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THE MITOCHONDRIAL PERMEABILITY TRANSITION PORE: CHANNEL FORMATION BY F-ATP SYNTHASE, INTEGRATION IN SIGNAL TRANSDUCTION, AND ROLE IN PATHOPHYSIOLOGY

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Bernardi P, Rasola A, Forte M, Lippe G. The Mitochondrial Permeability Transition Pore: Channel Formation by F-ATP Synthase, Integration in Signal Transduction, and Role in Pathophysiology. *Physiol Rev* 95: 1111–1155, 2015. Published August 12, 2015; doi:10.1152/physrev.00001.2015.—The mitochondrial permeability transition (PT) is a permeability increase of the inner mitochondrial membrane mediated by a channel, the permeability transition pore (PTP). After a brief historical introduction, we cover the key regulatory features of the PTP and provide a critical assessment of putative protein components that have been tested by genetic analysis. The discovery that under conditions of oxidative stress the F-ATP synthases of mammals, yeast, and *Drosophila* can be turned into Ca^{2+}-dependent channels, whose electrophysiological properties match those of the corresponding PTPs, opens new perspectives to the field. We discuss structural and functional features of F-ATP synthases that may provide clues to its transition from an energy-conserving into an energy-dissipating device as well as recent advances on signal transduction to the PTP and on its role in cellular pathophysiology.

I. INTRODUCTION

This review follows a previous *Physiological Reviews* article on the mitochondrial transport of cations and on the permeability transition (PT) (41). Progress in the field over the last 15 years has been astonishing, with the molecular identification of the Na^+/Ca^{2+} exchanger NCLX (447), of the essential component of the mitochondrial Ca^{2+} uniporter MICU1 (466), of the Ca^{2+} uniporter MCU itself (36, 152), and of the channel function of F-ATP synthases and their role in the PT (218). Channel formation by F-ATP synthase has been demonstrated in mammals (9, 218), yeast (96), and *Drosophila melanogaster* (611) and appears to be a novel property of the eukaryotic complex. The features of the F-ATP synthase channel in mammals (activation by Ca^{2+} and thiol oxidants, inhibition by Mg^{2+}/ADP, conductance properties) perfectly match those of the mitochondrial megachannel (MMC) (317, 475), which is the electrophysiological equivalent of the permeability transition pore (PTP) (569, 572, 573). While these findings leave little doubt that the PTP forms from the F-ATP synthase under conditions of oxidative stress (51), the mechanism of pore formation remains an open question (9, 218), as is the role of outer mitochondrial membrane (OMM) proteins in PTP modulation (51). In this review we cover regulation of both the PTP and of the F-ATP synthase and point to potential mechanisms that could turn the key enzyme of energy conservation into an energy-dissipating device. In addition, we discuss signaling pathways and posttranslational modifications that may affect pore formation and provide an update on the PTP in pathophysiology. It is our hope that the review will stimulate experiments eventually leading to a structural understanding of PTP formation.

A. Brief Historical Account

Increased permeability of the inner mitochondrial membrane (IMM) to solutes can be easily observed in isolated mammalian mitochondria, usually based on the onset of swelling (485). Stimulation by Ca^{2+} and coincidence of the permeability increase with loss of the ability to synthesize ATP has been recognized and studied very early (24, 26, 74, 100, 279, 354, 355, 390, 477, 485, 486, 582, 607, 636, 656). The term *permeability transition* was coined by Haworth and Hunter, who proposed that the permeabiliza-

tion process was dependent on the opening of a regulated channel (the PTP) and that the process was regulated and potentially reversible (263, 276–278). This idea did not gain much consensus, also because of the acceptance of the chemiosmotic hypothesis, which had just been recognized with the award of the Nobel Prize to Peter Mitchell in 1978 (414). It was widely felt that the presence of a large pore within the IMM would contradict the basic principles of chemiosmosis because its opening would disrupt the proton gradient and prevent the synthesis of ATP. Furthermore, the estimated pore radius of 1.4 nm (390), which is large enough to allow diffusion of ions and solutes with molecular masses up to about 1,500 Da, suggested that the permeability pathway was unspecific and could not be mediated by a protein (41). These concerns substantially contributed to the widespread view that the PT was an in vitro artifact rather than a process of relevance to pathophysiology (52).

This attitude was to change radically toward the end of last century following the key discovery that the PT is inhibited by cyclosporin (Cs) A (76, 134, 144, 194). CsA binds matrix cyclophilin (CyP) D, a peptidyl-prolyl *cis-trans* isomerase whose enzymatic activity is inhibited by CsA with a matching inhibition of the pore (121, 122, 432, 638). These discoveries gave great impulse to the field because they provided a protein regulator to the PTP and a drug to test its occurrence in cells and living organisms. Through the use of CsA, the occurrence of PTP opening in cell death (133) could be tested in a series of pioneering studies in hepatocytes subjected to oxidative stress (75, 285), anoxia (459), or treatment with ATP (662) as well as in cardiomyocytes (168) and isolated hearts (232) exposed to ischemia followed by reperfusion. The discovery that release of cytochrome *c* and of additional proteins of the intermembrane space is a key determinant for activation of the intrinsic pathway to apoptosis (165, 172, 266, 362, 373, 565) rapidly made the PT one of the most popular areas of mitochondrial research.

A second key finding was the demonstration that mitochondrial ion channels can be studied by electrophysiology (540) and that the IMM is endowed with the MMC, a high-conductance channel (317, 475) possessing all the basic regulatory features of the PTP (49, 569, 572, 573). Electrophysiology has greatly contributed to our understanding of the PTP (149, 150, 475, 569, 572, 573, 575) and to the recent demonstration that the PTP forms from F-ATP synthase (9, 96, 218). The reader is referred to a recent *Physiological Reviews* article for an update on the tremendous advances made in the field of mitochondrial ion channels (575).

A third important observation was that both the membrane potential and matrix pH modulate the probability of PTP opening (40, 433, 468), as confirmed at the single-channel level (664). Control by the proton electrochemical gradient provided a conceptual framework to reconcile the PTP with chemiosmosis (41). Since the threshold voltage for PTP opening is affected by many effectors (469, 470), this finding also allowed accommodation of many individual agents affecting the PT (44, 238, 239). Regulation of the PTP and its role in cell death have been the subject of intense and fruitful research over the last 30 years, as testified by nearly 5,000 publications. We refer the reader to a number of reviews for primary references that could not be cited here (41, 47, 48, 52, 112, 130, 131, 167, 217, 226, 233, 239, 240, 247, 252, 254, 313, 318, 357, 436, 438, 476, 534, 549, 558, 665, 667, 668). On the other hand, the structure of the PTP has long remained a mystery (51). Before discussing facts and hypotheses about its molecular nature, we present an overview of the key features of PTP regulation. This overview is mostly based on the features of the pore in mammalian mitochondria, but we will also mention relevant data on the PTP of *Drosophila* and yeast mitochondria with the important proviso that multiple conductance pathways may exist in yeast (see Refs. 381, 483, 484, 506, 595).

B. Consequences and Regulation of the Permeability Transition

The consequences of PTP opening depend on the open time of individual pores and on the number of open pores per mitochondrion at any given time. The complex relationship between channel kinetics and population dynamics has been discussed in detail (41) and will only be briefly summarized here. The PTP flickers between closed and open states both at the single channel and at the organelle level (317, 471, 475, 569, 572), but occurrence of transient, reversible openings can go undetected in a population of mitochondria unless openings are synchronized or individual mitochondria are monitored. Measurements of the membrane potential resolved in individual mitochondria indeed demonstrated transient and asynchronous cycles of depolarization-repolarization, which over time tended to become long-lasting and were accompanied by permeabilization to calcein (281). Both reversible and long-lasting depolarizations could be traced to PTP opening (281). Conditions have been described under which the mammalian PTP undergoes synchronized cycles of opening-closure and is permeable to ions but not sucrose, suggesting a lower conductance state (284). Importantly, the *Drosophila* PTP is permeable to H^+ and Ca^{2+} but not to sucrose (610), consistent with its lower conductance of 53 pS (611).

In isolated mitochondria PTP opening is affected by a large variety of compounds that act either as inducers (like thiol oxidants) or inhibitors (like Mg^{2+}, adenine nucleotides and acidic matrix pH; see Ref. 239 for an exhaustive list). "Induction" must be intended in the sense that the lag phase between accumulation of Ca^{2+} (which is an essential permissive agent for pore opening) and onset of permeabilization decreases, with a matching increase of the rate of

spreading of the process through a mitochondrial population. Conversely, "inhibition" means that the lag phase between Ca^{2+} accumulation and onset of permeabilization increases, with a matching decrease of the rate of spreading of the process through a mitochondrial population (41). Since no true blockers of the PTP are known, the effect of inhibitors is best described as desensitization, and that of inducers as sensitization (52).

When the PTP open probability increases, depolarization can be measured both in intact mitochondria and in situ (473). Collapse of the proton gradient prevents ATP synthesis as long as the pore is open, and ATP hydrolysis by the F-ATP synthase worsens ATP depletion. The consequences of the PT on respiration in vivo, and the related production of reactive oxygen species (ROS), depend on the extent of pyridine nucleotide depletion (52). In mammalian mitochondria, increased probability of PTP opening is also followed by equilibration of ionic gradients and solutes, which may cause swelling, cristae unfolding, and eventually OMM rupture (48).

PTP opening may also contribute to selective release of cytochrome *c* with an intact OMM. Two pools of cytochrome *c* can be identified in isolated rat liver mitochondria (42). About 15% of total cytochrome *c* can be reduced by added NADH (42) through the OMM rotenone-insensitive NADH-cytochrome b_5 reductase (543), suggesting that this pool resides in the intermembrane space; while 85% can only be reduced by electrons fed by the IMM electron transfer chain, suggesting that most cytochrome *c* resides within the intracristal compartments (196). After proapoptotic stimulation, PTP-dependent, CsA-sensitive cristae remodeling occurs with widening of cristae junctions and increase of the fraction of cytochrome *c* that can be released through OMM Bax/Bak channels (524). Thus the Bax/Bak-dependent and PTP-dependent pathways for cytochrome *c* release are synergistic rather than mutually exclusive (47).

1. Matrix effectors

Divalent cations are key to PTP regulation. As already mentioned, the PT requires matrix Ca^{2+}; although Ca^{2+} alone may not be sufficient to trigger a PT, it is an essential "permissive" factor (41). The threshold Ca^{2+} load required for PTP opening varies with the experimental conditions, in particular the presence of P_i (see below). Matrix Mg^{2+} desensitizes the PTP, and the effect is synergistic with that of adenine nucleotides. The desensitizing effect is also seen with other divalent cations (such as Sr^{2+} and Mn^{2+}) that are transported by the MCU and therefore are taken up in energized mitochondria. It appears likely that Ca^{2+}, Mg^{2+}, Sr^{2+}, and Mn^{2+} compete for the same binding site(s) (49). A second binding site for divalent cations is accessible from the intermembrane space (i.e., it does not require cation uptake) and mediates PTP desensitization with a K_i of ~0.2 mM with all divalent cations tested (49).

The PTP is extremely sensitive to oxidation-reduction events. Pore opening is promoted by oxidation of matrix pyridine nucleotides and dithiols, and by treatment with dithiol reagents like phenylarsine oxide and arsenite (470). The inducing effects can be individually reversed with appropriate reducing agents (124) and can be blocked by 1-chloro-2,4-dinitrobenzene, suggesting involvement of matrix glutathione (107). It appears likely that oxidation of critical PTP thiols mediates the inducing effects of peroxides and redox-cycling agents, which are indeed prevented by low concentrations (K_i ~5 μM) of *N*-ethylmaleimide (NEM) and monobromobimane (123, 470). PTP modulation by these redox-sensitive sites could also be the basis for the inducing effects of p66Shc, which oxidizes intermembrane cytochrome *c* producing superoxide anion (215, 590).

The PTP open probability increases with electron flux within complex I (191), and this finding led to the discovery that the PT is regulated by quinones, possibly through a specific binding site (615). Ubiquinone 0 or decylubiquinone prevent pore opening with all tested inducing agents, and their inhibitory effects (unlike those of CsA) can be relieved by pore-inactive quinones (615). It appears likely that PTP modulation by complex I is mediated, in part at least, by production of ROS. Indeed, oxidation of succinate, which induces reverse electron flow at complex I (548), greatly favors ROS production and PTP opening, both events being prevented by rotenone (361).

The PT is modulated by matrix pH. In de-energized mitochondria, the optimum for opening was at matrix pH 7.4, with a decrease both below and above this value (433). Inhibition by acidic pH occurs through reversible protonation of His residues that can be blocked by diethylpyrocarbonate (433, 525), while the basis for inhibition above pH 7.4 remains unknown. His126 of CyPA plays an important role both in ligand binding and catalysis (652), but PTP modulation by matrix pH was not affected by genetic ablation of CyPD (i.e., in *Ppif*$^{-/-}$ mice), demonstrating that the PTP-regulatory His are not located on CyPD (34). It should be mentioned that the PTP can also be affected by pH indirectly through compounds whose accumulation is affected by pH. For example, in energized mitochondria, an acidic external pH can promote rather than desensitize the PTP because it increases the rate of P_i uptake, worsening PTP opening and tissue damage in ischemic and postischemic acidosis (337). PTP regulation by matrix CyPD and CsA will be discussed specifically in an upcoming section.

P_i is the most puzzling PTP effector. In spite of the fact that increasing concentrations of P_i decrease matrix free [Ca^{2+}] (661), in mammalian mitochondria P_i acts as an inducer (239). Interestingly, P_i instead desensitizes the PTP of yeast and *Drosophila melanogaster*, species where the PTP is in-

sensitive to CsA (96, 236, 297, 507, 508, 610, 647). We found that P_i can inhibit the PTP in CyPD null mouse liver mitochondria (35), suggesting that the inducing effects of P_i may depend in part at least on CyPD binding, a mechanism that is supported by recent data on the Pi-dependent interaction of CyPD with the F-ATP synthase subunit oligomycin-sensitivity conferring protein (OSCP) (216, 218). It is possible that some of the in situ effects of P_i are mediated by formation of polyphosphate, which has been shown to be a potent activator of the PTP (3, 463, 530).

2. CsA and CyPD

As mentioned above, the PTP is modulated by CsA (76, 194, 253), a powerful immunosuppressive agent that targets CyPs, a class of ubiquitous proteins endowed with peptidyl prolyl *cis-trans* isomerase activity (189, 464, 579). Sixteen isoforms of CyPs have been found in humans; the most abundant is cytosolic CyPA (619). The enzymatic activity of all CyPs is inhibited by CsA (66), and the CsA/CyPA complex inhibits the cytosolic phosphatase calcineurin (371). As a result, NFAT is no longer dephosphorylated, an event that prevents its nuclear translocation causing immunosuppression (117, 614). It should also be mentioned that calcineurin inhibition prevents translocation of the pro-fission protein Drp-1 to mitochondria, an effect that does not relate to that of CsA on the PTP (98). Mammals possess a unique mitochondrial species called CyPD, which in the mouse is encoded by the *Ppif* gene (see Ref. 217 for a review). CyPD is the mitochondrial target for CsA and modulates the PTP by decreasing the Ca^{2+} load required for pore opening, an effect that is prevented in the presence of CsA (253). We, and others, successfully created $Ppif^{-/-}$ mice and examined the properties of the PTP in mitochondria lacking this protein (27, 34, 426, 518). CyPD-null mice showed no overt phenotype and no obvious changes in mitochondrial function. As expected, mitochondria from these mice lacked CyPD protein and were desensitized to Ca^{2+}, as opening of the PTP required about twice the Ca^{2+} load necessary in strain-matched, wild-type mitochondria (27, 34, 426, 518). In other words, the PTP in CyPD-null mitochondria is desensitized, and its opening requires higher Ca^{2+} levels that match those of wild-type mitochondria treated with CsA. As would be predicted, mitochondria lacking CyPD were insensitive to CsA (27, 34, 426, 518). Other than for the requirement of higher Ca^{2+} loads, the PTP response to a variety of modulators was similar in mitochondria from wild-type and CyPD null mice, thereby demonstrating that CyPD has all the key aspects of a PTP modulator but is not an essential structural component of the PTP (27, 34, 426, 518). Consistently, the electrophysiological features of the PTP from CyPD-null mitochondria are indistinguishable from those of wild-type individuals (149). Importantly, largely through the use of CyPD-null mice, a wide variety of murine models of human degenerative diseases were shown to have their basis in mitochondrial pathogenesis and misregulation of the PTP (27, 166, 193, 200, 383, 410, 426, 445, 454, 518, 623).

CP3 is the *Saccharomyces cerevisiae* mitochondrial CyP isoform, but its genetic ablation does not affect the PT, indicating that it does not regulate the PTP in this species (96). The *Drosophila melanogaster* genome encodes 14 different CyPs (464). Of these, CyP1 has an NH_2-terminal sequence that according to Mitoprot (115) confers high probability of mitochondrial import. Yet, full sequence analysis led to the conclusion that no mitochondrial CyP is present in this species (464). Consistent with this conclusion and the above data on yeast mitochondria, yeast and *Drosophila* PTPs are insensitive to CsA (297, 610).

3. Inner membrane

The inside-negative membrane potential tends to stabilize the PTP in the closed conformation, while depolarization favors pore opening (40). We have proposed that changes of both the transmembrane voltage and of the surface potential are decoded into changes of the PTP open probability by a voltage sensor (469) that may comprise critical Arg residues, as suggested by modulation of the PTP voltage dependence by Arg-selective reagents (175, 289, 368). This mechanism can explain pore opening by H^+ and K^+ currents (40, 527) and the effects of membrane-perturbing agents (77, 469) like amphipathic anions (which favor the PT) (465, 469, 526), polycations (343), amphipathic cations (77), and positively charged peptides (500) (which inhibit pore opening) (see Ref. 44 for review). We note that modulation by the surface potential could also account for the effects of atractylate (80) and bongkrekate (268), both ligands of the IMM adenine nucleotide translocase (ANT). Both compounds inhibit the ANT yet have opposite effects on pore opening, which is favored by atractylate and inhibited by bongkrekate (276). This set of observations was one of the bases for the proposal that the PTP forms from the ANT (637), an issue on which we shall return later in the review. Here we would like to observe that transition of the very abundant ANT from the "m" (bongkrekate-bound) to the "c" (atractylate-bound) conformation is accompanied by major structural rearrangements (378) that significantly perturb the surface potential (505, 516), which in turn may affect the PTP open-closed transition through the voltage sensor (see Ref. 44 for discussion).

4. Outer membrane

There is no doubt that the PT is primarily an IMM event, as it also occurs in mitoplasts, i.e., mitochondria stripped of the OMM (536). Yet, the OMM plays a role in pore modulation, as indicated by two sets of observations. The first is that PTP induction by rather high concentrations (0.5–1.0 mM) of NEM (478) and other substituted maleimides re-

quires an intact OMM (347). The effect of NEM is due to secondary oxidation of thiol groups that can also be triggered by copper-ortho-phenantroline (125). We have fully confirmed that PTP opening by NEM is abolished in mitoplasts, and thus that its inducing effect depends on OMM proteins (535). We have demonstrated the "sensitizing" role of the OMM in a second paradigm, where the PTP is modulated by dicarboxylic porphyrins plus irradiation with visible light, a treatment that leads to the production of singlet oxygen (499). Low doses of light inactivate the PTP through degradation of His residues, which in turn prevents matrix Cys oxidation (511). In contrast, higher doses of light activate the PTP through the direct oxidation of Cys residues in the OMM (474). The inducing effect of hematoporphyrin plus high light doses was completely lost in mitoplasts, indicating that it requires an intact OMM (536). Largely based on the effect of its ligands on the PTP, we, as well as others, had proposed that the protein responsible for PTP sensitization was the peripheral benzodiazepine (Bz) receptor, an OMM protein today called TSPO (101, 181, 319, 334, 399, 458, 536), but based on recent experiments on TSPO-null mice, this conclusion turned out to be incorrect (535). The target of NEM and of photooxidative stress thus remains unknown, but a potential candidate is Abcb6, an ATP binding cassette transporter of the OMM involved in heme and porphyrin homeostasis (335).

5. Models for pore formation

In 1993, Kinnally et al. (319) reported that nanomolar concentrations of several ligands of TSPO affected the channel properties of the MMC. These ligands included Ro5-4864, PK11195, and protoporphyrin IX, one of the most powerful inducers of the PTP (458). Independent work from the Snyder laboratory had shown that TSPO copurified with the ANT and the OMM voltage-dependent anion channel (VDAC) in protocols based on detergent extraction followed by hydroxylapatite chromatography. In these studies, radiolabeled high-affinity ligands were recovered in fractions where TSPO could be detected together with VDAC and ANT (399). These data suggested that formation of the PTP could involve the OMM proteins TSPO and VDAC, and the IMM protein ANT.

A few years later, this suggestion was strengthened by the Brdiczka laboratory, who were characterizing OMM and IMM "contact sites," specialized structures where the two membranes form close contacts mediated by protein-protein interactions (329). These dynamic sites would include hexokinase (HK) bound to the cytosolic surface of the OMM, VDAC within the OMM, creatine kinase and nucleoside diphosphate kinase in the intermembrane space, and ANT in the IMM. Contact sites were proposed to mediate channeling of adenine nucleotides to and from mitochondria, thus limiting the need for its diffusion (7, 83, 329). HK-enriched fractions from low-detergent extracts of mitochondria also formed channels with the conductance expected of the PTP (55). These fractions were not enriched in VDAC and/or ANT (55) and contained an extremely large number of proteins including members of the Bcl-2 family (387), which makes assignment of the channel activity to a specific species quite problematic. Furthermore, and unlike the case of PTP, currents were inhibited rather than induced by atractylate (55). This set of observations led to a model where the PTP would be a multiprotein complex spanning both mitochondrial membranes and comprising ANT, VDAC, TSPO, CyPD, as well as HK and Bcl-2 proteins (653).

As we will review more in detail below, this model did not stand the test of genetics as a CsA-sensitive PT could be easily detected in the absence of ANT (325), VDAC (28, 333), as well as of TSPO (535). Furthermore, mitochondria from the brine shrimp *Artemia franciscana* do not undergo a PT despite the presence of ANT, VDAC, and CyPD (406). An alternative model has been proposed where the PTP is formed by the P_i carrier (PiC) following its interaction with CyPD and ANT (359). However, results obtained by patch-clamp analysis of the reconstituted PiC do not match the electrophysiological PTP features (269), and genetic deletion of PiC does not support the idea that this protein is essential for PTP formation (339).

He and Lemasters (265) have proposed a model where the PTP would originate from misfolded membrane proteins that have been damaged by various forms of stress, which is similar to an earlier suggestion that the PT is due to oxidative damage of membrane proteins rather than a consequence of the opening of a preformed pore (330). Conductance through misfolded protein clusters would be normally blocked by chaperone-like proteins like CyPD. When the number of these protein clusters exceeds available chaperones opening of "unregulated" pores would occur, which would no longer be sensitive to CsA (265). The model accounts for both CsA-sensitive and -insensitive pores, as well as for the lack of selectivity of the permeability pathway. On the other hand, it is hard to see how a permeability increase mediated by denatured proteins would be regulated by voltage and matrix pH.

Ca^{2+}-dependent, PT-like activities have also been observed in reconstituted systems with both fatty acids (413, 560) and 3-hydroxybutyrate/polyphosphate (3, 463, 530), suggesting that channels can also form in the absence of specific proteins. Lipids may participate in PTP formation and regulation as shown by earlier work from the Pfeiffer laboratory, who suggested that fatty acids produced by activation of Ca^{2+}-dependent phospholipases profoundly affect the PTP and its sensitivity to CsA (77, 478).

A novel mechanism for PTP formation was recently proposed by our laboratories and first presented at the 17th European Bioenergetics Conference in 2012 (45). To iden-

tify the binding partners of CyPD, we monitored its associations to mitochondrial proteins after low-detergent extraction followed by protein separation by blue native gel electrophoresis. We found that CyPD comigrates with the F-ATP synthase, and the interaction was confirmed by immunoprecipitation of complex V followed by Western blotting (216). Binding of CyPD to the F-ATP synthase required P_i and caused a decrease of the enzyme's catalytic activity. This decrease was counteracted by CsA, which displaced CyPD and increased the catalytic activity (216). Cross-linking experiments with bifunctional reagents indicated that CyPD interacts with the lateral stalk subunits b, d, and OSCP (216). We could then immunoprecipitate each subunit individually and demonstrate that CyPD interacts with the OSCP subunit, from which it can be displaced by Bz-423 (218). Bz-423 was originally characterized as an apoptosis-inducing agent acting through mitochondria (59). OSCP was identified as its target through the unbiased screening of a human phage display library, and it was shown that Bz-423 is an inhibitor of the F-ATP synthase (290, 291, 551), an activity that is shared by several ligands of TSPO including PK-11195 (116). The demonstration that Bz-423 induces the PTP, and that its binding site on helices 3 and 4 of OSCP may coincide with that of CyPD (218) paved the way to the demonstration that F-ATP synthase can form channels with the features of the PTP-MMC.

Gel-purified dimers of F-ATP synthase from bovine heart were incorporated into lipid bilayers. Following treatment with Ca^{2+}, Bz-243 and phenylarsine oxide, a vicinal dithiol cross linker that is a powerful PTP inducer (49, 358), opening of channels with a unit conductance of ~500 pS was observed, which is compatible with that of the bona fide mammalian PTP-MMC (218). Monomers of F-ATP synthase, which have the same subunit composition as the dimers (588), were instead devoid of channel activity (218). The MMC has been consistently described as a multiconductance channel exhibiting many substates and a typical maximal (fully open) conductance of 0.9–1.0 nS, although a value of ~1.3 nS in symmetrical 150 mM KCl can also be observed (475, 572). Electrophysiological studies have produced clear evidence for a binary structure of the pore, which displays a prominent half-conductance level (374, 569, 574). A channel of 0.4–0.5 nS can also be observed whose frequency of appearence is inversely related to that of the higher "full conductance" MMC, and which may correspond to one-half of the MMC (151, 663). Based on these data, we suspect that the 500-pS current observed in our study with purified F-ATP synthase (218) is mediated by dimers and that the full conductance in the native IMM originates from F-ATP synthase tetramers.

Channels were inhibited by Mg^{2+}/ADP and by the F-ATP synthase inhibitor γ-imino ATP (a nonhydrolyzable ATP analog). Like the MMC of *Ppif*$^{-/-}$ mitochondria (149), and in keeping with the lack of CyPD in the preparations, channels were insensitive to CsA. Consistent with the absence of ANT, channel openings could not be induced by atractyloside and were still observed in the presence of bongkrekic acid (218). The channel-forming property is shared by purified F-ATP synthase dimers of mitochondria from *Saccharomyces cerevisiae*, which displayed Ca^{2+}-dependent currents of ~300 pS (96), and from *Drosophila melanogaster*, in which the conductance is 53 pS (611) in keeping with earlier results on solute permeation (610). Channel formation by F-ATP synthase has been confirmed in human cells (9) and is supported by a study where the c subunit was downregulated by siRNA in HeLa cells, which resulted in PTP inactivation (64). Potential mechanisms for PTP formation from F-ATP synthase is discussed in section III, while **TABLE 1** summarizes the key features of the PTP of *Saccharomyces cerevisiae*, *Drosophila melanogaster*, and mammals.

C. Genetic Analysis of Putative Pore Components

As already mentioned, the hypothesis that the PTP is composed by ANT (and/or PiC) in the IMM and VDAC in the OMM, plus a variety of modulators including TSPO (653), has been put to a rigorous test by genetic inactivation of its putative components. The rationale is simple: inactivation of the gene(s) encoding key structural element(s) should eliminate PTP activity while genetic inactivation of a modulator should allow the formation of the pore, albeit with altered functional characteristics that may suggest a specific role for each modulator. As discussed below, the results leave little doubt that ANT, PiC, VDAC, and TSPO are not essential for PTP formation. Genetic analysis of F-ATP synthase subunits will be discussed after description of its structure and function.

Table 1. *Properties of the F-ATP synthase channel across species*

	Conductance, pS	Matrix Ca^{2+}	Matrix Mg^{2+}/ADP	P_i	CsA	Matrix CyP
S. cerevisiae	300	Activates	Inhibits	Inhibits	No effect	Yes
D. melanogaster	53	Activates	Inhibits	Inhibits	No effect	No
Mammals	500	Activates	Inhibits	Activates	Inhibits	Yes

The conductance of F-ATP synthase channels and the effects of some PTP effectors in the indicated organisms are summarized. CsA, cyclosporin A; CyP, cyclophilin.

1. Adenine nucleotide translocator

In respiring mitochondria, the ANT, the most abundant IMM protein, catalyzes efflux of ATP from the mitochondrial matrix and uptake of ADP into the matrix. Binding of the ANT to a CyPD affinity matrix has been reported (135, 637), but the relevance of this observation to PTP regulation remains unclear because CyPD bound equally well the ANT purified from rat liver or yeast (637) in spite of the fact that in yeast mitochondria the PT is not regulated by CyP or inhibited by CsA (96, 297). ANT from bovine heart mitochondria reconstituted in liposomes does exhibit high-conductance channel activity that is stimulated by Ca^{2+} and insensitive to CsA (81). Like the MMC, the channel displays prominent voltage-gating effects (81) with conductance ranging between 50 and 700 pS in 100 mM KCl (81) and low probability of current fluctuation at voltages lower than 150 mV. Unlike the MMC, however, currents could only be inhibited by ADP and bongkrekate together, while ADP alone had a marginal effect (73, 81, 82).

The Wallace laboratory conditionally inactivated the liver *Ant2* gene in an *Ant1*$^{-/-}$ background, which generated mice with ANT1/ANT2-deficient liver mitochondria where respiration could not be stimulated by ADP (325). These mitochondria underwent CsA-sensitive PTP opening, but required a higher matrix Ca^{2+} load. The PT was inhibited by CsA and stimulated by H_2O_2 and diamide, indicating that the ANT is not the site of action of these oxidants or the relevant partner for CyPD binding; of note, the PTP was resistant to atractylate and ADP (325).

The survival of animals lacking all ANT isoforms is unexpected (249), and the main conclusion of this study has been challenged (65, 250, 254). Sequence analysis has indeed shown that, besides the genes encoding for ANT1 and -2, in most mammals two additional isoforms are present, SLC256A (ANT3) and SLC25A31 (ANT4). On these grounds, most recent reviews have called into question the idea that mitochondria prepared from ANT1/ANT2-null mitochondria were devoid of any ANT isoform, justifying its continued inclusion as a key structural element of the PTP (e.g., Refs. 65, 250). However, analysis of the mouse genome database (e.g., http://www.informatics.jax.org/) demonstrates that mice (and all rodents) lack a gene for ANT3 and hence can only express ANT1, ANT2, and ANT4. Moreover, ANT4 appears to be expressed specifically in germ cells and may be primarily compartmentalized to the sperm flagellum in all mammals (314, 364). One possible explanation would be the compensatory (mis)targeting of alternate isoforms normally directed to restricted locations (250, 251). While the liver may not normally express appreciable levels of ANT4, in this unique situation the viability of mice missing ANT1 and ANT2 may depend on higher levels of ANT4 in the liver (203). Even in this scenario, it is very difficult to see *1*) how these putative ANT molecules would not transport adenine nucleotides yet promote a CsA-sensitive PT (325), and *2*) why these putative pore-forming ANT4 molecules would not respond to atractylate and ADP (325), given that in wild-type mitochondria ANT4 is fully sensitive to both carboxyatractylate and bonkgrekate (163). Thus all available evidence points to the fact that ANT can modulate the PTP, possibly through its effects on the surface potential (44), but is not a core and required structural component.

2. Phosphate carrier

It has been pointed out that PTP formation in mitochondria missing major ANT isoforms could be due to its compensation by other members of the mitochondrial carrier family, PiC in particular (250). The PiC of the IMM is encoded by a single gene in mammals and is critical for ATP synthesis by serving as the primary means for mitochondrial P_i transport across the IMM. While long suspected as playing a role in PTP formation (e.g., Ref. 332), interest in the PiC was renewed following the realization that antibodies critical for the earlier studies on the ANT outlined above were, in fact, primarily directed toward the PiC (360). Additional biochemical studies demonstrated the CsA-sensitive binding of CyPD to the PiC and its modification by chemical reagents (e.g., by phenylarsine oxide and NEM) correlated with PTP opening and inhibition. These and a suite of additional biochemical studies led to the idea that PiC may be a key component of the PTP that undergoes a Ca^{2+}-induced change in conformation to induce pore formation in cooperation with the ANT (250, 360). Consequently, in addition to the ANT, recent models of the PTP also include PiC (e.g., Refs. 65, 228, 254). Genetic tests of the role of PiC in PTP formation initially involved the application of siRNA techniques in either mammalian cells or transgenic animals (242, 601). In both situations, mitochondria lacking up to 60% PiC expression showed no alteration in PTP function, and no effects on PTP activity were observed after overexpression of PiC (242, 601). Furthermore, in mitochondria prepared from cells where the gene encoding PiC had been genetically inactivated (resulting in over 90% reduction in PiC levels), the PTP could still form and displayed marginally reduced sensitivity to activators (339). Finally, patch-clamp experiments with the reconstituted, functionally active PiC revealed an anion channel function with a mean conductance as low as 40 pS which was further decreased to 25 pS by Ca^{2+} and Mg^{2+}, inhibited by P_i, and unaffected by ADP (269). Taken together, these experiments indicate that PiC, like the ANT, cannot constitute a key structural element of the PTP.

3. Voltage-dependent anion channels

VDAC is a major OMM protein which functions as a general diffusion pore for small hydrophilic molecules; in mammals, distinct genes encode three variants of the protein,

VDAC1, -2, and -3 (487). VDAC1 displays channel activity that is similar to that of the PTP (571, 574). As already mentioned, because of its presumed preferential location at sites of interaction between the OMM and IMM (132) and of its copurification with TSPO and ANT, VDAC has been included in traditional models of the PTP (65, 254). The putative role of VDACs as a structural element or regulator of the PTP has been tested by genetic analysis. A thorough study of *Vdac1*$^{-/-}$ mitochondria lacking the major isoform VDAC1 demonstrated that the properties of the PTP (response to Ca^{2+} and P_i, sensitivity to oxidants and NEM, inhibitory profile with CsA and quinones) were identical to those obtained from wild-type mice of matched genetic background, indicating that VDAC1 is fully dispensable for PTP formation (333). Similar results were obtained with VDAC1/3 null mitochondria, while whether VDAC2 could compensate for the absence of VDAC1 and/or 3 was more complex to assess because ablation of *Vdac2* results in embryonic lethality (106). This result is likely the consequence of the fact that VDAC2, while serving as a diffusion pore in the OMM like all VDACs, is also a potent inhibitor of the pro-apoptotic effects of OMM Bak (106). As a result, in the absence of VDAC2, Bak is unrestricted in its ability to induce cell death, likely leading to early embryonic lethality. To assess the role of VDAC2 in PTP formation, MEFs prepared from *Vdac1*$^{-/-}$/*Vdac3*$^{-/-}$ mice were treated with VDAC2 siRNAs, which did lead to a decrease of VDAC2 protein levels to 2% of normal, but to no alteration in PTP onset and properties (28). As a result, there is currently no evidence, either biochemical or genetic, that supports a role for VDAC either as a structural element of the PTP or as a key modulator. Consequently, models that imply a role for VDAC in PTP formation must be called into question (65, 254). Regulation of the PTP through binding of HK to OMM will be discussed in section IV.

4. TSPO

TSPO was initially identified as an OMM protein that bound a series of Bz analogs that do not target Bz receptors in the central nervous system. Hence, it was initially referred to as the peripheral Bz receptor (451, 510). Early studies appeared to demonstrate that TSPO is intimately involved in two separate functions, mitochondrial transport of cholesterol and protoporphyrin IX, and PTP regulation. In cells that produce steroid hormones (e.g., in the adrenal cortex), TSPO was thought to promote the transport of cholesterol into the mitochondrial matrix, the rate-limiting step in steroid synthesis (411). However, the ubiquitous expression of TSPO suggested that it could serve more general functions, one of which was long thought to be the regulation of PTP activity. TSPO was initially linked to PTP function based on its association with other proteins thought to be required in traditional models of the PTP (i.e., VDAC and ANT) (319, 399, 458). In addition, experiments with Bz specifically targeting TSPO suggested that its ligands promoted the opening of the PTP, a view extended by recent studies (53, 101). However, important aspects of these studies remained confusing (for details, see Ref. 535). Ensuing studies have employed natural and synthetic ligands to assess the role of TSPO function in a number of natural and pathological circumstances. Largely through the use of these compounds and biochemical associations, TSPO was proposed to play a role in the PTP activity associated with cell death in many human pathological conditions. Since initial studies suggested that nonconditional inactivation of the nuclear gene encoding TSPO resulted in embryonic lethality (450), we assessed the role of TSPO in PTP function through the generation of mice in which the *Tspo* gene had been conditionally eliminated (535). These studies demonstrated that TSPO plays no role in the regulation or structure of the PTP (535). Consequently, we suspect that endogenous and synthetic ligands of TSPO regulate PTP activity because of their direct effects on the F-ATP synthase (116), as demonstrated for Bz-423 (218). Consistent with this conclusion, hearts lacking TSPO are as sensitive to damage caused by PTP opening following ischemia-reperfusion injury (see below) as are hearts from wild-type mice, in contrast to mice missing CyPD (27, 426). Consequently, OMM regulation of PTP activity must occur though a mechanism that does not require TSPO and is based on proteins that have yet to be identified. These results call into question a wide variety of studies implicating TSPO in a number of pathological processes through its actions on the PTP, and the validity of placing TSPO in recent representations of the structure of the PTP (65). Interestingly, in a separate study of conditional *Tspo* mice, the role of TSPO role in cholesterol metabolism has also been questioned (423, 591); consequently, the precise OMM function of TSPO has yet to be established.

5. Bcl-2 family members

Apoptosis regulators of the Bcl-2 family are evolutionarily related molecules that govern mitochondrial OMM permeabilization and can be either pro-apoptotic (e.g., Bax, Bad and Bak) or anti-apoptotic (e.g., Bcl-2 proper and Bcl-xL) (578). Genetic tests of the requirement of members of this family in the formation and regulation of the PTP have been carried out with pro-apoptotic Bax, Bak, and Bad. In mitochondria from MEFs in which genes encoding Bax and Bak have been genetically inactivated, OMM permeability decreased without significantly altering PTP function in the IMM (306). Indeed, physiological analysis of IMMs and biochemical studies showed no difference between wild-type and Bax/Bak-null mitochondria while the PTP was still inhibited by CsA. Thus Bax and Bak do not directly regulate IMM aspects of the PTP. In addition, studies on MEFs missing the BH3-only family member Bad have indicated that the basic characteristics of the PTP are no different in Bad-null cells, but they appear sensitized to various stress factors such as ceramide (537). These stress factors are proposed to lead to a complex cascade of kinase/phosphatase reactions that enable productive association, and inactiva-

tion, with the anti-apoptotic protein Bcl-xL. In sum, critical genetic studies provide no evidence for Bcl-2 family members in the direct regulation, or formation, of the PTP, while Bax and Bak are required for PTP-dependent OMM permeabilization, given that in their absence organelle rupture and cell death are prevented (306).

II. STRUCTURE AND FUNCTION OF F-ATP SYNTHASES

Given that PTP formation critically depends on the F-ATP synthase, a review of its structure and function may be useful to direct future research in the field. In the following discussion, we will also highlight issues related to the PTP that may inspire new experiments and foster progress in this developing area of research.

F-ATP synthase is responsible for the synthesis of most of ATP in living cells, a task achieved by coupling the chemical reaction ADP + P_i = ATP to transmembrane proton translocation from the intermembrane space to the matrix. This reaction is reversible, and in the absence of a H^+ gradient, glycolytic ATP is hydrolyzed with coupled proton extrusion to the intermembrane space (for reviews, see Refs. 70, 187, 202, 298, 553, 628). In addition to the IMM, the F-ATP synthase is found in the thylakoid membrane of chloroplasts and in the plasma membrane of bacteria. Recent evidence supports its location also in the plasma membrane of mammalian cells, where orientation is opposite to what found in bacterial plasma membrane (385, 600).

A. Structure

The F-ATP synthase has a multi-subunit architecture whose overall structure and arrangement is extremely well conserved in spite of the early divergence of bacteria, plants, and animals. The complex is composed by a membrane-embedded F_O subcomplex, through which the protons flow, as well as by a soluble catalytic F_1 subcomplex linked to F_O (and thus to the IMM) by central and peripheral stalks, which are clearly resolved by cryotomography of single particles (29, 345, 509). The overall arrangement of mitochondrial F-ATP synthase in its physiological dimeric form (see also sect. II*B*) is reported in **FIGURE 1**.

The F_1 subcomplex always consists of three copies of subunits α and β and one copy of subunits γ, δ, and ε. The homologous α and β subunits carry the nucleotide binding sites and alternate to form a pseudo-hexameric ring around the coiled-coil structure of the γ subunit, which constitutes the central stalk along with subunit ε in bacteria, and with subunits ε and δ in chloroplasts and mitochondria [bacterial subunit ε is homologous to mitochondrial subunit δ (606)]. The steady-state catalytic mechanism for ATP synthesis/hydrolysis requires activity of all the catalytic sites,

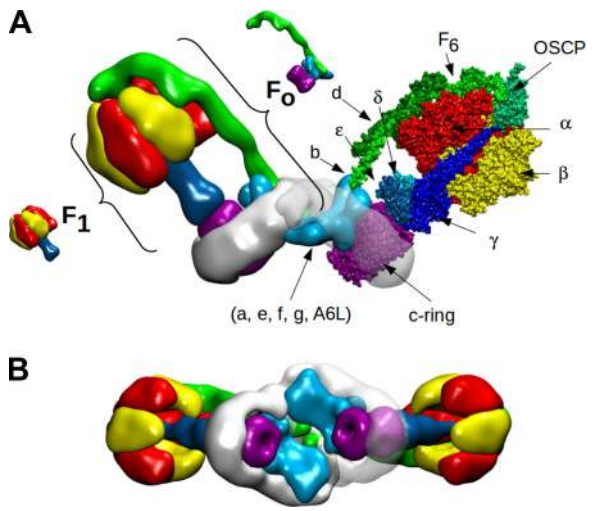

FIGURE 1. Model of F-ATP synthase dimer viewed from the lateral side (*A*) and from the intermembrane space (*B*). *A*: left monomer, the F_1 and F_O sectors are highlighted. Right monomer, the F_1 and F_O subunits are shown. In the F_1 sector, the front α and β subunits have been removed to reveal the central stalk. The F_1 α and β subunits are colored in red and yellow, respectively. The F_1 γ, δ, and ε subunits are colored in shades of blue, the peripheral stalk subunits b, d, F6 and OSCP in shades of green, and the c-ring in purple. The remaining F_O subunits a, e, f, g, and A6L are colored in light blue. The intramembrane F_O is surrounded by detergent, shown in white. The image has been built starting from the yeast dimer molecular model (146) (PDB id. 4b2q) to which the cryoelectron microscopy (cryo-EM) map of bovine F-ATP synthase (29) (EMD id. EMD-2091) has been superimposed. The fit of molecular models to cryo-EM map was performed using the program ADP_EM (208). 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 (237). The lateral stalk was taken from the yeast dimer (PDB id. 4b2q) which has been modeled using the bovine subunits. *B*: cryo-EM maps are rotated 180° to be viewed from the intermembrane space.

which are located in the β subunits at each α-β interface and interact in a highly cooperative manner. ATP (and sometimes ADP) molecules can also bind the α subunits at the α-β interfaces, but these nucleotides are not rapidly exchanged during catalysis; therefore, these sites, whose role is still unknown, are considered as noncatalytic (320).

The subunit composition of the F_O subcomplex is more variable. The simplest form is present in bacteria, where it consists of 1 copy of subunit a, 2 copies of subunit b, and between 10 and 15 copies of subunit c depending on the species (202), which implies a H^+/ATP stoichiometry between 3.3 and 5, respectively (626). The c subunits form a ring structure connected to F_1 by the central stalk, where the central region is probably occupied by phospholipids (626). The a subunit associates with the c-ring peripherally, and at the interface, two half transmembrane water channels form through which H^+ are transported via conserved carboxylic residues present in each c subunit. This residue is Asp_{61} in *Escherichia coli* and almost invariably Glu_{58} in other spe-

cies, including mammals (202). Subunit a also associates with the lateral stalk, which is formed by a homodimer of subunit b and by δ subunit located at the top of F_1. Single molecule approaches demonstrated that the catalytic and transport mechanisms are coupled by rotational catalysis at the rotor (constituted by the γ-ε-c-ring subcomplex) which is transmitted to the stator (formed by the α, β, δab subcomplex) (202). The lateral stalk of the stator is essential to prevent corotation of the α-β subunits with γ, which would curtail the catalytic activity.

The F_O sector of mitochondria has a more complex structure than that of bacteria. Subunits a, b, and c share high homology with the bacterial subunits, with the important difference that the c-ring has 10 copies of the c subunit in yeast and only 8 copies in all vertebrates and most if not all invertebrates, which decreases the H^+/ATP stoichiometry to 2.7 thus maximizing the ATP yield (626). Seven additional subunits have been identified in mitochondria, i.e., subunits d, e, f, g, F_6, and A6L, which are unique to the mitochondrial complex, and OSCP, which is similar to the bacterial δ subunit (606). The membrane-embedded part of F_O comprises subunits e, f, g, and A6L and is connected to F_1 by a complex peripheral stalk (160, 495), which is composed by one copy each of subunits b, d, F_6, and OSCP, the latter being located on top of F_1 as is the δ subunit in bacteria. When phospholipids have not been extracted, the hydrophobic proteins MLQ/6.8-kDa proteolipid (105, 408) and AGP/DAPIT (441) can also be detected in the F_O of mammals including humans (441), and their sequences are conserved in the genomes of vertebrates and metazoans (351). Conversely, the yeast enzyme additionally contains subunits i and k (19, 596) and a recently characterized subunit l, a homolog of subunit k (372). When all subunits are considered together, the mitochondrial F_O membrane domain is constituted by ~30 transmembrane α-helices (97), and the molecular mass of the whole complex varies between 540 and 585 kDa depending on the source.

The mitochondrial complex can also bind inhibitory factor 1 (IF_1), a protein conserved from yeast to mammals. IF_1 is a unidirectional inhibitor of ATP hydrolysis (31) that reversibly binds F_1 with a 1:1 stoichiometry, resulting in full inhibition of enzyme activity (94, 227, 259). Along with IF_1, in yeast mitochondria the enzyme can be regulated by two additional proteins, stabilizing factor 1 and 2 (90, 605). The subunit composition of the F-ATP synthase from prokaryotes and eukaryotes, along with the subunit homology and the corresponding nomenclature is reported in **TABLE 2**. These subunits are encoded by both nuclear and mitochondrial genes, and their assembly is a very complex process that is still under investigation (267). In yeast, the three F_O core proteins 6, 8, and 9 are encoded by mtDNA, while in mammals only subunits a and A6L are encoded by the mitochondrial genome (267).

Table 2. *Equivalence of subunits of F-ATP synthase from different sources based on sequence homology*

Mitochondria		
Bovine	**Yeast**	***E. coli***
α	α	α
β	β	β
γ	γ	γ
δ	δ	ε
ε	ε	
OSCP	OSCP	δ
b	4 or b	b*
A6L	8	
F6	h	
a	6 or a	a
c	9 or c	c
d	d	
e	e	
f	f	
g	g	
	i/j	
	k/l	
MLQ/6–8 kDa		
AGP/DAPIT		

*F-ATP synthase from *Escherichia coli* has two copies of the b subunit.

B. Supramolecular Organization: Clues to Pore Formation

Supramolecular organization of F-ATP synthase in situ was first reported by Allen (12), who observed that in *Paramecium multimicronucatum* F-ATP synthase complexes form a paired row around the outer curve of helical tubules, and noted that the complexes are closely and uniformly associated together within this helical band. Within the yeast, mammal, and plant IMM, F-ATP synthase is organized in dimers associated to form long rows of oligomers located at the cristae ridges, which are essential to maintain a high local curvature and normal cristae morphology (29, 147, 169, 246, 555). F-ATP synthase dimers have also been observed in chloroplasts of algae, where they appear to be susceptible to environmental effects like the P_i concentration (529), but not in bacteria. Dimers interact within the IMM through the F_O subunits (29, 147, 169, 246, 555) with the peripheral stalks turned away from each other **(FIGURE 1)**. Additional proposed roles of F-ATP synthase oligomers are higher efficiency and higher stability (57, 555, 586).

Studies in yeast established that preferential interactions in dimers occur through subunits 6 (635), 4 (546), e (20, 179), and g (86), which formed 6/6, 4/4, and e/g associations, and also through subunit h and the yeast-specific subunit i

(460). In keeping with their general presence, subunits a (632), e (58, 246), and g (246) were shown to play a role in F-ATP synthase dimerization also in mammals. A stabilizing effect on mammalian F-ATP synthase dimers has also been reported for the inhibitor protein IF_1 (56, 93, 206) and for the matrix metalloprotein Factor B (or subunit s), which interacts with e and g subunits of F_O as well as with the ADP/ATP carrier (294, 352). A second interface (the oligomerization interface) stabilized through e/e and g/g interactions allowing oligomer formation has been characterized in yeast (246) **(FIGURE 2)**. However, its existence is still debated because the distance between dimers appears variable in electron cryotomography of yeast mitochondria, making direct protein contacts difficult (146, 147). Furthermore, these latter studies showed fixed angles of >70° between two F_1 in all species examined (146, 147), rather than the angles ranging between 40° (127, 412) and 70–90° (169, 586) observed in previous single-particle electron microscopy images of dimers.

Both in yeast (246, 635) and mammals (56, 245, 632), the stabilizing contribution of the different subunits seems to be additive. Mutants lacking one or more of the above-mentioned subunits, such as ρ^0 cells (632) and yeast and human cells totally or partially depleted of e and g subunits (245, 461), still possess lower amounts of F-ATP synthase dimers and oligomers as detected by native gel electrophoresis. It is striking that downregulation of e and g subunits in HEK and HeLa cells not only affected F-ATP synthase dimerization/oligomerization, but also overall oxidative phosphorylation and structure of the mitochondrial network (245).

The PT has been observed in ρ^0 cells (389), while it was markedly inhibited in yeast mutants lacking subunits e and/or g (ΔTIM11, ΔATP20 and ΔTIM11;ΔATP20 strains) (96). These data support the hypothesis that dimerization of F-ATP synthase is necessary for PTP formation, but also suggest a different contribution of the F_O subunits in forming the PTP. Based on the observation that PTP is conserved from human to yeast and *Drosophila* (25), it seems reasonable to suggest that subunits e and g of the F_O subcomplex, which are involved in the dimerization and oligomerization interfaces, may also favor PTP formation possibly also through other F_O/peripheral stalk subunits, such as subunits 4, h, and i which stabilize the dimers independently of the e and g subunits (199). F-ATP synthase contains several

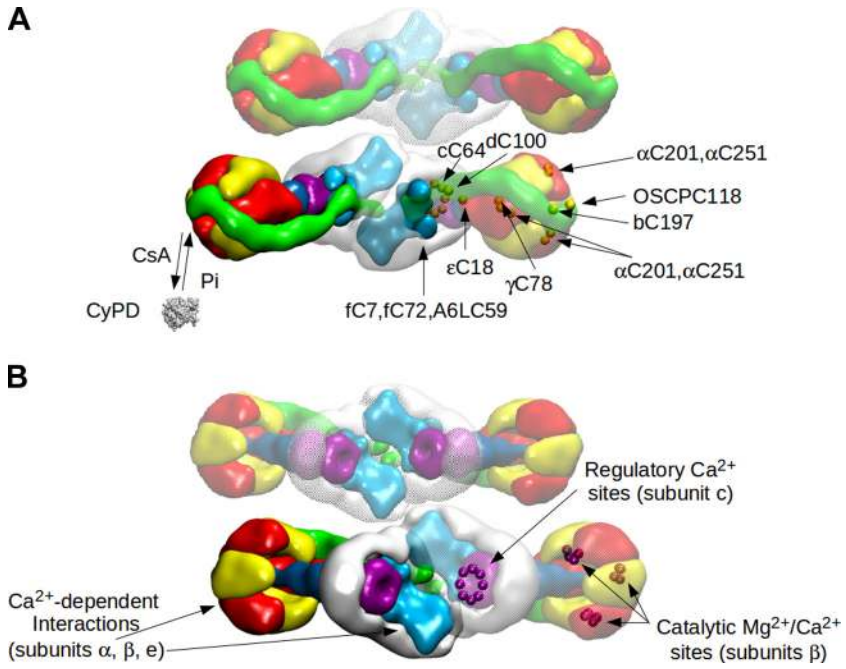

FIGURE 2. Model of F-ATP synthase dimer viewed from the matrix (*A*) and from the intermembrane space (*B*). *A*: the model (top view) was built and fitted to cryo-EM maps as described for **FIGURE 1** to illustrate the region where the PTP could form between paired monomers. A second dimer is also depicted to illustrate another possible region for PTP formation in the area defined by two paired dimers. Left monomer: reversible binding of CyPD to F-ATP synthase is shown. Right monomer: the position of human Cys residues on the specified subunits (dots) is mapped onto the 3D structure of the bovine F_1-c-ring complex and of the bovine lateral stalk (fC7 and A6LC59 are missing in the bovine complex). *B*: the same model shown in *A* is rotated 180° (view from the intermembrane space). 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 (62) (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 (17). Right monomer: Ca^{2+}-regulatory sites located in the c-ring (23, 402) and the residues (T163, R189, E192, D256) of β subunits interacting with the catalytic metal ions are mapped onto the 3D structure of the bovine F_1-c-ring complex. Numbering does not include the import sequences.

Cys residues, and consistently, treatment of mitochondria with $CuCl_2$ stabilized preexisting dimers by formation of disulfide bridges between adjacent monomers in ΔTIM11, ΔATP20, and ΔTIM11;ΔATP20 yeast strains (96).

Numerous atomic structures have been resolved of the following: *1*) various conformations of the bovine (1, 2, 33, 67, 68, 72, 214, 221, 301, 405, 442, 496, 598) and yeast F_1 sector (299, 300); *2*) the soluble part of the peripheral stalk (bdF6sol) (160) and bovine F_1 in complex with full-length or truncated subunits of the stator (OSCP, F6; bT and dT) (495); and *3*) the bovine F_1 c8 (626) and yeast F_1 c10 (143, 219, 554). A recent study of dimeric F-ATP synthase from the green alga *Polytomella* at 6.2 Å resolution shows that subunit a is arranged in two membrane-intrinsic hairpins at an angle of ~70° relative to the c-ring helices (11). It remains to be established whether this arrangement is conserved in other F-ATP synthases (382). The structure and arrangement of most of the remaining membrane subunits has not been defined yet, and the available data derive from cross-linking experiments (20, 38, 86, 462, 544, 547, 552, 596) and from the cryo-electron tomography structures of monomeric bovine (29, 509) and yeast F-ATP synthase (345).

Yeast e and g "nonessential" subunits are small hydrophobic proteins exclusively associated with dimers of the enzyme (along with the species-specific subunit k) (19). In dimers, the presence of the e subunit is essential for preserving the g subunit, while the e subunit is not affected by deletion of the g subunit (461). They are located at the periphery of F_O in the cryo-electron tomography difference maps (345), and their stoichiometry has been estimated to 2 subunits e and g per dimer (19). Subunit g could be cross-linked to subunit i (462) and to the membrane-embedded part of subunit 4 (544), which is anchored to the IMM by two transmembrane segments and contacts subunits f, 6, and 8. Both e and g subunits have one putative transmembrane domain inserted with the same orientation, and a soluble domain localized on opposite sides of the membrane with the COOH terminus of subunit e exposed to the intermembrane space. Both harbour a Cys residue ($eCys_{28}$ and $gCys_{75}$) and form e/g interactions in the dimerization interface through GXXXG motifs (20). As already mentioned, they also participate in the putative oligomerization interface though e/e and g/g interactions (246), as established in a yeast $gCys_{75}Ser/Leu_{109}Cys$ mutant, which formed $eCys_{28}$-$eCys_{28}$ and $gLeu_{109}Cys$-$gLeu_{109}Cys$ cross-links (20, 86). It is tempting to speculate that Ca^{2+} and dithiol reagents might induce conformational changes in e and g subunits, which could then contribute to PTP formation from F-ATP synthase dimers/tetramers. This may occur with or without association with the intramembrane parts of the two b subunits, whose proximity at the short hydrophilic loops linking their transmembrane domains has been demonstrated in the enzyme dimers (547). The involvement of other subunits, such as the species-specific subunit i (596) that is in contact with subunit g, cannot be excluded. On the other hand, subunit i and subunit f, which have one putative transmembrane domain, are in close proximity with subunits 6 (462) and 8 (552), respectively, whose absence did not preclude PTP opening (389), thus suggesting that i- and f-subunits do not contribute to channel formation.

A similar arrangement of e and g subunits in the IMM of bovine heart has been hypothesized based on cross-linking experiments (38), although, at variance from yeast, bovine e and g subunits remain associated to the enzyme monomers (118). Moreover, recent cryo-electron tomography data of intact bovine F-ATP synthase at 18 Å resolution (29) confirmed that these subunits extend from the a subunit density distal to the c_8-ring. Most importantly, this map highlighted that e and g subunits deviate from the expected plane of the lipid bilayer with a bend of ~43°, suggesting that two monomers placed in contact with their e and g subunits would bend the lipid bilayer at ~90°, consistent with the electron tomography of intact mitochondria. The same map revealed that the b subunit spans the membrane without contacting the c_8-ring in the enzyme monomer, suggesting to the authors that two subunits b are in close proximity in the dimer (29). We think that these observations support the hypothesis that the conserved subunits e, g, and b might contribute to PTP formation also in mammals.

It has been proposed that a latent H^+-translocating pathway exists within F_O, which would be formed by subunits e, f, g, and A6L, as well as the ANT. This pathway would be occluded in the matrix by the NH_2 terminus of factor B, which is conserved in animal mitochondria (37). Consequently, the conductance of this pathway would be markedly increased upon displacement of factor B from F_O subsequent to oxidation of Cys residues to the corresponding disulfide leading to mitochondrial uncoupling, as observed following treatment of IMM preparations with thiol modifiers (293). Although fascinating, this mechanism probably cannot account for PTP formation because *1*) the proposed latent H^+ pathway is sensitive to oligomycin, which binds to F_O subunit c and fully inhibits enzyme catalysis (567), while the PTP is not inhibited by oligomycin; and *2*) factor B is not present in yeast and *Drosophila melanogaster*, where the PTP is modulated by SH reagents, as in animal mitochondria (96, 610).

Interestingly, in F-ATP synthase from bovine heart mitochondria, another set of mono- and dithiols located in F_O has been described, whose environment was altered by membrane energization and whose oxidation resulted in complete and reversible uncoupling (645, 646). Based on the observation that uncoupling was not inhibited by oligomycin, the authors proposed that the permeability pathway is located on the cytosolic side of the oligomycin inhibitory site (645). 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 (626). 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 heart, whose modification also affects enzyme function (140, 370, 654). Oxidation of αCys_{294} and γCys_{103}, which results in the formation of an intersubunit inhibitory disulfide bridge, has been observed in canine heart failure (620) and could be involved in PTP modulation. However, these residues are located far away from one another in the assembled complex **(FIGURE 2)**, suggesting that the disulfide may form only in misfolded/aggregated enzymes (620). All together, the F_O subunits form ~30 transmembrane α helices (97), which might potentially contribute to PTP. Further genetic approaches are needed to test their potential role in PTP formation. A comparison of the Cys residues of human, *Drosophila*, and yeast F-ATP synthase is presented in **TABLE 3**.

C. Catalytic Mechanism

Rotational catalysis as the coupling mechanism of F-ATP synthases was suggested at the end of the 1970s by Paul Boyer (69), who in 1993 proposed the binding change mechanism for ATP synthesis. His proposal was strongly supported by the X-ray structure of bovine heart F_1 resolved in 1994 by the group of Sir John Walker (2), and by visualization of the ATP-driven rotation of bacterial F_1 immobilized onto a glass surface (6, 434) made possible through a fluorescent actin filament or gold beads attached to the γ subunit (202). Rotary subunit movements within the whole bacterial H^+-ATP synthase were also monitored in real time at subnanometer resolution through single-molecule fluorescence resonance energy transfer, which uses a double-labeled enzyme incorporated in liposomes driving either ATP hydrolysis or ATP synthesis (162, 177). It is now widely acknowledged that during ATP synthesis, the c-ring rotates clockwise (viewed from the matrix side of the membrane) powered by H^+ translocation to the mitochondrial matrix. H^+ translocation takes place through half channels located at the interface of the a and c subunits, and drives rotation of the γ subunit within the $\alpha_3\beta_3$ F_1 subcomplex at a rate of ~100 revolutions/s. This rotation takes each of the three catalytic sites through at least three major functional states denoted as βE, βDP, and βTP, thereby synthesizing 3 ATP molecules from ADP and P_i per each 360° rotation. When the enzyme works in the direction of ATP hydrolysis, the transition between βE, βTP, and βDP functional states drives the counterclockwise rotation of the γ subunit and of the c-ring, thereby causing H^+ translocation to the intermembrane space. Based on single-molecule measurements of bacterial F_1, it has been established that rotation of the γ subunit is not continuous, but rather proceeds in 120° steps, each step being driven by hydrolysis of one ATP molecule. The 120° steps can be resolved into substeps of 80° and 40°, which are driven by ATP binding and by release of ADP or P_i, respectively (6). However, recent rotational studies show that in mammals the catalytic cycle can differ significantly from the 80° to 40° substeps seen in bacteria, in that with human F_1 the substeps are rather ~65°, 25°, and 30°, corresponding to ATP binding, P_i release, and catalytic dwell, respectively (566). The remaining challenge is now to understand how the chemical energy of ATP is converted into mechanical work, i.e., the cooperativity of the three β subunits in driving the rotation of γ (388, 428).

Table 3. *Cys residues in human,* Drosophila, *and yeast F-ATP synthases*

Subunit	Position		
	Human	*Drosophila*	Yeast
OSCP	141	137	117
α	244,294	243,293,489	238
β			
γ	103	105,131,197,246	117
δ			106
ε	19		
a		112,144	33
b	239	78,193,229	
c	125	123	65
d	101	48	
e			28
f	72		
g		88	75
A6L (yeast 8)	59	24	
F6 (yeast h)			
k (yeast)			
j (yeast)			

Numbering includes the import sequences. Cys residues within import sequences are not included.

1. Me^{2+}, P_i, and adenine nucleotides

Mg^{2+} is essential for catalysis, which requires the binding of the nucleotide as a complex with metal (195). Together with the γ subunit, Mg^{2+} contributes to determine the asymmetry of the catalytic sites necessary for the binding-change mechanism (69). X-ray crystallography clearly established that in the absence of Mg^{2+} and nucleotide, the α,β complex of thermophilic *Bacillus* PS3 shows a threefold symmetry (532) and that in yeast F_1 the adenine-binding pocket of βTP is disrupted (300). Moreover, kinetic studies with *Escherichia coli* F_1 mutants containing Trp replacements at the catalytic sites showed that in the absence of Mg^{2+}, ATP binds the three catalytic sites with the same low affinity (627), highlighting the key role of Mg^{2+} in shaping the high-affinity catalytic sites of the enzyme. X-ray crystallography of bovine heart F_1 loaded with Mg^{2+} and nucleotides demonstrated the presence of six metal binding sites, each coordinated to the bound nucleotides in the three α

and β subunits (2). In the βTP precatalytic state and βDP catalytic state of ATP hydrolysis Mg^{2+} is hexa-coordinated by βThr_{163} (bovine numbering), by the COOH-terminal residue of the P-loop, by the oxygen atoms βO_2 and γO_2 of γ-imino ATP, and by three ordered water molecules (hydrogen-bonded to βArg_{189}, βGlu_{192}, and βAsp_{256}) (496).

Uncoupled ATP hydrolysis can be induced by membrane de-energization (185, 190), by incubation with the already mentioned modifiers of SH groups located in the F_O sector (645), by F-ATP synthase inhibitors (377, 421), by Ca^{2+}-ATP (429, 452), by Mg^{2+}-nucleotide complexes different from ATP (260), or when F_1 contains two empty catalytic sites (139). Interestingly, uncoupled hydrolysis caused by Mg^{2+}-nucleotide complexes different from ATP is inhibited much more by Mg^{2+}-ADP than coupled hydrolysis (260). Indeed, mitochondrial matrix proteins able to revert ATP synthase uncoupling have been identified, such as the already-mentioned factor B (294, 352). Mutagenesis studies suggest that coupling between catalytic activity and H^+ translocation (419) is due to the interaction between the DELSEED loop of β in a closed conformation with γ subunit at the bottom of the α,β cavity. In particular, the rigidity of the DELSEED loop, which is in an "up" or "down" position in β closed and β empty, respectively, seems important to transfer the torque from the nucleotide binding domain to the γ subunit (581). Nevertheless, how the above-mentioned proteins may restore enzyme coupling remains to be defined.

Mg^{2+} can be replaced by other divalent cations, including Ca^{2+}, and the ionic radius is the chief determinant of the ability to activate F-ATP synthase (531). Intriguingly, Ca^{2+} ions, at variance from other divalent cations with similar ionic radii, support ATP hydrolysis but not H^+ translocation both in bacteria (429) and in mammals (452). However, Ca^{2+}-ATP was as effective as Mg^{2+}-ATP at powering rotation of the γ subunit attached to an actin filament, as demonstrated in a highly active hybrid F_1 consisting of α,β subunits from *Rhodospirillum rubrum* and γ subunit from spinach chloroplasts (592). Replacement of the equivalent βThr_{163} (bovine numbering) with Ser in *Rhodospirillum rubrum* F-ATP synthase produced a mutant unable to support the proton-decoupled, Ca^{2+}-dependent ATP hydrolysis while maintaining proton-coupled ATP synthesis as well as Mg^{2+}- and Mn^{2+}-dependent ATP hydrolysis (429). Interestingly, this Thr residue is also involved in release of the inhibitory Mg^{2+}-ADP (288), which remains entrapped during uncoupled ATP hydrolysis (659). Conversely, substitution of the conserved βPhe_{174} for a Ser in an *Escherichia coli* mutant caused 90% loss of Mg^{2+}-dependent ATPase activity, while maintaining the Ca^{2+}-dependent ATPase activity (303, 437). These results strongly suggest that the catalytic site has a different conformation state when it is occupied by Ca^{2+} and that this conformational state is unable to couple the chemical catalysis to H^+ translocation. Our working hypothesis is that this specific coordination chemistry of Ca^{2+} ions in the catalytic site is able to induce PTP formation from F-ATP synthase dimers by long-range conformational changes in the F_O sector that remain to be defined (218). Equivalent mutants in mammals will represent a unique test of our hypothesis, which could be (dis)proved by their propensity to form a PTP. It is interesting that Sr^{2+}, which cannot substitute for Ca^{2+} as a trigger for PTP opening (49, 569), is unable to support ATP hydrolysis by soluble F_1 (531), suggesting an explanation for the quite distinct effects of these two cations on the PTP.

Consistent with the involvement of F-ATP synthase in PTP formation, the latter is inhibited by Mg^{2+}-ADP, which is also a strong inhibitor especially of uncoupled ATP hydrolysis (260). Because the inhibitory Mg^{2+}-ADP is promptly expelled at the onset of ATP synthesis, but not hydrolysis, its binding to a catalytic site might be responsible for the higher sensitivity of PTP to Ca^{2+} during ATP synthesis compared with ATP hydrolysis that has been observed in intact mitochondria (218). In mammalian mitochondria PTP opening is favored by P_i, as well as by arsenate and vanadate, which revert the Mg^{2+}-ADP inhibited form of F-ATP synthase (54, 85, 425, 659). In addition to this effect, P_i increases the binding of CyPD to the OSCP subunit (216). A "coupling" effect of F-ATP synthase activity by P_i at fairly low concentrations (in presence of very low concentration of ADP) has been described in membrane fragments obtained from *Rhodobacter capsulatus* and *Escherichia coli* by evaluating the efficiency of H^+ transport coupled to ATP hydrolysis. A model has been proposed where this high-affinity site could coincide with a transition state of the hydrolysis/synthesis reaction (139). It is tempting to speculate that if such a mechanism was present in all F-ATP synthases, it could be responsible for PTP inhibition by low P_i, which has been selectively observed in the absence of CyPD (35). Interestingly, based on the atomic structure of bovine F_1 inhibited by the P_i analog thiophosphate (32) and by use of molecular dynamics simulations, it has been proposed that during the 360° rotation cycle, P_i remains bound to β_E after ADP release, where it blocks γ rotation (428). Finally, it should be recalled that the inducing effect of high concentrations of P_i is not seen in yeast and *Drosophila melanogaster* mitochondria, which rather inhibit the PTP. In summary, at present, it is not easy to sort the effects of P_i on the F-ATP synthase from those that may depend on the P_i-dependent decrease of the Ca^{2+} and Mg^{2+} concentrations.

In analogy with its binding partner CyPD, OSCP strongly influences the threshold Ca^{2+} required for PTP opening, which decreases in mitochondria with decreased OSCP levels (218). The role of OSCP in enzyme catalysis has been established by numerous studies (155) and matches its potential role in modulating accessibility to the catalytic sites of Ca^{2+} ions necessary for PT induction. Fluorescence res-

onance energy transfer measurements have indeed documented that stress develops between F_1 and OSCP during ATP hydrolysis (210). Moreover, yeast OSCP mutants $Gly_{166}Asn$ (a highly conserved residue) showed partially uncoupled F-ATP synthase complexes that were more susceptible to dissociation than complexes containing native OSCP (71). In keeping with its regulatory role, OSCP has recently been recognized as the binding target of a variety of compounds and proteins. These include 17β-estradiol, whose binding promotes an intrinsically uncoupled state of F-ATP synthase (421); Bz-423, an apoptosis-inducing agent which inhibits both synthesis and hydrolysis of ATP (290, 551); sirtuin3, which mediates deacetylation of α and OSCP subunits in a nutrient-sensitive manner (603, 640); and the transcription factor p53, which has been proposed to take part in the assembly or stabilization of the mature F_OF_1 complexes (39). We found that CyPD affects both the synthetic and hydrolytic activity of F-ATP synthase (216) and possibly shares a common binding site with Bz-423 on OSCP (218) **(FIGURE 2)**. In a striking analogy, both Bz-423 and CyPD decreased the threshold matrix Ca^{2+} required for PTP opening (218).

Although PTP formation is influenced by modulators of F-ATP synthase catalysis, in electrophysiology experiments we observed PTP opening using preparations of bovine (218), yeast (96), and *Drosophila* (611) F-ATP synthase dimers that presumably contained very low levels of endogenous nucleotides. Addition of Ca^{2+} was essential for PTP opening, which was also favored by the dithiol reagent phenylarsine oxide, while it was inhibited by Mg^{2+} and ADP (96, 218). ATP and ADP are equally effective at inhibiting the PTP in intact mitochondria, and phenylarsine oxide does not inhibit ATP hydrolysis catalyzed by F-ATP synthase (645). These findings suggest that Ca^{2+}-ATP hydrolysis is not necessary to induce PTP opening. Furthermore, the PTP is not inhibited by oligomycin, while Ca^{2+}-ATP hydrolysis has been reported to be, at least partially, inhibited by oligomycin (452). The propensity of the PTP to open is affected by pH, with a maximum at pH 7.4, while opening is strongly inhibited as pH decreases to pH 6.4 through reversible protonation of still unidentified His residues, as well as when pH increases to pH 8.0 (432, 433). This is quite different from the pH profile of ATP hydrolysis (at least in the presence of Mg^{2+}), which has a maximum at about pH 8.0 (260). Such discrepancy suggests that the two events, ATP hydrolysis cycle and PTP opening, are not strictly related although, to the best of our knowledge, the pH profile of Ca^{2+}-ATP hydrolysis has not been defined yet. As already mentioned, pore inhibition by low pH has been initially ascribed to unbinding of CyPD from the PTP (432), but the observation that in mitochondria devoid of CyPD the PTP response to pH was maintained suggests that the PTP-modulating His residues (which still remain to be identified) are located within F-ATP synthase (34).

2. Inhibitory factor 1

In principle, the low open probability of PTP at low pH might be due to the binding of the inhibitor protein IF_1 to F_1 (94, 227, 259), which is strongly favored by low pH when the inhibitory dimeric IF_1 is formed (89, 90). This endogenous inhibitor could be responsible for the beneficial effects of F-ATP synthase inhibition during ischemia both in in vitro experimental models as well as in vivo (159, 192). Unexpectedly, mice with genetic ablation of IF_1 have no overt phenotype (427). The mechanism of inhibition by IF_1 is quite complex, and fundamental insights have been recently obtained by point mutations and X-ray crystallography of the bovine (31, 33) and yeast species (503). Considering that in mammalian mitochondria Mg^{2+}-ADP is a PTP inhibitor, it might be hypothesized that the final inhibited IF_1-F_1 complex, which contains two Mg^{2+}-ADP in the catalytic sites of β_{DP} and β_{TP} (31), is unable to properly bind Ca^{2+}, necessary for its switch to the PTP. Intriguingly, the His reagent diethylpyrocarbonate, whose addition to mammalian mitochondria restores the ability to induce PTP opening at low pH (432, 433), also prevents IF_1 binding to the inner membrane at acidic pH when preincubated with the isolated bovine heart IF_1 (608). This result suggests that the His residues of IF_1 involved in the pH modulation of mammalian IF_1 activity (89) might be involved in PTP inhibition at low pH as well. On the other hand, the inhibited IF_1-F_1 complex does not contain P_i (31, 503), which is a PTP inhibitor at low concentrations, suggesting that IF_1 binding might mask an inhibitory site favoring, rather than preventing, PTP opening. Modification of isolated β subunit from *Rhodospirillum rubrum* with diethylpyrocarbonate fully blocked P_i binding (310), suggesting that IF_1 and diethylpyrocarbonate might act in a similar way.

IF_1 has been also described to favor formation of F-ATP synthase dimers, although IF_1 is not essential for dimerization either in yeast (161) or mammals (588). However, how IF_1 binding improves the stability of the dimer structure remains to be clarified. In fact, overexpression of IF_1 in HeLa cells promoted dimer/oligomer stabilization and increased ATP synthesis, but also inhibited ATP hydrolysis at low pH and was protective against ischemic injury (93). Conversely, in cardiomyoblasts undergoing differentiation, IF_1 binding promoted dimer/oligomer stabilization and increased both ATP synthesis and ATP hydrolysis (56). These apparently conflicting data suggest that IF_1 can associate with F-ATP synthase dimers in different conformations. This hypothesis would be consistent with the intrinsically disordered structure of IF_1, which makes it potentially able to recognize, and interact with, more than one partner (31). Intriguingly, a pH-independent interaction of IF_1 with OSCP has been described (655). Such additional interactions might favor PTP formation, but the recently reported protecting effect of IF_1 overexpression against apoptotic cell death, which appeared related to F-ATP synthase dimerization, may rather suggest that IF_1-mediated dimer

stabilization counteracts PTP opening (180). We note, however, that in this study the amount of IF_1 bound to F-ATP synthase dimers was not quantified, and that the pH profile for PTP opening was not addressed. In conclusion, PTP modulation by IF_1 remains an intriguing possibility that should be further explored.

3. Ca^{2+} in regulation of F-ATP synthase

It has long been known that F-ATP synthase is activated by Ca^{2+} in normal heart and skeletal muscle (259), in parallel with the Ca^{2+}-sensitive dehydrogenases of the Krebs cycle and pyruvate dehydrogenase (154, 248, 256, 397). Moreover, the response to Ca^{2+} is rapid enough to support steep changes in myocardial work load (585). Although an allosteric mechanisms for activation has been postulated already in the 1990s (259), how Ca^{2+} affects the F-ATP synthase remains unclear, yet these putative regulatory interactions are of obvious interest to the mechanism of PTP control.

Several mechanisms have been proposed to explain F-ATP synthase activation by Ca^{2+} **(FIGURE 2)**. In cardiomyocytes a Ca^{2+}-dependent interaction of the F_1 subcomplex with the S100A1 protein, which is expressed predominantly in cardiac muscle, reportedly leads to increased ATP production (62). In rat liver and enamel cells, the β (but not the α) subunit displayed low-affinity binding of up to 7–18 mol Ca^{2+}/mol in sites different from the nucleotide-binding sites, including the acidic sequence DELSEED (275), which is not present in the α subunit. Intriguingly, a *Bacillus* PS3 mutant where all the negative charges of the DELSEED sequence had been removed showed significantly higher ATP synthesis activity (419). However, no measurements have been performed in rat liver and enamel cells to confirm that Ca^{2+} binding induces enzyme activation.

F_O subunit e has also been hypothesized to be a Ca^{2+}-dependent activating region of F-ATP synthase, based on the homology of its residues 34–56 to the Ca^{2+}-dependent tropomyosin-binding region for troponin T, and on the ability to mediate an increase of F-ATP synthase activity upon interaction with a specific antibody (17). Interestingly, these residues are exposed at the C-site of F_O, potentially ensuring the rapid decoding of changes of cytosolic Ca^{2+}. Evidence that this Ca^{2+}-dependent regulatory region of subunit e is involved in Ca^{2+}-dependent F-ATP synthase regulation, and that tropomyosin-like molecule(s) exist in the mitochondrial intermembrane space, is still lacking. Moreover, as for subunit β, subunit e seems to be a common rather than an activation mechanism preferentially occurring in heart and skeletal muscle, although its differently regulated stoichiometry among species and tissues (17, 58) might suggest different responses to Ca^{2+} ions. Due to exposure of the Ca^{2+}-binding site to the mitochondrial intermembrane space, subunit e may be involved in Ca^{2+}-dependent inhibition of PTP opening (49). Subunit e is important for F-ATP synthase dimer formation both in mammals and in yeast (58, 246, 461), suggesting that Ca^{2+} regulation could target the dimer structure/function, which is essential for PTP formation (218) as recently confirmed in yeast (96).

The NH_2 terminus of F_O subunit c, which is also exposed to the intermembrane space (568, 626), also contains a conserved Ca^{2+} binding site formed by two Asp residues, which could represent another candidate for PTP inhibition by Ca^{2+} in the intermembrane space. Purified subunit c from neuronal plasma membrane is able to form a voltage-sensitive pore that carries monovalent cation currents regulated by Ca^{2+} and cGMP (402), suggesting that Ca^{2+} could alter the c ring conformation (402). In bacteria and chloroplasts, Ca^{2+} binding to subunit c at the periplasmic/luminal site has been reported to block H^+ translocation in both directions (599). 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 (23, 599).

III. F-ATP SYNTHASE AND THE PERMEABILITY TRANSITION

A. Genetic Manipulation of F-ATP Synthase Subunits

In mammalian cells, genetic manipulations addressing the PTP-forming features of F-ATP synthase have been performed with siRNAs for OSCP (218) and c subunits (9, 64), and in ρ^0 cells lacking subunits 6 and A6L (389). In *Saccharomyces cerevisiae*, the role of genetic inactivation of the "dimerization" subunits e (TIM11) and g (ATP20) on PTP formation has also been assessed (19).

Decreased expression of OSCP after treatment with specific siRNAs in human cells led to a parallel decrease of CyPD binding to F-ATP synthase, and to a matching decrease of the threshold matrix Ca^{2+} required for PTP opening in the face of a conserved catalytic activity and of a normal buildup of the proton gradient (218, 290). Accessibility of the metal binding sites of the catalytic F_1 sector could be influenced by OSCP, which would then affect the probability of replacing Mg^{2+} with Ca^{2+} and thus to cause PTP opening (218). According to this hypothesis, OSCP would be a "negative" modulator whose effects can be counteracted by binding of the "positive" effector CyPD, which does increase the apparent Ca^{2+} affinity of the PTP. Under this scenario, CyPD binding to OSCP, or ablation of the latter, would induce equivalent effects causing increased probability of PTP opening (218).

Treatment with siRNA against the c subunit decreased PTP opening in response to various death stimuli (9, 64), while overexpression of Myc-tagged c subunit enhanced PTP opening (64). As also noted by Halestrap (254), while con-

sistent with a role of the F-ATP synthase in PTP regulation, these experiments cannot determine whether the effects are linked to a direct involvement of the c subunit, are an indirect consequence of decreased ATP production by the knockdown (9, 64) or by a misfolded protein response by overexpression (64). Also, it is not clear whether and to what extent biogenesis of the F-ATP synthase was actually suppressed in this study, which only relied on an antibody against the uncleaved immature form of the c subunit, and where expression of the F_1 subunits was not investigated (64). The proposal by Jonas and co-workers that the PTP channel may form within the c-ring (9) will be discussed in section III*B*.

ρ^0 cells lack mtDNA (315) and therefore assemble an F-ATP synthase that lacks mitochondrially encoded subunits a and A6L (632). The F-ATP synthase of ρ^0 cells does not pump protons but is catalytically active as an ATP hydrolase. This activity is essential to maintain a gradient of ATP (almost entirely produced by glycolysis) between the cytosol and the matrix, allowing the electrogenic exchange of extramitochondrial ATP for matrix ADP which generates a $\Delta\psi_m$ (84) of about −67 mV (16). Wittig et al. (632) have shown that dimers of F-ATP synthase do form in ρ^0 cells. In these studies, the F-ATP synthase is almost fully assembled in spite of the absence of subunits a and A6L but results in the apparent lower stability of the complexes after digitonin treatment. While the levels of F-ATP synthase complexes were smaller, dimeric and even tetrameric forms of the large assembly intermediate were preserved, suggesting that the F-ATP synthase associated further into higher order structures in the mitochondrial membrane (632). ρ^0 cells almost entirely depend on glycolysis for ATP production and upregulate HK II, which is largely bound to the OMM (389). Occurrence of the PT has been demonstrated in intact ρ^0 cells based on mitochondrial depolarization following displacement of mitochondrial HK II (389) with clotrimazole or with an HK II-HIV-1 TAT fusion peptide (109). Since the assay for PTP detection was based on in situ depolarization (389), it is not known whether the properties of the PTP formed in the absence of the a and A6L subunits (in particular its conductance) are the same as those of wild-type F-ATP synthase.

Given that dimeric, but not monomeric, F-ATP synthase forms channels in mammals (218), yeast (96), and *Drosophila* (611), we have explored the role of the yeast dimerization subunits e and g in PTP formation by their genetic inactivation. Strains lacking subunits e (220) or g (146) display abnormal morphology, with balloon-shaped cristae and F-ATP synthase monomers distributed randomly in the membrane, but develop a normal membrane potential (96). In turn, this allowed measurements of their propensity to open the PTP following Ca^{2+} uptake in the presence of the ionophore ETH129, which catalyzes electrophoretic Ca^{2+} uptake in energized yeast mitochondria (297) when the concentration of P_i is optimized (647). Yeast strains lacking the e and/or g subunit showed a remarkable resistance to PTP opening, which suggests that dimer formation is important for pore formation in situ (96). In these experiments, onset of PTP opening is measured as the spontaneous Ca^{2+} release following Ca^{2+} uptake, which was observed also in the e and/or g subunit-null mitochondria at higher Ca^{2+} loads (96). One possible explanation is that dimers can transiently form even in the absence of e and g subunits, as shown in intact cells (211) and after treatment of mitochondria with copper, which promotes dimerization through formation of disulfide bridges between adjacent cysteine residues of the monomers (199, 246, 604). A second explanation should also be kept in mind, however. Yeast mitochondria lack the MCU (95), and therefore, the ionophore ETH129 must be added to allow Ca^{2+} uptake and determination of the Ca^{2+} sensitivity of the PTP (96, 297, 647). Onset of Ca^{2+} release can be due to PTP opening but also to increased H^+ permeability, which would be followed by Ca^{2+} release via ETH129 itself. In other words, it cannot be excluded that the Ca^{2+} release occurring at higher Ca^{2+} loads in the yeast e and g null mutants (96) may not be due to the PTP, and thus that pore formation may strictly require the presence of the e and/or g subunits.

B. Mechanism of Channel Formation

The mechanism for channel formation within the F-ATP synthase is an open question. One recent proposal is that the actual channel forms within F_O (9), while we have suggested that the pore forms in F-ATP synthase dimers (or higher order structures) at the interface between monomers (96, 218, 611).

1. F_O and the c-ring

The first evidence that the F_O sector can form a high-conductance voltage-dependent channel has been obtained in 1989 (541), but its functional role remained elusive. Subsequent patch-clamp studies of highly purified c subunit, which self-assembles into annular structures (18, 404), demonstrated the presence of channels inhibited by Ca^{2+} and activated by cGMP (400–402). Attention to subunit c as a potential regulator of the PTP was drawn by the observations that the PT could be induced by a phosphorylated peptide derived from the c subunit and by subunit c itself (22, 23).

Recently, Jonas and co-workers reconstituted the c subunit or the purified F-ATP synthase in liposomes, and measured Ca^{2+}-activated channels (9) with properties similar to those described for purified dimers (218). They suggested that the channel of the PTP forms within the c-ring itself after Ca^{2+}-dependent extrusion of F_1, i.e., of the $\gamma/\delta/\varepsilon$ subunits (9). The "F_O channel" could not be closed by subunits γ, δ, or ε, while it was blocked by subunit β, suggesting that this is the mechanism through which pore closure occurs in situ (9).

Displacement of F_1 from F_O requires very drastic conditions, such as treatment with 2 M urea (119, 302), yet a functional F_OF_1 complex can be easily reconstituted (178, 370), indicating that the $\gamma/\delta/\varepsilon$ subunits do reinsert into F_O. It is hard to envision a plausible mechanism through which matrix Ca^{2+} would cause release of F_1, and then create a channel within F_O that cannot be closed by subunits $\gamma/\delta/\varepsilon$ (9). The reported closure of the "F_O channel" by the β subunit is of some concern, because subunit β does not interact with the c-ring **(FIGURE 1)** (2) and the source of free β subunit is not obvious given that the F_1 subcomplex is extremely resistant to denaturation. Furthermore, if PTP opening depends on expulsion of F_1, yet F_1 itself cannot reintegrate within F_O through subunits $\gamma/\delta/\varepsilon$, each event of pore opening would denature the corresponding F-ATP synthase, while the PTP flickers between open and closed states (280, 317, 475, 575) and is readily and fully reversible in mitoplasts (569), intact mitochondria (472), as well as in reconstituted dimers of F-ATP synthase (218). Of note, after PTP-dependent swelling, pore closure is followed by shrinkage and full recovery of mitochondrial structure and function, provided that the experiment is performed in K^+-based media to allow K^+ extrusion by the K^+/H^+ exchanger (41) and that cytochrome *c*, which is lost during swelling, is added back (472).

In the protocols of Alavian et al. (9), channel openings strictly required CyPD, were inhibited by CsA after the addition of Ca^{2+}, and could also be detected with F-ATP synthase preparations. Like the MMC in the native IMM (149), the mammalian F-ATP synthase does not require CyPD for channel formation (218). Furthermore, neither the yeast nor the *Drosophila* PTPs are regulated by CyPs, yet their F-ATP synthases do form Ca^{2+} channels (96, 611). Thus the structural bases for the CyPD requirement and CsA sensitivity of "F_O channels" remain obscure.

Mammals possess three isoforms (P1, P2, and P3) of subunit c of F-ATP synthase, which differ only in their mitochondrial targeting peptides, so that the mature c subunits are identical (170, 648). The targeting sequences are not redundant, however, and may have specific functions in maintenance of the respiratory chain (609). During F-ATP synthase biogenesis, the membrane-embedded c-ring attaches to F_1 at an early stage (267), i.e., before the subunits of the peripheral stalk (b, d, F6, and OSCP), the membranous subcomplex (e, f, g, and other species-specific subunits), and the mtDNA-encoded subunits a and A6L are incorporated (632). The level of this complex (P1 isoform) appears crucial for defining the final content of F-ATP synthase, at least in mammals (273, 331). When present in "excess," subunit c forms hydrophobic aggregates in the IMM as well as in lysosome-derived organelles that can be observed in Batten disease (401, 446). Importantly, these hydrophobic aggregates in the IMM do not increase membrane permeability, as recently observed after silencing of the ε subunit in HEK293 cells, which blocked the biogenesis of F-ATP synthase with accumulation of subunit c (262). Mitochondria in these cells were more coupled (262), which is unexpected if the c-ring can form high-conductance membrane channels.

A final point is our recent finding that the *Drosophila melanogaster* F-ATP synthase forms channels with a unit conductance of 53 pS (611). As already mentioned, vertebrates and most invertebrates have 8 c subunits per F-ATP synthase monomer, while the number of c subunits varies between 10 and 15 in prokaryotes, chloroplasts, yeasts, and fungi (626). In all c_8-ring members, *1*) Ala residues are conserved at positions 13, 19, and 23 (bovine numbering) of the NH_2-terminal α-helix, which is essential to avoid clashes of the side chains (626), and *2*) the COOH-terminal α-helix invariably has a Lys residue at position 43, which binds cardiolipin and allows tight packing of the c subunits that is essential for activity and stability (171, 222). These critical residues are conserved in *Drosophila* (626), which is also affected by loss of cardiolipin (4), indicating that F-ATP synthase of *Drosophila melanogaster* belongs to the c_8-ring set. It is quite difficult to see how c-rings of the same size can form 500-pS channels in mammals and 53-pS channels in *Drosophila*. The lack of correlation between conductance of the channel and composition of the c-ring is also supported by measurements in yeast, where the c-ring has 10 subunits and the conductance is 300 pS (626). In summary, the ensemble of these findings is not consistent with the idea that the PTP channel forms within the c-ring, whose actual conductance and Ca^{2+} dependence remains controversial (9, 400–402).

2. F-ATP synthase dimers

We have observed channel formation after incorporation of gel-purified F-ATP synthase dimers of mitochondria from bovine heart, *Saccharomyces cerevisiae*, and *Drosophila melanogaster* in asolectin bilayers, while channel formation was not observed when monomers were used (96, 218, 611). Although dimers and monomers are functionally distinct in the native membrane (57), it is not known whether this difference is maintained after incorporation into an artificial membrane. Furthermore, it is not known whether dimers would maintain the dimeric structure, dissociate into monomers, or rather form higher-order structures like tetramers, a process that may also depend on the conditions used to obtain the F-ATP synthase preparations. Jonas and co-workers (9) have observed channel formation after incorporation of F-ATP synthase monomers into liposomes, but it is difficult to exclude that higher-order structures were formed, possibly because of the membrane curvature that may favor dimer and/or oligomer reassociation, which could be harder to obtain in planar bilayers. As already discussed, we think that the dimer/tetramer hypothesis is also strongly supported by the effects of genetic ablation of the e and/or g subunits on the yeast PTP (96) and by the

maximal conductance of the reconstitued dimer, which corresponds to half the maximal conductance of the MMC in the native inner membrane (575).

We have proposed that the PTP channel forms at the interface of two monomers (associated into dimers) also because we were inspired by an atomic force microscopy study of native IMM (88). In this study, Buzhynskyy et al. (88) demonstrated the existence of two classes of dimers characterized by a stalk-to-stalk distance of 15 and 10 nm, respectively, with the latter presumably allowing binding of IF_1. The authors concluded that the torque generated during ATP synthesis stabilizes the interfaces within each F-ATP synthase, within dimers and oligomers, and as a consequence stabilizes cristae morphology with a stalk-to-stalk distance of 15 nm. In contrast, ATP hydrolysis would destabilize dimers by pulling the stators apart, thus allowing the monomers to get closer to one another at a stalk-to-stalk distance of 10 nm, favoring IF_1 binding (88). Since the sense of rotation of F-ATP synthase (synthesis vs. hydrolysis) strongly affects the threshold Ca^{2+} required for PTP opening in intact mitochondria (218, 611), the different conformation of dimers may affect the probability of replacing Mg^{2+} with Ca^{2+} at the F-ATP synthase catalytic site, triggering the conformational change that results in PTP opening (218). We would like to stress that the "dimer hypothesis" is only a working model that sets pore formation at a membrane-protein or protein-protein interface that does not directly involve the c-ring. This is consistent with the fact that the catalytic activity of F-ATP synthase can be inhibited (e.g., with oligomycin) without affecting PTP formation. Conversely, PTP formation can be inhibited (e.g., by CsA) without affecting, or even stimulating, activity of F-ATP synthase (216).

Structural work on the F-ATP synthase is fully consistent with the idea that substituting Ca^{2+} for Mg^{2+} at the catalytic site has a major conformational effect on the complex structure. We have already mentioned that, together with subunit γ, Mg^{2+} contributes to create the asymmetry of the catalytic sites that is essential for ATP synthesis (69, 195). In the absence of Mg^{2+} (and nucleotide), the α,β complex shows a threefold symmetry (532), while the adenine-binding pocket of βTP is disrupted (300). When Ca^{2+} replaces Mg^{2+}, ATP hydrolysis takes place, but this is apparently not coupled to H^+ translocation (429, 452), indicating that Ca^{2+} occupancy of the catalytic site causes a conformational change that decouples chemical catalysis from H^+ translocation. If this conformational change is related to PTP opening, the lack of apparent H^+ translocation with Ca^{2+}-ATP hydrolysis may be rather due to backflow of H^+ through the open pore. Our working model is that at the prevailing matrix Me^{2+} concentrations (millimolar Mg^{2+}, micromolar Ca^{2+}), the chances that Ca^{2+} replaces Mg^{2+} at the catalytic sites are minimal, and a PT does not occur. In mitochondria where the pore is modulated by CyPD, its binding to OSCP, which is favored by P_i (216), would cause a conformational change affecting accessibility of the Me^{2+} binding sites, but not necessarily causing the PT if matrix $[Ca^{2+}]$ remains low. Accessibility of the Me^{2+} binding site would be independently increased by thiol oxidation and counteracted by thiol reduction, making replacement of Mg^{2+} with Ca^{2+} more likely irrespective of whether the PTP is modulated by mitochondrial CyP. Once the PT occurs, ion/solute permeation would occur at the F_O interfaces within dimers or higher order structures like tetramers. Note that this model predicts that the PT can occur at individual F-ATP synthase units, accounting for the fact that PTP-dependent depolarization can occur asynchronously in a mitochondrial population (281) **(FIGURE 2)**.

Some recent PTP models have tried to incorporate the F-ATP synthase into the old two-membrane picture of the PTP, which requires an alteration of relative protein dimensions and of their topology (65, 479). We think that such cartoons do less to illuminate than serve as points of confusion as to which molecules play key structural roles, which are modulators and which have no function at all. This also applies to a recent review according to which the PTP would be related to the "ATP synthasome" (228). We note that the "ATP synthasome" (i.e., the physical association between F-ATP synthase, PiC, and ANT) has been seen after detergent extraction under conditions where only the monomeric form of F-ATP synthase is present (103). Moreover, recent studies have demonstrated that the ANT migrates as a monomer, and that higher molecular weight species are only observed under less favorable solubilization conditions, consistent with aggregation of the protein (129). Finally, these complexes are not apparent in cryo-electron tomography studies of mitochondria from various sources, where the density maps perfectly fit F-ATP synthase dimers (29, 146, 147, 509, 555).

IV. SIGNAL TRANSDUCTION TO THE PERMEABILITY TRANSITION PORE

Mitochondria dynamically integrate a variety of signals to adjust their metabolic activities to the changing cellular needs and conditions. Over the last few years, it has become clear that all complexes of oxidative phosphorylation (OXPHOS), including F-ATP synthase, undergo posttranslational modifications (PTMs) that may guarantee fine tuning of their activity, for instance, during cell differentiation or along the cell cycle (128, 283). Similarly, the PTP can be regulated by proteins that undergo complex PTMs by molecular cascades of signal transduction (493). Thus, for the first time, identification of F-ATP synthase as the key to PTP formation allows us to draw a tentative unifying picture connecting available data on PTMs of pore components and F-ATP synthase.

A. Posttranslational Modification of Pore Regulators

Given the lack of information on PTP structure, efforts had so far focused on PTP modulation by signals converging on pore regulators. This field of investigation is extremely complex for several reasons: crucial components of PTMs depend on the frequency, duration, and intensity of the signals, which in turn depend on the cell type and metabolic conditions. In addition, the subcellular localization of regulatory proteins and of multimeric enzyme complexes can further modulate PTMs of the PTP. Furthermore, most signal transducing enzymes (e.g., kinases and phosphatases) are located in multiple subcellular compartments, which demands a careful purification of mitochondria under conditions that preserve PTMs. Despite these difficulties, a complex regulatory network composed by phosphorylation, acetylation, and nitrosylation events that impact on PTP opening is emerging.

One key connection between the PTP and the metabolic status of the cell is provided by HK, the first enzyme of glucose metabolism. HK isoforms I and II have high affinity for glucose and possess hydrophobic NH_2 termini that allow their docking to the OMM (394, 457, 502). In particular, HK II is the sole isoenzyme endowed with two catalytic domains, which maximize the rate of glucose metabolism to glucose-6-phosphate. This arrangement is strictly required in tissues such as brain and heart, insulin-sensitive muscle or fat depots, and in rapidly growing cancer cells (395). HK II expression is induced in most malignancies, where it crucially contributes to the Warburg effect, i.e., to the uncoupling between increased glycolysis and oxygen availability required for tumor cell survival during primary growth, when blood supply is scarce (274, 624). We have found that in different cell models with a high rate of glucose utilization, including neoplastic cells, cardiomyocytes, and cells lacking mtDNA, detachment of HK II from the mitochondrial external surface by the use of a cell-permeant, selective displacing peptide prompts PTP induction, which rapidly leads to cell demise (109, 389, 449).

HK II binding to mitochondria is controlled by kinase signaling. The Ser/Thr kinase AKT, a central transduction node that delivers anabolic and survival signals downstream to several growth factors, and whose dysregulation contributes to metabolic diseases and tumors (380), promotes HK II binding to mitochondria by phosphorylating HK II itself (418) or its upstream regulator GSK3 (394, 502). The exact mechanisms through which GSK3, a constitutively active Ser/Thr protein kinase that is inhibited by all major anabolic signaling cascades (292), controls the mitochondrial localization of HK II remain unclear (discussed in Ref. 493) as it remains to be established how HK II, which is externally associated to mitochondria, might regulate the PTP on the IMM. As a first step in elucidating this point, we have recently reported that HK II forms a multimeric complex with the myotonic dystrophy protein kinase and the active form of the Tyr kinase Src on the OMM (449). In these experiments, mitochondrial binding of HK II protects the PTP from opening independently of its enzymatic activity, and its detachment amplifies the oxidative damage caused by placing cells in conditions of serum and glucose depletion (449). Therefore, mitochondrial HK II could play an important antioxidant function (see also Ref. 564) possibly following interaction with another glycolytic enzyme, the fructose-2,6-bisphosphatase TIGAR (108), thus protecting cells from ROS-induced PTP opening. Taken together, these observations indicate that mitochondrial HK II might be an important "porekeeper," which would act as a key survival device in conditions of adequate nutrition controlled by growth factor stimulation, or when kinase pathways are aberrantly induced by neoplastic transformation. Independently of the mode of PTP regulation by HK II, these data directly link the pore to crucial signal transduction pathways, providing an important example of how information on the status of the cell can be conveyed to the PTP.

Furthermore, PTMs serve as PTP regulators through mitochondria-restricted kinases, among which GSK3 plays a central role (111). Indeed, mitochondrial GSK3 (mGSK3) is inhibited by the survival kinases AKT and ERK1/2, PKCε, PKG, and p70s6K (261) and acts as the final effector of these transduction pathways in the regulation of PTP opening (295). A large body of information highlights the importance of PTP regulation by mGSK3 in pathological conditions, such as ischemia-reperfusion of the heart (see below). Notably, maintenance of cardiomyocyte viability during ischemic pre- and postconditioning requires phosphorylation-dependent inhibition of mGSK3, which in turn inhibits the PTP in response to ROS or Ca^{2+} overload (296, 415, 417). mGSK3 activation is dramatically increased during heart ischemia (416). Accordingly, administration of a GSK3 inhibitor together with CsA reduces infarct size in mice (99), and expression of a constitutively active GSK3 could not rescue myocytes from oxidative insults, whereas transfection with an inactive enzyme was protective (296, 440). Moreover, ROS-dependent activation of mGSK3 enhances cell death in neurons, probably following PTP induction (316, 467). Taken together, these observations suggest that mGSK3 coordinates diverse signaling pathways to connect PTP opening with stress and survival signals by changing the ROS and/or Ca^{2+} threshold of PTP opening. At present, it is unknown whether the pool of mGSK3 can directly phosphorylate any F-ATP synthase components, but we and others have reported that CyPD is a target of GSK3 phosphorylation in tumor cells (492, 589) and in cells lacking mtDNA (389).

By in silico analysis, we identified Ser/Thr residues on CyPD that are possible GSK targets, and we showed that recombinant GSK3 can phosphorylate CyPD in vitro (492). When

moving to in-cell experiments, we found that mGSK3 could be inhibited by a mitochondrial-restricted fraction of the Ser/Thr kinase ERK (492). ERK activation by the protooncogene Ras delivers crucial signals that govern cell growth, proliferation, motility, and survival (398); Ras dysregulation is mandatory in a number of diseases, notably in the process of neoplastic transformation (156, 520). Our data demonstrate that in several cancer cell models mitochondrial ERK is constitutively active, and it confers resistance to PTP inducers through GSK3 inhibition, which in turn leads to the negative regulation of CyPD phosphorylation. Accordingly, PTP inhibition could be ablated by inducing GSK3-dependent phosphorylation of CyPD through treatment of cells with ERK inhibitors. This enhancement of PTP opening was increased after CyPD overexpression and absent in CyPD knockout cells; conversely, pharmacological inhibition of GSK3 protected cells from PTP opening (492). Therefore, inhibition of mGSK3 by active mitochondrial ERK prevents CyPD phosphorylation and desensitizes PTP opening. These data provide a mechanistic link between the ability to escape death, a hallmark of cancer cells (255), and PTP regulation by kinase signaling (discussed in Refs. 493, 494). Further evidence supports the importance of pore regulation by PTMs acting on CyPD: *1*) the signal transducer and activator of transcription 3 (STAT3), which is regulated by several kinase pathways, interacts with CyPD and inhibits PTP opening (61); *2*) PTP sensitization by acetylation is prevented by AMPK activation of the sirtuin-3 deacetylase, which targets CyPD (533); and *3*) nitrosylation targeting a specific Cys residue of CyPD inhibits PTP opening (324, 431) **(FIGURE 3)**.

CyPD is not the only molecular chaperone involved in PTP regulation. In the last few years, the mitochondrial pools of Hsp90 and Hsp60 and the mitochondria-restricted TRAP1 chaperone were reported to prevent PTP opening in neoplastic cells following interaction with CyPD (213, 305). As the activity of all these molecules can be modulated by PTMs (491, 504), these observations raise the intriguing possibility that multichaperone platforms might maintain the appropriate folding and activity of pore components and regulators, dynamically adjusting PTP modulation to the requirements of the cell by conveying signaling through phosphorylation changes.

It is also possible that other kinase pathways can modulate the PTP through GSK3-independent regulation, even if mechanistic details are still missing. One example is provided by the Ser/Thr kinase PINK1. PINK1 can inhibit PTP-dependent cell death (617), whereas its ablation lowers the threshold for PTP opening (205). Importantly, kinase activity is always balanced by phosphatases, but information on mitochondrial phosphatases and on their role as PTP regulators is scarce. It was reported that a mitochondrial matrix-targeted protein phosphatase 2C family member, PP2Cm, blocks PTP opening and acts as a survival element (375), whereas PTP-mediated cell death is inducible by the protein phosphatases 2B (649) and SH2-containing tyrosine phosphatase 1 (286).

B. F-ATP Synthase as a Signaling Target

Our understanding of how F-ATP synthase enzymatic activity is finely tuned is still incomplete. The enzyme is regulated by CyPD, IF_1, and a variety of regulatory peptides (62, 216, 294, 352), but nothing is known about PTMs that might regulate its transition and function as the PTP. Importantly, no tissue-specific isoforms except for the heart and liver type of γ subunit (396) have been reported for F-ATP synthase, but some evidence suggests that the enzyme cannot be regulated only by substrate availability. For instance, in the heart, ATP levels do not dramatically change with activity (mainly due to buffering by creatinine kinase), yet the F-ATP synthase fully responds to increased ATP expenditure, indicating that it can be regulated by

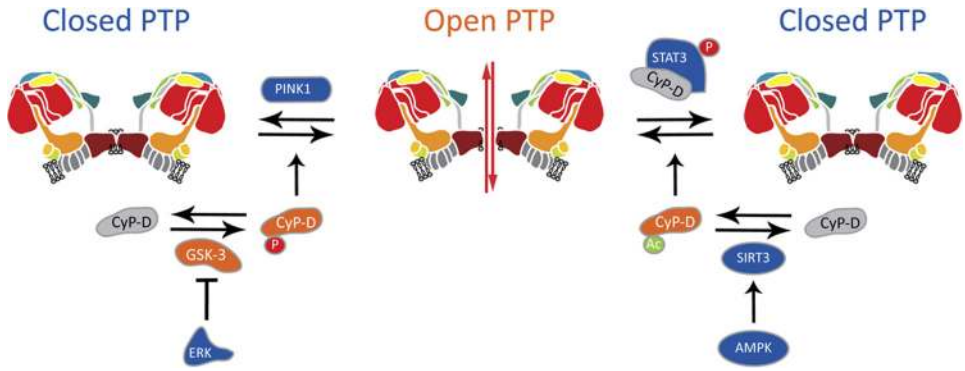

FIGURE 3. Regulation of PTP opening by posttranslational modifications of CyPD. CyPD is a molecular terminal of many signaling axes that regulate the PTP (inducers are indicated in orange, inhibitors in blue): *1*) CyPD phosphorylation by GSK3β facilitates PTP opening, and GSK3β activity is abrogated by induction of the Ser/Thr kinase ERK; *2*) phosphorylated STAT3 binds to CyPD and inhibits PTP opening; and *3*) CyPD acetylation sensitizes the PTP to opening and is prevented by AMPK activation of the SIRT3 deacetylase. In addition, the Ser/Thr kinase PINK1 inhibits PTP opening through poorly defined mechanisms. [Modified from Rasola and Bernardi (490) with permission.]

biochemical events, such as PTMs, in vivo. Accordingly, a surprisingly high number of modifications have been identified in the various subunits of F-ATP synthase, including phosphorylation, acetylation, trimethylation, nitration, *S*-nitrosylation, and tryptophan oxidation. In the last few years, the utilization of powerful technical approaches, such as the combination of cell incubation with [^{32}P]γ-ATP, followed by two-dimensional gel analysis, detection of phosphorylated residues with anti-phospho antibodies, and MALDI-MS/MS mapping of specific phosphorylation sites is further increasing the identification of PTMs (282). It was recently estimated that 67 different phosphorylation sites exist in 12 of the 16 subunits of mammalian F-ATP synthase (128). However, in most cases, it remains completely unknown what are the signaling pathways responsible for these PTMs, in which tissues or biological conditions these modifications occur, how they impact on the biochemical activity of the target protein and of the holoenzyme, and how much of the target protein can be modified (304). Indeed, most sites were detected by a targeted MS/MS approach, suggesting that the abundance of phosphopeptides was very low and raising doubts on their possible biological effects.

In spite of these limitations, some PTMs were reported to affect the F-ATP synthase enzymatic activity, while others might regulate its ability to form dimers and/or multimers, both changes that could impact on PTP modulation. Among PTMs that regulate F-ATP synthase activity, it was reported that *1*) phosphorylation of the d subunit of the lateral stalk by PKCδ inhibits F-ATP synthase activity in neonatal cardiac myocytes (430); *2*) cell stimulation with platelet-derived growth factor induced phosphorylation of the δ subunit (323); and *3*) phosphorylation of Tyr_{213} in the β subunit might play a role in modulation of the F-ATP synthase, and it is deregulated in type 2 diabetes (272). Remarkably, most F-ATP synthase phosphorylation sites are found in the F_1 sector, mainly in the hexamer formed by the α and β subunits as well as in the lateral stalk of the enzyme (OSCP, b, d, and F6 subunits) (128). However, little conformational changes during the catalytic cycle of the enzyme are observed in the upper region of the F_1 hexamer. Yet, most phosphorylated residues were found in this region, with the exception of four phosphorylation residues located near the substrate binding pocket of subunit β (68), and four phosphorylation sites in subunit γ (128, 158). These PTMs could affect its rotation relative to the α-β hexamer (613).

The functional role of PTMs other than phosphorylations has also been investigated. Several F-ATP synthase subunits, including α, β, γ, b, d, g, OSCP, 8, and F6, are acetylated, and these acetylation events dynamically follow fasting/feeding cycles in mice, suggesting a functional effect that remains poorly understood (304). However, *S*-glutathionylation and *S*-nitrosation (also known as *S*-nitrosylation) of subunit α decrease the ATP hydrolytic activity of the enzyme under oxidative stress (104, 207, 562), with possible implications during ischemic conditions when these PTMs might contribute to preserve intracellular ATP levels (563, 621).

Dimerization and oligomerization of F-ATP synthase, together with the formation of respiratory supercomplexes including other OXPHOS components, might affect not only formation of the PTP, but also substrate channeling, stabilization of OXPHOS components, sequestration of reactive intermediates, and IMM morphology, crucially contributing to formation of cristae (634). It is therefore conceivable that such delicate biochemical activities might be finely regulated by PTMs. As already mentioned transmembrane helices of subunit a, subunits of the stator stalk and accessory subunits (e, g, b, h, and i) are predicted to form the structural basis for dimerization by stabilizing the monomer-monomer interface (19, 21, 58, 545, 632, 633). Accordingly, phosphorylation of a Ser residue of subunit g has been proposed to regulate F-ATP synthase dimerization (497), yet the signaling pathways that control subunit g phosphorylation remain unknown. In addition, it was shown that phosphorylation of a Tyr residue in the γ subunit of F_1 might take part in the regulation of dimer formation, since it was only observed in the monomeric form of the F-ATP synthase complex of bovine heart by the use of phosphotyrosine antibodies and MS/MS (158). A further candidate potentially involved in F-ATP synthase dimerization is IF_1, where two phosphorylation sites that may serve regulatory functions have been detected (128). In particular, phosphorylation of Ser_{39} (658) might interfere with the binding to the β subunit, as it is found inside F_1 in the dimeric form (33). Future studies are needed to understand whether PTMs of IF_1 could impact on PTP activity through modulation of F-ATP synthase dimeric conformation.

C. Redox Signaling

ROS are potent PTP inducers through several mechanisms: oxidants increase intracellular Ca^{2+} levels by modulating the activity of IP_3 and ryanodine receptors on the ER/SR, and also of SERCA pumps and Ca^{2+} channels on the plasma membrane (92). Above a certain threshold level, mitochondrial Ca^{2+} or membrane hyperpolarization might also produce ROS through inhibition of respiratory complexes or displacement of cytochrome *c* from cardiolipin in the IMM (444). These events would induce ROS generation by decreasing the rate of respiration and by PTP induction, which completely stops electron flow along the respiratory chain and causes release of mitochondrial GSH (240). Transient PTP inductions might prompt short bursts of ROS and propagating waves of short PTP openings in the surrounding mitochondria. These events would produce additional ROS in the so-called ROS-induced ROS release (667), with possible signaling functions through the modulation of

chaperones, kinases, and gene expression (490). Above a certain degree of mitochondrial ROS rise, this can augment the Ca^{2+} surge, exhausting the high intramitochondrial redox buffering capacity and resulting in an unrestrained elevation of mitochondrial ROS that eventually prompts prolonged PTP opening and cell death.

Changes in respiratory activity are an important factor in causing alterations of the redox equilibrium of a cell also through modulation of ROS production (328). Dozens of PTMs have been reported in OXPHOS complexes (128), which could contribute in tuning ROS production and eventually PTP opening. For instance, Tyr phosphorylation of both cytochrome oxidase and cytochrome *c* inhibits respiration, shielding mitochondria from hyperpolarization and the subsequent increase in ROS levels (282). Cytochrome *c* can trigger PTP induction also following reduction by the mitochondrial pool of phosphorylated p66Shc, which prompts electron transfer directly from cytochrome *c* to oxygen to generate hydrogen peroxide, which may lead to pore opening (215). Induction of IF_1 expression, which is observed in some pathological conditions such as neoplastic transformation, promotes an increase in mitochondrial membrane potential, thus contributing to trigger ROS generation (512). Recent observations highlight the importance of respiratory complex II, succinate dehydrogenase (SDH), in ROS generation. SDH subunit A, the catalytic flavoprotein of the holoenzyme (312), can be phosphorylated by the Src-family tyrosine kinase Fgr in response to ROS stimulation (5), whereas SDH activity can be further enhanced independently of ROS by deacetylation on several lysine residues mediated by sirtuin 3 (188). The following increase in SDH enzymatic activity leads to a secondary wave of ROS generation, with important implications in metabolic adaptations of cells to conditions in which the substrate shift from glucose to fatty acids requires a greater electron flux from FAD, such as during hypoxia/reoxygenation or starvation (5).

In keeping with the importance of SDH in maintaining the redox equilibrium of the cell, we have recently shown that SDH activity is crucial in inducing ROS-dependent PTP opening following nutrient depletion in some tumor cell models. These cells escape death elicited through PTP induction by the mitochondrial chaperone TRAP1, which inhibits SDH activity (243). TRAP1 is a member of the Hsp90 protein family that displays an antioxidant activity, which prevents PTP opening following ischemic damage (641, 642). This is also observed in several tumor types, where TRAP1 expression is generally increased and correlates with malignant progression (491). TRAP1 was reported to inhibit respiration following interactions with both SDH (523) and cytochrome oxidase (651). Thus it is conceivable that TRAP1 might display multiple chaperone functions that converge on blocking PTP induction, following its interaction with several OXPHOS components and with CyPD (see above). The interplay between SDH activity and ROS generation has recently been demonstrated to be crucial in ischemic tissues, where an excess of succinate is produced. Indeed, during ischemia SDH can act in reverse, generating succinate from its usual downstream metabolite fumarate. When reperfusion occurs, succinate is rapidly oxidized causing over-reduction of the coenzyme Q pool and driving "reverse" electron flow towards respiratory complex I, which generates a high amount of ROS leading to ischemia/reperfusion injury (113). It is highly likely that cell death in these conditions is triggered by ROS-dependent PTP opening; accordingly, the complex I inhibitor rotenone acts as an important PTP inhibitor (361).

In vitro, F-ATP synthase is susceptible to ROS (120, 369, 657) and to oxidative/nitrative stress associated with disorders of the central nervous system (482, 561), caloric restriction (561), and aging (234, 264). Hydrogen peroxide selectively inactivates F_1 from bovine heart through formation of iron-protein adducts that generate high ROS levels (369). A specific target of singlet oxygen and hydrogen peroxide has been recently identified in a methionine-cysteine cluster of the γ subunit of chloroplasts. This cluster is highly conserved and is essential for enzyme coupling, as shown by F-ATP synthase inactivation following oxidation (264). Since the cluster is conserved, it is tempting to speculate that it may also mediate ROS-dependent PTP opening. Other selective targets of ROS that could be involved in PTP formation are three Trp residues of d subunit in mammals (583).

V. PATHOPHYSIOLOGY

A. Potential Role in Ca^{2+} Homeostasis

Mitochondria are critical regulators of Ca^{2+} homeostasis (593). There is no question that the PTP can undergo fast cycles of opening/closure both in isolated mitochondria and intact cells (280, 281, 471, 473, 666), a likely result of the flickering detected by patch-clamp (9, 569, 572) and bilayer experiments (96, 218, 611). If a $[Ca^{2+}]$ concentration gradient exists between the matrix and the intermembrane space, PTP-dependent depolarization will be followed by Ca^{2+} release. Whether this event provides a pathway for physiological Ca^{2+} release from mitochondria, and therefore participates in physiological Ca^{2+} homeostasis, has been discussed in the field (46, 50, 239) but remains a difficult problem to address. Indeed, opening of individual pores is usually not synchronized, and polarized mitochondria may take up the Ca^{2+} released by depolarized mitochondria (280, 281). Furthermore, the fraction of F-ATP synthase units undergoing channel formation under "resting" conditions must be very small, and it is not easy to predict what level of inhibition is required to observe an effect. Since the large size of the pore allows charge compensation within the channel itself (41), flux is presumably

limited by the Ca^{2+} concentration gradient, suggesting that most if not all PTP units may need to be inhibited to observe an effect on Ca^{2+} release. These considerations raise some doubts about the conclusions of a recent study where the problem has been tackled by monitoring cytosolic and mitochondrial Ca^{2+} transients after treatment of HeLa cells with siRNAs against the c subunit. No differences were observed, leading the authors to conclude that the PTP does not play a role in mitochondrial Ca^{2+} efflux under basal conditions (148). We note that the experimental evidence that the F-ATP synthase was actually downregulated is a single Western blot reporting a partial decrease of the precursor of subunit c against actin (148). This problem adds to our concerns about the experimental design and should induce some caution in considering the authors' far-reaching conclusions (148).

Since no pore blockers are available, most experiments addressing the role of the PTP in Ca^{2+} homeostasis have used CsA, and this demands additional cautions. The first problem is that the inhibitory effect of CsA obviously depends on the expression level of CyPD, which is rarely assessed. A study from the Fontaine laboratory has revealed that the variation can be extremely large with a matching difference in sensitivity to CsA, which for example was totally absent in NIH3T3 fibroblasts and HL60 cells (361). The second issue is the relative expression of CyPD and of F-ATP synthase. Cross-linking experiments in beef heart mitochondria indicate that there is much less CyPD than b, d, and OSCP subunits (216). If these results can be extrapolated to other tissues, many F-ATP synthase channels will be insensitive to CsA even if CyPD is expressed. Finally, not all the mitochondrial effects of CsA are mediated by PTP inhibition. Even when the most common artifacts are avoided (47, 48), inhibition of calcineurin can prevent mitochondrial fission by preventing Drp1 translocation (98). This event can affect mitochondrial shape, which in turn influences Ca^{2+} homeostasis (501). Thus, while with proper precautions an effect of CsA indicates that the PTP may be involved in the event under study, a negative result does not always allow safe conclusions to be made (48, 52).

In 1992, Altschuld et al. (13) reported that CsA increased net Ca^{2+} uptake and decreased Ca^{2+} efflux in isolated cardiomyocytes, through a mitochondrial effect that had no impact on cell morphology or viability. On the other hand, no effect on Ca^{2+} homeostasis was found in a study on hormonally stimulated rat livers perfused with CsA where the PTP was demonstrably inhibited after in vivo treatment (176). Ca^{2+} homeostasis has also been addressed in *Ppif*$^{-/-}$ cells and mice. The hearts of *Ppif*$^{-/-}$ mice displayed functional changes that are consistent with increased Ca^{2+} retention due to decreased PTP activity, such as decreased contractile reserve, increased shortening and relaxation times, and longer decay of cytosolic Ca^{2+} transients (173). Mitochondria displayed an increased Ca^{2+} content that was matched by larger Ca^{2+} transients in wild-type individuals treated with CsA. CsA also decreased the rise time required for Ca^{2+} accumulation and prolonged the recovery time, consistent with operation of the PTP as a Ca^{2+} release channel preventing Ca^{2+} overload (173). In keeping with this idea, in adult cortical neurons from wild-type and *Ppif*$^{-/-}$ mice, cytosolic [Ca^{2+}] increases induced by either ATP or by depolarizing concentrations of KCl gave comparable, transient increases of mitochondrial [Ca^{2+}], while application of the two stimuli together resulted in much higher levels of mitochondrial [Ca^{2+}] in the *Ppif*$^{-/-}$ neurons. This result suggests that the threshold for PTP activation had been reached in the wild-type but not in the CyPD-null mitochondria in situ (30). Consequently, the regulatory role of PTP opening in Ca^{2+} homeostasis, which may be of relevance to synaptic plasticity (593), may become apparent only for relatively large mitochondrial Ca^{2+} loads (30). Mice expressing amyotrophic lateral sclerosis-linked mutants of superoxide dismutase 1 develop a motor neuron disease with many pathological hallmarks also seen in patients (241). Spinal chord mitochondria display decreased Ca^{2+} retention capacity long before onset of motor weakness and neuronal death (141), and this was corrected by ablation of the *Ppif* gene (454). In these mice, improved mitochondrial Ca^{2+} buffering was matched by improved ATP synthesis and reduced swelling, attenuation of glial activation, reduction of misfolded aggregates in the spinal cord, and significant suppression of motor neuron death throughout disease, although survival was not improved (454).

Our recent findings that the F-ATP synthase of *Drosophila* mitochondria forms 53 pS channels (611) responsible for mitochondrial Ca^{2+}-induced Ca^{2+} release (610), and that the channels may be selective for Ca^{2+} and H^+ (610), are of relevance to the present discussion. Indeed, while the mammalian PTP could be involved either in Ca^{2+} homeostasis or in apoptosis induction depending on its open time (473), due to its small size the *Drosophila* PTP could be involved only in Ca^{2+} homeostasis (50, 610). Consistent with this fact, the mitochondrial pathway is not essential in most cases of *Drosophila* apoptosis (164, 403, 439, 616, 643).

Transient PTP openings are also intimately linked to mitochondrial ROS production in the process of ROS-induced ROS release (666). This process overlaps with Ca^{2+}-induced Ca^{2+} release, to which it appears to be mechanistically connected, as thoroughly discussed by Zorov et al. (667). "Flashes" related to transient PTP openings have also been observed in mitochondria expressing a circularly permuted yellow fluorescent protein (622). The flashes have been attributed to bursts of local production of the superoxide anion, but there is an ongoing debate on whether they are partially or totally due to changes of matrix pH (521, 522), although matrix alkalinization appears way too small to account for the observed fluorescence changes (629).

Irrespective of the nature of the detected species, there is little doubt that the flashes document the occurrence of transient PTP openings, which have been also detected in living animals (183). In summary, the role of transient PTP openings in physiological Ca^{2+} and ROS homeostasis is supported by increasing experimental evidence and deserves further study.

B. Pore Opening in Cell Death

Persistent PTP opening may constitute the point of no return in cell commitment to death (489). As already mentioned, prolonged PTP opening causes mitochondrial depolarization by equilibration of the proton gradient, followed by loss of pyridine nucleotides and respiratory inhibition (157, 607). Equilibration of ions and of solutes with molecular masses below the pore size induces mitochondrial swelling, given the colloidal osmotic pressure exerted by matrix proteins, leading to breaches in the OMM and release of intermembrane, apoptogenic proteins like cytochrome *c* (472). In addition, Ca^{2+} equilibrates across the open pores, and mitochondria can no longer maintain a Ca^{2+} concentration gradient (50). The combined effect of these dramatic events initiates an apoptotic routine, if enough ATP is available to supply the required energy, or induces necrosis (15). PTP dysregulation is a crucial element in many pathological conditions, such as degenerative diseases, where excessive PTP opening induces tissue damage, and cancer, where insensitivity to PTP induction contributes to the cellular ability to escape apoptosis that characterizes neoplastic cells (243, 322). As a corollary, identification of drugs that modulate PTP induction is a promising area of investigation for the potential treatment of many diseases (182, 550, 587, 631). We will briefly cover diseases where an increased propensity to PTP opening is part of pathogenesis and discuss in some detail emerging mechanisms that may link PTP resistance to cancer onset and progression.

1. Diseases with increased propensity to pore opening

Treatment with CyPD inhibitors, or CyPD ablation, generally confers remarkable protection against ischemia-reperfusion injury of the heart (27, 231, 426, 480, 538), brain (327, 353, 363, 518, 594, 623), kidney (453), and liver (271, 422). In the heart, protection extends to preconditioning and postconditioning protocols (366) and organ failure (367); in brain to hypoglicemic damage (186, 197, 311), ammonia neurotoxicity (10, 435), and trauma (558, 559). It is easy to predict that these fields will enormously benefit from novel PTP inhibitors acting independently of CyPD (182) and from increased understanding of the posttranslational modifications of F-ATP synthase that may be the basis for preconditioning, where IF_1 binding to F-ATP synthase persisted after the end of the short ischemia (159, 612), and postconditioning.

Muscular dystrophies are a group of genetic diseases characterized by progressive loss of muscle tissue, which leads to progressive skeletal muscle weakness, impairment of contractility, disability often associated to inability to walk, respiratory problems, and cardiomyopathy (488). A severe form of dystrophy, Ullrich congenital muscular dystrophy, and the milder Bethlem myopathy are caused by mutations that hamper maturation of collagen VI, a key component of the extracellular matrix of skeletal muscle. Collagen VI deficiency impairs anchoring of skeletal muscle fibers to the matrix, exposing muscles to extensive mechanical stress that eventually leads to death of muscle fibers (341). We have shown that PTP induction is the effector mechanism responsible for the loss of muscle fibers in collagen VI null mice (287) in a process that involves ROS production by mitochondrial monoamine oxidases (539). Mitochondrial dysfunction and cell death could be prevented both in mice and in myoblasts from Ullrich patients by treatment with CsA and other CyPD inhibitors devoid of immunosuppressive activity, paving the way to the use of PTP inhibitors for the treatment of patients (43, 407, 669). Several pieces of evidence point to a role of the PTP in the pathogenesis of other muscular dystrophies as well. Genetic ablation of CyPD reduces the dystrophyc phenotype in both skeletal muscle and heart of mice lacking δ-sarcoglycan, and the premature lethality associated with the absence of the α2 chain of laminin, which causes congenital muscular dystrophy (410). Moreover, treatment with CyPD inhibitors rescues mitochondrial dysfunction and muscle fiber necrosis in mdx mice, a model of Duchenne muscular dystrophy (498).

Deregulated Ca^{2+} influx into neurons through receptors for *N*-methyl-D-aspartate and glutamate causes excitotoxicity, leading to activation of degradative enzymes, loss of redox equilibrium, and eventually mitochondrial depolarization and cell death (436, 576), probably induced by PTP opening. Accordingly, several features of the PT can be observed during excitotoxic injury (481), which can be blocked by CsA (517). Moreover, after induction of excitotoxicity, a secondary rise of cytosolic Ca^{2+}, named "delayed Ca^{2+} deregulation," occurs (78), which probably leads to cell demise via stable PTP opening (481).

Many diseases of the central nervous system are characterized by derangements of Ca^{2+} and ROS homeostasis that induce PTP opening as an important pathological feature. Alzheimer's disease, the most common form of dementia in aged people, is characterized by deposition of amyloid plaques formed by the amyloid β peptide, a cleavage product of the amyloid precursor protein (244). It has been shown that amyloid β oligomers alter Ca^{2+} homeostasis (153). In addition, amyloid β can be imported in mitochondria (257), where it interacts with CyPD and enhances PTP induction, whereas neurons from CyPD knockout mice are protected from cell death induced by amyloid β-dependent PTP (166). Interestingly, a novel association

with Alzheimer's disease risk, which reached genome-wide significance, has been recently identified in the F-ATP synthase ATP5H locus, which encodes subunit d of the lateral stalk (60).

Huntington disease is caused by mutations that lead to translation of an expanded polyglutamine tract in the gene encoding the protein huntingtin. Patients display progressive motor and cognitive deficits that are invariably lethal (342). Pathogenic huntingtin mutations cause enhanced mitochondrial sensitivity to Ca^{2+} that eventually can lead to PTP induction both in cells from patients (448) and in animal models of the disease (212, 580). Accordingly, neurons carrying the mutant huntingtin gene display mitochondrial Ca^{2+} elevation with increased oxidative stress and mtDNA damage (618) and increased PTP sensitivity to Ca^{2+} (365).

Amyotrophic lateral sclerosis is an appalling neurodegenerative disease characterized by loss of spinal motor neurons, which is rapidly progressive and lethal (456). In a small subset of patients, the disease is genetically inherited and caused by mutations in superoxide dismutase 1 (63). Transgenic mice expressing the mutant protein display mitochondrial alterations including swelling, respiratory inhibition, reduced Ca^{2+} buffering capacity and fragmentation, and an elevated generation of ROS (235, 309). Either CsA or genetic ablation of CyPD increases Ca^{2+} buffering capacity and ATP synthesis in mitochondria of mouse models of amyotrophic lateral sclerosis, indicating that PTP opening might be the effector of neuron cell death (63, 456). The PTP-protective effects initially reported for minocycline (660) led to a major trial on patients that unfortunately had to be terminated because of lack of efficacy and presence of serious side effects (224), which can probably be traced to the permeabilizing effects of the drug on mitochondria (338, 519).

In multiple sclerosis, the myelin sheath of neurons in the central nervous system is destroyed as a consequence of an idiopathic disease leading to a variety of neurological symptoms and to progressive disability (556). The disease is worsened by axonal degeneration, which is associated to mitochondrial Ca^{2+} overload and bioenergetic dysfunction (198). Experimental autoimmune encephalomyelitis is widely used as an animal model of multiple sclerosis, as mice develop an inflammatory demyelinating disease of the central nervous system after administration of myelin components. When CyPD-null mice are treated, they display a marked protection from axonal degeneration and a milder clinical picture despite a normal inflammatory response, thus providing strong evidence that PTP is involved in disease pathogenesis (193). A further indication of the central role played by the PTP in multiple sclerosis is provided by the observation that axonal damage of mice undergoing experimental autoimmune encephalomyelitis is reduced by genetic inactivation of p66Shc, which abrogates an important pathway of ROS-induced PTP opening (see above) (514).

The most frequent form of neurodegenerative disorder affecting movement, Parkinson's disease, is caused by death of dopaminergic neurons in the mesencephalic region called substantia nigra pars compacta. A fine tuning of intracellular Ca^{2+} fluctuations and of the Ca^{2+} storage capacity of mitochondria is particularly important in these neurons, as the release of the neurotransmitter dopamine is under the control of voltage-dependent L-type Ca^{2+} channels (630). Indeed, sensitization to PTP opening has been proposed as a major cause of neurodegeneration in several models of the disease characterized by altered homeostasis of intracellular Ca^{2+} (87, 376, 384), including the forms caused by complex I inhibition (229, 528) and by inactivation of the Ser/Thr kinase PINK1 (597), in which changes in Ca^{2+} storage capacity (8, 205), impairment of respiratory complex I (209), and altered mitophagy (307) are observed. Moreover, the PTP can be induced in dopaminergic neurons by inability to buffer increased intracellular ROS levels (79). Interestingly, the decline of motor scores is much slower in patients treated with dopamine agonists (such as pramipexole and ropinirole) rather than with L-dopa. This effect could be explained by a neuroprotective inhibition of the PTP by dopamine agonists, which has been reported in rat mitochondria (455, 515), and would be similar in mechanism to PTP-dependent protection of the penumbra zone in oxygen-glucose deprivation protocols by treatment with melatonin (14).

Mitochondrial dysfunction occurs in several lysosomal storage diseases (321), and some evidence is available to suggest that the PTP is involved. Loss of mitochondrial Ca^{2+} homeostasis was proposed as the unifying mechanism and, indeed, in a mouse model of human GM1-gangliosidosis, accumulation of GM1-ganglioside caused mitochondrial Ca^{2+} overload, PTP opening, and apoptosis (513). Mitochondrial dysfunction is also key to Gaucher's disease, the most common lysosomal storage disorder (443). This genetic disease causes loss of activity of glucocerebrosidase and, intriguingly, confers a high risk of developing Parkinson's disease (443).

The PTP is involved in liver failure models that mimic fulminant hepatitis (356). Protection by CsA has been reported after treatment with lipopolysaccharide of *Escherichia coli* (136–138) and heat-inactivated *Propionibacterium acnes* (625) or D-galactosamine (308, 542), with anti-Fas antibody (184), and in an animal model of α-1 antitrypsin deficiency with liver injury (584). Interestingly, encouraging results have also been obtained in one cohort of cases of fulminant viral hepatitis treated with CsA (650). Ca^{2+} deregulation and PTP opening are also involved in toxicity of diclofenac (391) and acetaminophen (258, 392), which accounts for about half of the cases of acute liver failure. Acetaminophen poisoning induces glutathione depletion and inhibition of mitochondrial respiratory complexes I and II, thereby increasing ROS generation

and altering the mitochondrial ability to sequester Ca^{2+}. Such events ultimately lead to PTP opening and to potentially fatal centrilobular hepatic necrosis (270). Notably, in cultured mouse hepatocytes, acetaminophen toxicity is abrogated by both CsA and by its nonimmunosuppressive analog NIM811 (326).

Mitochondrial dysfunction due to PTP dysregulation is considered to be an important effector mechanism in sepsis (137, 138, 344) and in the subsequent multiple organ dysfunction syndrome (142, 642), which derives from decreased oxygen utilization and may also be a consequence of damage to endothelial mitochondria (145). Finally, a causal link with the PTP is emerging in diabetes (336), which may be related both to endothelial damage and its vascular complications, and to the demise of insulin-secreting β cells (200, 201, 340, 379, 577).

2. Cancer and decreased propensity to pore opening

Cancer cells are extremely refractory to cell death following a variety of stress stimuli. PTP desensitization is emerging as an important strategy used by neoplastic cells to escape death. An example is provided by the induction of liver tumors in rats by administration of the arylamine 2-acetylaminofluorene, which closely mimics the diverse stress conditions (alcoholic liver disease, chronic hepatitis B and C, and cholestasis) that predispose to hepatocellular carcinoma in humans. We have found that 2-acetylaminofluorene-treated rats become resistant to liver apoptosis and necrosis by desensitizing the PTP before onset of transformation, which indicates that the pore is an early target of adaptive responses that promote tumorigenesis in vivo (322).

Changes in metabolic functions and in transduction pathways, together with their exposure to an environment where oxygen and nutrient resources continuously fluctuate (230), lead to important changes in the redox status of tumor cells. In particular, increased levels of ROS must be strictly controlled to avoid oxidative stress and eventually pore opening and cell death (91). A deeper understanding of how tumor cells protect themselves from ROS-induced PTP opening offers the possibility for novel selective antineoplastic strategies. Indeed, as cancer cells must walk a delicate tightrope between ROS generation and scavenging, they may become extremely sensitive to further oxidative insults that overtake their antioxidant defenses (225).

Liver cancer cells are protected from oxidative stress elicited by chemotherapeutic treatment by inhibiting respiratory complex I through overexpression and mitochondrial localization of SERPINB3, a serine protease inhibitor of the serpin family (223), thus blocking ROS generation and protecting cells from PTP opening (114). As reported above, mitochondrial binding of HK II delivers a survival signal that stabilizes the PTP in the closed conformation (109). We (449) and others (564, 639) have observed that mitochondrial HK II displays an antioxidant function that explains its survival function, independently of HK II enzymatic activity (discussed in Ref. 449). Notably, the kinase AKT has an extremely relevant role in installing the antiapoptotic phenotype of tumor cells (174) by phosphorylating HK II and thus favoring its OMM binding. In turn, this inhibits Ca^{2+}-induced cytochrome *c* release and apoptosis (418). Thus the design of drugs that target mitochondrial HK II, such as membrane-permeable peptides that compete for HK II binding to the OMM, is highly warranted, as this approach could selectively kill neoplastic cells by ROS-dependent PTP induction.

Another important survival strategy employed by tumors that impacts on the PTP involves the mitochondrial chaperone TRAP1. Indeed, TRAP1 displays an antioxidant activity in several pathological conditions, such as cerebral (644) and heart ischemia (641). In addition, it inhibits PTP opening and death of hypoxic cardiomyocytes (641). In several tumor types, the expression of TRAP1 is increased during malignant progression (reviewed in Ref. 491). Augmented TRAP1 expression enhances tumor malignancy by protecting neoplastic cells from toxicity elicited by several antineoplastic agents (126, 393, 420), likely through inhibition of ROS-dependent PTP opening (243). Therefore, the design of compounds that selectively inhibit TRAP1 chaperone activity constitutes a promising antitumor approach. Interestingly, the use of molecular dynamics simulations has been applied to the closely related chaperone Hsp90, allowing the identification of potential allosteric inhibitors of its chaperone activity cycle (424). A similar strategy can be envisioned for TRAP1, exploiting structural differences between TRAP1 and Hsp90 (346) to design novel classes of allosteric inhibitors that selectively target the chaperone cycle TRAP1.

Inhibition of Kv1.3, a voltage-dependent K^+ channel located in the inner mitochondrial membrane, induces ROS generation and PTP opening (570). It was recently shown that membrane-permeant, selective Kv1.3 inhibitors such as Psora-4, PAP-1, and clofazimine are effective on several cancer cell lines and on B lymphocytes derived from B-chronic lymphocytic leukemia (348, 349). Notably, clofazimine reduced by 90% the size of a melanoma in an orthotopic mouse model, without any side effects (348).

It is becoming increasingly clear that some widely used antineoplastic drugs are able to prompt a ROS surge that induces the PTP, which could explain at least in part the toxicity associated to their use. For instance, taxol (paclitaxel), which is known to induce apoptosis by stabilizing microtubules, also prompts ROS-dependent PTP induction in liver cancer cells (602). Cisplatin, on the other hand, causes the formation of adducts with DNA that induce apoptosis (204) and can also elicit CyPD-dependent necro-

sis in pancreatic cancer cells (102). Importantly, we have recently found that cisplatin, doxorubicin, and the BH3 mimetic EM20-25 (409) prompt a rapid ROS increase and PTP opening in hepatoma cells (114). These observations suggest that the efficacy of compounds designed on the structure of cisplatin will increase with the decrease of the toxic side effects (111). We have characterized one such compound, the Gold(III)-dithiocarbamato complex AUL12, that is endowed with high selectivity towards tumor cells and xenograft tumors but with extremely low nephrotoxicity and acute toxicity (386). We found that AUL12 strongly inhibits respiratory complex I, inducing a rapid burst of mitochondrial ROS levels that leads to activation of the mitochondrial pool of GSK3 and to the ensuing phosphorylation of CyPD (110). Therefore, these data provide a biochemical mechanism that connects chemotherapeutic activity with ROS generation, kinase activation, and PTP induction in mitochondria. Significantly, several plant-derived natural compounds that act by increasing intracellular ROS levels, thereby serving as PTP inducers, have been tested in tumor cells and in animal models, and some of them are currently undergoing clinical or preclinical trials (350, 557). A summary of the signaling pathways described in this paragraph can be found in **FIGURE 4**.

VI. CONCLUSIONS

Major progress has been made in understanding the nature of the PTP. Conservation of the channel-forming property of F-ATP synthases from yeast to *Drosophila* and mammals, with distinctive conductance features, suggests a function that has been acquired in eukaryotes, and maintained and exploited in evolution. The PTP may have initially evolved as a Ca^{2+}-release channel and then acquired a role in cell death that became part of the mitochondrial pathway to apoptosis. In this perspective, it is very striking that the F-ATP synthase, the point of integration of Mitchell's proton gradient with ATP synthesis or hydrolysis, may also be the point of integration of death signals. The finding that the PTP forms from F-ATP synthase is so recent that key mechanistic issues still need to be clarified. Yet, it is easy to predict that, 60 years after its discovery, the years to come will see a solution to the mystery of the PTP and the dissection of the molecular mechanisms that mediate the transition of the F-ATP synthase from an energy-conserving into an energy-dissipating device.

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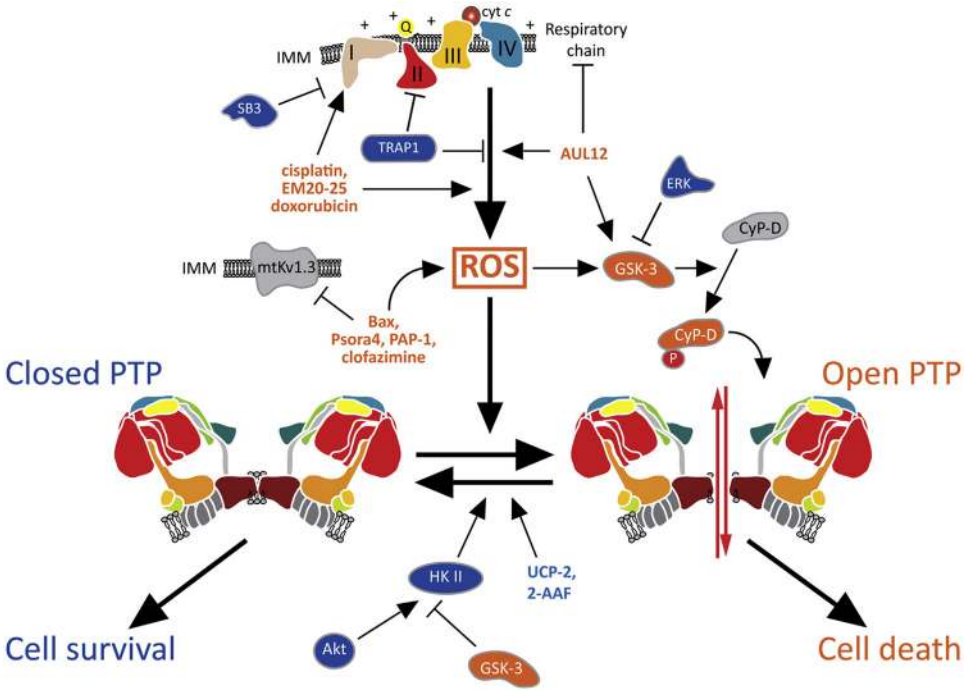

FIGURE 4. Mechanisms of PTP regulation in tumor cells. A variety of factors control tumor cell viability by modulating PTP opening, mainly by modulating ROS levels. A ROS surge elicits PTP opening and cell death, whereas ROS inhibition keeps the pore locked and protects cells from noxious stimuli. PTP inducers are indicated in orange, PTP inhibitors in blue. I–IV, respiratory complexes; 2-AAF, 2-acetylaminofluorene; Q, coenzyme Q; cyt *c*, cytochrome *c*; HK II, hexokinase II; IMM, inner mitochondrial membrane; SB3, serpin B3; UCP-2, uncoupling protein 2. [From Rasola and Bernardi (494) with permission.]

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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