be further stabilized by interaction with IF_1 favoring ATP synthesis in cells overexpressing IF_1,[142,143] but this proposal has been criticized based on biochemical studies in isolated ox heart membranes,[144] as well as on the lack of effect on dimer stability of IF_1 silencing in human osteosarcoma cells.[145] Moreover, dimers seem to be stabilized by binding of the matrix metalloprotein Factor B (or subunit s of F-ATP synthase),[146] 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 appears variable in electron cryotomography of mitochondria from different sources, which would make direct protein contacts difficult.[132] An interesting aspect of the dimeric/oligomeric structure is that the stabilizing contribution of the different subunits seems to be additive. Indeed, mutants lacking one or more of the above mentioned subunits, such as $ρ^0$ cells (which lack mitochondrial DNA[147] and therefore do not synthesize subunits a and A6L)[136] and human cells totally or partially depleted of e and g subunits,[148] still form lower amounts of F-ATP synthase dimers and oligomers as detected by native gel electrophoresis.[136]

Catalysis requires the binding of the nucleotide as a complex with Mg^{2+},[149] which plays a key role in shaping the high affinity catalytic sites located in the β subunits, where Mg^{2+} is hexa-coordinated by $βThr_{163}$ (bovine numbering), by the oxygen atoms $βO_2$ and $γO_2$ of ATP, and by 3 ordered water molecules (hydrogen-bonded to $βArg_{189}$, $βGlu_{192}$ and

$\beta Asp_{256})^{117}$ (Figure 3). Mg^{2+} can be replaced by other divalent cations, including Ca^{2+}, and the ionic radius is the chief determinant of the ability to support catalysis.[150] However, Ca^{2+} ions support γ subunit rotation and ATP hydrolysis, but not H^+ translocation and ATP synthesis, as documented both in bacteria[85] and in mammals,[86] strongly suggesting that the catalytic site has a different conformation state when it is occupied by Ca^{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[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 PT has been detected in ρ^0 cells lacking subunit a and A6L,[151] while it was markedly inhibited in yeast mutants lacking subunits e and/or g,[52] suggesting a different contribution of the F_O subunits in forming the PTP. Based on the observation that PTP has been conserved from yeast to Drosophila and mammals,[152] it seems reasonable to suggest that the conserved subunits b, e and g of the Fo subcomplex, which mediate the dimer/oligomer formation, may also favor PTP formation in the presence of Ca^{2+} ions and thiol oxidants, possibly also through other subunits.

In the heart Ca^{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 5–10 fold.[124] Indeed, it is now well established that F-ATP synthase does not respond simply to the bioenergetic parameters, i.e. to the levels of ATP, ADP, Pi and proton motive force, and reversible and rapid stimulation of F-ATP synthase by physiological intramitochondrial Ca^{2+} levels has been clearly demonstrated in isolated pig heart mitochondria[14] and cultured rat cardiomyocytes.[153] These changes were parallel to stimulation of the Ca^{2+}-sensitive dehydrogenases of the Krebs cycle and rapid enough (100 ms) to potentially support steep changes in myocardial workload.[14] Intriguingly, the ability to up-regulate the F-ATP synthase is lost in hypertension and hyperthyroidism,[124] although its relationship to the genesis of these disease states is not clear. Although a direct allosteric mechanisms mediated by Ca^{2+} ions was ruled out already in the 1990s,[124] how Ca^{2+} modulates the F-ATP synthase activity has not been established yet.

In cardiomyocytes a Ca^{2+}-dependent interaction of F_1 with the S100A1 protein, which is expressed predominantly in cardiac muscle, has been reported to lead to increased ATP production.[154] The F_O subunit e has been hypothesized to contain a putative Ca^{2+}-dependent activating region exposed at the cytosolic site of F_O sector, potentially ensuring the rapid decoding of increases of cytosolic $[Ca^{2+}]$.[155] Indeed, residues 34–56 of e subunit are highly homologous to the Ca^{2+}-dependent tropomyosin-binding region for troponin T, and their interaction with a specific antibody mediates an increase of F-ATP synthase activity. Due to exposure of the Ca^{2+}-binding site to the mitochondrial intermembrane space, subunit e may be also involved in the Ca^{2+}-dependent inhibition of PTP opening[92]. Subunit e is important

for F-ATP synthase dimer formation,[138,139] suggesting that Ca^{2+} inhibition could target the dimer structure and function (Figure 3).

Another conserved Ca^{2+} binding site has been described in the N-terminus of F_O subunit c,[156,157] which could also represent a candidate for PTP inhibition by Ca^{2+} in the intermembrane space. Ca^{2+} binding to subunit c purified from neuronal plasma membrane and reconstituted in artificial membranes was able to block monovalent cation currents[157], and Ca^{2+} binding to subunit c inhibited H^+ translocation in bacteria and chloroplasts,[158] suggesting that Ca^{2+} could alter the c ring conformation. However, while the Ca^{2+}-binding capacity of subunit c from bacteria and chloroplasts is well established, that of the mammalian subunit c is debated.[158] These data emphasize the complexity of the Ca^{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. [120,159] Interestingly, we observed that the sense of rotation of F-ATP synthase strongly affects the threshold of Ca^{2+} required for PTP opening in intact mitochondria.[51,53] Because continuous ATP hydrolysis markedly reduces the Ca^{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., who demonstrated the existence of two classes of dimers characterized by different stalk-to-stalk distance in native IMM.[160]

It is well established that heart mitochondria of both slow- and fast-beating species possess sufficiently high levels of IF_1 relative to the F-ATP synthase to completely inhibit ATPase activity with an effect equivalent to the action of oligomycin.[124] 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_1, thus mitigating ATP depletion. Indeed, in ischemic hearts of dog,[161] goat[162] and rabbit[163] the loss of ATPase activity correlates with an increased IF_1 content. Down-regulation of F-ATP synthase activity is also observed in anoxic cardiomyocytes, although the IF_1 contents were not measured.[153] In HeLa and C2C12 cells transient IF_1 overexpression preserves ATP and protects from cell death induced by oxygen and glucose deprivation, a finding that would enlist IF_1 in prosurvival proteins.[143] Nevertheless, the response to cell injury seems to depend on the cell type, as IF_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_1 than astrocytes.[125] However, it is not yet clear how IF_1 expression is modulated. Two modulators seem to be implicated, i.e. hypoxia inducible factor 1α and the immediate early response gene X-1, the latter targeting IF_1 for degradation.[164,165]

IF_1-F_1 interactions are governed by the factors promoting IF_1 release and rebinding, i.e. membrane potential and MgATP. The existence of a dynamic equilibrium, in which binding

and release occur simultaneously involving the entire population of F-ATP synthase, has been demonstrated in energized ox heart mitochondria.[124] In addition, IF_1 binding is strongly favored by low pH when the inhibitory dimeric IF_1 is formed[166] so that in ischemia, when pH and membrane potential decrease, a new dynamic equilibrium is reached. In principle, the binding of IF_1 might also contribute to decrease the open probability of PTP during ischemia (Figure 2). Indeed, the crystal structure of the IF_1-F_1 complex from ox heart has shown that the initial low-affinity binding of IF_1 to the F_1 surface is followed by the final inhibitory binding in response to ATP hydrolysis, so that two Mg^{2+}-ADP molecules are contained in the F_1 catalytic sites of β_{DP} and β_{TP}.[167] Considering that Mg^{2+}-ADP is a PTP inhibitor, it is conceivable that the final inhibited IF_1-F_1 complex may be unable to properly bind Ca^{2+}, which is necessary for the switch of F-ATP synthase to the PTP. Intriguingly, the His reagent diethylpyrocarbonate, which restores the ability of mammalian mitochondria to undergo PTP opening at low matrix pH,[100,168] also prevents IF_1 binding at acidic pH.[169] On the other hand, the inhibited IF_1-F_1 complex does not contain Pi,[167] which is a PTP inhibitor at low concentrations, suggesting that IF_1 binding might mask an inhibitory site favoring, rather than preventing, PTP opening.

Although direct modulation of PTP by IF_1 remains an intriguing possibility that should be further explored, IF_1 could also affect PTP formation as a consequence of its role in ischemic preconditioning. It is well established that massive PTP opening - which occurs during reperfusion when membrane potential is restored and Ca^{2+} overload occurs - is prevented by short ischemic periods before long-lasting ischemia and reperfusion, a phenomenon called ischemic preconditioning. During ischemic,[162,170] as well pharmacological preconditioning,[171,172] IF_1 seems to mediate a peculiar, long-lasting ATPase inhibition in dog, goat and rat hearts (until 30–120 min 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[173]. As preconditioning results in sparing of ATP, lowering of membrane potential and Ca^{2+} accumulation, it may appear that IF_1 favors conditions that inhibit PTP formation during reperfusion. Moreover, based on the observation that the lack of IF_1 in Luft's disease is associated with uncoupled mitochondria,[174] long-lasting IF_1 binding during ischemia might maintain tight coupling supporting oxidative phosphorylation during reperfusion. Clarification of the molecular events involving IF_1 in preconditioning hold great promise for understanding preconditioning and for devising pharmacological strategies for cardioprotection.

Post-translational modifications of F-ATP synthase and PTP formation

Until recently, there were few reports regarding the PTMs of F-ATP synthase, which have been associated with specific biological function/processes in a very limited number of cases. Considerable information has been obtained about oxidative modifications of F-ATP synthase. Experiments with isolated mitochondria from bovine heart demonstrated that F-ATP synthase contains a set of thiols located in F_O (Figure 4), whose environment senses membrane energization and whose oxidation results in complete and reversible mitochondrial uncoupling.[175,176] 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.[175] This position would suggest involvement of the unique Cys

residue of subunit c, which is located near the Glu_{58} residue essential for proton translocation.[118] It is tempting to speculate that oxidation of this Cys residue may favor PTP formation in another part of F_O. 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.[177] In animal F-ATP synthase Factor B contains two vicinal Cys residues, whose oxidation leads to mitochondrial uncoupling.[178] However, Factor B is not present in yeast and *Drosophila melanogaster*, where the PTP is also modulated by SH reagents,[52,53,91] thus suggesting that Factor B is probably not involved in PTP formation. We showed that isolated F_1 from bovine heart F-ATP synthase is selectively inactivated by hydrogen peroxide through redox-active iron-protein adducts probably generating highly reactive oxygen species in proximity of the catalytic nucleotide binding sites.[179] Due to 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/nitrative stress associated with heart failure,[180] CNS disorders,[181,182] caloric restriction[183] and aging.[184,185] Specifically, the α subunit is S-nitrosylated in response to GSNO treatment of a mouse heart membrane fraction.[186] Moreover, in the same subunit Tyr nitration was increased following I/R of mouse heart[187] (Figure 5), suggesting that α subunit is actively involved in various oxidative modifications. Consistently, the formation of an inter-subunit disulfide bridge between αC251 and γC78 has been observed in canine dyssynchronous heart failure, which reverted following cardiac resynchronization therapy,[188] and disulfide formation was proposed to be involved in PTP activation.[95] However, these residues are quite 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 PT during reperfusion in spite of an impaired ability to accumulate Ca^{2+} 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/or PTP formation.[189] Moreover, a pharmaco-proteomic approach demonstrated that myocardial infarction in dogs caused several nitric oxide-related chemical modifications of d subunit, i.e., nitration of Y114 and hydroxylation of K71 (numbering according to PDB id 4b2q), followed by its myristoylation.[190] 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, which plays key roles in I/R, is a modulator of F-ATP synthase in cardiomyocites through direct binding to subunit d, which requires cardiolipin[191]. Subunit d also contains three Trp residues, which have been classified as "hot spots" for oxidation in human heart being found in normoxic conditions[192] (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 PTM concern Lys43 of the c subunit, which is completely trimethylated in all vertebrates and in the majority of invertebrates[118,119] (Figure

5). 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 F_1 sector[193] where they may be functionally relevant. The modified residues include αS184, αS419,[194] γY52[195] and γS121[196] (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[197] (Figure 5).

Conclusions

Identification of the F-ATP synthase as the most likely channel-forming element of the PTP[51–53,83] 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.[51–53] 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Non-standard abbreviations and acronyms

CsA	cyclosporin A
CyP	cyclophilin
IF$_1$	inhibitor factor 1
IMM	inner mitochondrial membrane
I/R	ischemia/reperfusion
MCU	mitochondrial Ca^{2+} uniporter
OMM	outer mitochondrial membrane
OSCP	oligomycin sensitivity conferring protein
PTM	posttranslational modifications
PTP	permeability transition pore

ROS	reactive oxygen species
TSPO	translocator protein of 18 kDa (TSPO)
VDAC	voltage-dependent anion channel

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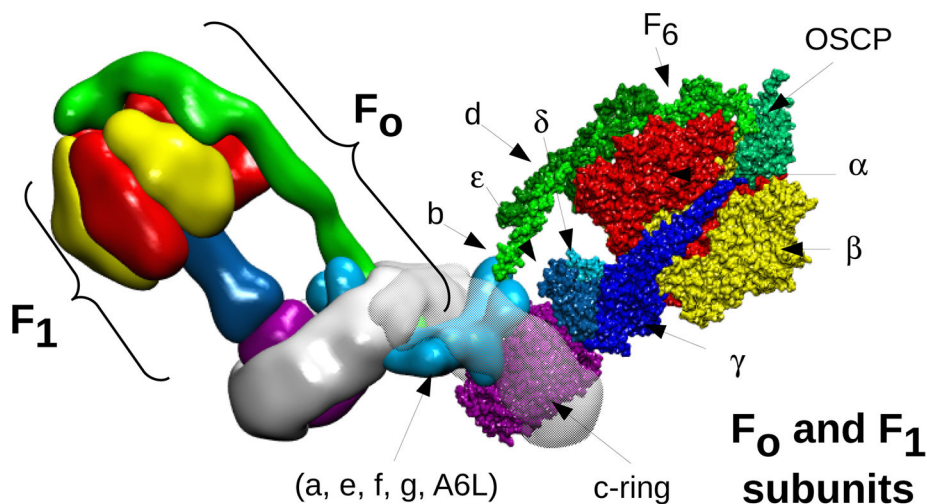
Figure 1. Structure of bovine F-ATP synthase

Left monomer, the F_1 and Fo sectors are highlighted. Right monomer, the F_1 and Fo subunits are shown. In the F_1 sector the front α and β subunits have been removed to reveal the F_1-rotor (central stalk). The F_1 α and β subunits are colored in red and yellow, respectively. The F_1-rotor γ, δ and ε subunits are colored in shades of blue, the peripheral stalk subunits b, d, F_6 and OSCP in shades of green, and the c-ring in purple. The remaining Fo subunits a, e, f, g, A6L, whose structure has not been defined yet, are located in the Fo subcomplex colored in light blue. The image (lateral view) has been built starting from the yeast dimer molecular model[133] (PDB id. 4b2q) and superimposing the cryo-electron microscopy map of bovine F-ATP synthase[141] (EMD id. EMD-2091). The fit of molecular models to cryo-electron microscopy map was performed using the program ADP_EM[198]. The molecular model for bovine F-ATP synthase was obtained by superimposing the 3D structure of the bovine F_1-c-ring complex (PDB id. 2xnd) onto each corresponding monomer of the yeast dimer. The superposition was performed using the swiss pdb viewer routine Iterative magic fit[199]. The lateral stalk was taken from the yeast dimer (PDB id. 4b2q) which contains the bovine subunits.
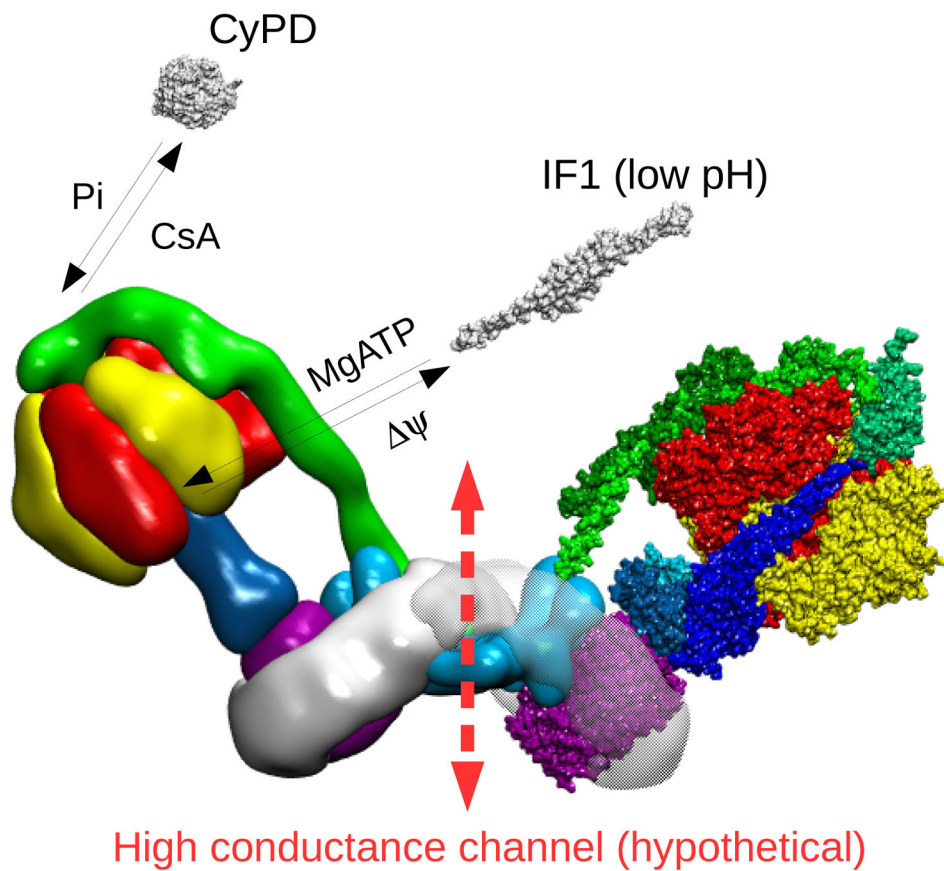
Figure 2. Interactors of F-ATP synthase and PTP formation

Reversible binding of CyPD and IF$_1$ 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.
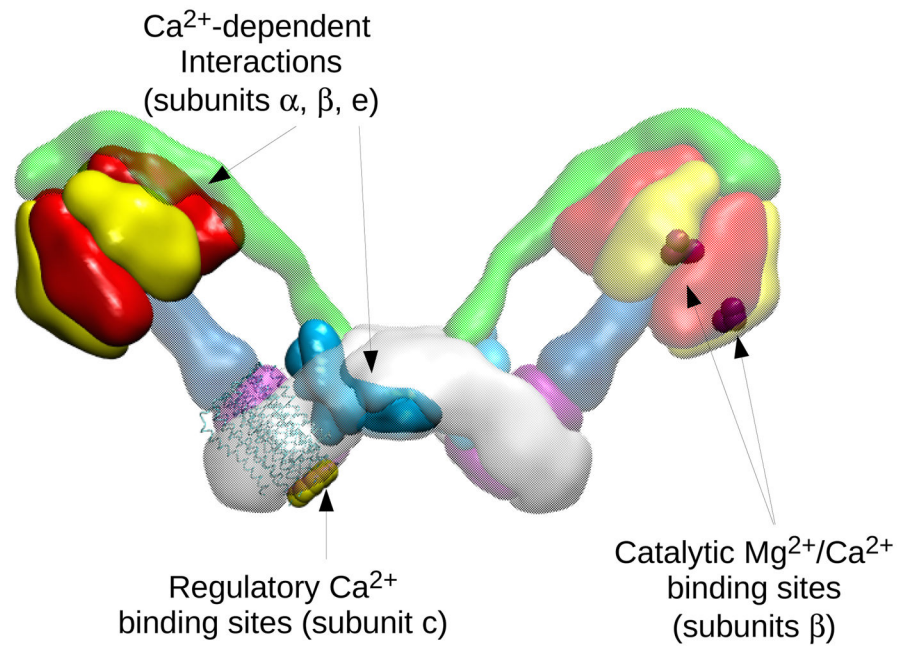
Figure 3. Map of Mg^{2+}/Ca^{2+} binding sites and Ca^{2+}-dependent interactions in bovine F-ATP synthase

Left monomer, the subunits involved in Ca^{2+}-dependent interactions are highlighted, i.e. subunits α and β, which interact with the matrix protein S100A1[154] (not shown in the picture), and the Fo region containing subunit e, which may interact with a hypothetical tropomyosin-like protein localized in the intermembrane space[155]. Ca^{2+}-regulatory sites located in the c-ring are also shown[156,157]. Right monomer, the residues (T163, R189, E192, D256) of β subunit interacting with the catalytic metal ions are mapped onto the 3D structure of the bovine F_1-c-ring complex.
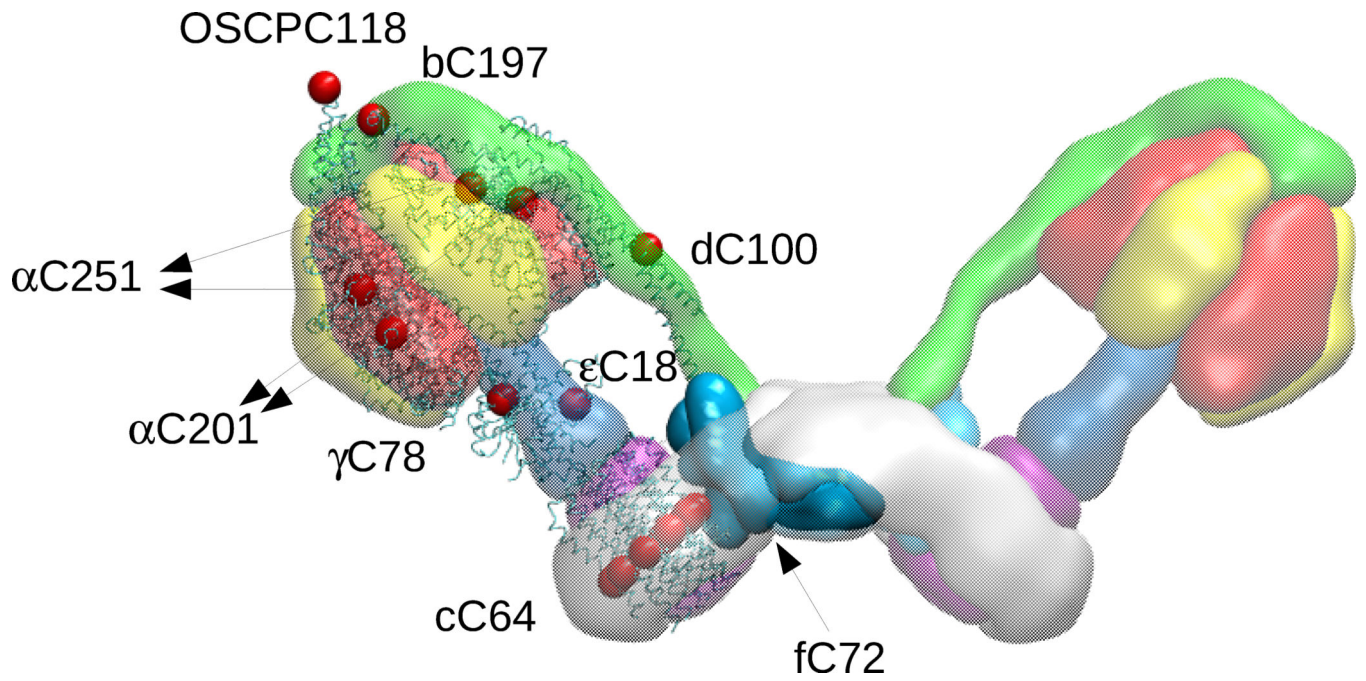
Figure 4. Map of Cys residues in bovine heart F-ATP synthase
Position of Cys residues on the specified subunits (red dots) is mapped onto the 3D structure of the bovine F_1-c-ring complex and of the bovine lateral stalk.
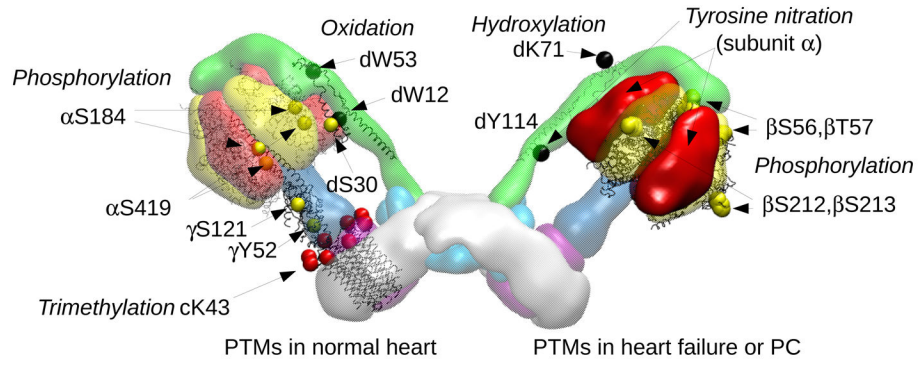
Figure 5. Posttranslational modifications of F-ATP synthase in the normal and failing heart
Left monomer, the residues involved in PTMs in normal heart are shown, i.e. complete trimethylation of cK43[118]; oxidation of W12, W53 of subunit d[192]; phosphorylation of αS184, αS419,[194] γY52[195] and γS121,[196] dS30.[193] Right monomer, residues involved in PTMs associated with preconditioning or heart failure are shown, i.e. phosphorylation of βS56, βT57, βT212, βS213[197]; nitration of d Y114 (numbering according to PDB id 4b2q) and hydroxylation of dK71[190]. Subunit α is also highlighted, being the target of tyrosine nitration[187].