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Review

Recent progress in elucidating the molecular mechanism of the mitochondrial permeability transition pore

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ABSTRACT

The mitochondrial permeability transition pore (MPTP) plays a key role in cell death, especially necrosis, and mediates the injury tissues such as the heart and brain experience following ischaemia and reperfusion. However, the molecular identity of the MPTP remains uncertain. Knockout studies have confirmed a role for cyclophilin-D (CyP-D) in pore opening, probably mediated by its peptidyl–prolyl *cis–trans* isomerase activity that facilitates a conformational change in an inner membrane protein. However, similar knockout studies have cast doubt on the central role of the adenine nucleotide translocase (ANT), previously regarded as a leading contender for the membrane component that forms the transmembrane channel of the MPTP. Here we review the evidence for and against a role for the ANT in MPTP opening and conclude that it usually plays a regulatory role rather than provide the transmembrane pore component. We suggest that the protein fulfilling the latter role is the mitochondrial phosphate carrier (PiC) and summarise recent evidence in support of this proposal. Our data are consistent with a model for the MPTP in which a calcium-triggered conformational change of the PiC, facilitated by CyP-D, induces pore opening. We propose that this is enhanced by an association of the PiC with the "c" conformation of the ANT. Agents that modulate pore opening may act on either or both the PiC and the ANT.

1. Introduction

The mitochondrial permeability transition pore (MPTP) is a non-specific pore, permeable to all molecules of less than 1.5 kDa, which opens in the inner mitochondrial membrane (IMM) under conditions of calcium overload. Opening is greatly enhanced by adenine nucleotide depletion, elevated phosphate and oxidative stress. These are conditions known to accompany reperfusion following a period of ischaemia, and in response to others stresses such as the application of certain metabolic poisons and toxins. Indeed the opening of the MPTP is now recognised to be a major cause of the necrotic cell death occurring under such conditions (see [1–4]). The role, if any, of the MPTP in healthy cells remains unclear since mice lacking cyclophilin-D, a component of the MPTP, show know obvious phenotype other than being protected against ischaemic injury [5–8].

1.1. The role of the MPTP in necrotic cell death

The role of the MPTP in necrotic cell death can be readily explained. Once the pore opens it allows free passage of protons across the inner membrane leading to a dissipation of the membrane potential and pH gradient that comprise the proton motive force. Not only does this prevent ATP generation by oxidative phosphorylation, but reversal of the ATPase occurs causing the breakdown of cytosolic ATP generated by glycolysis. As a result tissue ATP levels become severely compromised and, left unchecked, these will lead to major perturbations in the ionic and metabolic homeostasis of the cell. Ultimately these changes will cause necrotic cell death through the activation of phospholipases, nucleases and proteases [4,9,10]. Perhaps the best documented example of the role of the MPTP in necrotic cell death is in reperfusion injury of the heart [1,9], liver [11] and brain [12,13]. Here, the ischaemic phase of the insult causes calcium concentrations to rise and the production of some reactive oxygen species (ROS) that leads to oxidative stress. Although ROS and calcium are potential triggers of MPTP opening, the enhanced glycolysis that occurs during ischaemia leads to accumulation of lactic acid and a decrease in intracellular pH. This prevents MPTP opening which is progressively inhibited as the pH drops below 7 [14]. However, upon reperfusion there is a burst of ROS formation and the pH returns to normal, stimulating pore opening and hence cell death [1,9].

The importance of the MPTP in the necrotic death of the heart, brain and liver under such conditions was initially recognised through the

Abbreviations: ANT, adenine nucleotide translocase; BKA, bongkrekic acid; CAT, carboxyatractyloside; CsA, cyclosporin A; CyP, cyclophilin; IMM, inner mitochondrial membrane; MPTP, mitochondrial permeability transition pore; NEM, *N*-ethylmaleimide; PAO, phenylarsine oxide; PiC, mitochondrial phosphate carrier; PPIase, peptidyl–prolyl *cis–trans* isomerase; ROS, reactive oxygen species; SfA, sanglifehrin A; VDAC, voltage dependent anion channel

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use of MPTP inhibitors such as cyclosporin A (CsA) and sanglifehrin A (SfA) [15–18]. Furthermore, it is the hippocampus of the brain that is most vulnerable to ischaemic and hypoglycaemic damage, and mitochondria from this region are more susceptible to MPTP opening in vitro than are mitochondria from the cortex or cerebellum [12,13,19]. Most recently, further proof of a critical role for MPTP opening in necrotic cell death has been provided by the use of mice in which the target of CsA and SfA, cyclophilin-D (CyP-D — see below) had been knocked out. These animals showed substantial protection from ischaemia/reperfusion damage (infarct size) of both the heart [6,7] and brain [8]. In addition, the use of these mice has shown that cardiac failure associated with chronic calcium overload also involves MPTP-dependent death of cardiac myocytes [20].

1.2. The role of the MPTP in apoptosis

Another consequence of MPTP opening is swelling of the mitochondrial matrix, and this may play a role in apoptotic cell death under some conditions. Swelling occurs because the MPTP is permeable to all solutes of <1.5 kDa and once open the non-protein components of the mitochondrial matrix will rapidly equilibrate across the IMM. However, the matrix proteins remain and because they are at a higher concentration than in the cytosol, they will exert a colloidal osmotic pressure. It is this that causes the matrix to swell, the folds of the cristae allowing this to occur without breaking the IMM. However, the outer membrane cannot accommodate much swelling of the matrix without the outer membrane rupturing. Once such rupture occurs the contents of the inter membrane space are released including cytochrome *c* and other pro-apoptotic proteins such as Smac/Diablo and Apoptosis Inducing Factor (AIF). Thus, even if MPTP opening is insufficient to deplete ATP levels and cause necrosis, apoptosis may result [4,4,10,21]. It should be noted, however, that release of pro-apoptotic proteins from the inter membrane space is normally mediated by specific permeabilisation of the outer membrane through the action of pro-apoptotic members of the Bcl-2 family such as Bax [22,23].

2. The molecular mechanism of the MPTP

In view of its critical role in cell death, it is clearly important to understand the molecular mechanism of the MPTP. Several models have been proposed and although there remains no consensus as to the exact mechanism, there is increasing certainty that cyclophilin-D (CyP-D) and the adenine nucleotide translocase (ANT) play important roles. The remainder of this review will summarise the evidence for their involvement and provide evidence that additional components remain to be identified. We will briefly outline evidence from our laboratory that one such component may be the mitochondrial phosphate carrier (PiC).

2.1. The role of cyclophilin-D

A key to elucidating the mechanism of the MPTP was provided by Crompton and colleagues who first reported that sub-micromolar concentrations of cyclosporin A (CsA) inhibit pore opening [24]. Studies from this laboratory revealed that the potency of several CsA analogues as inhibitors of the pore correlated with their ability to inhibit the activity of a matrix peptidyl–prolyl *cis–trans* isomerase (PPIase) [25,26]. We subsequently purified this PPIase and identified it as cyclophilin-D (CyP-D) [27]. CyP-D is a protein encoded by the *PPIF* gene of the nuclear genome. It is synthesised in the cytosol and enters the mitochondria using a mitochondrial targeting sequence which is subsequently cleaved off [28,29]. The importance of CyP-D has recently been confirmed by the demonstration that mitochondria from CyP-D knockout mice do not exhibit CsA-sensitive MPTP opening [6,7,30]. However, it is important to note that MPTP opening can still be demonstrated in these mitochondria if the calcium loading of the mitochondria is increased sufficiently. This is also the case for wild-type mitochondria that have been treated with CsA [31]. These data support the hypothesis that pore opening involves a conformational change in a membrane protein that is facilitated by the PPIase activity of CyP-D but that can occur in the absence of CyP-D at higher [Ca^{2+}].

Cyclosporin A has some disadvantages as an inhibitor of CyP-D since it forms a complex with cytosolic cyclophilin-A (CyP-A) that inhibits the calcium activated protein phosphatase calcineurin. Indeed, it is through this pathway that the drug mediates its immunosuppressive effects [32]. Thus when used in vivo CsA has the potential to exert many other effects on cellular function independent of its inhibition of MPTP opening (for examples see [33,34]). Consequently, several CsA analogues have been developed that lack the ability to inhibit calcineurin but which are still active as inhibitors of the PPIase activity of CyP-D. These include 6-methyl-ala-CsA, 4-methyl-val-CsA (NIM-811) and 3-D-methyl-ala-4-ethyl-val-CsA (DEBIO-025) [35–37]. In addition we have shown that an unrelated immunosuppressant, sanglifehrin A (SfA), is also a potent inhibitor of the PPIase activity of CyP-D and inhibits the MPTP whilst being inactive against calcineurin, [18]. However, SfA shows some differences from CsA in the way that it inhibits MPTP opening. The concentration dependence of MPTP inhibition by SfA is sigmoidal unlike CsA which shows a linear relationship [18]. Furthermore, SfA does not prevent CyP-D binding to the inner membrane component of the MPTP whilst CsA inhibits binding. Thus it would seem that SfA acts to inhibit MPTP opening by inhibiting the conformation change catalysed by the bound CyP-D rather than by CyP-D binding [18].

The data reviewed above established beyond doubt the role of CyP-D in facilitating the opening of the MPTP. However, the identity of the membrane protein that binds CyP-D is less certain. Indeed it is possible that there is no specific protein involved and such a view underlies the model for MPTP formation proposed by He and Lemasters [38]. In this model it is proposed that the MPTP forms as a result of the aggregation of misfolded integral membrane proteins that have been damaged by oxidant and other stresses. CyP-D will normally block conductance through these protein aggregates, but when protein clusters exceed the CyP-D available to block conductance, unregulated pore opening occurs that is stimulated by calcium and inhibited by CsA binding to CyP-D. The apparent involvement of the adenine nucleotide translocase (ANT) in pore formation (see below) is explained in terms of the high amount of this protein in the inner mitochondrial membrane and its susceptibility to oxidative damage. Thus it is the protein most likely to form aggregates. However, as outlined below, the activation and inhibition of MPTP opening by different ligands of the ANT that cause opposite changes in its conformational state argues against such a non-specific effect.

2.2. The adenine nucleotide translocase

2.2.1. Evidence for a role of the ANT in MPTP formation

The most widely accepted candidate for the membrane component of the MPTP, first proposed by us in 1990 [25], is the adenine nucleotide translocase (ANT). The evidence for its involvement is considerable; it is reviewed extensively elsewhere [39] and will only be summarised here. Early observations from several laboratories, including our own, showed that MPTP opening is enhanced by adenine nucleotide depletion and inhibited by addition of ATP or ADP. Opening is also modulated by other specific ligands of the ANT including carboxyatractyloside (CAT) and bongkrekic acid (BKA). CAT induces the "c" conformation of the ANT and sensitises pore opening to [Ca^{2+}]. Conversely, BKA enhances the "m" conformation" and inhibits pore opening [25,31,40,41]. In addition, the ability of nucleotides to inhibit pore opening correlates with their ability to act as transportable substrates for the ANT [31].

More direct evidence that the ANT might bind CyP-D in a CsA-sensitive manner was provided through the use of a CyP-D affinity

column (glutathione-*S*-transferase-tagged CyP-D coupled to a glutathione sepharose support). Both we [42] and Crompton et al [43] demonstrated that the ANT can bind to CyP-D, although in other respects different conclusions were reached. In our own studies ANT binding to CyP-D was found to be CsA-sensitive whilst Crompton et al failed to detect CsA inhibition of binding. However, these workers detected binding of the voltage dependent anion channel (VDAC) that we did not observe. These differences may reflect the different detergent solubilisation routine and our use of liver mitochondria rather than the heart mitochondria used by Crompton et al.

We recently obtained further evidence for the specific binding of CyP-D by the ANT using co-immunoprecipitation. For this purpose the ANT from solubilised liver mitochondria was immunoprecipitated using a polyclonal antibody against the whole rat liver ANT, purified by a published method [44]. Using this antibody, CyP-D was found to co-immunoprecipitate with the ANT in a CsA-sensitive manner whilst SfA did not prevent the interaction [10]. These data are in agreement with our earlier studies using GST-CyP-D pull-down [45]. Taken together, accumulating data would seem to provide strong evidence for a CsA-sensitive interaction between CyP-D and the ANT. However, very recently we have established that the protein detected by the polyclonal ANT antibody used in the immunoprecipitation experiments is not actually the ANT but rather the mitochondrial phosphate carrier (PiC). It appears that this must have been a contaminant in the original purified rat liver ANT used to raise the antibody [46]. As we discuss further below, we now believe that it is the PiC rather than the ANT that is the major protein to bind CyP-D and form the MPTP.

Another strong line of evidence in favour of the involvement of the ANT in MPTP opening comes from the ability of oxidative stress and certain thiol reagents such as the vicinal thiol reagent phenylarsine oxide (PAO) to stimulate MPTP opening. We have shown that such treatments act by overcoming the ability of adenine nucleotides to inhibit the MPTP [31]. Since the ANT from solubilised rat liver inner mitochondrial membranes binds to immobilised-PAO, it was proposed that this effect of PAO, and that of oxidative stress, was mediated by cross-linking of vicinal thiols on the ANT [31]. Subsequent experiments enables us to identify these thiol groups as Cys^{160} and Cys^{257} of the ANT (numbering for rat ANT1), with modification of Cys^{160} alone by eosin-5-maleimide being sufficient to overcome the MPTP inhibition by adenine nucleotides [45]. We have further suggested that the well-established inhibition of MPTP opening by high membrane potential [47] may reflect greater binding of adenine nucleotides to the matrix surface of the ANT leading to a higher preponderance of the "m" form of the ANT [31,39]. In contrast, we have proposed that the inhibitory effect of low pH on MPTP opening [14] is probably mediated by protons competing for Ca^{2+} at the calcium trigger site on the matrix surface of the ANT [39].

The best circumstantial evidence that the ANT may contain this trigger site for calcium comes from our demonstration that in energised heart mitochondria sub-micromolar concentrations of calcium induce the "c" conformation of the ANT, as does CAT, whilst this effect is reversed by BKA or ADP which inhibit MPTP opening [25,48,49]. Although the identity of this site has not been established, there are several glutamate and aspartate residues on the inner surface of the ANT that might bind calcium [39]. However, the recently published three dimensional structure of the ANT in its CAT-bound form [50] has not provided any further clues as to which, if any of these residues may be involved. One feature that does emerge from the published structure is that it has a large channel on the cytosolic surface that penetrates deep into the membrane and is blocked by a relatively narrow gate at the bottom. This would be consistent with MPTP opening being mediated by a conformation change that wedges this gate open.

The evidence presented so far is largely circumstantial and for a definitive proof it would be necessary to show MPTP activity in a reconstituted preparation of purified ANT. Although there have been several reports of reconstitution of the MPTP from partially purified solubilised inner membrane proteins that include the ANT and CyP-D, and other reports of reconstitution using purified ANT and CyP-D, none of these is very persuasive as reviewed elsewhere [39]. The most convincing data comes from Brustovetsky and Klingenberg who used the purified and reconstituted adenine nucleotide translocase of *Neurospora crassa* to demonstrate the formation of non-specific channels at high calcium concentrations [51]. These data are consistent with observations from many laboratories that the MPTP can open at very high $[Ca^{2+}]$ in CyP-D deficient mitochondria [5–7,30] just as it can in control mitochondria treated with CsA [31]. The data are also consistent with the proposal discussed above, that the effect of CyP-D is to facilitate the pore-forming conformational change such that it occurs at lower $[Ca^{2+}]$. Furthermore, subsequent experiments revealed that the opening probability of these pores at high membrane potential was increased by the presence of cyclophilin from *N. crassa* and by oxidative stress [52]. These results might appear to confirm that CyP-D can facilitate a calcium-induced change in the conformation of the ANT that produces a channel, and as such might appear to offer definitive proof of the model. However, as we review below, recent data cast doubt on this. It should also be noted that these experiments were performed with the ANT from *N. crassa* and it is not clear whether these mitochondria exhibit a conventional CsA-sensitive MPTP since yeast mitochondria do not (see [39]).

2.2.2. Evidence against a role for the ANT in MPTP formation

Despite the strong evidence summarised above, an absolute requirement for the ANT in MPTP formation has been ruled out by the observation that mouse liver mitochondria lacking the two major isoforms of ANT (ANT1 and ANT2) still exhibit a CsA-inhibitable MPTP, although much higher calcium concentrations are required to initiate pore opening [53]. We have expressed some concerns about these data since export of mitochondrial ATP from the mitochondria to the cytosol via the ANT is essential for many aspects of liver metabolism, and especially urea synthesis and gluconeogenesis [54]. Yet these mice have no obvious disturbances of their liver metabolism. The explanation for this may lie in published proteomic studies [55] which reveal the presence of a novel ANT isoform, ANT4 [56], in mouse liver mitochondrial inner membranes. It seems likely that it is this ANT4 that this is capable of maintaining sufficient ATP/ADP transport to support metabolism. Nevertheless, the data of Kokoszka et al cannot be explained purely on the basis of such residual ANT being responsible for MPTP formation in these mitochondria since pore opening was found to be insensitive to ligands of the ANT [53].

The ANT is the most abundant member by far of a large family of mitochondrial carriers with common structural motifs [57], and one explanation of the data of Kokoszka et al is that in its absence another member of the family is capable of forming the MPTP [54]. An alternative explanation is that the ANT is not normally the major pore-forming component of the MPTP, but rather plays only a regulatory role [10]. Recently we have investigated the interaction between CAT and PAO on MPTP opening and our new data provides further evidence in support for this second possibility. Our previous studies had demonstrated that the binding of the ANT to a PAO-affinity matrix is abolished by pre-treatment of mitochondria with CAT [31] and this is consistent with the published structure of ANT1 with CAT bound. This reveals that when CAT is bound Cys^{160} and Cys^{257} should be too far apart to be cross-linked by PAO [50]. Thus it would be predicted that if PAO was exerting its activation of MPTP opening purely by binding to the ANT, no additional activation should be observed by PAO following CAT treatment. Yet, as illustrated in Fig. 1, this is not what we observed. As we showed previously [31], CAT treatment greatly diminished the sensitivity of the MPTP to inhibition by ADP, whilst an even greater effect was observed following PAO treatment. This effect of PAO was induced even in mitochondria pre-treated with CAT which stops the ANT binding to a PAO-column. These data imply that PAO must reduce the ADP-sensitivity of the pore at least in part independently of its

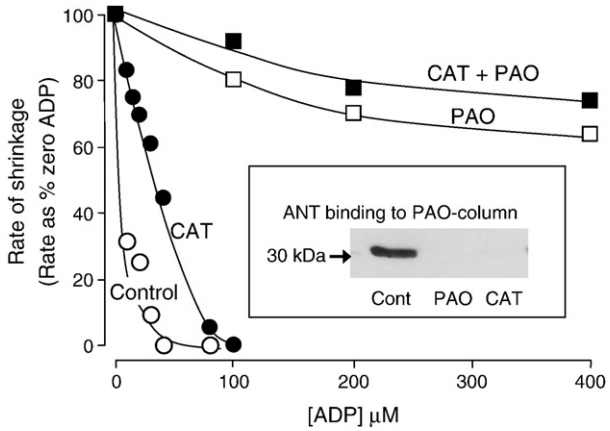

Fig. 1. Phenylarsine oxide can activate MPTP opening in the presence of CAT. Mitochondria were pre-swollen in the absence and presence of 5 μM CAT, 20 μM PAO, or both as indicated and the sensitivity of MPTP opening to inhibition by ADP determined from the rate of shrinkage (increase in A_{520}) upon addition of the polyethylene glycol [31]. When present, CAT was added 1 min prior to addition of 20 μM PAO. The free [Ca^{2+}] was set at 70 μM in the assay buffer and [ADP] varied as shown. The inset shows the effect of CAT on the binding of the ANT in detergent-solubilised rat liver inner mitochondrial membranes to immobilised-PAO determined as described previously [31]. Data are taken from [46].

binding to the ANT. We will describe below (section 2.5) how this may involve the mitochondrial phosphate carrier (PiC).

2.3. The voltage dependent anion channel

Several other proteins have been proposed to be components of the MPTP, including the voltage activated anion channel (VDAC, also known as porin) and the peripheral benzodiazepine receptor (see [4,9,23]). The possibility that these proteins might be involved was originally proposed [58] because they co-purify as a complex with the ANT under some conditions [59]. The same proteins are also thought to interact at contact sites, points of intimate contact between the inner and outer mitochondrial membranes [60]. Since that time the groups of Kroemer and Brdiczka have suggested that other proteins associated with the contact sites, such hexokinase, creatine kinase and the anti- and pro-apoptotic proteins Bcl-2 and Bax may also be involved in the regulation of the MPTP [61–64]. However, the evidence that any of these components play an essential role in pore formation (as opposed to a potential regulatory role) is weak.

Until recently, perhaps the strongest case could be made for an involvement of VDAC in MPTP formation since Crompton and colleagues had shown that both ANT and VDAC from detergent-solubilised heart mitochondria could bind to GST-CyP-D [43], although our own data did not confirm this [42]. Subsequently, it was reported that the ability of ubiquinone analogues such as UQ_0 and Ro 68-3400 to inhibit MPTP opening [65,66] was associated with labelling of a 32 kDa protein of the inner mitochondrial membrane by [^{3}H] Ro 68-3400, and this protein was initially identified as VDAC1 [67]. However, it was later demonstrated that Ro 68-3400 labels the same 32 kDa protein in mitochondria lacking VDAC1, thus revealing that VDAC1 was not the target of ubiquinone analogues [68]. Furthermore, very recently it has been shown that mitochondria lacking all isoforms of VDAC exhibit normal pore opening, thus proving that VDAC is not an essential component of the MPTP [69].

Our own very recent data [46] suggest that ubiquinone analogues can bind to both the ANT, already implicated in the MPTP as noted above, and the mitochondrial phosphate transporter (PiC) that we now believe may also be involved in MPTP formation (see below). Thus both UQ_0 and Ro 68–3400 induce the "m" conformation of the ANT and inhibit the mitochondrial phosphate transporter at similar concentrations to those inhibiting the MPTP. Since both the ANT and PiC proteins run on SDS-PAGE in the 30–34 kDa region, they represent potential candidates for the protein labelled by [^{3}H]-Ro 68–3400 [67,68].

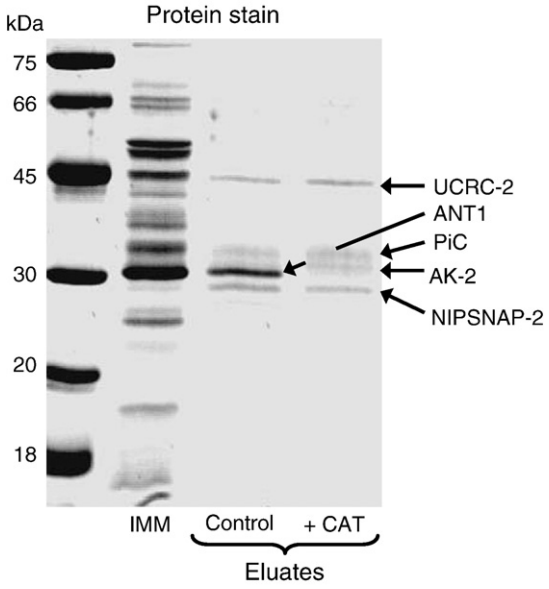

Fig. 2. Identification of inner mitochondrial membrane proteins that bind to a phenylarsine oxide affinity matrix. Inner mitochondrial membranes prepared from control and CAT pre-treated beef heart mitochondria were solubilised in Triton X-100, applied to the PAO-affinity matrix and then eluted with dithiothreitol. Proteins were separated by SDS/PAGE and revealed by Sypro-Ruby protein stain prior to identification by mass spectrometry. From order of highest molecular weight these proteins were ubiquinol–cytochrome *c*-reductase core protein II (UCRC-2); phosphate carrier protein (PiC); adenylate kinase-2 (AK-2); NIPSNAP-2 (a protein of unknown function). Data are taken from [46].

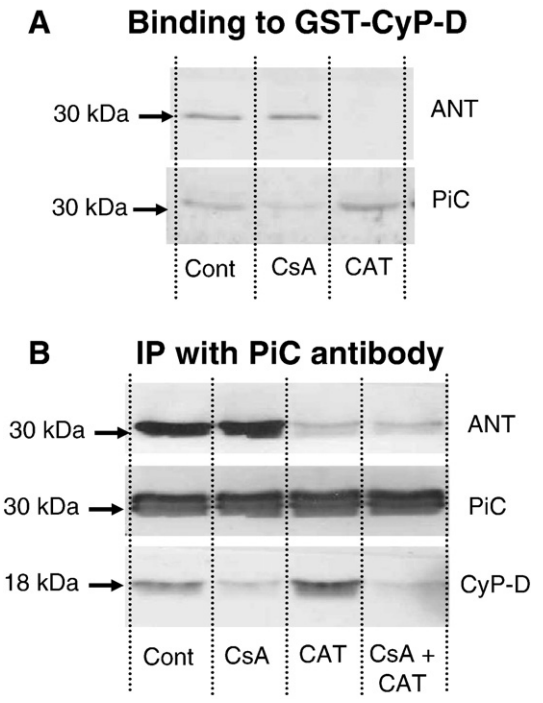

Fig. 3. The mitochondrial phosphate carrier rather than the ANT binds to CyP-D. In Panel A, solubilised inner mitochondrial membranes prepared from mitochondria pre-treated with 5 μM CsA or10 μM CAT were passed through a GST–CyP-D affinity column and after washing specifically bound proteins eluted with glutathione as described previously [42,45]. Samples were separated by SDS/PAGE and analysed by Western blotting with PiC and ANT antibodies. In Panel B, mitochondria were treated 2 μM CsA or 2 μM CAT for 10 min, before their solubilisation and incubation with immunoprecipitating PiC antibody. Immuno-complexes were separated by SDS/PAGE and analysed by Western blotting with the antibodies indicated. Data are taken from [46].

In the light of the evidence discussed above, we can conclude that VDAC normally plays no role in MPTP formation, CyP-D definitely plays a facilitating role in MPTP opening whilst the ANT plays some role, probably regulatory, but is dispensable. However, we are still left with the critical question as to what protein(s) normally form(s) the inner membrane pore component of the MPTP. The data we describe below suggest that the mitochondrial phosphate carrier (PiC) may fulfil this role.

2.4. The mitochondrial phosphate carrier

We noted above that in the CAT-bound form the ANT no longer binds to a PAO-column, yet MPTP opening in CAT-treated mitochondria can still be activated by PAO treatment (see also Fig. 1). Thus we investigated what solubilised inner membrane proteins from CAT-treated mitochondria bind to a PAO-affinity matrix [46]. We confirmed that the major protein of non-CAT-treated inner membranes to bind to the PAO-column was the ANT but that four additional proteins were also bound (see Fig. 2). Unlike the ANT, the binding of these proteins to the PAO-column was uninfluenced by CAT treatment. Three of the proteins were in the 30–35 kDa region characteristic of mitochondrial membrane transporters and were identified as NIPSNAP-2 (Swiss-Prot O75323), a protein of unknown function also identified in mouse liver mitochondria [55]), adenylate kinase 2 (AK2 — an intermembrane space enzyme) and the mitochondrial phosphate carrier (PiC). The fourth protein band ran at 45 kDa and was identified as ubiquinol–cytochrome *c*-reductase core protein II. Of these proteins the most likely candidates to play a role in MPTP formation or regulation would seem to be either AK-2, since it has adenine nucleotide binding sites, or the PiC since phosphate has been known as a potent activator of MPTP opening for many years [70]. However, AK-2 is a soluble protein and thus cannot be the membrane component of the MPTP. Furthermore, its involvement in regulation was eliminated by demonstrating that the potent inhibitor of adenylate kinase, P1,P5-di(adenoside-5′) pentaphosphate had no effect on pore opening at concentrations that inhibited AK2 activity by more than 90% [46].

A role for the PiC is supported by the observation that mitochondrial phosphate transport can be inhibited by ubiquinone analogues that inhibit pore opening (see above) and by *N*-ethylmaleimide (NEM) [71], which is known to be a potent inhibitor of MPTP opening at low concentrations [45,72]. Interestingly, Krämer and colleagues have demonstrated that the PiC of yeast mitochondria can be converted into a non-specific anion channel by dithiol cross-linking between two Cys^{28} residues within trans membrane helix 1 to form a PiC dimer [73]. An equivalent cysteine residue (Cys^{27}) is also present in mammalian PiC and could account for the observed binding of the PiC to the PAO-column that is CAT-insensitive (Fig. 2). It would also provide an additional site of activation of the MPTP by PAO.

If the PiC acts as the pore-forming component of the MPTP it would be predicted that it would bind CyP-D in a CsA-sensitive manner. We have confirmed this to be the case by both GST-CyP-D pull-down experiments and co-immunoprecipitation [46]. Typical data are given in Fig. 3. Furthermore, we were able to show that the ANT from CAT-treated mitochondria also co-immunoprecipitated with the PiC, suggesting the possibility that the MPTP might be formed as a complex between the PiC, ANT and CyP-D. Indeed, there is evidence that the PiC and ANT may interact together when associated with the ATP synthase within "the ATP synthasome" [74,75].

In addition to the data described above there is some circumstantial evidence to support a critical role for the PiC in MPTP formation. It has been observed that the knockdown of the PiC in HeLa cells reduces their sensitivity to apoptosis induced by staurosporine [76] and others have

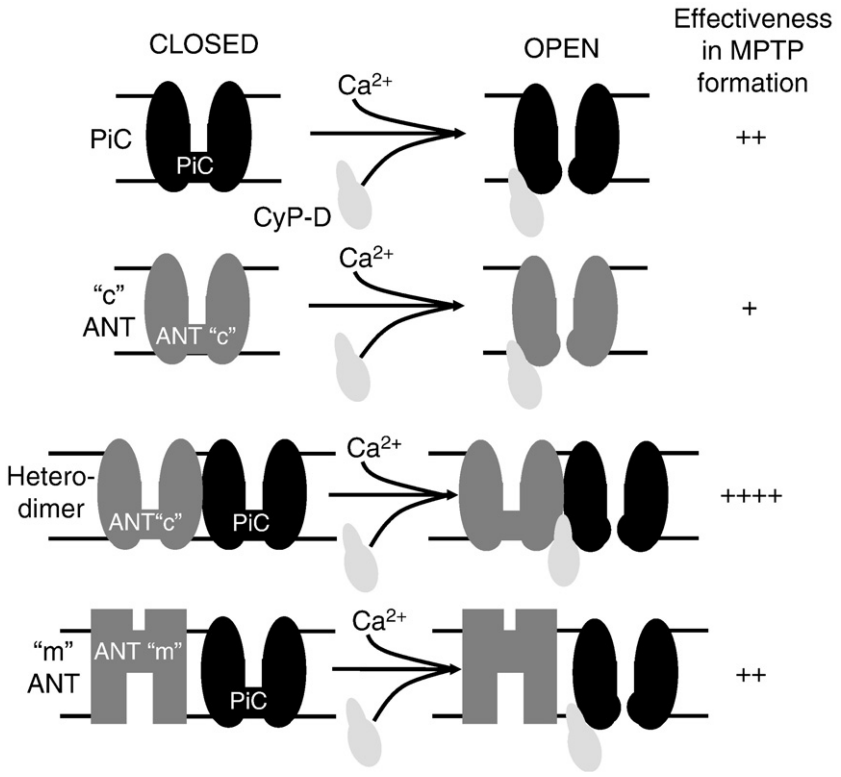

Fig. 4. Scheme illustrating the proposed role of CyP-D, ANT and PiC in the formation of the MPTP. The usual pore-forming component is proposed to be the PiC rather than the ANT, but binding of the ANT in the "c" conformation to the PiC enhances pore formation. Some pore formation by the ANT is not precluded but it is suggested that this is relatively ineffective compared to the PiC. The conformational change of the PiC (ANT) responsible for pore formation is facilitated by CyP-D binding in all cases, but can occur at high calcium in its absence. Adenine nucleotides are proposed to inhibit by binding to the ANT and preventing its interaction with the PiC and any pore formation it might undergo in its own right. It is suggested that PAO may modify cysteine residues on the ANT to prevent adenine nucleotide binding as well as directly modifying the PiC to increase its probability of entering the pore-forming conformation.

shown that the MPTP mediates apoptosis under these conditions [77]. Furthermore, PiC over expression in cells can induce apoptosis [76].

2.5. Conclusions — a new model for the MPTP

Our data lead us to propose a model for the MPTP in which the pore-forming component is the PiC rather than the ANT. This undergoes a calcium-triggered conformational change to induce pore formation which is facilitated by the peptidyl–prolyl *cis–trans* isomerase activity of CyP-D. This allows for pore opening in the absence of CyP-D or the presence of CsA but at a much higher calcium load as observed [6,7,30,31]. An interaction of the PiC with the "c" conformation of the ANT is proposed to enhance the sensitivity of the PiC to this conformational change, whereas the "m" conformation would either exert no such effect or inhibit the process. This would explain the ability to demonstrate pore opening in mitochondria containing no ANT1 or ANT2, but with reduced sensitivity to calcium and no sensitivity to ligands of the ANT [53]. The activation of the MPTP by PAO may involve both a direct effect of PAO binding to the PiC and an additional effect to prevent the adenine nucleotide inhibition mediated via the ANT. Ubiquinone analogues and NEM may work both by enhancing the "m" conformation of the ANT and via binding to the PiC. Fig. 4 presents a scheme summarising our new proposals.

In order to prove the role for the PiC in pore formation it will be necessary to use siRNA knockdown or a transgenic mouse approach using a conditional knockout. Such studies are underway. Further proof of the involvement of both the PiC and ANT in the formation and regulation of the MPTP could in principle be obtained through their reconstitution into proteoliposomes and demonstration of calcium mediated, CsA-sensitive pore opening. Work to this end is in progress, but is hampered by the instability of mitochondrial membrane carriers during purification [39].

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