The mitochondrial permeability transition pore

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Abstract

This chapter reviews recent advances in the identification of the structural elements of the permeability transition pore. The discovery that cyclosporin A (CsA) inhibits the pore proved instrumental. Various approaches indicate that CsA blocks the pore by binding to cyclophilin (CyP)-D. In particular, covalent labelling of CyP-D \textit{in situ} by a photoactive CsA derivative has shown that pore ligands have the same effects on the degree to which CsA both blocks the pore and binds to CyP-D. The recognition that CyP-D is a key component has enabled the other constituents to be resolved. Use of a CyP-D fusion protein as affinity matrix has revealed that CyP-D binds very strongly to 1:1 complexes of the voltage-dependent anion channel (from the outer membrane) and adenine nucleotide translocase (inner membrane). Our current model envisages that the pore arises as a complex between these three components at contact sites between the mitochondrial inner and outer membranes. This is in line with recent reconstitutions of pore activity from protein fractions containing these proteins. The strength of interaction between these proteins suggests that it may be a permanent feature rather than assembled only under pathological conditions. Calcium, the key activator of the pore, does not appear to affect pore assembly; rather, an allosteric action allowing pore flicker into an open state is indicated. CsA inhibits pore flicker and lowers the binding affinity for calcium. Whether adenine nucleotide translocase or the voltage-dependent anion channel (via inner membrane insertion) provides the inner membrane pore has not been settled, and data relevant to this issue are also documented.

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Introduction

When Ca$^{2+}$ is added to respiring mitochondria \textit{in vitro} in the presence of inorganic phosphate (Pi) and ATP there is a brief stimulation of respiration, as the Ca$^{2+}$ is accumulated, followed by a return to the resting rate. Without ATP, however, respiration continues at the stimulated rate as energy transduction is permanently uncoupled. This old, seemingly trivial observation has, nevertheless, spawned thousands of investigations. Whereas many of the early studies were undoubtedly driven by sheer curiosity, the motive today is likely to reflect the emerging relevance of the phenomenon to cell death. It is now generally accepted that the impairment of energy transduction reflects the Ca$^{2+}$-induced opening of a large pore in the mitochondrial inner membrane, the so-called permeability transition (PT) pore. Recognition of the true nature of the lesion formed a turning point in the course of the investigations since it enabled attention to be focused on the components of the pore and how these may be influenced by pathological constraints. The purpose of this article is to distil from these studies a possible outline structure of the PT pore. Remarkably, it seems that the basic components of the pore are normally ‘law-abiding’ proteins with defined roles in the life of the cell. Under pathological conditions, however, they coalesce into a complex that can kill the cell. It is appropriate to begin, therefore, by outlining the pathological factors that activate the pore, and which lead to its proposed involvement in necrotic cell death.

The PT pore and its possible role in ischaemia/reperfusion injury

The first evidence for the existence of the PT pore was obtained by Haworth and Hunter in the late 1970s ([1] and references therein), but there was little interest in cell death at that time and the possible cellular implications of pore opening were not appreciated. Indeed, almost a decade passed before a case was made that the PT pore might have a critical role in cell necrosis in connection with tissue reperfusion injury [2–5]. The reasoning went as follows: tissue ischaemia results in dissipation of ATP, but little change in ADP as there is a net loss of adenine nucleotides to nucleosides and bases [6]. There is also a many-fold increase in tissue Pi [7], and a progressive rise in resting (unstimulated) cytosolic free Ca$^{2+}$ [8–10]. On reperfusion, reactive oxygen species (ROS) are generated, leading to oxidation of redox couples (oxidative stress) ([11] and references therein). The same set of factors trigger PT pore opening, at least in isolated mitochondria. Of these, mitochondrial Ca$^{2+}$ overload is essential. Mitochondria are immune to rapid cytosolic Ca$^{2+}$ transients, but become overloaded with Ca$^{2+}$ when external Ca$^{2+}$ is maintained above a critical limit [5]. This limit (termed the set point [12]) is about 1 μM, and is similar to the resting cytosolic free [Ca$^{2+}$] that needs to be exceeded for irreversible cell injury associated with anoxia/re-oxygenation [9,10]. This close correspondence between mitochondrial and cellular tolerance to Ca$^{2+}$ suggests that mitochondrial Ca$^{2+}$ overload is a factor in this form of cell death. Besides Ca$^{2+}$,
PT pore opening requires a co-inducer, either P_i [1] or oxidative stress [2,4]. Under any condition, pore opening is blocked by ATP, but 1 mM ATP is required [13], a condition not satisfied after prolonged ischaemia [6]. As a possible scenario (Figure 1), reperfusion after prolonged ischaemia (when resting cytosolic Ca^{2+} exceeds the set point) would produce mitochondrial Ca^{2+} overload which, with high P_i, oxidative stress and low ATP could lead to pore activation. If this occurred, then H^+ backflow through the pore would uncouple mitochondrial energy transduction and initiate a vicious cycle leading to cell death (Figure 1). Contributory factors could include the stimulation of mitochondrial ROS production by high Ca^{2+} [14] and the opening of Ca^{2+} release channels of the sarcoplasmic reticulum by ROS [15].

Measurements *in vivo* reveal that pore opening occurs during reperfusion (heart [16]) and oxidant stress (hepatocytes [17]). The pore inhibitor cyclosporin A (CsA) also affords some protection against anoxia/re-oxygenation- or hydroperoxide-induced injury in some studies, e.g. of heart [18,19].

![Figure 1 Possible scenario for the involvement of the PT pore in events leading to cell necrosis associated with reperfusion injury.](image-url)
liver [20] and brain [21]. Single cardiomyocytes loaded with tetramethylrhodamine ethyl ester provide both a continuous fluorescent assay of pore activation (collapse of the mitochondrial inner membrane potential) and a source of intramitochondrial ROS [22]. In this experimental model, ROS-induced injury was first apparent as a localized mitochondrial depolarization, which became widespread before cell death; again, CsA retarded the onset of potential loss and cell death. These data clearly lend support to the hypothesis that pore opening has a crucial role in the development of irreversible injury leading to cell necrosis.

It has been proposed that PT pore opening may also be an early event in apoptosis. By inducing mitochondrial swelling and rupture of the outer membrane, PT pore opening is seen as a possible means for the release of pro-apoptotic cytochrome c and apoptosis-inducing factor from the intermembrane space [23–25]. Many cellular insults share the capacity to induce both apoptosis and necrosis, the outcome being determined by the intensity of the insult [26]. Certain mechanisms, therefore, may be common to both, but a fundamental difference seems to be that whereas early apoptosis maintains ATP and includes energy-requiring steps, necrosis is associated with rapid ATP dissipation [6,26]. Thus the notion that pore opening may be the critical step at which cell injury leads irreversibly to cell necrosis [3–5] sits easily with indications that at least 80% of cell ATP would need to be dissipated before pore opening could occur. On the other hand, pore opening during apoptosis would require a mechanism for overriding ATP inhibition. Moreover, the logical, inevitable consequence of pore opening would seem to be the very rapid loss of residual ATP and cell viability (Figure 1), again rather difficult to rationalize within apoptosis.

Structural components of the PT pore

**Adenine nucleotide translocase (ANT)**

ANT is a major protein constituent of the mitochondrial inner membrane, responsible for the export of ATP in exchange for ADP. ANT operates as a gated pore that flips between two extreme conformations in which the substrate-binding site faces the cytoplasmic side of the inner membrane (c-state conformation) or the matrix side (m-state). Three isoforms have been recognized, coded by different genes [27]. Interestingly, ANT-2 is mainly expressed in liver and kidney, tissues from which mitochondria most readily display PT pore activity, but whether this correlation reflects specific ANT-2 involvement in the PT pore awaits clarification. The proposed involvement of ANT originated at least a decade ago with the finding that PT pore opening is very susceptible to ANT ligands. Ligands that bind to the c-state (attractylate, pyridoxal phosphate) activate pore opening, whereas m-state ligands (e.g. bongkrekate) inhibit it ([28–30] and references therein). These observations gave rise to the concept that the c-state conformation is susceptible to Ca\(^{2+}\)-induced deformation into an open-pore state, whereas the m-state is not. This is consistent with the blockade of PT pore opening in isolated mitochondria by exogenous ANT substrates, since in their absence ANT would be arrested in
the c-state, and prone to deformation, whereas exogenous ADP or ATP would allow reconversion to the m-state. Two laboratories have now reported that purified ANT can be deformed into an open pore when treated with high Ca\(^{2+}\) and atractylate [31,32]. However, earlier experiments detected no Ca\(^{2+}\)-induced opening in submitochondrial particles [33]. At the time this was interpreted to indicate the involvement of other components (which appears to be true, see later), but in the light of the work with purified ANT, it may also indicate that deformation by Ca\(^{2+}\) is suppressed in the intact inner membrane.

**Cyclophilin-D**

Cyclophilins (CyPs) are a family of proteins that catalyse the \textit{cis–trans} isomerization of accessible prolyl peptide bonds in proteins (reviewed in [34]). They are so-called since they bind CsA, a cyclic undecapeptide isolated originally from the fungus \textit{Tolypocladium inflatum}. CsA inhibits peptidylprolyl \textit{cis–trans} isomerase activity. CyPs present several isoforms with defined subcellular locations, e.g. CyP-A (cytosol), CyP-B and CyP-C (endoplasmic reticulum), and CyP-D (mitochondria). The cellular functions of CyPs are not established, but most probably one role is to catalyse the folding of nascent proteins. For example, the folding of newly imported mitochondrial proteins occurs more slowly in deletion mutants (yeast) of mitochondrial (matrix) CyP [35]. Remarkably, CyPs gain another property when bound to CsA, since the CyP–CsA complex, but neither component alone, inhibits the protein phosphatase, calcineurin [36]. A native equivalent of CsA has not been found.

As with ANT, the involvement of CyP in the PT pore was first suggested from the effects of the respective ligand. In 1988, we observed that pore opening (sucrose permeability) induced by Ca\(^{2+}\), with either P, or oxidative stress as co-inducer, was blocked by CsA [3]. The amount of CsA needed for complete pore blockade (approx. 50 pmol/mg of mitochondrial protein) was much less than the amount of ANT (>1 nmol/mg in heart mitochondria) and it was clear that another protein, a CsA-binding protein, was involved. A role for mitochondrial CyP was proposed, based on the similarity between the relative potencies of different cyclosporins as inhibitors of CyP and the pore, and from the amounts of bound CsA needed to inhibit the pore and CyP [29,33].

A further approach made use of a photoactive, radiolabelled CsA derivative to tag covalently the relevant protein [37–39]. The derivative contained a diazirine group linked to position 8 of the cyclosporin ring via an aminobutanoyl spacer. The amino acid at position 8 is not involved in binding to CyP ([40] and references therein), so that the substituted CsA binds and photolabels CyP (e.g. [38]). One would anticipate that it might also tag proteins binding to the CyP–CsA complex. In an attempt to pinpoint the relevant photolabelled component(s), the effects of Ca\(^{2+}\) on photolabelling were also investigated; the rationale being that since there is a competitive interrelation between Ca\(^{2+}\) (pore activator) and CsA (inhibitor) [33,41], Ca\(^{2+}\) might be expected to interfere specifically with photolabelling of the pore-related component. In the event, Ca\(^{2+}\) selectively depressed the photolabelling of two components [38]. Of these, the major component was identified as CyP-D, the mitochondrial CyP isoform, from internal peptide sequences [39]. Moreover, as a further indi-
cator, CyP-D photolabelling was enhanced by ADP [39]. The opposing effects of these two pore ligands on CyP-D photolabelling correlate with their opposing actions on pore activity (Ca\(^{2+}\), activator; ADP, inhibitor). This correlation between ligand effects on photolabelling and on pore activity provides a strong case that CyP-D is indeed the CsA ‘receptor’ of the PT pore.

CyP-D (19 kDa; migrating as 20–21 kDa on SDS/PAGE) is located in the matrix space [39]. An N-terminally truncated form of CyP-D (18 kDa) is also found in mitochondrial extracts [42,43] and may correspond to the CyP-18 recovered in the intermembrane-space fraction after mitochondrial subfractionation [39].

**A 12 kDa protein (P-12)**

The second component photolabelled by the CsA derivative in a Ca\(^{2+}\)- and ADP-sensitive manner (see earlier) was a 12 kDa protein (P-12) [38]. This component is membrane-located, but has not yet been identified. Possibly, P-12 associates with the CsA–CyP-D complex, and becomes photolabelled as a result.

**Porin or voltage-dependent anion channel**

The voltage-dependent anion channel (VDAC) provides a non-selective pore in the mitochondrial outer membrane. It allows metabolite and ion access to the solute-specific transport systems of the inner membrane. For example, VDAC-deletion mutants in yeast show a greatly reduced outer membrane permeability to NADH and other solutes [44].

Other aspects of VDAC behaviour are enigmatic. It has been known for some time that VDAC is localized preferentially at points of contact between the inner and outer membrane [45] and that VDAC can associate tightly with the inner membrane [46]. The point of contact was clarified by McEnery et al. [47], who isolated VDAC complexed to ANT. VDAC can also bind water-soluble proteins, in particular kinases (hexokinase, creatine kinase). Recently, Brdiczka, Wallimann and co-workers have reconstituted pore activity in both planar bilayers and proteoliposomes with ANT–VDAC complexes [48–50]. One such complex, containing ANT, VDAC, hexokinase and CyP-D, yielded a non-selective pore (malate, ATP) when activated by atracyloside and Ca\(^{2+}\), and the pore activity was blocked by CsA [48]. Intriguingly, proteoliposomes containing the creatine kinase–VDAC–ANT complex did not show pore activity unless the octameric creatine kinase was dissociated into dimers [49].

VDAC comprises about 0.3% of mitochondrial protein [51]. This is equivalent to about 100 pmol of VDAC/mg of mitochondrial protein, or roughly 1/10 of the abundance of ANT. Thus only a small fraction of ANT can be complexed to VDAC. Whether complex formation is isofrom specific is not known, but cDNAs encoding three VDAC homologues have been identified in mouse [52].
Which component provides the pore?

The first estimate of pore size was made from the osmotic behaviour of Ca\(^{2+}\)-treated mitochondria in solutions of poly(ethylene glycols) (PEG) [1]. There was a sharp cut-off in permeability around molecular mass 1500 Da, with PEG 1500 just able to permeate. Subsequently, a rapid pulsed-flow technique was used to measure solute fluxes [4,53,54]. From the relative permeabilities of mannitol, sucrose and arsenazo III, a pore diameter of about 2.3 nm was estimated. This corresponds quite well with the osmotic data, the hydrodynamic radius of PEG 1500 being about 1.2 nm [55].

How do these estimates compare with those for defined components of the ANT–VDAC complex? At low transmembrane voltages, VDAC just admits PEG 3400 [56], and has an estimated diameter of 2.5–3.0 nm [57]. At higher voltages, VDAC closes to a form that excludes PEG 1500 but admits gamma cyclodextrin, and has an estimated diameter of about 1.8 nm [58]. Thus, the PT pore is about the same size as VDAC, but one cannot readily correlate the PT pore to either state of VDAC in particular.

Conductance measurements ought to allow a more accurate comparison. These measurements have been made on mitochondrial mitoplasts in which the outer membrane is largely absent, but with some contact sites retained. A 1.3 nS (nanoSiemens; 150 mM KCl) channel, with a 600 pS substrate, has been detected [30,59]. By comparison, the maximally open state of VDAC in planar bilayers yields a conductance equivalent to 600 pS in 150 mM KCl [58]. A single VDAC suffices for a transmembrane pore [60], but co-operative behaviour between reconstituted VDAC molecules has been observed [61]. It is not unreasonable, then, to consider that the 600 pS/1.3 nS channel may reflect co-operative behaviour between two VDAC molecules [30], although this raises the question of how VDAC may insert, or be present in, the inner membrane.

Whether the 600 pS/1.3 nS channel is indeed the PT pore has not been settled, since the channel displays certain PT-pore-like properties, but not others. Activation of the channel by Ca\(^{2+}\) [62] and blockade by CsA [63] clearly echo PT-pore properties. On the other hand, the channel has been detected in mitoplasts prepared from yeast mutants lacking all ANT isoforms [64]. The channel is also unaffected by atractylate [64]. Taken as a whole, therefore, the electrophysiological data favour the idea that VDAC provides the actual channel.

Purified ANT reconstituted into planar bilayers is reversibly activated by high [Ca\(^{2+}\)] to produce a large channel with a conductance equivalent to 900 pS in 150 mM KCl [31,32]. Like the PT pore, the channel is blocked by bongkrekate, but not by atractylate. If ANT does provide the translocation pathway through the pore, does this arise because ANT is deformed in such a way that the previously selective conduit for ADP and ATP is opened into a non-selective pore? The fact that atractylate does not decrease the Ca\(^{2+}\)-activated ANT conductance [31] indicates that bound atractylate does not protrude significantly into the open channel. Clearly, if ANT occupied the ADP/ATP binding site then an alternative conduit would be predicted. But the atractylate and ADP-binding sites do not appear to be the same. Thus Roux et
al. [65] used engineered ANT variants, in which selected (fluorescent) tryptophans were replaced, to map residues involved in the binding and conformational changes associated with these ligands. They found a clear disparity between the tryptophans affected by ADP and atractylate. It may be, therefore, that atractylate binds and stabilizes a form in which the normally selective ADP/ATP conduit is enlarged into an open, non-selective pore.

However, if the PT pore is a deformed ANT, it is clear nevertheless that the deformation must occur reversibly during pore opening/closure. Pore opening/closure means pore flicker at a frequency dependent on matrix free \([\text{Ca}^{2+}]\). Reversibility in this context means, therefore, restoration of the ANT native state between flickers. This becomes clear, as follows.

A fundamental property of a gated pore, like ANT, which normally mediates obligatory exchange between solutes, is that the conformational change between the c- and m-states only occurs when the solute-binding site is occupied by transportable solute; in essence, the complementarity between the transported solute and the intermediate (between c- and m-) state of the carrier provides binding energy for the conformational change to take place. For example, purified ANT naturally adopts the m-state and binds bongkrekate, but not atractylate. However, if ADP is added to allow the conformational switch to the c-state, then atractylate is bound [66]. Intriguingly, pulsed-flow measurements of PT-pore state have revealed a similar ADP-catalysed switch during pore opening and closure, i.e. that an ADP-catalysed step is a prerequisite of pore flicker [54]. This substantiates the involvement of ANT. But ADP would only be able to catalyse the conformational switch (to the c-state) of native ANT; in a non-selective open pore, on the other hand, binding energy for the conformational change is not available since there is no binding interaction between the transported solute and the pore protein. ADP catalysis of pore opening implies, then, that ANT must revert to its native (presumably m-) state between pore flickers.

Clearly, ADP catalysis of pore flicker [54] is also consistent with a purely ancillary role for ANT as part of a supermolecular complex. In this case, the pore-forming unit would be provided by some other component (VDAC?), ANT would remain in its native state, and the c-state/m-state switch would determine the propensity of the other component to flicker into an open pore. Other data consistent with such a model are discussed by Zoratti and Szabo [30].

**A stable pore complex**

CyP-D is a water-soluble protein which is believed to have a basic function in catalysing the folding of newly imported proteins in the mitochondrial matrix. One assumes, then, that it can diffuse within the matrix compartment and interact with diverse protein targets. This has led to the prevailing concept that under pathological conditions of high matrix \(\text{Ca}^{2+}\) CyP-D may bind to a novel protein target with pore-forming properties and stabilize this protein in an open-pore state [29,33]. According to this model the CyP–CsA complex would be unable to bind to the membrane target, thereby accounting for the
CsA inhibition of pore opening. Suppression of CsA photolabelling of CyP-D by high matrix Ca\(^{2+}\) [38] is quite consistent with this model since it predicts that at high [Ca\(^{2+}\)] the target protein would in effect compete with CsA for CyP-D.

Recent findings question this model. We have used a CyP-D fusion protein for the detection of CyP-D-binding proteins in mitochondria. The CyP-D moiety of the fusion binds specifically to 32 kDa membrane proteins, which react with antibodies against VDAC and ANT (Figure 2). VDAC and ANT have very similar subunit molecular masses, but on high resolution the 32 kDa band can be resolved into a doublet (results not shown). It appears, then, that the CyP-D target is the VDAC–ANT complex. Using the fusion-detection system, we have observed no effect of Ca\(^{2+}\) (up to 300 \(\mu\)M) on the interaction between VDAC–ANT and CyP-D. In other words, the VDAC–ANT–CyP-D complex appears to be stable irrespective of the presence or absence of Ca\(^{2+}\).

Rather than the pore-producing complex being assembled only under extreme, pathological conditions, it seems that the basic complex may exist all the time, albeit in a pore-inactive state.

The model that emerges is depicted in Figure 3. According to this, the VDAC–ANT–CyP-D complex is a physiological entity whose function has yet to be clarified. In the presence of Ca\(^{2+}\) the complex can undergo a fundamental change that allows it to flicker into an open-pore state for brief intervals. When ATP is bound to ANT (for simplicity, results not shown) this change is prohibited, thus preventing pore flicker. The likelihood of pore flicker is determined by the concentration of the 'calcified' complex (i.e. VDAC–ANT–CyP-D–Ca\(^{2+}\)) and, therefore, by matrix free [Ca\(^{2+}\)]. This
accounts for the observation that, in adenine-nucleotide-depleted mitochondria, a critical matrix \([\text{Ca}^{2+}]\) is not required; rather, pore opening (i.e. the incidence of pore flicker) is directly proportional to matrix free \([\text{Ca}^{2+}]\), even down to 2\(\mu\)M [67]. When CsA binds to CyP-D in the complex, CyP-D remains associated with the complex, but the complex is no longer able to form a pore. As depicted, CsA and \(\text{Ca}^{2+}\) binding may be mutually exclusive, resulting in a competitive interrelation between these two ligands, thereby explaining the capacity of \(\text{Ca}^{2+}\) to depress CsA photolabelling of CyP-D [38,39]. The ability of the complex to accommodate CyP-D–CsA, in addition to CyP-D alone, would explain why the CsA-photolabelled CyP-D was invariably recovered in the membrane fraction of sonicated mitochondria [38,39]; if CsA binding to CyP-D had induced CyP-D dissociation, then recovery of photolabelled CyP-D in the soluble fraction would have been anticipated. The model also accounts for the ability of CsA not only to prevent, but also to reverse, the ‘permeabilized’ state of \(\text{Ca}^{2+}\)/oxidant stress-treated mitochondria [33], since this state simply reflects a propensity for pore flicker. The model envisages no catalytic (peptidylprolyl cis–trans isomerase) function of CyP-D in pore opening. This accords with pulsed-flow studies of pore opening/closure [33], which detected no positive indication for a catalytic role; but this particular issue is not settled.

Whether CyP-D binds to ANT or VDAC in the complex has not been resolved. CyP-D is primarily a matrix protein, making ANT the logical target. If VDAC bound to matrix CyP-D, then some VDAC would need to insert, or be resident in, the inner membrane. Residual VDAC is found in submitochondrial particles [68], but whether this is contaminatory or resident is not known. In view of the mounting evidence for complex formation, it seems most probable that VDAC recovered in inner membrane fractions is bound to ANT. However, the arrangement of any such VDAC is obscure, whether it is surface-associated or whether it partitions into the inner membrane. Zoratti and Szabo [30] have listed a number of positive indications of VDAC insertion, but the question remains open and, on balance, a model involving matrix CyP-D may be reasonably represented, as in Figure 4(b).
There is also an alternative. An 18 kDa, N-terminally truncated CyP-D may reside in the intermembrane space. We have found that VDAC–ANT binding to CyP-D is not prevented by peptides corresponding to the CyP-D N-terminus. This indicates that the N-terminal region is unlikely to be involved in binding and, consequently, that an N-terminally truncated form would bind to VDAC–ANT. One cannot rule out, therefore, that the VDAC–ANT complex interacts with a CyP-D(18) of the intermembrane space (Figure 4a).

Both models imply that the PT pore resides at intermembrane contact sites. They are reinforced, therefore, by evidence that contact sites are enriched in both VDAC and ANT [69], that the abundance of contact sites is increased by Ca²⁺ [70] and atractylate [69], and that PT-pore activity can be reconstituted from complexes containing the outer and inner membrane components, VDAC and ANT [48–50].

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