The mitochondrial permeability transition: Recent progress and open questions

Paolo Bernardi[1] (iD), Michela Carraro[1] and Giovanna Lippe[2]

1 Department of Biomedical Sciences and CNR Neuroscience Institute, University of Padova, Italy
2 Department of Medicine, University of Udine, Italy

Correspondence

P. Bernardi, Department of Biomedical Sciences, University of Padova, Via Ugo Bassi 58/B, I-35131 Padova, Italy
Tel: (+039) 049 827 6365
E-mail: paolo.bernardi@unipd.it

Major progress has been made in defining the basis of the mitochondrial permeability transition, a Ca^{2+}-dependent permeability increase of the inner membrane that has puzzled mitochondrial research for almost 70 years. Initially considered an artefact of limited biological interest by most, over the years the permeability transition has raised to the status of regulator of mitochondrial ion homeostasis and of druggable effector mechanism of cell death. The permeability transition is mediated by opening of channel(s) modulated by matrix cyclophilin D, the permeability transition pore(s) (PTP). The field has received new impulse (a) from the hypothesis that the PTP may originate from a Ca^{2+}-dependent conformational change of F-ATP synthase and (b) from the reevaluation of the long-standing hypothesis that it originates from the adenine nucleotide translocator (ANT). Here, we provide a synthetic account of the structure of ANT and F-ATP synthase to discuss potential and controversial mechanisms through which they may form high-conductance channels; and review some intriguing findings from the wealth of early studies of PTP modulation that still await an explanation. We hope that this review will stimulate new experiments addressing the many outstanding problems, and thus contribute to the eventual solution of the puzzle of the permeability transition.

A bird's eye view on the permeability transition

Although the term 'permeability transition' (PT) was introduced in 1976 [1] and further defined in 1979 [2–4], occurrence of an unselective permeability increase of mitochondria and its detrimental consequences on energy conservation had been known since the very first studies on isolated organelles [5,6]. The permeability increase leading to swelling was widely considered to be a form of membrane damage [7] possibly caused by long-chain fatty acid(s) [8] and by lysophospholipids generated by the Ca^{2+}-dependent activation of phospholipase A_2, as suggested by the remarkable protective effects of nupercaine [9] and of N-ethylmaleimide [10]. The far-reaching hypothesis that the PT could rather be caused by opening of a regulated inner mitochondrial membrane (IMM) channel playing a role in physiology (the PT pore, PTP) [2–4] was generally met with scepticism also because of the acceptance of the chemiosmotic hypothesis of

Abbreviations

ANT, adenine nucleotide translocator; ATR, atractylate; BKA, bongkrekate; Bz, benzodiazepine; CsA, cyclosporine A; CyP, cyclophilin; DDM, dodecyl maltoside; EM, electron microscopy; IMM, inner mitochondrial membrane; IMS, intermembrane space; LMNG, lauryl maltose neopentyl glycol; OMM, outer mitochondrial membrane; OSCP, oligomycin sensitivity conferral protein; PT, permeability transition; PTP, permeability transition pore; TSPO, translocator protein; Ub, ubiquinone; UCP1, uncoupling protein 1; VDAC, voltage-dependent anion channel.

energy conservation [11]. Indeed, permeability defects with an estimated pore radius of 14 Å [7] seemed hard to reconcile with the low permeability of the coupling membrane to solutes and ions, as discussed in detail in previous reviews [12–14]. The channel hypothesis gained traction (a) with the electrophysiological identification of an IMM high-conductance channel named mitochondrial multiconductance channel [15] or megachannel [16], which shares the key features of the PTP [17,18], see [19] for a review and (b) with the discovery that the PT could be inhibited by nanomolar concentrations of cyclosporine A (CsA) [20–23], a finding that was instrumental in establishing a pathogenic role for the PTP in cell and organ injury [24–28]. CsA had no effects on phospholipase A$_2$ [29] while it inhibited a matrix peptidyl prolyl *cis-trans* isomerase [23] that was later shown to be mitochondrial cyclophilin (CyP) D [30,31]. There is no question that CyPD favours onset of the PT [30] because, like treatment with CsA, deletion of the *Ppif* gene (which encodes CyPD) desensitises the PTP to Ca^{2+} [32–35]; and yet both CsA-sensitive and CsA-insensitive PTs with distinct features could be identified, with a synergistic effect of free fatty acids on PTP opening [36,37].

The molecular nature of the PTP is still an open issue, but significant progress has been made in the recent years. Based on the effects of the selective inhibitors of the adenine nucleotide translocator (ANT) atractylate (ATR, which favours PTP opening) and bongkrekate (BKA, which favours PTP closure) the first proposed candidate for channel formation was the ANT itself [2]. Consistent with this hypothesis, ANT from bovine heart and *Neurospora crassa* formed Ca^{2+}-activated channels [38,39] with many of the features displayed by the PTP at the patch-clamp [17,40,41], including CsA-inhibitable stimulation by yeast cyclophilin [39]. At about the same time, the ANT was shown to copurify with the outer mitochondrial membrane (OMM) proteins voltage-dependent anion channel (VDAC) and translocator protein (TSPO, formerly called peripheral benzodiazepine receptor) [42]; and it was found that nanomolar concentrations of TSPO-binding ligands (like benzodiazepines) promoted channel opening [43]. Although puzzling, given that the PT takes place at the IMM [44], these findings shifted the attention of the field to sites of contact between the OMM and IMM, with the former also providing accommodation for hexokinase II [45,46] and for proteins of the Bcl-2 family, linking the PTP to apoptosis and to regulated necrosis [47,48].

Modulation by CyPD and inhibition by CsA considerably strengthened the idea that the PT was mediated by a single molecular entity. Given that genetic ablation of each of the putative components of the PTP (i.e., ANTs, VDACs and TSPO) did not prevent occurrence of the PT [49–53], we searched for novel PTP candidates using mitochondrial CyPD as the bait. A most interesting interactor turned out to be the mitochondrial F-ATP synthase [54]. This observation was the starting point for a set of studies addressing the question of whether F-ATP synthase can form a Ca^{2+}-dependent channel with the features of the PTP. Results were apparently conflicting.

On one hand, it was shown that partially purified F-ATP synthase can generate Ca^{2+}-activated channels in a variety of eukaryotic mitochondria [55–60]; that knockdown of subunit c [61] or point mutations that do not affect enzyme complex assembly or ATP synthesis caused specific changes in the properties of the PTP [56,59,62–68]; and that highly purified F-ATP synthase preparations display features expected of the PTP in electrophysiological experiments [69,70]. Remarkably, it was also discovered that benzodiazepines are actually not selective ligands of TSPO, as they also inhibit F-ATP synthase [71,72] while promoting the PT [55,69], effects that mimic those of CyPD through a shared binding site on subunit oligomycin sensitivity conferral protein (OSCP) [55,73].

On the other hand, ablation of individual subunits of F-ATP synthase (which prevented its assembly) in HAP1 cells was not followed by disappearance of the PT, which persisted and maintained its sensitivity to CsA [74–76]. Yet, in the same cells ablation of subunit c led to the appearance of a channel sensitive to both CsA and BKA, while the channel of wild-type cells was sensitive to CsA only [77]. These results can be explained by the existence of two CsA-sensitive channels modulated by CyPD. One channel is formed from the F-ATP synthase, the other from the ANT. Our recent work fully confirmed the dual nature of the PT and suggested that in wild-type cells the F-ATP synthase predominates, yet the two PTPs are interconnected [67] possibly by physical association at the 'ATP synthasomes', which may also involve the Pi carrier [78–80].

Despite these advances, many open questions remain including the molecular mechanisms through which the ANT and F-ATP synthase can form high-conductance channels, the role of the OMM, and the assignment of a variety of agents and factors that critically modulate the PT. The following discussion is meant to address these issues, with the hope that it can stimulate new experiments to solve the longest-standing mystery of mitochondrial biology. Space limitations preclude discussion of the physiology and pharmacology of the PTP, and of its role in degenerative diseases, cancer and aging, important topics for which we refer the reader to published reviews [14,81–92].

Adenine nucleotide translocator

The ANT is a 30 kDa protein of the IMM that was first identified in the early 1960s [93,94]. It catalyses the equimolar exchange of Mg^{2+}-free ADP and ATP across the IMM. Transport of adenine nucleotides is determined by their concentration gradient and by the membrane potential existing across the IMM, since ADP/ATP exchange is electrogenic. In respiring mitochondria this results in uptake of ADP from the cytosol and release of newly synthesised ATP. Humans have four ANT isoforms, AAC1-AAC4, with AAC4 being specific to germline and to pluripotent stem cells [95,96]. ANTs belong to the SLC25 mitochondrial carrier family, which is the largest solute transporter family comprising 53 carriers in humans [97]. These carriers transport metabolites, inorganic ions and cofactors across the IMM and are thought to operate through a common mechanism, defined as alternating-access mechanism [98]. It is based on three main functional elements, that is, one central substrate-binding site and two gates with salt bridge networks on either side of the carrier [99].

Structure and catalytic mechanism

Most structural properties of the SLC25 family have been defined from studies of the ANT, which were favoured by its abundance and by the availability of two specific inhibitors, ATR [100] and BKA [101], which lock the carrier in two different conformational states. ATR traps the ANT in the cytoplasmic-open state (c-state) with the adenine-binding site facing the cytosol, while BKA locks the ANT in the matrix-open state (m-state) with the adenine-binding site facing the matrix [102]. It should be kept in mind that no structures of substrate-bound states have yet been defined, so that the events taking place during adenine nucleotide translocation are not known. Information at atomic resolution was first provided by the crystallographic structure of the carboxyATR-inhibited bovine ANT [103]. The protein contains three homologous domains of about 100 amino acids, each comprising an odd-numbered transmembrane α-helix (H1, H3 and H5), a loop with a short matrix α-helix (h12, h34 and h56) lying in the plane of the IMM, and an even-numbered transmembrane helix (H2, H4 and H6). This basic structural fold was confirmed for the yeast isoforms Aac2p and Aac3p [104].

In the c-state (which favours the PT) the odd-numbered transmembrane α-helices, which contain the conserved Px[DE]xx[KR] motif, are close together towards the matrix side of the membrane, enabling the charged residues to establish inter-domain salt-bridges (the matrix salt-bridge network) and closing the central substrate-binding site from the matrix side [104]. In the m-state (which inhibits the PT) the even-numbered α-helices make inter-domain salt-bridges (the cytoplasmic salt-bridge network) through highly conserved [YF][DE]xx[KR] motifs, closing the central cavity from the cytoplasm side, while the matrix helices on the membrane surface are rotated outward opening the central cavity to the matrix side [105].

The proposed transport mechanism involves six mobile elements, two per domain. During the switch from the c- to the m-state triggered by substrate binding, the core elements composed by the cytoplasmic ends of the H1, H3 and H5 helices would move outward as rigid bodies, opening up the substrate-binding site to matrix, while the cytoplasmic gates formed by the H2, H4 and H6 helices rotate inwards, closing the cytoplasmic side. The reverse movements would occur in the m- to c-state transition [99,102]. According to this structural model, the carrier is monomeric because the large conformational changes preclude the formation of a stable dimerisation interface during the transport cycle. An outline of the ANT and of the overall ATP/ADP exchange process is reported in the left panel of Fig. 1.

Possible mechanism of high-conductance channel formation

Some clues on the requirements for channel formation by ANT may come from recent work on H^+ transport by the structurally related uncoupling protein 1 (UCP1) and by ANT itself. In thermogenic brown fat H^+ permeation is a highly regulated process under hormonal control requiring fatty acids and mediated by UCP1 [106,107], which like ANTs is a member of the SLC25 mitochondrial carrier family [97]. H^+ ions are transported by the fatty acyl anion, which shuttles within UCP1 itself [108]. Of note, deletion of amino acids 261–269 converts UCP1 into a pore allowing permeation of species with molecular mass up to 1 kDa [109]. The IMM has a measurable permeability to H^+ (the 'H+ leak') also in tissues that do not express UCP1. The H^+ leak is widely considered to result from passive H^+ permeation through the lipid bilayer, which is compensated by basal respiration (oxygen consumption not coupled to ATP synthesis). Basal respiration is increased by fatty acids, an effect that can be prevented by ADP or by inhibitors of the ANT [110]. This interesting finding led to the suggestion that the H^+ leak may be due, in part at least, to H^+ transport through the ANT either through an allosteric effect of
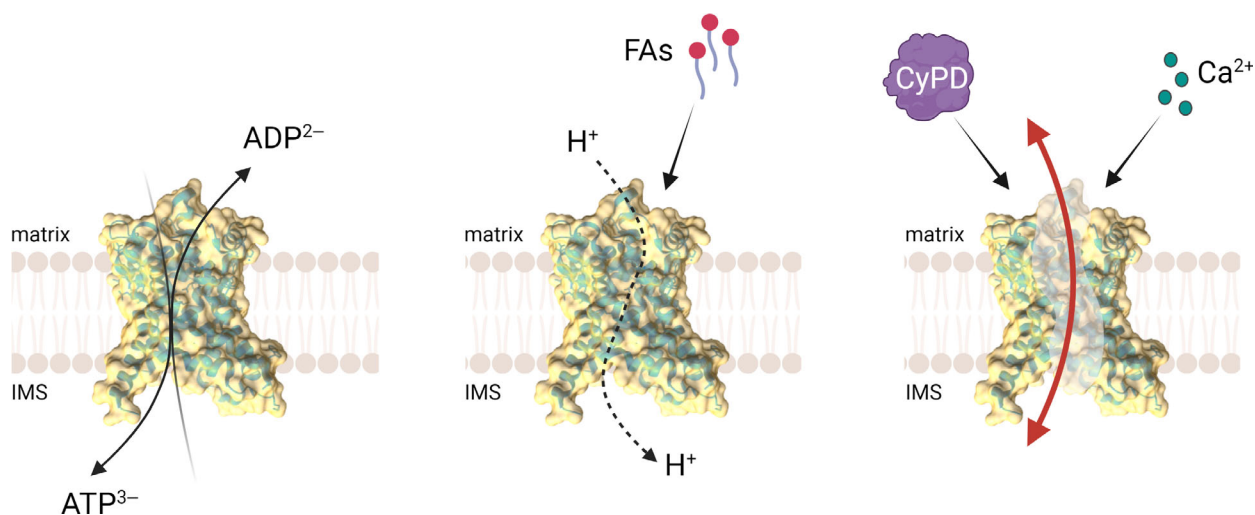
Fig. 1. Schematic representation of the possible functions of ANT. (Left) The established function of ANT is to exchange adenine nucleotides, transporting matrix ATP to the cytosol and cytosolic ADP to the matrix in energised mitochondria. (Center) In response to fatty acids ANT mediates H^+ currents, suggesting that nucleotide exchange and H^+ transport are not mutually exclusive functions. (Right) In the presence of Ca^{2+} and CyPD, ANT may undergo a still undefined conformational change which allows the formation of a high-conductance channel.

the fatty acid, or through transport of the fatty acyl anion coupled to passive diffusion of the protonated fatty acid through the lipid bilayer [110]. A recent electrophysiological study has demonstrated that a relevant fraction of the H^+ leak indeed takes place through the ANT acting as a channel in which fatty acids play an essential role as cofactors in H^+ transport without being actually translocated [111]. These studies demonstrate that the ANT can act as a (H^+-selective) channel (middle panel of Fig. 1).

At present, there is no obvious mechanism to explain channel formation by ANT. Inspection of the protein does not reveal the presence of Ca^{2+}-binding sites, suggesting the possible requirement for additional factors, such as cardiolipin [112] or for post-translational modifications, for example, Ca^{2+}-dependent limited proteolysis. It has been suggested that the PT is favoured by oxidation of ANT residues C57 [113,114] and C160 [114], which would cause formation of disulphide bridges followed by enhanced binding of CyPD [114]; yet, the PTP could be induced by thiol oxidants without dimerisation of the ANT [115] and Ca^{2+}-induced ANT channel opening has been documented in the absence of oxidants [38,39]. It is hopeful that the contribution of ANT Cys residues will be studied by site-directed mutagenesis and electrophysiology, and possibly by structure determination through high-resolution cryo-EM in the presence of Ca^{2+}. It appears conceivable, however, that CyPD and Ca^{2+} could favour the channel transition of ANT, as seen in reconstitution experiments with the bovine [38]

and yeast species [39], possibly in cooperation with fatty acids, which are well-known activators of the PTP [116] (right panel of Fig. 1).

F-ATP synthase

F-ATP synthase is an abundant complex located in the IMM that functionally cooperates with ANT and the Pi carrier to produce ATP from cytosolic ADP in aerobic conditions. F-ATP synthase makes ATP from ADP and Pi by rotary catalysis using the proton motive force generated by H^+ pumping by the respiratory chain complexes. It consists of the water-soluble, F_1 head exposed to the mitochondrial matrix, which is responsible for the synthesis of ATP; and the membrane-embedded F_O sector involved in proton traslocation. The two sectors are connected by a central stalk rotating within F_1 and a stationary peripheral stalk essential in maintaining complex stability [117].

Structure and catalytic mechanism

Crystallography has revealed the structure of many F_1 and F_O sub-complexes from bovine heart [118,119] and yeast [120]. Cryo-EM studies have reported structures of the entire complex from *Yarrowia lipolytica* [121], *Saccharomyces cerevisiae* [122], *Sus scrofa* [123], *Bos taurus* [124] and *Ovis aries* [125], providing high-resolution maps of all the F_O subunits (upper panel of Fig. 2). The F_1 sector comprises three αβ pairs
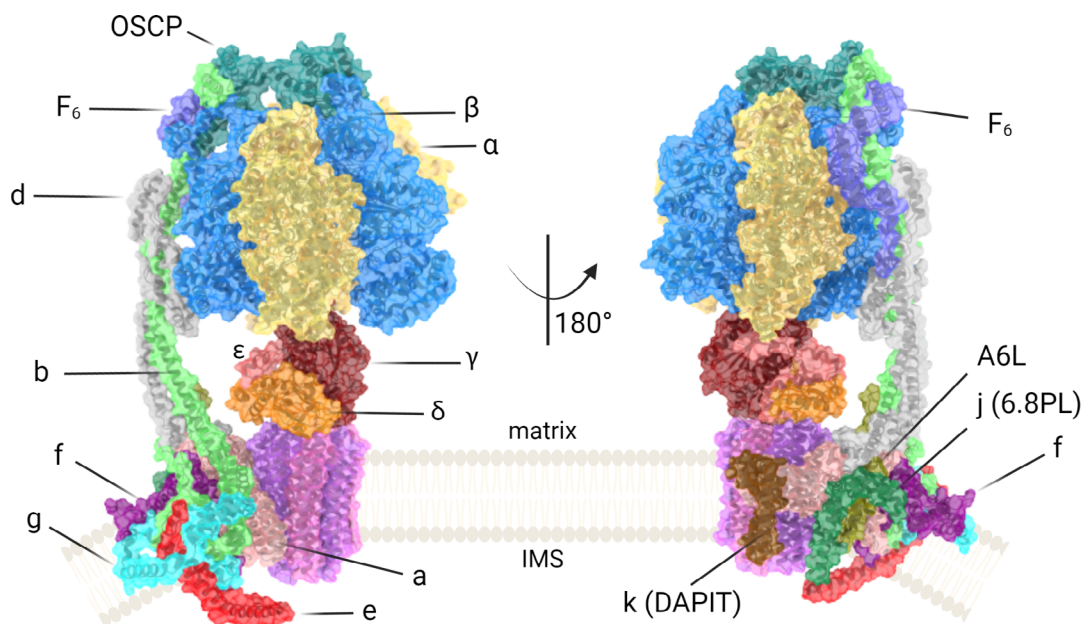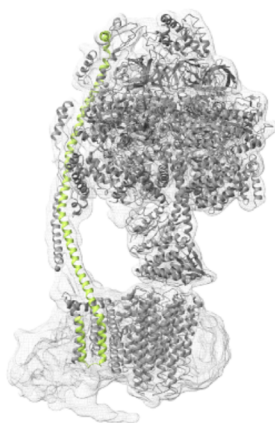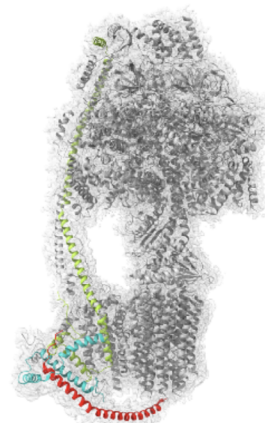
Fig. 2. Structure of the F-ATP synthase monomer. (Upper panel) F-ATP synthase structure is based on the atomic model of Spikes *et al.* [124]. (Lower panel) Density maps of F-ATP synthase derived by cryo-EM in the indicated studies, with fitted atomic models. Subunits b, e and g are green, cyan and red, respectively.

surrounding the γ subunit, which forms the central stalk by associating at the foot with subunits δ and ε. The three αβ pairs contain six nucleotide-binding sites, only three of which are catalytically active and mostly contributed by the β subunits. A rotor ring with a variable number of copies of subunit c (8 in metazoans, 10 in yeast and a record 17 in *Burkholderia pseudomallei*) is located in the F_O sector. The cavity of the c-ring is

occupied by phospholipids [125], in keeping with previous proposals [126]. The lipids at the intermembrane space (IMS) and matrix end of the c-ring are functionally and chemically distinct. Only the matrix-side lipids rotate with the c-ring, and thus providing a 'lubricated' plug facilitating rotation [125]. The outer surface of the c-ring is attached to subunit a, which is arranged in a concave four-helix horizontal bundle around the c-ring forming

two aqueous half-channels separated by a conserved arginine [117]. In mammals, subunit a is also tightly bound to subunits j (6.8PL), k (DAPIT) and 8 (A6L) forming a structure that has been denoted as 'proton translocation cluster' [124,125]. The two half-channels allow H^+ to reach an essential ionised glutamate residue of subunit c that, once protonated, moves to a more hydrophobic environment generating rotation of the c-ring and consequently of the central stalk. Rotation of γ subunit forces each of the three catalytic sites into three major conformations with different affinities for nucleotides, denoted as β_E (empty), β_{DP} (occupied by Mg^{2+}-ADP and Pi) and β_{TP} (occupied by Mg^{2+}-ATP), thereby catalysing the synthesis of one ATP molecule during each 120° rotation of the γ subunit [127]. The enzyme can work in reverse pushing the central stalk and the c-ring backward with the energy deriving from ATP hydrolysis, generating a H^+ gradient. Catalysis requires the nucleotide in complex with Mg^{2+} (or with other metals with decreased efficiency) [128]. Of specific interest to the topic of this review, Ca^{2+} has the unique property to catalyse ATP hydrolysis not coupled to formation of a H^+ gradient [129,130] despite its ability to generate rotation of the γ subunit [131], a finding that suggests onset of permeabilisation.

The mitochondrial F_O contains an additional subset of four conserved subunits (b, e, f, g) with subunit b reaching out of the membrane to form the core of the peripheral stalk. Through its helices H2 and H3 within the IMM, subunit b makes a 'U-turn' forming a hairpin able to interact with the horizontal bundle of subunit a on one side, and on the other side to form a compact triple transmembrane helix bundle with subunits e and g [125]. This bundle is packed through GXXXG motifs located at N- and C-termini of subunits e and g, respectively, with the C-terminus of subunit e reaching out and attaching to the c-ring lipids from the IMS to form a 'hook' [124,125]. A structure protruding into the IMS, thought to arise from subunits e and g in contact with subunit b, had originally been shown in the cryo-EM analysis of the bovine heart F-ATP synthase (lower left panel of Fig. 2); this structure first revealed flexibility of the intact enzyme due to bending and twisting of the central and peripheral stalks [132]. Subunit b also contributes to form the membrane domain of the peripheral stalk together with subunits e, f, g, 8 and j [124]. In its extrinsic part subunit b forms a long, continuous α-helix stiffened by interactions with subunits 8, F6 and d, finally reaching out to the C-terminus of OSCP subunit on top of the F_1 sector. OSCP forms many tight interactions with F_1 at three α, two β and with F6 and b subunits. These interactions result in a strong link between F_O and F_1, which prevents co-rotation of the $\alpha\beta$ pairs with subunit γ. Anchoring is further strengthened by the already mentioned connection of subunit e with the lipid plug of the c-ring at the opposite side [124,125] (lower middle and right panels of Fig. 2). This complex structure makes F-ATP synthase a 'perfect chemomechanical coupling device' between H^+ translocation, rotor rotation and ATP synthesis/hydrolysis [133]. Based on the cryo-EM structures of different rotational states [132,134], cooperation between F_O and F_1 appears ensured by elastic power transmission mediated by the flexible peripheral stalk, primarily at the inter-domain hinge of the OSCP subunit, a well-established target of drugs and matrix proteins including CyPD [135].

Oligomeric states of F-ATP synthase

As mentioned earlier, a notable feature of the F-ATP synthase complexes is their association into dimers that self-assemble into long rows of oligomers to develop the typical cristae [136,137], which have a direct impact on mitochondrial bioenergetics [138]. However, the structural details and functional roles of such supramolecular structures are still debated. Pioneering experiments in yeast established the existence of (a) a dimerisation interface stabilised through interactions of subunits a, b, e, g, F6 and i/j, which contribute in an additive way to the association of monomers; and (b) an oligomerisation interface mainly stabilised through e/e and g/g interactions [139]. Later, *in situ* cryo-EM structures demonstrated that F-ATP synthase forms V-shaped (type I) dimers at an angle of 80–90° between the two central stalks, with the peripheral stalks turned away from one another [117]. The recent cryo-EM structure of dimeric complexes from bovine heart shows that the interaction is mainly mediated by the two j subunits and is highly dynamic, that is, it changes over time to accommodate motions both dependent on and independent of catalysis [140]. This study also proposed that dimers associate into oligomers mainly through homo-interactions between the matrix-exposed part of subunit g together with subunit k, forming a 'fluid interface' able to follow the cristae curvature. Conversely, cryo-EM analyses of the ovine and porcine tetrameric complexes showed that the 'neighbours in a row' monomers are linked by two IF_1 molecules, and that the resulting tetrameric structures have a higher stability compared to the dimers [123,125]. Such results suggest that different F-ATP synthase oligomeric structures can coexist within a mitochondrion at the cristae edges. In addition, the presence of a sub-population of monomeric F-ATP synthase complexes probably prevailing in the inner

boundary membrane has been proposed [141]. These monomeric complexes are characterised by higher mobility compared to the dimeric complexes localised at the cristae edges and may be working as ATPases. F-ATP synthase thus seems able to undergo not only functional but also spatial and structural reorganisation to respond to different metabolic conditions [141], which also involve interactions with the cristae-shaping protein OPA-1 [142]. Finally, it has been proposed that F-ATP synthase, ANT and the Pi carrier associate in a supramolecular structure, the 'ATP synthasome' [78,79], the formation of which would depend on CyPD association and on the bioenergetic state of the mitochondria [80].

Possible mechanisms of channel formation

As mentioned in the Introduction, the first clue that the F-ATP synthase could be involved in the PT was the demonstration that CyPD interacts with the peripheral stalk of F-ATP synthase [54]. CyPD binding required relatively high concentrations (10 mM) of Pi and resulted in partial inhibition (about 30%) of the rate of oligomycin-sensitive ATP synthesis and hydrolysis; CsA dissociated CyPD from the F-ATP synthase removing this inhibitory effect [54]. A few years later clear evidence was obtained that Ca^{2+}-dependent channel formation from F-ATP synthase can actually take place [55,56] as now supported by a variety of studies based on reconstitution of highly purified preparations from bovine [69] and porcine hearts [70].

Based on extensive mutagenesis [56,61–68], reconstitution [55–59,69,70] and structural work [123–125] a plausible mechanism through which F-ATP synthase could generate a channel is beginning to emerge. First proposed by Gerle as the 'death finger' hypothesis [143,144], this model combines elements of two previous hypotheses, that is, that the PTP may form at the dimer interface of monomers through a conformational change originating at OSCP [55] or at the c-ring after dissociation of F_1 [56,61]. The following putative sequence of events takes into account most of the available information obtained in several laboratories (Fig. 3A).

a) Under basal conditions (enzyme turnover with Mg^{2+} at the catalytic site) rotation of the γ subunit is smooth, and elastic force is dissipated through OSCP and the peripheral stalk with little effects on the 'hook apparatus' connecting with the outer side of the lipid plug in the c-ring (Fig. 3A, left).

b) Binding of Ca^{2+} occurs at the catalytic metal site contributed by both α and β subunits (Fig. 3A, centre), which is usually occupied by Mg^{2+}; the larger van der Waals radius of Ca^{2+} causes a spatial rearrangement of the F_1 sector with increased overall rigidity [62].

c) The rearrangement is transmitted to the crown region, that is, the β-barrel-shaped 'ring' at the F_1/ OSCP interface [119] through the long connecting loop made up by residues 82-131 of subunit β [62].

d) The decreased compliance causes more mechanical stress on OSCP, overcoming its 'shock absorber' function during rotation of subunit γ [134] in a process that is favoured by binding of CyPD (Fig. 3A, centre) or of its chemical mimic benzodiazepine (Bz)-423 [55], which decreases OSCP flexibility; CsA desensitises the PTP by displacing CyPD from OSCP rather than by a direct effect on F-ATP synthase [54,55].

e) The mechanical energy is transmitted from OSCP to the peripheral stalk relaying it at the point of entrance into the membrane [121,132], where subunit b forms a hairpin that makes a tight association with the C- and N-termini of subunits g and e, respectively, forming a strong 'wedge' [124] or 'bundle' [125].

f) The C-terminus of subunit e, which extends outside the bundle region to make contact with the c-ring in the 'hook apparatus' [125], exerts a pulling effect on the lipids allowing formation of a channel within the c-ring by displacing the outer lipid plug [125].

g) At physiological, low levels of matrix Ca^{2+} the PTP oscillates between the closed and open states (Fig. 3A, centre); as matrix Ca^{2+} increases, or as the result of additional inducing agents, openings become more stable and lead to displacement of the inner lipids and of the central stalk (Fig. 3A, right). Of note, also the fully open state is reversible when Ca^{2+} is removed [145].

Cryo-EM structures of F-ATP synthase prepared in the presence of 5 mM Ca^{2+} yielded 3D density classes specific to the Ca^{2+} dataset, that is, never observed with Mg^{2+}. These could be arranged in a plausible sequence that may provide snapshots of the different stages in the process of PTP opening [125]. The mechanism through which the channel would reversibly form within the c-ring [56], which in the fully open state also implies some rearrangement or even the displacement of F_1, remains shrouded in mystery. Importantly, it is not clear whether all conductance substates of the PTP can be explained by the above hypothesis.

In patch-clamp experiments with native membranes the PTP exhibits a large variety of substates ranging from as little as 45 pS to as high as > 1000 pS [15,16]. All these substates are also observed in highly purified dimeric F-ATP synthase preparations reconstituted in
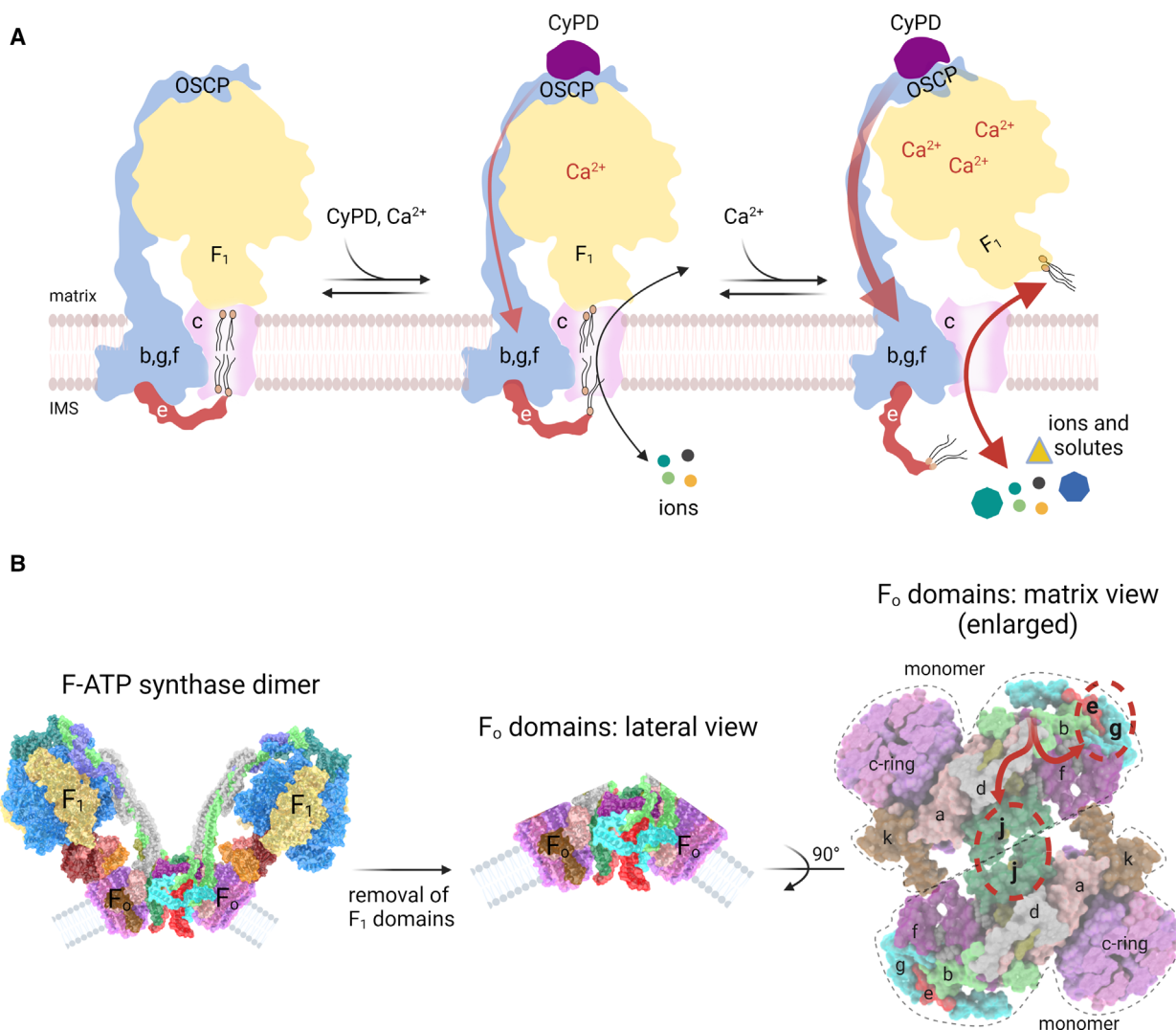
Fig. 3. Hypothetical models for PTP formation by F-ATP synthase. (A) The 'death finger' model proposes the transition of the c-ring into the PTP channel on a conformational change of the peripheral stalk that eventually perturbs subunit e, the final transducer of pore opening. The C-terminus of subunit e makes contacts with lipids of the plug within the c-ring from the IMS (left). In the presence of physiological, low Ca^{2+} concentrations with CyPD bound to OSCP subunit (middle), the peripheral stalk transmits mechanical force generated by Ca^{2+} binding to subunit e (red arrow), which may exert a pulling action dragging some lipids out of the plug. This condition, together with a secondary relaxation of the central stalk/c-ring connections would accommodate a low-conductance mode of channel opening mediating the passage of ions but not of larger solutes, representing the 'flickering' mode of the PTP (middle). As the matrix Ca^{2+} levels rise, the mechanical force exerted on subunit e becomes stronger allowing removal of the lipid plug from the c-ring and displacement of F_1 with formation of the high-conductance PTP, which remains fully reversible if Ca^{2+} is removed [145]. (B) Entire F-ATP synthase dimer (left), after removal of the F_1 domains (centre) and following a 90° rotation to show the two F_O domains as viewed from the matrix side (right). The conformational change transmitted through the peripheral stalk (red arrows in the right panel) may affect both subunits g/e and the monomer–monomer interface, with possible channel formation at the point of contact of subunits j, which undergo a pivoting motion at their interface during catalysis [124].

lipid bilayers [69]. As just mentioned, it is possible that the low-conductance flickering reflects reversible oscillations of the lipid plug within the c-ring. An interesting (and not mutually exclusive) alternative, however, is suggested by the reconstruction of the dimer interface from the high-resolution structure of monomers (Fig. 3B), where adjacent subunits j form a cavity that appears not to be filled by lipid [124] and that may provide an interface for channel formation in dimers (Fig. 3B). This could contribute to the reversible, transient flickering of the PTP observed both in isolated mitochondria and intact cells [146–149].

It has been proposed that PTP formation requires dissociation of the dimers, in a study where isolated mitochondria were subjected to Ca^{2+}-dependent PTP opening leading to a small decrease of the dimer/monomer ratio, as measured by activity staining in native gels where the presence of oligomers and of partially assembled forms of the complex was not assessed [150]. The prevalent state of F-ATP synthase was still the dimeric form, so that assigning occurrence of the PT to the monomers appears arbitrary. The key issue, however, is that these experiments cannot tell whether the partial disassembly of the dimers is the cause or rather the consequence of swelling, a problem that also applies to the interpretation of the *in situ* studies [150]. While PTP formation in monomers is possible, whether this is a requisite for pore opening remains very difficult to assess even in reconstituted systems, as will be discussed later.

Species-specific features

The F-ATP synthase is a highly conserved enzyme with a substantial degree of sequence identity from bacteria to mammals, which is particularly evident for the catalytic domain, where more than 60% residues of subunit β have been conserved [151]. In eukaryotes, the enzyme contains unique so-called 'supernumerary' subunits anchored to the F_O region, which in yeast include subunits e and g that are strictly associated with dimers [152,153] and appear to mediate dimerisation of monomers in mammals as well [139]. Cryo-EM images of dimers from different species confirmed a wide conservation of the central structure, while revealing extensive variations in the composition and structure of the peripheral stalk, especially in the dimeric interface [121,154,155].

The molecular connections between monomers or dimers vary among species, and most data were gathered by structural analysis of the yeast and bovine enzymes. In *Yarrowia lipolytica*, the dimeric interface was reported to be primarily occupied by the C-terminus of subunit f, to include subunits a and 8 while the resulting wedge-shaped space in between appears to be filled by lipids [121]. In the structure of the *Saccharomyces cerevisiae* F-ATP synthase dimer, the density of subunit f was reassigned to subunit i/j (j in mammals), while occupancy of the dimer interface by subunit a was confirmed [156]. In both structures subunits e and g, which are connected with the N-terminus of subunit b in a well-defined bundle, locate at a more distal position. They confer a peculiar curvature to the membrane and do not participate in dimer formation but rather in dimer–dimer contacts.

Subunit e protrudes straight out of the F_o domain with its C-terminus towards the IMS without any obvious implications in the organisation of higher-order structures [121]. In *Saccharomyces cerevisiae,* it connects with subunit k in an arrangement that may be specific for yeast, given that subunit e has a completely different orientation in the mammalian enzyme [124,125,132]. However, it is important to note that the density for yeast subunit e (and g) was reconstructed using poly-alanine models, which may camouflage the real structure and position of the protein. In the bovine and ovine F-ATP synthases, the dimer interface includes subunit j, which spans from the matrix to the IMS and together with subunit f generates a cavity where the membrane has reduced thickness. Interestingly, subunit a appears to be completely covered by subunits j, 8 and partially by cardiolipin. As in yeast, subunit e locates in a proximal domain and coordinates dimer–dimer contacts, but it bends toward the lipid plug of the c-ring.

Subunit e shows species-specific sequence features (Fig. 4). As expected, the well-characterised GXXXG motif (position 23-27 in *Homo sapiens*), which allows tight packing with the α helices of subunits g and b, is highly conserved both in terms of sequence and of position within the protein. Sequence similarity is also visible in the transmembrane region. Prominent variations are instead present in the C-terminus, with terminal sequences unique to various strains of *Drosophila* and yeast. These overall differences may provide an explanation for the different orientation of subunit e relative to the mammalian protein, and perhaps also for the channel-forming ability of F-ATP synthases. Indeed, it is of interest that the PTP displays distinct properties in different species [157] including the mean conductance, which ranges from 500 pS (bovine) [55] to 300 pS (yeast) [57], to a mere 53 pS in *Drosophila* [58], where the PTP operates as a highly selective Ca^{2+} release channel [158]. Whether these properties can be ascribed to the unique C-termini of subunit e and/or to the specific features of the monomer/monomer interfaces is being actively investigated in our laboratories. These studies should provide interesting insights into the mechanism(s) of PTP formation and a potential test of the 'death finger' hypothesis [144].

Effect of detergents

As already discussed, in the native IMM the F-ATP synthase is organised in complex multimeric structures [159]. After assembly from preformed F_1/c-ring and peripheral stalk subcomplexes [160] monomers associate into dimers [152], the actual building blocks that
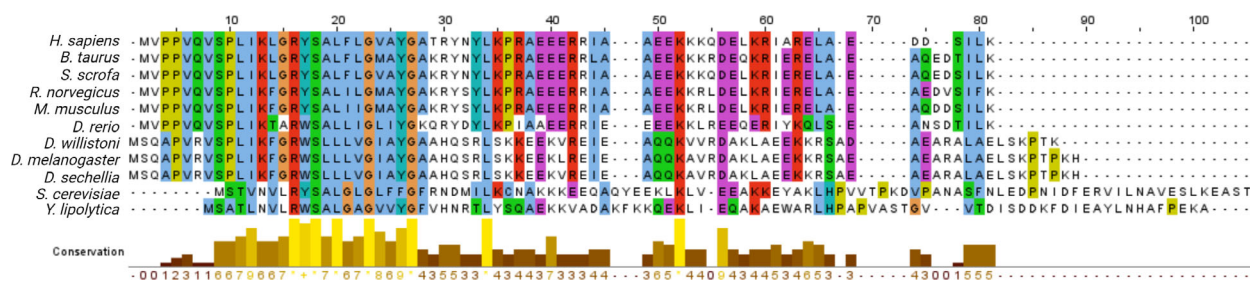
Fig. 4. Sequence alignment of subunit e from various species. Accession number of sequences used for the alignment: *H. sapiens* (P56385), *B. taurus* (Q00361), *S. scrofa* (Q9MYT8), *R. norvegicus* (P29419), *M. musculus* (Q06185), *D. rerio* (A7YY99), *D. willistoni* (B4N947), *D. melanogaster* (O77134), *D. sechellia* (B4HEN9), *S. cerevisiae* (P81449) and *Y. lipolytica* (B5FVG3). The multiple sequence alignment was performed with the CLUSTALW program and analysed by JALVIEW software.

by lateral association form long rows of oligomers, which in turn help to shape the IMM foldings at the cristae [161]. Purification of F-ATP synthase disrupts its native conformation affecting the properties of the resulting enzyme complex in a manner that depends on the detergent(s) used, an issue that should always be kept in mind in the design and interpretation of reconstitution experiments.

Extraction with digitonin [162] or lauryl maltose neopentyl glycol (LMNG) [69] generated monomeric, dimeric and oligomeric states when F-ATP synthase was analysed by non-denaturing blue-native [163] or clear-native PAGE [164]. After elution, F-ATP synthase dimers but not monomers displayed Ca^{2+}-induced channel activity in lipid bilayers [55,69]. Protein elution from the gel slices was carried out with *n*-heptyl β-D-thioglucopyranoside [55,69], however; and we suspect that this step may have removed components of the hook apparatus (like subunit e) from monomers but not dimers, and thus preventing channel formation in the former.

In contrast with these results, it was convincingly shown that reconstituted, monomeric F-ATP synthase generates currents similar to those of the PTP [70]. In these protocols, mitochondria were extracted with dodecyl maltoside (DDM), which removes the labile subunits j and k [165] together with the 'dimerisation' subunits e and g [70], thus generating the monomeric form only [166]. At variance from the case of the *bona fide* PTP, currents did not require added Ca^{2+} and were inhibited by oligomycin [70]. As mentioned earlier, the subunits removed by DDM are critically located in the hook apparatus [70,165]; and yet, channel opening was seen [70]. A possible explanation is the delipidating effect of DDM [163], which may perturb the lipid plug thus bypassing the Ca^{2+} requirement for PTP activation. Preservation of the lipids by the milder detergents digitonin and LMNG could explain lack of channel formation in the reconstituted system with our monomer preparations [55,69].

Cyclophilin D

CyPD is the unique mitochondrial isoform of the cyclophilins, a family with more than 15 mammalian members that exhibit peptidyl-prolyl *cis-trans* isomerase activity and share the ability to bind the inhibitory drug CsA. In mammals, CyPD is encoded by the *Ppif* gene [167,168]. CyPD has emerged as central in the mitochondrial proteome. Early work documented an interaction between CyPD and the PTP putative components ANTs, VDACs and TSPO [23], the Pi carrier [169] and the F-ATP synthase [54,55]. Additional partners have been discovered including components of the electron transport chain [170], proteins involved in cellular signalling pathways including ERK [171,172], GSK3β [171,173] and SIRT3 [174,175], and proteins involved in stress response pathways including HSP90 and TRAP1 [176]. Interestingly, only some of these interactions are sensitive to CsA, suggesting the existence of multiple binding modes that are yet to be explored [167]. The best-characterised function of CyPD is sensitisation of the PTP through binding to OSCP [54,55]. This association requires Pi, which probably acts by charge neutralisation favouring the electrostatic interactions between the two proteins [63]. Through this effect Pi is a PTP inducer [177] despite its lowering effect on the concentration of matrix free Ca^{2+} [178]. It is remarkable that in yeast and *Drosophila* mitochondria, where the PTP is insensitive to CsA, Pi is a PTP inhibitor [57,158,179]; and that in the absence of CyPD (or in the presence of CsA) Pi becomes an inhibitor also for the PTP of mammalian mitochondria [180].

Getting a crystal of CyPD suitable for X-ray analysis has been challenging, due to the high water

solubility and pI of the protein. A high-resolution structure was obtained of a K133I mutant of human CyPD lacking the first 13 amino acids. The structure confirmed the high homology of CyPD with other CyPs, which all consist of eight antiparallel β-sheets, two α-helices and one 3_{10} helix enclosing the sheets [181]. CyPD does not undergo conformational changes on binding of CsA, an interaction that mainly involves hydrophobic and hydrogen bonds [181]. It is important to note that the unique N-terminus missing in the structure may impart specific features to the protein, also because this region is the target of many post-translational modifications.

Post-translational modifications in pore modulation

CyPD can undergo several post-translational modifications that alter PTP regulation [167,168]. CyPD is the target of phosphorylation, with functional effects that depend on the phosphorylated site and may result in opposite outcomes. In mice lacking the mitochondrial Ca^{2+} uniporter, phosphorylation at S42 increased association of CyPD with F-ATP synthase resulting in PTP sensitisation [182]. Conversely, PI3K- and Akt2-mediated phosphorylation at S31 preserved mitochondrial function; while expression of the CyPD-S31A phosphomimetic mutant caused mitochondrial dysfunction consistent with PTP activation [183], as also observed after phosphorylation by GSK3β [171]. By combining *in silico* analysis of potential CyPD phosphorylation sites for GSK3β with genetic manipulations, a crucial role of CyPD residue S191 has been recently demonstrated in mouse hearts, with phosphorylation increasing CyPD binding to OSCP, increased PTP opening and myocardial damage [184].

CyPD can undergo (de)acetylation reactions, and these modifications significantly affect the PTP [174,185]. Indeed, in *Sirt3*$^{-/-}$ mice CyPD is hyperacetylated at K166 (a residue conserved in the human protein) leading to PTP activation and cell death in a model of aortic constriction [174]. Consistently (a) hypoxia increased CyPD acetylation while SIRT3 overexpression was protective in rat heart-derived H9C2 cells; (b) the acetylation mimic K166Q increased PTP sensitisation and cell death, while the K166R variant had the opposite effect; and (c) cardiac ischaemic postconditioning did not reduce infarct size and CyPD acetylation in mice lacking SIRT3, suggesting that attenuation of CyPD acetylation by SIRT3 could prevent lethal injury at reperfusion [186]. Finally, acetylation of OSCP residue K70 caused by NAD^+ redox imbalance promotes interaction of F-ATP synthase with CyPD and sensitises the PTP to opening in mouse hearts [187].

CyPD contains several Cys residues, some of which (C82 and C104 in the human protein) strongly influence the enzyme's isomerase activity [188]. Site-directed mutagenesis identified human C203 as an important redox-sensitive residue, possibly through formation of a unique disulphide bridge with an yet-undefined partner [188]. Consistently, mutation of the corresponding mouse C202 to a Ser residue desensitised the PTP in cells [189] and in Langendorff perfused mouse hearts subjected to ischaemia/reperfusion injury, with a matching decreased association of CyPD-C202S with F-ATP synthase [190]. CyPD C202 is also the target of S-nitrosylation [191] and of S-palmitoylation [190], and it has been proposed that oxidation of C202/3 favours PTP opening leading to ischaemia-reperfusion damage, while its S-nitrosylation/acylation confers cardioprotection [190].

Open questions

The progress discussed earlier has been substantial, and yet key open questions remain. These include the role of the OMM, the basis for modulation by quinones and activation by free fatty acids, the assignment of regulatory sulfhydryl residues mediating the effect of oxidants, and the existence of additional permeability pathways.

Outer membrane

The PT is an IMM event as it can take place in mitoplasts, which lack an intact OMM [44]; and yet there is evidence that the OMM (a) is involved in mediating the effect of maleimides because their inducing activity on the PTP is no longer seen in mitoplasts [192] and (b) is required for the inducing effects of photosensitisers like porphyrins [193]. Porphyrins can be excited by visible light to produce singlet oxygen, $^1O_2$, which primarily affects amino acids (Trp, Tyr, His, Cys, Met) causing a variety of alterations in target proteins [194]. Porphyrins (like haematoporphyrin) tend to concentrate in mitochondria [195]. Remarkably, it was known that F-ATP synthase and ANT are the most vulnerable targets of $^1O_2$ [195,196]. Photoirradiation of haematoporphyrin-loaded mitochondria has been used to study the effects of $^1O_2$ on the PTP [197–199]. At variance from the inducing effect generally observed with oxidants, short (≤ 100 s) irradiation times caused desensitisation of the PTP to Ca^{2+}, the first example of pore inactivation by an oxidant [197]. If the irradiation time was extended to more than 100 s, a process of

PTP reactivation ensued that was mediated by the OMM [199], as confirmed by the finding that mitoplasts were completely refractory to this reactivation of the PTP by high light doses [44]. These results are interesting also because they bear on the question of whether the PTP may form at the sites of contact between the OMM and IMM, as was suggested relatively early in the history of the PT [46]. Indeed, in the photoirradiation paradigm it is very unlikely that PTP activation is mediated by a diffusible species because $^1O_2$ is very short-lived particularly in biological systems, where it has an estimated lifetime of 100-250 ns and a diffusion distance of the order of 10–20 nm [200,201].

The major proteins of the OMM are VDACs [202], which have long been suggested to take part in PTP formation [45,46,203–210], an issue that generated considerable discussion [211–213]. Ro 68-3400, the first high-affinity inhibitor of the PTP to be identified by functional screening of a chemical library, labelled a 32 kDa protein originally identified as VDAC1 by mass spectroscopy after hydroxyapatite chromatography [207]. Subsequent studies failed to confirm the identity of the labelled protein as VDAC1, 2 or 3 [50]; and a thorough characterisation of the PTP in mitochondria from VDAC1-null mice revealed no differences in the effects of a variety of inducers and inhibitors, including CsA and ubiquinone (Ub) 0 [50]. The possible compensatory role of VDAC2 and 3 was ruled out by experiments in cells where all three VDAC isoforms had been genetically ablated [51]. As a result of these studies we think that VDACs cannot be considered essential components of the PTP. However, together with the earlier work mentioned earlier [203–210], some recent studies are consistent with a modulatory role of VDACs, which may be the targets of signalling pathways and of drugs [214–216].

A role in the PT has also been described for other proteins that can reside in the OMM such as pro- and anti-apoptotic members of the Bcl-2 family [47,48] and hexokinases [212,217–219]. In the case of Bcl-2 family members, the effect may be exerted more on PTP-dependent swelling than on PTP modulation as such. Indeed, it has been shown that these proteins exert a mechanical effect on OMM resistance to stretching, which is decreased by proapoptotic Bax and Bak and increased by antiapoptotic Bcl-2 [48]. In the case of hexokinase, the mechanism may instead be linked to Ca^{2+} diffusion through contact sites between the endoplasmic reticulum and the OMM, because displacement of the protein leads to PTP opening through a large yet localised process of Ca^{2+} influx [220].

Quinones and lipids

Quinones are among the most interesting modulators of the PT. After the discovery that Ub0 is a potent inhibitor [221], studies were performed to define the structure-activity relationship of quinones with various side chains [222,223], see [224] for a review. From these studies, three classes could be identified, that is, PTP inhibitors, PTP inducers and PTP-inactive quinones that are able to compete with both inhibitors and inducers [224]. The structure-activity relationship is extremely complex given that seemingly minor structural modifications profoundly affect the effects on the PTP. The most striking examples are perhaps those of decylUb (inhibitor) and OH-decylUb (inducer); and of the isomers 3-EtO-decylUb (inhibitor) and 2-EtO-decylUb (inactive). It appears that the 1,4-benzoquinone ring as such is not sufficient for pore regulation, and that specific substituents at carbons 2, 3, 5 and 6 may be essential for PTP modulation. It is also noteworthy that quinones with very different chemical structures may have similar effects on PTP regulation, suggesting that the spatial conformation may be more important than the nature of the substituents *per se*. The correlation between quinone structure and effects on the PTP, as well as their target(s), remain elusive although it is clear that neither the redox potential nor the hydrophobicity of the side chain are crucial factors [221–224]. Overall, data are consistent with the existence of a common binding site, which however remains undefined [224].

A recent study with photoreactive, PTP-inhibiting derivatives of Ub1 in *Saccharomyces cerevisiae* mitochondria has shown labelling between residues Phe221 and Lys234 (a segment that connects the 15th and 16th β-strand sheets) in the C-terminal region of VDAC1 [215]. No labelled proteins were detected in VDAC1-null yeast, indicating that the interaction is selective for VDAC1 [215]. Whether quinone-sensitive PTP opening occurred in yeast mitochondria lacking VDAC1 was not assessed, however, so it cannot be excluded that quinones bind to both VDAC1 and to a component of the IMM involved in the PT.

Another intriguing observation is that the crystallised, 14 subunit c-ring of spinach chloroplasts resolved at 2.3 Å presents internal electron densities forming circles parallel to the membrane plane, which based on a variety of criteria were attributed to plastoquinones [225]. Similar structures have been observed in the c-ring of other organisms [226–230], which could be formed by coenzyme Q in mitochondria and by menaquinone in bacteria [225]. Given the prominent effects of quinones on the PTP and the likely role of

the c-ring in PTP formation [56,60,61], it is tempting to speculate that short-chain quinones may compete for binding with endogenous ubiquinone, affecting in turn the probability of channel formation by the PTP. The lipid plug of the c-ring might also be the site of action of fatty acids, which have long been known to uncouple oxidative phosphorylation [8–10] with an effect largely mediated by the PTP [116,231,232].

Assigning a site of action to pore modulators

The number and variety of PT modulators remains puzzling. In their still very useful review of 1990, Gunter and Pfeiffer listed 43 classes of compounds and conditions affecting the PT [177], a list that has continued to grow and still represents a challenge to any mechanistic hypothesis to explain the PT. Significant progress was made with the discoveries that the PTP is voltage-dependent [233] and inhibited by mildly acidic matrix pH [18,234], and that the voltage threshold for opening is modulated by many agents [235] with a prominent effect of oxidative events affecting the status of critical thiols [236] and of matrix pyridine nucleotides [237]. These findings suggested that many inducers and inhibitors could converge on a smaller number of effector sites determining the stochastic probability of PTP opening [12]. The existence of more than one mechanism for the PT [67,77] has complicated the picture, but some specific sites of regulation have been convincingly assigned to F-ATP synthase (a) by genetic ablation or downregulation of subunits OSCP [55,67], c [61], e and b [67], f [238] and of the bundle region made up by subunits b, e and g [59]; and (b) by site-directed mutagenesis which led to identification of the Ca^{2+}-binding site in subunit β [62], of the H^+-sensing His residue in OSCP [63], of the conserved Arg residue modified by glyoxals in subunit g, which may play a role in voltage sensing [239], of the regulatory interaction between one Arg of subunit e and one Glu of subunit g [65], of the OSCP Cys residue responsible for the effects of the dithiol oxidant diamide [66] and of Gly residues involved in channel formation in subunit c [56,68]. The location of at least two additional classes of Cys residues [115,237] and of a Me^{2+}-binding site located on the cytosolic side, the occupancy of which results in PTP inhibition [240], remains to be identified.

Rotenone is a very effective inhibitor of the PTP, as reproducibly observed in succinate-energised mitochondria [241,242]. One possible explanation is that it inhibits reverse electron transfer through respiratory complex I, a major source of reactive oxygen species that may overwhelm antioxidant defences particularly in the phase of reperfusion after ischaemia [243]. However, the effects of rotenone and CsA are additive and complementary, in the sense that inhibition by rotenone increases as inhibition by CsA decreases and *vice versa*, with maximum inhibition being constant [242]. Genetic ablation of CyPD restores PTP inhibition by rotenone in tissues that are otherwise resistant to its effects, suggesting that inhibition by rotenone and CsA occurs through a common, still unidentified site masked by CyPD [242].

Conclusions and perspectives

We think that progress in defining the molecular bases of the PT has been substantial, particularly over the last 10 years; but it is also clear that several key questions still await an answer. The number of pores appears to be very limited compared with the abundance of F-ATP synthase and ANT [244]. Can all F-ATP synthase and ANT molecules form a PTP or rather a priming event, possibly a posttranslational modification, is required? Can the pores form anywhere, or are there privileged locations, for example, the interface with the OMM? What is the role of the interactions of F-ATP synthase and ANT at the ATP synthasomes, given that lack of F-ATP synthase leads to activation of the ANT channel [67,77]? Are there additional mechanisms for the PT based on other proteins or molecular species such as polyphosphate [245,246]? We would also like to mention a very important paper demonstrating that the salt- and anoxia-tolerant brine shrimp *Artemia franciscana* does not undergo the permeability transition in spite of the presence of both ANT and F-ATP synthase [247]. We expect that sequence analysis of the corresponding genes will provide an invaluable tool to address the basis for this resistance and to further test the molecular mechanisms of the PTP. Finally, we would also like to report a very elegant paper that appeared at the time of our submission, and that bears on the importance of the PTP in the lifespan of *Caenorhabditis elegans*. The study demonstrates that genetic ablation of OSCP in adult worms triggers the PTP and shortens lifespan, which can be restored by pharmacological or genetic pore inhibition [248]. We hope that this review will help address the numerous open questions and eventually help solve the 70-year-old mystery of the PT.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

All authors contributed to writing of the paper. PB edited the paper and reviewed its final version.

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