The Adenine Nucleotide Translocase: A Central Component of the Mitochondrial Permeability Transition Pore and Key Player in Cell Death

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Abstract: In addition to its normal function, the adenine nucleotide translocase (ANT) forms the inner membrane channel of the mitochondrial permeability transition pore (MPTP). Binding of cyclophilin-D (CyP-D) to its matrix surface (probably on Pro_{61} on loop 1) facilitates a calcium-triggered conformational change converting it from a specific transporter to a non-specific pore. The voltage dependent anion channel (VDAC) binds to the outer face of the ANT, at contact sites between the inner and outer membranes, and together VDAC, ANT and CyP-D probably represent the minimum MPTP configuration. The evidence for this is critically reviewed as is the structure and molecular mechanism of the carrier in its normal physiological mode. This provides helpful insights into MPTP regulation by adenine nucleotides, membrane potential and ANT ligands such as carboxyatractyloside and bongkrekic acid. Oxidative stress activates the MPTP by glutathione-mediated cross-linking of Cys_{159} and Cys_{256} on matrix-facing loops of the ANT that inhibits ADP binding and enhances CyP-D binding. Molecular modeling of the loop containing the ADP binding site suggests an arrangement of aspartate and glutamate residues that may provide a calcium binding site. There are other proteins that may bind to the ANT, modulating MPTP opening and hence cell death. These included members of the Bax/Bcl-2 family (both oncoproteins and tumor suppressors) and viral proteins. Vpr from HIV-1 can bind to ANT and convert it into a pro-apoptotic pore, whereas vMIA from cytomegalovirus interacts to inhibit opening. Thus the ANT may provide a molecular link between physiopathological mechanisms of infection and the regulation of MPTP function and so represents a potential therapeutic target.

Key words Cyclophilin**,** voltage dependent anion channel, oxidative stress, carboxyatractyloside, bongkrekic acid, Bax/Bcl-2 family, viral infection, apoptosis.

INTRODUCTION

The mitochondrial permeability transition pore (MPTP) is now recognised to play a major role in both necrotic and apoptotic cell death. Although not proven beyond doubt, there is extensive evidence that the major components of the MPTP are the adenine nucleotide translocase (ANT) in the inner membrane (IM) of the mitochondria, cyclophilin D in the matrix and the voltage dependent anion channel (VDAC – also known as porin) in the outer membrane. These proteins are thought to come together at intermembrane junctions to form the MPTP, whose opening is triggered by elevated matrix [Ca^{2+}] and sensitised by a range of other effectors such as adenine nucleotide depletion and oxidative stress that often accompany cell death. Several other proteins have been proposed to play some role in MPTP action, including the peripheral benzodiazepine receptor on the outer membrane, creatine kinase in the intermembrane space and hexokinase binding to the cytosolic face of the outer membrane. However, the evidence for these proteins being critical for MPTP function is lacking [1-3]. The major emphasis of this review will be on the critical role of the ANT in the formation of the MPTP and how it acts as a target for a wide range of agents, both endogenous and exogenous, that can modulate cell death.

However, before focusing on this aspect of ANT function it is important to recognise that the normal physiological role of the ANT is the exchange of adenine nucleotide across the inner mitochondrial membrane. This is of fundamental importance for normal cellular metabolism as is well illustrated by the potent toxicity of the two "classic" inhibitors of ANT function, carboxyatractyloside (CAT) and bongkrekic acid (BKA) [4]. The former is found in the leaves of the Mediterranean thistle *Atractylis gummifera* and kills those animals that are unwise enough to eat it. The latter is obtained from the bacteria *Pseudomonas cocovenenans*. This occasionally contaminates Tempe Bongkrek, the now illegal fermented coconut dish from Central Java that, until recently, killed many people each year (http://www.tourismindonesia.com/articles/tempe-1.asp). Indeed, it is the fundamental role of the ANT in metabolism that may well place constraints on its suitability as a pharmacological target.

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THE PHYSIOLOGICAL ROLE OF THE ANT

In most mammalian cells, the major site of ATP synthesis is in the mitochondria, through oxidative phosphorylation, whilst the major use of ATP is in the cytosol and other intracellular organelles. Thus it is essential to have a rapid mechanism for transporting ATP out of the mitochondria and ADP back in. This is the major physiological role of the ANT. It is one of the most abundant mitochondrial proteins, and in energy demanding tissues such as heart it is present at about 1.2 nmoles per mg protein, representing up to 10% of the protein of the inner membrane, and about one third that amount in liver [4,5]. The ANT catalyses the electrogenic exchange of ATP^{4-} with ADP^{3-}, the charge imbalance enabling the large negative membrane potential that is normally maintained across the inner mitochondrial membrane (about –180mV) to drive ATP out of the mitochondria into the cytosol in exchange for ADP entering. This accounts for the much higher (at least fifty fold) ATP/ADP ratio present in the cytosol than the mitochondria that is of fundamental importance for cellular metabolism [5]. In cells in which mitochondrial respiration is compromised, for example by respiratory chain inhibitors or genetic defects, the transporter can reverse and allow the entry of ATP into the mitochondria. Here ATP hydrolysis by the proton translocating ATPase can generate a modest proton motive force and maintain some aspects of normal mitochondrial function and metabolic compartmentation in the absence of a functional respiratory chain.

STRUCTURAL AND FUNCTIONAL CHARACTERISATION OF THE ANT

General Topological Features

The ANT (or more accurately ANT isoforms as discussed below) is a member of the large mitochondrial carrier family, all of which are nuclear encoded. The ANTs share common structural features with other members of the family. All contain a tripartite repeat of approximately 100 amino acid residues and are predicted to have 6 putative transmembrane spanning domains (2 in each repeat) of 18-22 residues, with both the amino- and carboxy- termini in the inter-membrane space. The transmembrane domains are joined by hydrophilic regions; three loops of about 40 amino acids face the matrix and two shorter loops of about 26 and 18 amino acids face the intermembrane space [6]. This is illustrated schematically in Fig. **1**. Another important feature of the ANT is that it is totally dependent on the very tight binding of 6 molecules of cardiolipin per molecule of protein for activity. This cardiolipin is not removed by normal detergent solubilisation, but if it is the protein denatures [7-9].

The ANT binds one molecule of CAT or BKA per 2 ANT molecules and the resulting complex, when solubilised, behaves as a 60kDa protein, suggesting that it exists as a dimer (see [4,10]). In support of this, a fusion protein of two tandem covalently linked yeast ANT molecules has been shown to be functional, with similar

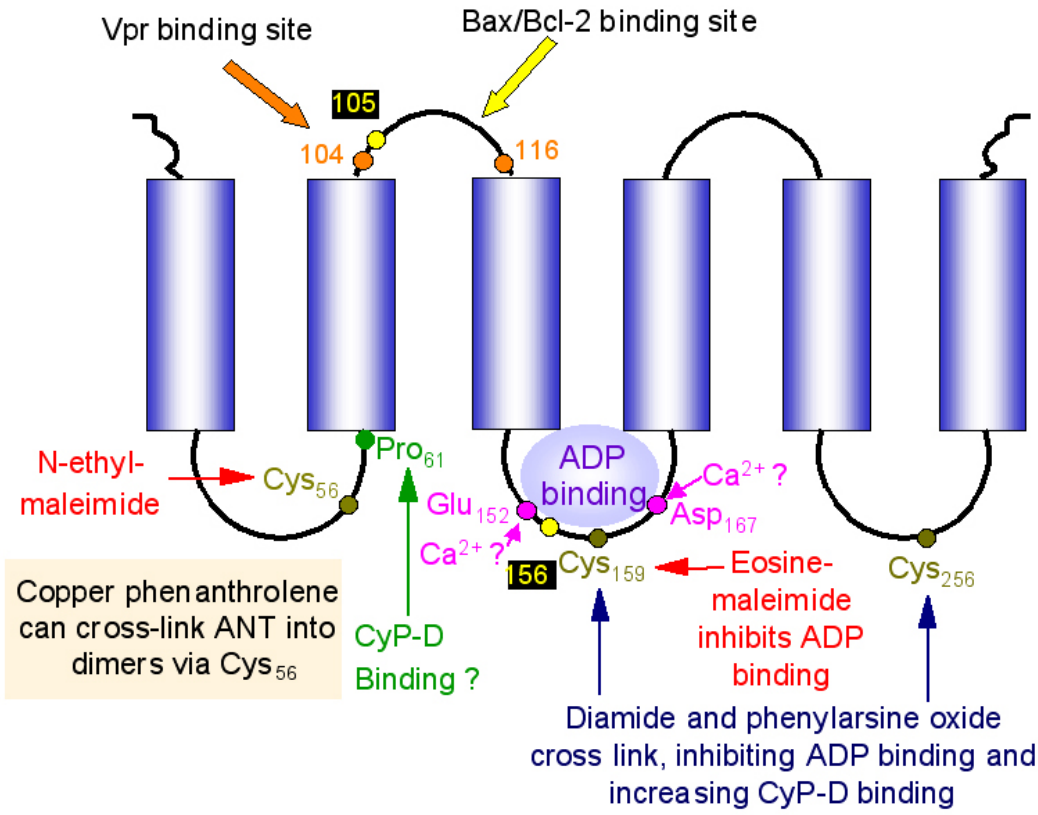

Fig. (1). The proposed topology of the ANT and site of action modulators of MPTP activity. Residue numbering is given for bovine ANT1 with the exception of the sequences identified as containing the binding sites for Vpr (104 to 116 delimited by dark grey circles) and Bax/Bcl-2 (105-156 delimited by light grey circles) for which numbering refers to human ANT2 and ANT1 respectively. However differences in numbering between bovine and human ANTs in this region are minimal.

activity and inhibitor binding properties to the normal ANT [11,12]. Furthermore, the two subunits can be covalently cross-linked between the matrix facing Cys_{56} residues of adjacent subunits using copper o-phenanthroline or bifunctional bimaleimides [13,14]. However, none of these observations rules out the possibility that a tetramer may be the minimal functional unit and some evidence for this has been presented [4]. Unlike matrix proteins and many other mitochondrial IM proteins, mitochondrial carrier proteins lack an N-terminal targeting sequence (see [10]). However, their import into the mitochondrial IM still involves translocase complexes of the outer membrane (Tom) and inner membrane (Tim). Each of the three tripartite repeat modules co-operates in recruitment to the Tom complex, translocation across the outer membrane, association with the Tim complex and integration into the IM [15].

ANT Isoforms

There are three isoforms of the ANT in humans, each about 300 amino acids in length (33kDa) and sharing about 90% sequence identity. Sequence differences are scattered throughout the protein and residue substitutions are of a conservative nature [4]. ANT1 (also known as T1) is primarily expressed in heart and skeletal muscle whilst ANT3 (confusingly also known as T2) is expressed in all tissues, at levels reflecting the demands of the tissue for oxidative phosphorylation [16]. In contrast, ANT2 (T3) is not expressed in normal adult tissue, but is expressed in rapidly proliferating cells such as myoblasts and tumours [17,18]. In rat and mouse there only two isoforms (equivalent to ANT1 and ANT2) with ANT2 present ubiquitously much as ANT3 is in humans. All the reported studies on the functional characterisation of purified and reconstituted ANT were performed on ANT1 (mainly from bovine heart) and there are no published data on the other isoforms. This is probably because ANT2 appears to be less amenable than ANT1 to purification and reconstitution of active transporter [19]. Thus it remains unclear what advantages arise from cells expressing the different isoforms in terms of their metabolism or sensitivity to MPTP opening, but this may be an important consideration if the ANT is to be considered as a pharmacological target. This is well illustrated by the demonstration that ANT1 knock-out transgenic mice survive, but exhibit ragged skeletal red muscle fibres and cardiac hypertrophy, with dramatic mitochondrial proliferation in both tissues [20]. Where both ANTs are found in the same tissue, as in heart mitochondria, there is some evidence that it is ANT1 that preferentially localises within the contact sites and associates with CyP-D to form the MPTP [21]. However, this is not easily reconciled with the observation that liver mitochondria are at least as sensitive to MPTP opening as heart mitochondria [22], yet contain little or no ANT1 [16,23,24].

The ANT Exhibits Two Distinct Conformations

Critical to the study of the structure and topology of the ANT is the ability of the two potent inhibitors of the ANT, CAT and BKA, to induce distinct conformations of the ANT. These are stable during solubilisation and can be purified as immunologically distinct proteins. The conformation induced by CAT, which is membrane impermeant and binds to the ANT from the cytosolic surface, is known as the "c" conformation, whilst BKA, that is membrane permeant and binds to the matrix surface of the ANT, stabilises the "m" conformation [6]. The interconversion between these two conformations induces such a profound change in the protein structure that it causes a change in the morphology of heart mitochondria that can be detected as a change in light scattering. BKA causes light scattering to increase and the matrix to appear condensed, whilst CAT has the opposite effect [22,25,26]. The conformational changes can also be studied using endogenous tryptophan fluorescence or fluorescent ADP analogues [4]. The proposed topology of the ANT, illustrated in Fig. **1**, has been confirmed experimentally by specific labeling of the two ANT conformations with a range of membrane permeant and impermeant thiol and lysine reagents (see [6,13,27-31]. Together with the use of photactivatable substrate analogues [32,33] these studies have also led to information on both the substrate binding sites and the conformational changes that occur during the translocation cycle.

Substrate Binding Sites and their Sensitivity to Thiol Reagents

Current evidence suggests that adenine nucleotide binding involves the two matrix loops nearest the C-terminal end of the molecule that undergo conformational changes during the translocation cycle. The region around Lys_{162} appears critical for ADP binding and is accessible to the membrane impermeant lysine reagent pyridoxal phosphate, an inhibitor of the ANT, only when the carrier is in the "m" conformation. This demonstrates that part of this loop must be exposed to the extramitochondrial surface in the "m" conformation [34]. However, the "c" conformation specific inhibitor arylazido-atractyloside also photolabels the same loop of the ANT [35] demonstrating that part of it must also be accessible in the "c" conformation. Taken together with the observation that the membrane impermeant substrate analogue 2-azido-ATP binds to the same region [32] these data suggest that movements in this loop forms part of the conformational change involved in moving ATP across the membrane. 2-Azido-ATP also labels residues in the most C-terminal matrix loop (loop 3) implying that some part of this sequence must also be accessible to the cytosolic surface [32]. Much additional data to support and extend these conclusions has come from Terada's laboratory through the use of thiol reagents to probe the accessibility and interaction between the three reactive cysteine residues in the matrix facing loops [13,29-31]. These are Cys_{56} in loop 1, Cys_{159} in loop 2 and Cys_{256} in loop 3 (residue numbers refer to the bovine sequence). Their modification by thiol reagents inhibits adenine nucleotide exchange by the ANT. In view of the importance of these cysteine residues in the regulation of MPTP opening, to be discussed below, the effect of their covalent modification will be considered in some detail.

In confirmation of earlier work by the groups of Klingenberg and Vignais, at low concentrations the membrane permeant thiol reagent N-ethylmaleimide (NEM) was found to attack Cys_{56} only when the carrier was in the

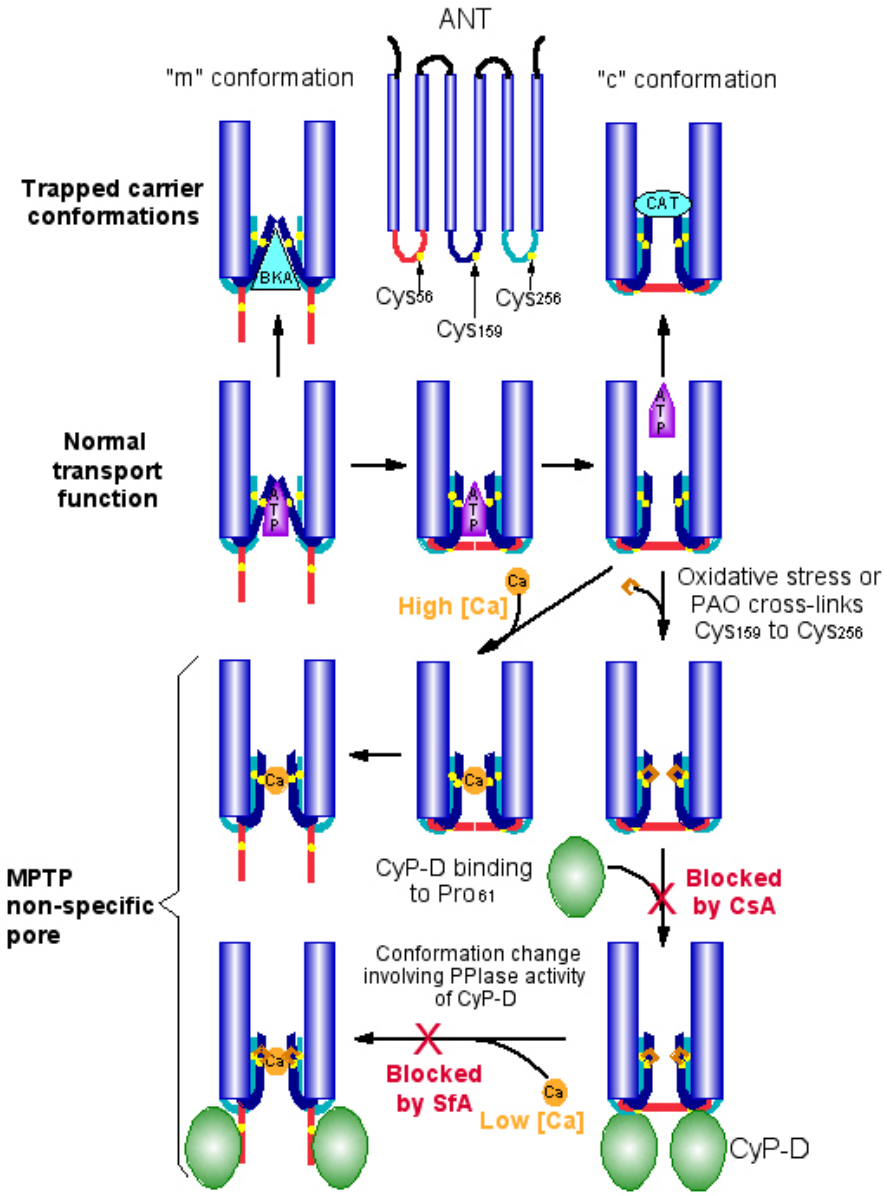

Fig. (2). Hypothetical scheme illustrating how the ANT may switch from its normal carrier function to a non-specific pore. The model for normal ANT function follows that of Terada and colleagues and is based upon the disposition of matrix loops predicted by from their labeling studies [29-31]. The binding of CyP-D to loop 1 and the resulting conformational change that convert the ANT into a non-specific pore is adapted from our own earlier models [22, 124, 137].

"m" conformation, labeling being abolished by CAT treatment. In sub-mitochondrial particles (SMPs) NEM also preferentially attacked Cys_{56} in a CAT sensitive manner, with a slower modification of Cys_{159} and very slow modification of Cys_{256}. The accessibility of Cys_{56} to the matrix in the "m" conformation is also suggested by the ability of copper o-phenanthrolene and bifunctional dimaleimides to cross-link two ANT molecules in SMPs through their Cys_{56} residues in a CAT sensitive manner [13,14]. Thus the first matrix loop of the ANT would appear to be well exposed and flexible in the "m" conformation but become inaccessible in the "c" conformation, perhaps by moving into the membrane. In the presence of BKA, but not CAT, copper o-phenanthrolene can also produce a cross-link between Cys_{56} in and Cys_{256} in the detergent solubilised ANT, suggesting the that loops 1 and 3 can come into close proximity in the "m" conformation but not the "c" conformation. However, this cross-link is not observed when the ANT is in its native environment in SMPs [31]. These data were interpreted by Terada et al as indicating that in the dimeric ANT, the first loop of each subunit act together to create a gate for the pore which opens ("m" conformation) and closes ("c" conformation) during the translocation cycle, thus regulating access to the substrate binding site on the matrix side [29-31]. This is illustrated in Fig. **2**. It will be noted below how CyP-D is thought to bind to a proline residue (Pro_{61}) in loop 1, and when triggered by calcium induces the conformational change responsible for MPTP formation. Maintaining an open gate would seem to be a potential means of doing this.

In contrast to the small water soluble NEM, the more bulky aromatic anionic maleimide, eosine-5-maleimide

(EMA) preferentially attacks Cys_{159} in loop 2 as well as modifying Cys_{56} in loop 1 more slowly and Cys_{256} in loop 3 slower still. Labeling of Cys_{159} and Cys_{256} but not Cys_{56} was greatly reduced by BKA and this was taken to indicate that BKA binds close to these residues [29,30]. In support of this it was found that Eosin Y, an analogue of EMA that does not possess the thiol-reactive maleimide group, is a high affinity substrate analogue of the ANT whose binding is also inhibited by BKA but not CAT [30,36]. These data are consistent with there being a binding site for adenine nucleotides close to Cys_{159} whose affinity for adenine nucleotides is conformationally dependent. Taken together with the observation that Lys_{162} is critical for ADP binding and is accessible to membrane impermeant pyridoxal phosphate only when the carrier is in the "m" conformation [34] it would appear that there is an ADP binding site close to Cys_{159}. The accessibility of this binding site to the matrix and intermembrane space alters as the ANT undergoes a conformational change as part of the translocation cycle [30]. In this context it is of interest that there is evidence for a slow but detectable net efflux of adenine nucleotides from mitochondria that can be blocked by BKA but enhanced by CAT, implying some slippage in this gating mechanism that normally ensures the ANT acts as an antiporter can occur when CAT is bound [25]. Furthermore, when treated with low concentrations of thiol-reactive organomercurial reagents, which react preferentially with Cys_{159} [37] the purified, reconstituted ANT can be converted from an obligate antiporter into a uniporter catalysing net adenine nucleotide transport rather than exchange [38]. Again, this is consistent with cysteine residues being closely associated with interaction between substrate binding and gating of the ANT.

Kinetic Models for ANT Mediated Transport

The transport kinetics and mechanism of ANT1 have been investigated in great detail using isolated mitochondria, SMPs and purified ANT reconstituted into proteoliposomes. In its native environment (beef heart mitochondria or SMPs) the ANT has been shown to be specific for ADP and ATP or, with 5-10 fold less affinity their deoxyribose equivalents. The magnesium complexes are not transported; nor are AMP or guanine, cytidine and thymine containing nucleotides. Pyrophosphate is a weak substrate for the ANT [39] and as a result can be used to deplete mitochondria of their adenine nucleotide pool [40,41]. The carrier has a turnover number of about 10 s^{-1} at 18°C and activation energy of about 45 $kJ.mol^{-1}$ [5]. The K_m values for ADP and ATP are very much dependent on the conditions used for their measurement, and values between 1-100 μM have been reported [5,42,43,43-48]. The nature of the translocation cycle is also debated. All other members of the mitochondrial carrier family that act as antiporters, such as the 2-oxoglutarate / malate, citrate / malate, dicarboxylate / phosphate and glutamate / aspartate transporters share an identical kinetic mechanism. This is a sequential mechanism in which a ternary complex is first formed between the carrier and the two substrates, one on the outside and one on the inside surface, which is followed by a concerted transport step and release of both substrates on the opposite sides of the membrane. The binding and release of substrates is random order and very rapid, whilst the translocation of substrates across the membrane is the rate determining step (see [10]). There is some kinetic evidence that the ANT displays the same mechanism [46] and if this is the case, it would require the ANT to have two distinct binding sites for adenine nucleotides on either side of the membrane. This is important because, as will be discussed below, there is evidence that adenine nucleotides can inhibit the MPTP at two distinct sites.

However, data from Klingenberg's laboratory has been interpreted in terms of a model in which there is a gated pore with a single gate that can bind adenine nucleotide from either side. In this model, which is also the one assumed by Terada in interpreting the labeling data discussed above, the two conformations of the carrier represent the gated substrate binding site facing outwards ("c" conformation) or inwards ("m" conformation) [5,6]. Most recently, further support for this model has come from measurement of transient membrane currents across the ANT reconstituted into black lipid membranes. These experiments have shown that the ANT can transport ATP unidirectionally but only for single site turnover, as would be predicted for the single gated pore model [49]. However, data on the kinetics of transport in mitochondria and SMPs do not fit this model; nor does the ability of impermeant fluorescent ADP analogues to bind to the ANT in both its "c" and "m" conformations [4]. Thus, although it is certain that BKA and CAT stabilise different conformations in the translocation cycle, it remains unclear as to whether or not these conformations are identical to an outward facing and inward facing single binding site. Another point of debate is how the membrane potential influences the transport, and this also has implications for the effects of membrane potential on the MPTP. Since ATP^{4-} is transported in exchange for ADP^{3-} the mitochondrial membrane potential favors ATP efflux from the mitochondria in exchange for ADP influx. This could be mediated by affecting the relative binding affinity of the ANT for ATP and ADP on both sides of the membrane in the sequential mechanism and there is some evidence in favor of this [42]. However, data from Klingenberg's laboratory have been analysed in terms of the single site gated pore model with the potential increasing the velocity of the translocation step [43,45]. There may be an additional effect of surface charge on ATP and ADP binding to the ANT [50], another factor of relevance to the role of the ANT in MPTP where surface charge has also been implicated as discussed below.

THE ANT ACTS AS THE INNER MEMBRANE PORE FORMING COMPONENT OF THE MPTP

Ligands of the ANT Modulate the MPTP

Chappell & Crofts [51] were first to note that the massive swelling of liver mitochondria induced by calcium in the presence of phosphate was inhibited by ADP and activated by carboxyatractyloside. This was more than a decade before it was recognised that this "permeability transition" was mediated by a protein channel (the MPTP) rather than a non-specific breakdown in the IM permeability

barrier caused by phospholipase A_2 activation. An involvement of the ANT in pore opening was first proposed formally by Hunter and Haworth [52] and subsequently more extensive evidence provided by LeQuoc and LeQuoc [53] and ourselves [22,54]. The early evidence was based largely upon the observation that in energised mitochondria, any reagent such as CAT that stabilised the "c" conformation of the ANT, sensitised the MPT to [Ca^{2+}], whilst any reagent such as BKA that stabilised the "m" conformation of the ANT, made the MPT less sensitive to [Ca^{2+}]. In addition, the potent inhibitory action of ADP on pore opening by decreasing the sensitivity of the calcium trigger site to [Ca^{2+}], provides additional evidence for a critical role of the ANT. The major effect of ADP appears to on the matrix surface because depeletion of mitochondria of matrix adenine nucleotides by pore opening and closing or by pyrophosphate treatment makes them hypersensitive to [Ca^{2+}] [41, 52]. However, there are two ADP binding sites involved in this inhibition, with K_i values of about 1 and 25 μM. The high affinity site is blocked by CAT with the same concentration dependence that inhibits adenine nucleotide translocation, and is therefore almost certainly associated with the ANT [52,54-58]. The identity of the second site is less clear but may well be an extramitochondrial binding site for adenine nucleotides on the ANT. This would be entirely consistent with the sequential kinetic mechanism discussed above which invokes simultaneous occupation of two distinct binding sites on either side of the membrane. However, it would not fit so readily with the gated pore model proposed by Klingenberg in which there is a single binding site for adenine nucleotides with access from either side of the membrane depending upon the conformation of the ANT. Further evidence for the involvement of the ANT is provided by a comparison of the ability of a range of nucleotides to inhibit MPTP opening with their ability to be transported by the ANT. Our own studies showed that apart from ADP, only ATP and deoxy-ADP inhibited the MPTP with $K_{0.5}$ values 500 and 20 times greater than ADP respectively. This correlates well with their affinity for the matrix binding site of the ANT under de-energised conditions. The affinity for ATP appeared to increase under energised conditions, consistent with the electrogenic nature of ATP export [54].

The ANT Can Act as the Membrane Potential Sensor of the MPTP

Bernardi and colleagues have provided strong evidence that the MPTP is voltage-regulated, being activated as the membrane potential becomes less negative [59-64]. We have suggested that the membrane potential is sensed by the ANT itself through an effect on adenine nucleotide binding as outlined above. In support of this hypothesis, we have demonstrated that in mitochondria depleted of adenine nucleotides by pyrophosphate treatment, not only is the MPT much more sensitive to [Ca^{2+}], but it is also no longer voltage sensitive [22,41]. Trifluoperazine is another inhibitor of the MPTP that changes the voltage sensitivity of the MPTP [65] and thus is only effective under energised but not de-energised conditions [54]. It has been proposed that the effects of this agent are mediated by changing the surface membrane charge of the membrane [65] which is entirely consistent with the documented effect of surface charge on ATP and ADP binding to the ANT [50] discussed above.

Evidence that the Trigger Site for Calcium is Integral to the ANT

Calcium is an essential trigger for the MPTP under most conditions, and where agents such as phenylarsine oxide or CAT have been shown to induce pore opening in the absence of added calcium this probably reflects sensitisation of the pore to the residual matrix calcium rather than a calcium independent mechanism [54]. The specificity of the calcium trigger site appears to be absolute and other divalent cations such as Sr^{2+}, Mn^{2+}, Ba^{2+} and Mg^{2+} act as inhibitors [55,66,67]. Protons also compete for Ca^{2+} binding at this site, accounting for the profound inhibition of MPTP opening as the pH drops below 7 [55,66,67]. Experiments from one of our laboratories have demonstrated that in energised heart mitochondria, increasing the matrix [Ca^{2+}] but not [Sr^{2+}] causes a conformational change in the ANT that is detected as a rapid decrease in light scattering, quite distinct from the large light scattering decrease that accompanies MPTP opening [22,68]. This decrease in light scattering is rapidly reversed by ADP or BKA, with the effect of ADP being overcome by CAT, implying that matrix calcium was enhancing the “c” conformation of the ANT [22]. In liver mitochondria a similar light scattering effect induced by calcium could only be demonstrated if dextran was included in the medium to mimic the colloidal osmotic pressure of the cytosol [69]. This may be significant since dextran is know to enhance contact site formation between the inner and outer membranes [70] where it has been suggested that VDAC and the ANT interact to form the MPTP [2,21,71]. In the purified reconstituted ANT, calcium can induce non-selective pore formation by the ANT without added CyP-D, but this requires millimolar concentrations [72]. Thus it is possible that an interaction of the ANT with VDAC in the contact sites greatly enhances the ability of calcium to induce this change in conformation of the ANT. Conversely, it has been shown that calcium can enhance contact site formation [73].

These effects of calcium all involve its binding to a matrix facing site on the ANT, whose affinity for calcium is increased in the “c” conformation and inhibited by ADP [54,56,74]. Little is known about which residues are important for calcium binding. Indeed it has been proposed that the tightly bound cardiolipin associated with the ANT may play a role by binding Ca^{2+} and thus releasing positive charges within the protein which might open the gate [72]. However, there are four-conserved aspartate and glutamate residues in matrix loop 1 and five residues in each of loops 2 and 3, which might be orientated in such a way as to produce a calcium-binding motif. Loops 2 and 3 would seem most likely since these contain the adenine nucleotide binding site as noted above, and ADP inhibits MPTP opening by decreasing the affinity for [Ca^{2+}]. Indeed, by molecular dynamics computational analysis of loop 2 it is possible to produce a theoretical model of the adenine nucleotide binding site that accounts for the observed substrate specificity [75]. In this model, Glu_{152} and Asp_{167} come into close proximity and it could be envisaged that within the dimer, the combination of four carboxylate

groups might create a calcium binding motif. This would leave loop 1 free to bind CyP-D, as will be described below. The arginine-specific reagents phenylglyoxal and 2,3-butanedione have been shown to inhibit the calcium trigger site and it is tempting to speculate that this might be through modification of Arg_{151} that is adjacent to Glu_{152} [76]. Higher concentrations of NEM that modify Cys_{159} in the same loop also decrease the sensitivity of the MPTP to $[Ca^{2+}]$ [77]. In addition to the matrix calcium-specific trigger site there is another inhibitory divalent cation binding site on the external surface of the IM with a K_i for Mg^{2+} of about 0.3mM [78].

CyP-D Binds Specifically to the ANT

In 1990 we proposed that the effects of cyclosporin A and ligands of the ANT on MPTP opening could be explained if CyP-D bound directly to the ANT and, through its peptidyl-prolyl cis trans isomerase activity, mediated a calcium activated conformational change that converted the carrier from a specific antiporter into a non-specific pore [22]. This hypothesis lies behind most current models of the MPTP, and is supported by an increasing body of evidence. Early experiments from one of our laboratories demonstrated that oxidative stress induced with t-butyl hydroperoxide (TBH) or glutathione oxidation induced by diamide treatment increased CyP-D binding to the inner mitochondrial membrane in parallel with increasing the sensitivity of pore opening to $[Ca^{2+}]$. This binding was prevented by CsA [77,79,80]. In order to show this effect consistently, the mitochondrial membranes had to be prepared in iso-osmotic KSCN medium to stabilise the complex between CyP-D and its membrane target protein. Stabilisation could also be achieved by the addition of low concentrations of guanidinium hydrochloride, implying that it was the chaotropic property of KSCN that was responsible for its stabilising effects [80]. This suggests that the CyP-D forms a complex with the target protein, inducing a conformational change that exposes more of the protein surface to the aqueous medium. Such an effect might be predicted for the formation of a channel.

Apart from oxidative stress and thiol reagents, other known modulators of the MPTP such as matrix $[Ca^{2+}]$, [ADP], pH or membrane potential had no effect on CyP-D binding implying that these agents act via the ADP binding site or Ca trigger site on the ANT [54,79,80]. In contrast, Bernardi and colleagues demonstrated an inhibitory effect of low pH on CyP-D binding to SMPs which was prevented by the histidine reagent diethylpyrocarbonate that also blocks the inhibitory effect of pH on the MPTP [81,82]. These experiments were performed in low ionic strength media where a large number of other matrix proteins also remained bound to the membrane, and thus it is possible that the effect of pH was on non-specific binding of CyP-D to charged groups on the phospholipids or membrane proteins. Crompton and colleagues [83,84] labeled the membrane bound CyP-D with photoactivatable CsA derivatives within intact mitochondria. Their data suggested that Ca^{2+} might enhance and ADP diminish CyP-D binding under such conditions, but our own experiments did not confirm this [80,85].

The data described above show that CyP-D can bind to an inner mitochondrial membrane component under conditions that favor MPTP opening, but they do not demonstrate that the binding partner is the ANT. This was achieved by passing detergent solubilised inner mitochondrial membrane proteins over a CyP-D affinity column and analysing proteins that remained bound after extensive washing. In this laboratory the only tightly bound protein that was detected was the ANT and this binding was inhibited by pre-treatment of the CyP-D affinity column with CsA and enhanced when the inner membranes were prepared from liver mitochondria subjected to oxidative stress [77,85]. In similar studies, Crompton and colleagues found that both VDAC and the ANT bound tightly to the GST-CyP column, but in contrast to our own studies they were unable to prevent binding with CsA [2,86]. These apparent discrepancies may be the result of major differences in the experimental protocols used. Crompton et al. solubilised whole heart mitochondria in the zwitterionic detergent CHAPS whilst in our own studies purified inner membranes from liver mitochondrial were solubilised in the non-ionic detergent Triton-X100. Since, as noted above, heart mitochondria and liver mitochondria possess different isoforms of the ANT (mainly ANT1 and ANT2 respectively), it is possible that the relative affinities of the isoforms for CyP-D and VDAC are different. In this context, it is of interest that Vyssokikh et al [21] have reported that in mitochondria that contain both ANT1 and ANT2, it is the former that preferentially binds VDAC at the contact sites. Thus it is not unreasonable that the use of heart mitochondria by Crompton et al would have led to binding of both ANT1 and VDAC to the CyP-D affinity column, whereas the ANT2 from liver mitochondria bound without VDAC.

Reconstitution of the MPTP Using Purified ANT and CyP-D

The critical proof that the MPTP is formed by a calcium-triggered conformational change of ANT, facilitated by bound CyP-D (and perhaps VDAC) requires reconstitution of CsA-sensitive MPTP activity from the purified components. Early work from Krämer's group had shown that treatment of mitochondrial carriers such as the ANT and the glutamate / aspartate antiporter with thiol reagents is able to convert them from specific antiporters to non-specific channels [38,87]. These pores exhibit similar permeability properties to the MPTP but differ in that they are not sensitive to either CsA or $[Ca^{2+}]$. Subsequently it was shown that the purified ANT from heart mitochondria can form similar non-specific pores when reconstituted into proteoliposomes and exposed to high (millimolar) concentrations of calcium [72,88]. Pore opening was inhibited by ADP and BKA but not by CAT. However, the sensitivity of the purified ANT to $[Ca^{2+}]$ was far lower than that of the MPTP within mitochondria, although this may relect the high abundance of the ANT in mitochondria with the need for only one dimer to enter the open state for the permeability transition to occur. CsA did not inhibit pore opening by the purified ANT, but this is not unexpected in the absence of CyP-D. Subsequently the groups of Brdiczka and Kroemer demonstrated that crude fractions of brain

homogenates enriched in proteins associated with mitochondrial contact sites, including VDAC, ANT, hexokinase, creatine kinase and CyP-D, could be reconstituted into proteoliposomes to form a calcium activated, CsA-sensitive pore [89-91]. However, which of the components were essential for pore formation could not be established by such techniques. Subsequently, Kroemer's group went on to use the purified, reconstituted ANT in studies on the MPTP (see Table 2 for references), whilst Crompton's group reported reconstitution of CsA-sensitive pore activity using the CyP-D/ANT/VDAC complex isolated by glutathione elution from GST-CyP-D affinity column [86]. The latter study provides perhaps the most compelling evidence for VDAC playing a role in MPTP formation although, as recognised by Crompton et al., it does not demonstrate whether VDAC is essential for MPTP formation or merely exerts a regulatory effect.

Although a range of controls were included in the experiments described above, such as the use of protein free liposomes, there are problems inherent in the techniques used that may limit their usefulness.

- The first problem is that of solubilising the membrane proteins in a suitable detergent without denaturing them and maintaining their activity through purification. For example, it is well documented that unless stabilized by binding of a high affinity ligand such as BKA or atractyloside, the ANT rapidly denatures when solubilised in detergent [19]. It may be significant that in laboratories specialising in measuring the activity of the reconstituted ANT, studies are performed at low temperature and over a millisecond timescale [19,49]. In contrast recent studies using the reconstituted ANT for investigating the MPTP have confirmed ANT activity by measuring transport over one hour at room temperature implying that the majority of the reconstituted ANT may be denatured (see e.g. [92]).
- The second problem is the need to obtain sealed, detergent-free proteoliposomes, with the pure ANT in a defined orientation, preferably with the matrix surface facing out to allow binding of exogenous CyP-D. A particular problem in many reported studies is the use of an inappropriate step for the removal of Triton-X100 following reconstitution of the solubilised proteins. The low critical micellar concentration and large micellar size of this detergent prevents its proper removal from proteoliposomes by dilution and gel filtration [93]. Since even very low concentrations of Triton X100 can exert profound effects on both the inhibitor sensitivity of VDAC [94] and on the general permeability of lipososomes [95], results obtained in this way must be treated with caution.
- The third problem is that a method must be found to assay pore opening in small amounts of reconstituted material. Most published studies have used the release of a radioactive, fluorescent or chromogenic marker molecule entrapped within the proteoliposome. In many cases the release was measured after 30-60 min at room temperature (e.g. [96,97]) yet total release of the marker from a proteoliposome would be expected to occur almost instantaneously once the MPTP had opened. Furthermore, this approach does not allow continuous recording of pore activity to determine kinetics of activation and regulation since the release of the marker is an all or none phenomenon.

Aware of the problems listed above, studies performed in one of our laboratories have employed a very rapid purification procedure for the ANT, using Triton-X100 solubilised heart mitochondria with hydroxyapatite chromatography followed by ion exchange FPLC [88] and immediate reconstitution of the pure protein fraction in proteoliposomes, using Biobeads to remove detergent. To assay the reconstituted MPTP we have developed a continuous spectrophotometric technique in which malate dehydrogenase is entrapped within the proteoliposomes and is only accessible to its substrates (NADH and oxaloacetate) when the pore opens. This is monitored by the decrease in A_{340} that accompanies oxidation of NADH, and thus it is possible to determine the rate at which pores open as well as the fraction of pores that are open under any particular condition. We have used this technique to demonstrate that in the presence of recombinant CyP-D, the purified, reconstituted ANT does form CsA-sensitive pores at micromolar [Ca^{2+}] [98]. These data are important because they confirm that the minimum configuration of the MPTP includes only the ANT and CyP-D, and any role for other proteins such as VDAC is likely to be regulatory rather than essential. However, we have found that the success rate for reconstitution of active MPTP in this manner is <10%, and this has prevented further progress. The cause of this inconsistency appears to be the inherent instability of the ANT during purification and faster purification procedures that maintain the ANT in a more native state are being developed. Brustovetsky et al have had greater success using electrophysiological techniques to measure current flow through channel formed by the reconstituted recombinant ANT from Neurospora crassa [99]. This behaved much as the bovine heart ANT and exhibited ADP-inhibitable pore opening at high (1mM) [Ca^{2+}]. When a *Neurospora* cyclophilin was added to the system, the ADP inhibition was abolished in a CsA sensitive manner. However, these data were all obtained at a much higher [Ca^{2+}] than is required to open the mammalian MPTP, although, as noted above, this may relect the high abundance of the ANT in mitochondria with the need for only one dimer to enter the open state for the permeability transition to occur. Another concern is that no evidence was presented that intact *Neurospora crassa* mitochondria can undergo a calcium-dependent CsA-inhibitable permeability transition. Indeed, if they behave like *Saccharomyces cerevisiae* it is unlikely that they do [85,100,101].

Thiol Groups on the ANT play a Critical Role in Pore Opening

Oxidative stress induced by reagents such as t-butylhydroperoxide (TBH) or diamide and also thiol reagents such as phenylarsine oxide (PAO) [102-104] greatly sensitise the MPTP to [Ca^{2+}] by two mechanisms. Firstly CyP-D binding is increased as noted above, and secondly the binding of adenine nucleotides is greatly impaired [54].

PAO has the greatest effect of the reagents tested, raising the $K_{0.5}$ for ADP inhibition of the MPTP to > 500μM [54] and can covalently bind to vicinal thiol groups on the ANT [54,77]. Oxidative stress and PAO also shift the voltage dependence of the MPT, allowing the pore to open at more negative potentials [61,62,105]. This is exactly what would be predicted if thiol modification inhibits adenine nucleotide binding to the matrix surface of the ANT whilst membrane potential enhances this binding.

Bernardi and colleagues have provided data that implicate two distinct thiol groups in modulating MPTP activity [106-108]. One is mediated by oxidation of glutathione, for example by TBH or diamide, and is protected by both monobromobimane and N-ethylmaleimide that alkylate reduced glutathione, preventing its oxidation. The other responds to the redox state of matrix NAD(P), and is protected by N-ethylmaleimide but not monobromobimane. It is the latter site that accounts for the well documented stimulatory effect of oxidation of matrix NADH on the MPT perhaps through the mediation of thioredoxin or lipoamide [109,110]. As outlined above, the ANT is known to have three cysteine residues that show differential reactivity to various thiol reagents and oxidising agents in a conformation dependent manner [13,29,30]. We have proposed that these cysteines represent obvious candidates for the thiol groups that regulate both CyP-D binding and the inhibitory effects of ADP and membrane potential on the MPTP, and have provided extensive data to support this hypothesis [54,77,91]. Specific modification of Cys_{159} within the adenine nucleotide binding site of the ANT with eosin maleimide not only inhibits adenine nucleotide binding and translocation by the ANT [29,36] but also almost totally abolishes the inhibition of the MPTP by ADP [54,77]. Furthermore, we have demonstrated that PAO chemically cross-links Cys_{159} with Cys_{256} whilst diamide causes a disulphide bond to form between these two cysteine residues through the mediation of oxidised glutathione [77]. This latter process is inhibited by low concentrations of N-ethylmaleimide that alkylates glutathione within in the matrix, and thus accounts for one of the thiol groups identified by Bernardi's laboratory. Since Cys_{159} is involved in the intramolecular cross-link induced by both PAO and oxidative stress, this modification can account for the ability of these treatments to antagonise ADP inhibition of the MPTP.

We were also able to demonstrate that both PAO and diamide treatment of mitochondria reduced the ability of copper phenanthrolene to cross link two ANT monomers through their Cys_{56} residues. These data were taken to indicate that this cross-linking induces a conformational change in the ANT that reduces the reactivity of Cys_{56}. The proximity of Cys_{56} to Pro_{61}, the putative binding site for CyP-D, suggests that this conformational change may provide an explanation for the increased CyP-D binding to the ANT induced by PAO and oxidative stress [77,85]. Our data provided no support for the previous suggestion that dimerisation of two ANT monomers through their Cys_{56} residues might be important in the effects of oxidative stress on the MPTP [54,91], although this cross-link is known to inhibit the conventional transport activity of the ANT [13]. However, Cys_{56} could be the other thiol group on the MPTP that was identified by Bernardi and colleagues as being blocked by NEM but not by monobromobimane [107] since the MPTP is inhibited by low concentrations of NEM (50 μM) that are known to selectively alkylate this residue [77]. Alternatively, this NEM- sensitive site may not be on the ANT itself but on the thioredoxin or lipoamide mediated processes that have been proposed to be responsible for the stimulation of the MPTP in response to oxidation of matrix NADH [109]. Indeed, thioredoxin reductase and glutathione reductase are likely to be intimately involved in the mechanism by which the disulphide bonds formed between Cys_{159} and Cys_{256} are reduced again [111]. Our current thinking on the role of thiol groups of the ANT in MPTP function is incorporated into the schemes shown in Fig. **1** and Fig. **2**.

Other Non-protein Effectors of the MPTP that may Act Directly on the ANT

Ubiquinone analogues can act as either as activators or inhibitors of the MPTP [112-114]. The probability of MPTP opening was observed to vary according to the rate of electron transfer through Complex 1 of the respiratory chain, and these data led Fontaine and colleagues to suggest that components of Complex 1 may be involved in the formation and/or regulation of the MPTP [113-115]. However, the recent observation that the uncoupling proteins UCP1, UCP2 and UCP3 require oxidised ubiquinone to function suggest an alternative mechanism [116,117]. Since the UCPs are close relatives of ANT it would seem quite likely that a ubiquinone binding site might also exist on the ANT. Fatty acids, and more especially fatty acyl CoA esters, are also potent ligands of the ANT that induce the "c" conformation, inhibit transport activity and, in the former case, induce proton translocating activity by the ANT [37,118-120]. This offers a potential explanation as to why fatty acids, and especially long chain unsaturated ones such as arachidonic acid and retinoic acid stimulate MPTP opening and enhance cell death [120-123].

Pro_{61} Represents the Likely Binding Site of the ANT for CyP-D

Cyclosporin A and Sanglifehrin A (SFA) are both potent inhibitors of the peptidyl-prolyl cis-trans isomerase (PPIase) activity of CyP-D and MPTP opening. However, CsA prevents CyP-D binding to the ANT whilst SFA has either no effect or enhances binding [124]. These data suggest that the opening of the MPTP involves binding of CyP-D (prevented by CsA) followed by a conformational change dependent on the PPIase activity of CyP-D (inhibited by SFA). This would demand that proline is an essential amino acid in the CyP-D binding site upon which the PPIase activity works to mediate the conformational change. We originally proposed that this residue is Pro_{61} of matrix loop 1, and there is increasing evidence to support this view. First, Pro_{61} is conserved in mammalian ANT isoforms that demonstrate a CsA-sensitive MPTP, but is not present in the ANT isoforms of Yeast in which any observed permeability transitions are CsA-insensitive [85,100,101]. For plant mitochondria, whose ANT also lacks Pro_{61}, there are conflicting data on the effects of CsA on a calcium induced permeability transition [125,126]. Second, matrix

loop 1 is the only one of the three loops that is exclusively facing the matrix and shows major changes in accessibility to the matrix in response to the "m" to "c" conformational change. Indeed, as outlined above, it has been proposed that in the dimeric ANT the first loops act together to create a gate for the pore. Binding of CyP-D to Pro_{61} might induce a conformational change that holds the gating mechanism in the open state, thus favoring MPTP formation. In contrast, the other two loops appear to penetrate into the translocation channel in the membrane and form the adenine nucleotide binding site [6,13,30,31]. The combination of an open gate caused by CyP-D binding and calcium in the adenine nucleotide binding site might provide the optimal conditions for MPTP formation. This is illustrated schematically in Fig. **2**.

Genetic Approaches to Demonstrate the Role of the ANT in MPTP Formation

A genetic approach to elucidating the mechanisms of the MPTP would be desirable in which proposed components of the MPTP are either knocked out totally or subjected to site-directed mutagenesis. Yeast would seem the organism of choice for such studies especially since it appears that their mitochondria do not display a conventional CsA-sensitive MPT [85,100,101] and yeast ANT knockouts are available. Unfortunately, expressing active mammalian ANT isoforms in yeast mitochondria has proved extremely difficult but recent reports suggest that it can be done if the N-terminus of the yeast ANT is spliced onto the mammalian isoform [127]. In conjunction with the expression mammalian CyP-

Table 1 Known Effectors of the the ANT that may Modulate Cell Death Through Effects on MPTP Opening. Where Many Papers Report Similar Phenomena, Review References are Given

Agent / Treatment	Action on ANT	Effect on MPTP	Effect on cell death	References
Calcium	Triggers conformational change	Activates	Activates	[1-3]
Sr^{2+}, Mg^{2+}, Mn^{2+}	Competes with Ca^{2+} for trigger site	Inhibits		[55, 66]
Low pH	H^{+} competes with Ca^{2+} for trigger site	Inhibits	Inhibits	[[55, 67, 137-137]
Adenine nucleotides	Bind to substrate site	Inhibits by decreasing affinity for Ca^{2+}		[1-3]
CAT and atractyloside	Locks in "c" conformation	Activates by overcoming AdN inhibition	Activates	[1-3; 129-131]
BKA	Locks in "m" conformation	Inhibits	Inhibits	[1-3, 129-131]
Depolarisation	Decreases affinity for ATP binding	Activates	Activates	[1-3]
AdN depletion; ischaemia, reperfusion injury	Less adenine nucleotide binding	Activates by overcoming AdN inhibition	Activates	[1-3, 137, 139]
Oxidative stress e.g. diamide, TBH, reperfusion injury, Verteporfin	Cross-links Cys_{159} to Cys_{256}	Activates by overcoming AdN inhibition and increasing CyP-D binding	Activates	[1-3, 54, 77 ,91,134-139]
Phenylarsine oxide	Cross-links Cys_{159} to Cys_{256}	Activates by overcoming AdN inhibition and increasing CyP-D binding	Activates	[1-3, 54, 77]
Eosine maleimide	Alkylates Cys_{159}	Activates by overcoming AdN inhibition	ND	[54, 77]
N-ethylmaleimide	Alkylates Cys_{56}	Inhibits by decreasing affinity for Ca^{2+}		[77]
Trifluoperazine	Modulates adenine nucleotide binding through surface charge effect	Decrease voltage sensitivity	Inhibits	[54, 65]
Long chain fatty acids	Inhibit ADP binding and induce proton channel	Activates	Activates	[37, 118-123]
Clodronate	Competitive inhibitor of transport	ND	Activates	[184]
MT-21	Conformational change blocking CyP-D and phenylarsine oxide binding	None	Activates	[132]
Arsenite, Lonidamine, CD437	Induce pore formation in reconstituted ANT	Activates	Activates	[141]
NO, peroxynitrite, HNE	Lipid peroxidation; formation by reconstituted ANT	Activates	Activates	[140]
Acetate, Propionate	Induce pore formation in reconstituted ANT	Activates	Activates	[142]

ND, not Determined; AdN, Adenine Nucleotide, TBH, t-Butylhydroperoxide

D this may provide an alternative approach to reconstitution in studying the molecular mechanism of the MPTP and has the advantage of being amenable to site-directed mutagenesis. However, it must always be borne in mind that over-expression of an IM protein may disrupt normal mitochondrial function and lead to cell death irrespective of whether or not its induces the formation of the MPTP. This may be the real basis of the report that overexpression of ANT-1 in mammalian cells can dominantly induce apoptosis [128].

THE ANT AS A TARGET FOR MODULATING CELL DEATH PATHWAYS

A number of small molecular weight agents have been shown to modulate cell death through an interaction with the ANT, as summarised in Table 1.

Ligands of the ANT

There have been many studies in which atractyloside (a cell-permeant CAT analogue) and BKA have been used as "specific" activators and inhibitors of the MPTP in cultured cell models of apoptosis (see [129,130]) and even perfused tissues and whole animals [130,131]. However, many such studies fail to recognise that the primary effect of these reagents will be to prevent ATP/ADP exchange across the mitochondrial inner membrane, with profound effects on cellular metabolism irrespective of any effect on the MPTP. Thus the ability of atractyloside [130], or the novel ANT inhibitor MT-21 [132] to induce apoptosis, may not be a direct effect of the drug binding to the ANT and opening the MPTP, but a secondary response to disturbed energy metabolism. It should also be noted that CAT and atractyloside bind to the ANT with very high affinity (K_d<1μM) and thus their use at 100 - 50000 μM in some experiments may well induce non-specific effects quite unrelated to their binding to the ANT such as inhibition of the ryanodine receptor in the sarcoplasmic reticulum [133]. It is probably more acceptable to take the ability of BKA to inhibit apoptosis as evidence for a role of the MPTP since inhibition of mitochondrial adenine nucleotide transport might be expected to be damaging rather than protective towards the cell. However, BKA can only be used effectively in cells, that are highly glycolytic and thus not dependent on oxidative phosphorylation for the majority of their ATP requirements. Although cultured cells and tumours often behave in this way, this is not the case for most normal tissues.

Table 2. Endogenous Molecules and Xenobiotics Whose Actions on the MPTP have been Demonstrated Directly on Purified ANT or MPTP Preparations Reconstituted into Artificial Membrane

Molecule	Experimental protocol	Effect	Inhibition of effect by	References
Bax	MPTP- liposome; ANT-liposome; ANT-PLB	P	Bcl-2, AdN	[89,96 ,148,151]
Bax Δα5-6, Bax ΔIGDE	MPTP - liposome; ANT-liposome; ANT-PLB	P	ND	[89,96 ,148,151]
BH3 Bax	ANT-liposome; ANT-PLB	P	BKA	[185]
BH3 Bcl-2	ANT-liposome; ANT-PLB	I	ND	[185]
Bid, tBid	MPTP - liposome; ANT-liposome; ANT-PLB	P	Bcl-2, AdN	[186]
Bcl-2	MPTP - liposome; ANT-liposome; ANT-PLB	I	ND	[89, 96, 148]
Bcl-2 Δα5-6, Bcl-2 G145A	MPTP - liposome; ANT-liposome; ANT-PLB	ND	ND	[89, 96, 148]
Atractyloside	MPTP - liposome; ANT-liposome; ANT-PLB	P	Bcl-2, AdN, BKA, CsA	[89, 148]
Calcium	MPTP - liposome; ANT-liposome; ANT-PLB	P	Bcl-2, AdN, BKA, CsA	[72, 86 , 89,99,148,151]
Diamide, DTDP, BMH, TBH	MPTP - liposome; ANT-liposome	P	AdN, BKA, CsA	[91]
Vpr 52-96	MPTP - liposome; ANT-liposome	P	Bcl-2, AdN, BKA	[97, 166]
Verteporfin	MPTP - liposome; ANT-liposome	P	Bcl-2, AdN	[92]
Arsenite, Lonidamine, CD437	MPTP - liposome; ANT-liposome	P	Bcl-2, AdN	[141]
NO, peroxynitrite, HNE	MPTP - liposome; ANT-liposome	P	Bcl-2, AdN	[140]
Acetate, Propionate	MPTP - liposome; ANT-liposome	P	Bcl-2, AdN	[142]
ANT 104-116	ANT-liposome	I	ND	[97]

Purified ANT or detergent solubilised fractions containing MPTP components were reconstituted in proteoliposomes (i.e. ANT-liposome and MPTP liposome, respectively) that had been previously loaded with a fluorochrome to measure the pore opening. Molecules were added exogenously to proteoliposomes or to ANT-contaning planar lipid bilayers (i.e ANT-PLB) for 30 min at room temperature. Atractyloside, calcium, inactive random peptides and plain liposomes were used as internal controls. AdN, adenine nucleotide; BKA, bongkrekic acid, CsA, cyclosporine A; P, permeabilizing effect; I, inhibitory effect; ND, not determined.

Oxidative Stress and Reperfusion Injury

Cells subject to profound oxidative stress induced by agents such as diamide or t-butylhydroperoxide (TBH) have been shown to undergo apoptosis associated with MPTP opening that can be inhibited by CsA. This has been taken to be a direct effect of the oxidative stress on thiol groups of the ANT or oxidation of its tightly bound cardiolipin, leading to MPTP opening (see [91,134,135]). However, a secondary effect following modification of other proteins, such as plasma membrane ion channels, cannot be ruled out [136]. Another example where MPTP opening is thought to play a major role in tissue damage (primarily necrosis) is upon reperfusion after an extended period of ischaemia. Such reperfusion injury is associated with oxidative stress, increased matrix [Ca^{2+}], decreased adenine nucleotide concentrations and elevated phosphate concentrations. These are exactly the conditions required to prime the ANT for MPTP opening, and this has been shown to occur after about 2 minutes of reperfusion. At this point, inhibition of MPTP opening by the low pH that occurs in ischaemia is removed as the intracellular pH is normalised [137-139]. Other agents that have been proposed to act directly on the ANT to cause MPTP opening and cell death include peroxynitrite, 4-hydroxynonenal, arsenite, lonidamine, CD437, verteporfin and short chain fatty acids [92,140-142],

Table 3. Proteins that may Interact with the ANT to Modulate Cell Death

Protein	Source	Effect on MPTP	Effect on cell death	References
Endogenous proteins				
VDAC	Outer mitochondrial membrane	Component / regulatory protein	Channel closure activates	[1-3, 187]
CyP-D	Mitochondrial matrix	Component / regulatory protein	Overexpression inhibits	[1-3, 188]
Bcl-2	Cytosol	Inhibition of MPTP and activation of ADP/ATP translocation	Inhibits	[143-148, 151]
Bax	Cytosol	Activation of MPTP and inhibition of ADP/ATP translocation	Activates	[143-148, 151]
Bid, tBid	Cytosol	Activation	Activates	[143-148]
ARL2 + BART	Cytosol	ND	ND	[182]
IκB-α	Cytosol	Release to cytosol on MPTP opening	Activates	[183]
Viral proteins related to Bcl-2				
BHRF1	Epstein-Barr virus	Mitochondrial localisation	Inhibits	[156,157]
E1B	Adenovirus	Interaction with Bax	Inhibits	[158]
NR13	Rous sarcoma virus	Unknown at a molecular level	Inhibits	[159]
5-HL	African swine fever virus	Unknown at a molecular level	Inhibits	[160]
HVS	Herpes viruses	Unknown at a molecular level	Inhibits	[161]-[163]
Other viral proteins acting on the MPTP				
HIV viral protein R (Vpr)	Human immunodeficiency virus (HIV-1)	Opens MPTP pore via an interaction with ANT	Activates	[97,166]
vMIA	Cytomegalovirus	Inhibits MPTP pore opening via an interaction with ANT	Inhibits	[140,167]
HBx	Human hepatitis B virus	Opens MPTP pore via an interaction with VDAC	Activates	[169]
M11L	Myxoma poxvirus	Inhibits MPTP pore opening via an interaction with PBR	Inhibits	[168]
Mitochondrial targeted viral proteins –unknown mechanism				
p13II	Human T lymphotropic virus	Loss of transmembrane potential	Induce	[164]
Tat	Human immunodeficiency virus (HIV-1)	Loss of transmembrane potential	Induce	[165]

ND, not determined

although once again it cannot be ruled out that other effects of these agents are more important as primary causes of cell death. Nevertheless, the death-inducing effect of these agents can be prevented by Bcl-2 overexpression in tumoral cells, and their ANT-pore opening capacity can be inhibited by BKA, ADP, ATP and recombinant Bcl-2 in proteoliposomes.

OTHER PROTEINS MAY INTERACT WITH THE ANT TO ENHANCE OR INHIBIT MPTP OPENING AND CELL DEATH

In recent years there have been several reports that a range of pro-apoptotic and anti-apoptotic proteins, including members of the Bax / Bcl-2 family viral proteins, interact directly with the ANT to exert their effects on apoptosis as summarised in Table 3.

Bax / Bcl-2 family members.

The Bax/Bcl-2 family is composed of pro-apoptotic proteins such as Bax, Bak, Bad and Bid and of anti-apoptotic proteins including Bcl-2 and Bcl-x(L) that act as tumor suppressors and oncogenes respectively [143]. Although their precise mode of action is still a matter of debate, they appear to regulate apoptosis through their capacity for homo- and hetero-oligomerization, channel formation, effects on intracellular calcium fluxes, and/or by increasing the level of cell tolerance to ROS damage (for review see [144,145]). In addition, although they can be found in different intracellular compartments, their modulation of apoptosis seems to be mediated at the mitochondrial level by increasing mitochondrial membrane permeabilisation (MMP) in the case of pro-apoptotic members, or by stabilizing the barrier function of mitochondrial membranes in the case of anti-apoptotic members [146]. Indeed, it has been reported that following mitochondrial translocation, Bax not only oligimerises but also interacts with integral mitochondrial membrane proteins such as VDAC [147] and the ANT [148]. These data suggest that Bax may bind to the ANT, converting it into a non-specific pore responsible for MMP increase during some conditions of apoptosis [148] and that this process may be antagonised by Bcl-2. In support of this, immunodepletion of Bax from the MPTP prior to reconstitution, or the use of MPTP purified from $Bax^{-/-}$ mice, revealed that the presence of Bax within the MPTP is required for its optimal response to opening in the presence of atractyloside [148]. Moreover, direct physical interactions between ANT, Bax and Bcl-2 were demonstrated by co-immunoprecipitation in various cancer cell lines, cardiomyocytes or neurons [148-151] and confirmed by means of the yeast two-hybrid system [148]. The latter experiments identified residues 105-156 of the ANT as being important for the interaction with BAX and as indicated in Fig. **1**, this region contains a transmembrane helix and parts of both an extramitochondrial and intramitochondrial loop. Whether these interactions are specific and occur in the physiological setting is hard to assess because of the inherent problems associated with studying interactions between membrane proteins that require detergent solubilisation. Yeast two hybrid results are especially hard to interpret with membrane proteins because the very nature of the transcription factor interactions that underlie the technique requires that both expressed proteins are soluble in the cytosol. Thus, when a transmembrane helical region of the ANT is found to associate with BAX it could merely reflect the natural hydrophobic tendencies of the sequence that might cause it to pair up with another hydrophobic sequence such as BAX. Such an interaction would produce a hydrophilic surface that keeps the proteins in solution whilst activating expression of the reporter gene. However, good evidence for the specificity of the BAX / ANT interaction is provided by binding competition studies that locate the region of the ANT to which Vpr (see below) and Bcl-2 bind as residues 104-116 [97]. This sequence overlaps with that identified by the yeast two hybrid screen and is contained within an extramitochondrial loop as might be expected for the binding of cytosolic proteins.

Further evidence for the interaction between ANT and Bax comes from in *vitro* experiments in which both proteins were reconstituted together into liposomes or planar lipid bilayers. Data are summarised in Table 2. Atractyloside was found to be more efficient at permeabilising the proteoliposomes than if either protein were reconstituted alone, suggesting that Bax-ANT interaction depends on the conformation state of ANT. Furthermore, this permeabilisation was inhibited by BKA [96,148]. These ANT-Bax channels were found to possess a higher opening frequency than those formed by ANT or by Bax alone, and the selectivity of ANT-Bax channels was cationic, whereas the selectivity of Bax channels alone was anionic. Bcl-2 and ligands of the ANT such as BKA, ATP and ADP closed the ANT-Bax channels opened by atractyloside [96,148]. Interestingly, inactive mutants of Bax or Bcl-2, which reportedly have lost their apoptosis-modulator function, failed to affect channel formation by the ANT [96,148]. Thus its appears that the ANT/Bax pair permeabilises artificial lipid bilayers in response to an atractyloside-induced conformational change of ANT, whereas the ANT/Bcl-2 pair does not. The endogenous signals that render ANT sensitive to Bax regulation in vivo have not been elucidated so far and plausible hypotheses would include matrix pH alteration, ATP concentration decline, conformation transitions, and/or protein-protein interaction changes.

VDAC (mitochondrial porin) has also been reported to play an essential role in the regulation of the outer membrane (OM) permeability by proteins from Bcl-2 family, independently of any effect on the ANT or MPTP (for review see [152]). Anti-apoptotic Bcl-2 family members were reported to close VDAC, whereas some pro-apoptotic members interact with VDAC to generate a protein-conducting channel through which cytochrome *c* can pass [147]. Although VDAC is known to be a component of the MPTP, its role in the regulation of OM permeability could be separated from the occurrence of permeability transition events. The region of Bcl-2 and Bcl-x(L) that interacts with VDAC was identified as their BH4 domain contained within the N-terminus [153]. Thus, VDAC-containing proteoliposomes treated with Bcl-x(L), but not Bcl-x(L) lacking the BH4 domain, displayed no channel activity, indicating a closure of VDAC channel. Furthermore, BH4 peptides of Bcl-2 and Bcl-x(L), but not mutant peptides, were able to inhibit both VDAC activity in proteoliposomes

even in the presence of Bax, and also apoptotic transmembrane potential loss in isolated mitochondria [153]. It should be noted that reservations have been raised about the techniques used to remove detergents in these studies that suggest caution may be needed in interpreting the results [69].

Despite the extensive data reported above that support an interaction of Bcl-2 family members with the ANT / VDAC to modulate MPTP activity, there are data on isolated mitochondria that fail to support this view. Thus it has been demonstrated that the MPTP opens in liver mitochondria from mice over-expressing Bcl-2 with almost the same sensitivity to inducers as mitochondria from control mice [154]. Indeed, in our hands, Bcl-2 protection (when endogenously expressed in liver mitochondria) was partial and observed only for moderate doses of MPTP inducers such as Ca^{2+}, atractyloside. Another report suggests that Bcl-2 prevents the permeability transition by enabling the reduced pyridine nucleotides to be maintained during oxidative stress rather than through a direct effect on the MPTP mechanism [155].

Viral Proteins Interacting with MPTP Members

Several different proteins from a number of human viruses have been reported to interact with mitochondria and in some cases, to modulate apoptosis. The first class of proteins is composed of Bcl-2-related proteins such as the Epstein-Barr virus-coded BHRF1 protein [156,157], the adenovirus E1B 19 kDa protein [158], the Rous sarcoma virus encoded protein NR13 [159], the african swine fever virus 5-HL protein [160], and proteins from human Herpes virus 8 [161], from Herpes virus saimiri [162] and from Herpes virus papio [163]. These Bcl-2 related proteins generally suppress apoptosis, some of them being involved in the malign transformation. The second class of proteins is composed of those that localize to and act on mitochondria via unresolved molecular mechanisms including p13II from human T lymphotropic virus (HTLV) [164] and Tat from human immunodeficiency virus type 1 (HIV-1) [165]. A third class of proteins has been described that are thought to regulate apoptosis via direct interactions with MPTP components. These include the viral protein R (Vpr) from HIV-1 [97,166], vMIA from cytomegalovirus (CMV) [140,167], M11L from myxoma poxvirus [168] and HBx from human hepatitis B virus (HBV) [169]. Some of these MPTP-interacting proteins are pro-apoptotic whereas others are anti-apoptotic suggesting the occurrence of different modes of action.

Among this ever-growing list of viral proteins acting on mitochondria, HIV-1 Vpr is one of the best characterized. Vpr is a 14-kDa accessory protein encoded by HIV-1 and is expressed in HIV-1-infected cells where it is incorporated into virions. It is pro-apoptotic and has been shown to induce MMP via a specific interaction with the MPTP [97,166]. Thus, a synthetic Vpr-derived peptide (Vpr 52-96), corresponding to the C-terminal moiety of the protein, uncouples the respiratory chain and induces a rapid permeabilisation of the IM to protons and NADH before cytochrome *c* is released. When added to isolated mouse liver mitochondria, Vpr52-96 induces matrix swelling, which is prevented by pre-incubation of mitochondria with recombinant Bcl-2 protein [97]. In ANT-containing proteoliposomes or planar lipid bilayers, Vpr52-96 and purified native ANT cooperate to form large conductance channels [97]. This cooperative channel formation is abolished by the addition of a peptide corresponding to the putative Vpr-binding site of ANT (amino acids 104-116 of human ANT2 as shown in Fig. **1**) suggesting that the interaction between the two proteins is specific. Recombinant Bcl-2 inhibits channel formation by the ANT-Vpr complex in planar lipid bilayers and reduces the ANT-Vpr interaction, as determined by affinity purification and plasmon resonance studies [97]. Thus it seems probable that Vpr increases the mitochondrial IM permeability through a direct structural and functional interaction with the ANT.

Human cytomegalovirus (CMV) is a Herpes virus that causes opportunistic infections in immunocompromised individuals. CMV inhibits apoptosis mediated by death receptors and a product of the immediate early gene UL37 exon 1 is a protein known as viral inhibitor of apoptosis, vMIA. This localizes to mitochondria and is capable of suppressing apoptosis induced by diverse stimuli ([170] and for review see [167]). vMIA inhibits Fas-mediated apoptosis at a point downstream of caspase-8 activation and Bid cleavage but upstream of cytochrome *c* release. vMIA appears to associate with ANT in the mitochondria, but not VDAC or Bax. Deletion mutagenesis identified two domains of vMIA (amino acids 5-34 and 118-147 of UL37) that are necessary for its mitochondrial localization and for its apoptosis-related function respectively. Interestingly, these two domains are conserved in multiple primary strains of cytomegalovirus [171]. The functional properties of vMIA resemble those ascribed to Bcl-2 [167]. However, the absence of sequence similarity to Bcl-2 or any other known cell death suppressors suggests that vMIA defines a previously undescribed class of anti-apoptotic proteins that prevent cell death by a direct interaction with ANT.

M11L is another anti-apoptotic protein that is essential for the virulence of the myxoma poxvirus and interacts with a MPTP member to modulate apoptosis. Indeed, the protein is directed specifically to mitochondria by a short COOH-terminal region that is necessary and sufficient for targeting [168]. This targeting region consists of a hydrophobic domain flanked by basic amino acid residues, adjacent to a positively charged tail. It has been reported by Everett et al. that M11L prevents the loss of mitochondrial membrane potential ($\Delta\Psi m$) during cell death by inhibiting opening of the MPTP [172]. Indeed, using a cross-linking approach, these workers found that M11L physically associates with the mitochondrial peripheral benzodiazepine receptor (PBR), an OM protein that some workers have suggested may be a component of the MPTP and which can be co-purified as a complex with the ANT and VDAC [173]. This interaction of M11L and the PBR was confirmed by fluorescence resonance energy transfer (FRET) analysis. Stable expression of M11L was able to prevent the release of cytochrome *c* induced by different apoptosis inducers such as staurosporine and protoporphyrin IX, a ligand of the PBR [172]. The presence of PBR protects cells from Myxoma virus infection and the associated expression of early proteins, including M11L. These observations support the hypothesis that M11L acts by direct interaction with the PBR leading to

inhibition of the MPTP and hence of apoptosis [172]. This might represent an important antiviral defense mechanism.

Human hepatitis B virus (HBV) is one of the principal causative agents of chronic hepatitis and can be associated with the development of hepatocellular carcinoma. The HBV genome encodes a protein, termed HBx, recognized primarily as a transcription activator. However, the role of HBx protein in hepatitis B virus induced carcinogenesis is unclear. Indeed, a diverse range of effects of HBx have been described, including modulation of cell growth, signal transduction, DNA repair and apoptosis [169,174-176]. In addition, it seems that HBx can stimulate the cytoplasmic Ras, Raf, and MAP kinase-signaling pathways, deregulate the cell cycle, activate the transcription factor AP-1 and associate with various cellular components [169,174,175,177-180]. Even more confusing, the pro- or anti-apoptotic status of HBx may depend on the hepatocellular context of infection [176] and its dependence on Bcl-2-apoptosis inhibition is still unclear as emphasized by contradictory findings noted above. Nevertheless, it has been reported that HBx may interact with the VDAC and cause the dissipation of $\Delta\Psi$ and the cell death [181]. This may provide additional evidence for a functional link between VDAC and the ANT to modulate MPTP opening.

OTHER MISCELLANEOUS PROTEINS REPORTED TO INTERACT WITH THE ANT

The ADP-ribosylation factor-like 2 (ARL2) GTPase, and its binding partner, binder of ARL2 (BART), although primarily cytosolic, have been shown to translocate into mitochondria where they interacted specifically with ANT1 (but not ANT2) to form a complex, the function of which is unknown. In ANT1 knockout mice the amount of ARL2 in heart and skeletal muscle mitochondria is increased, suggesting that the ANT / ARL2 / BART complex inhibits further ARL2 uptake. The significance of this interaction is unknown, but it has been suggested that it might play a role in signal transduction pathways between mitochondria and cytosol that reflect the energy status of the cell [182]. Another protein that has been shown to interact with the ANT by yeast two hybrid analysis, glutathione-S-transferase pull down assays and co-immunoprecipitation experiments is IκB-α, an inhibitory subunit of the transcription factor NFκB that promotes cell survival. Sub-cellular fractionation studies, electron microscopy and resistance to proteolysis confirmed a mitochondrial location, most probably within the intermembrane space suggesting interaction with an outer facing loop of the ANT. Upon induction of apoptosis, for example through Fas-receptor activation, IκB-α was released from mitochondria along with other pro-apoptotic proteins, providing a mechanism for down-regulating cell survival pathways coincidentally with stimulating apoptosis [183].

CONCLUSIONS AND FUTURE DIRECTIONS

The evidence that the ANT forms the IM channel of the MPTP is impressive and the ability to explain the actions of many modulators of the MPTP through their effect on the ANT is convincing. However, the published reports claiming reconstitution of the MPTP from purified ANT and CyP-D, although persuasive, lack the necessary rigour to constitute proof. A major goal of future research must be to improve this reconstitution procedure. Only then will it be possible to define with certainty the minimal composition of the MPTP and how other proteins such as members of the Bcl-2 family and viral proteins interact to regulate its function. Key questions to address are the following. Does VDAC interact with the ANT as an essential structural component of the MPTP or merely in a regulatory capacity? Are there other proteins that are essential for MPTP function or regulation that have yet to be identified? Can both the major ANT isoforms form the MPTP and do they exhibit similar affinities for CyP-D, VDAC and other regulatory proteins? Can protein-protein interactions within the MPTP complex change during the apoptosis process? Can other transporters take the place of ANT in MPTP formation ? Is the role of cardiolipin bound to the ANT strictly structural or does it also play a functional / regulatory role? Progress in these areas promises the development of new therapeutic strategies for a range of conditions from ischaemic heart disease, through stroke to toxic liver damage and infectious diseases. A "pure" reconstituted MPTP would also provide the ideal system on which to screen for novel drugs that inhibit the MPTP without the undesirable side effects of agents such as CsA. However, it may prove difficult to target the ANT and avoid toxicity to healthy tissues unless a site can be found on the ANT that prevents MPTP formation without inhibiting its normal translocase activity. Solving the 3D structure of the ANT in its normal physiological state and within the MPTP would clearly help in this process. However, this is not easy for flexible and lipid-binding proteins such as the ANT. In the mean time molecular modeling such as that performed by Terada's group [75] may also provide some useful insights into potential target sites for drugs.

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ABBREVIATIONS USED

ANT	=	Adenine nucleotide translocase
CyP-D	=	Cyclophilin D
CsA	=	Cyclosporin A
$\Delta\Psi$m	=	Transmembrane potential
IM	=	Inner membrane
MMP	=	Mitochondrial membrane permeabilisation
MPTP	=	Mitochondrial permeability transition pore
NEM	=	N-Ethylmaleimide
OM	=	Outer membrane

PPIase = Peptidyl-prolyl cis-trans isomerase

SFA = Sanglifehrin A

SMP = Submitochondrial particle

VDAC = Voltage dependent anion channel

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