Reconstituted adenine nucleotide translocase forms a channel for small molecules comparable to the mitochondrial permeability transition pore

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Abstract Highly purified adenylate translocase (ANT) from rat heart mitochondria was functionally reconstituted as ATP/ADP exchange carrier in asolectin/cardiolipin vesicles. The ANT preparations used were free of porin, cyclophilin D, and Bax as analysed immunologically and by activity measurements. After pre-loading the ANT-containing proteoliposomes with ATP, malate or AMP, a gradual release of the trapped compounds by increasing the external Ca^{2+} concentrations could be demonstrated. *N*-Methyl-Val-4-cyclosporin did not inhibit the Ca^{2+} dependent release of internal substances from ANT liposomes. This inhibitor was found to be specific for the mitochondrial permeability transition pore (MTP) in intact mitochondria or reconstituted MTP-like protein complexes (e.g. hexokinase, porin, ANT complex). However, ADP in concentrations >20 μM inhibited the liberation of internal compounds, while in contrast, atractyloside (30 μM) and $HgCl_2$ (5 μM) both induced permeability of the ANT-containing liposomes resulting in a release of trapped substances. These results strongly suggest that ANT itself is capable to adopt a pore-like structure under conditions known to induce the permeability transition in mitochondria.

Key words: Adenine nucleotide translocase; Mitochondrial permeability transition pore; Porin; Creatine kinase; Hexokinase

1. Introduction

Mitochondria from a variety of sources exhibit a Ca^{2+}-dependent increase of inner membrane permeability for molecules with $M_r < 1500$ Da, the 'permeability transition'. Responsible for this behavior is a regulated inner membrane channel, the mitochondrial permeability transition pore (MTP). Pore opening is dependent on the transmembrane potential difference, on matrix pH, redox state of SH groups and a variety of effectors acting on multiple sites within the mitochondrion (for reviews see [1–3]). Cyclosporin A (CsA) [4] is a very potent (apparent I_{50} in the range of 50 nM) selective inhibitor of the MTP.

In recent publications, evidence was presented indicating that mitochondrial permeability transition constitutes an important step in the apoptotic process. In a cell free system containing isolated mitochondria and nuclei, opening of the MTP was necessary to induce DNA fragmentation typical for nuclear apoptosis [5,6].

So far, the proteins constituting the channel are not known. The observation by Halestrap et al. [7,8] that atractyloside opened the MTP, while bongkrekate and ADP closed it, led to the assumption that ANT could be at least one constituent of the channel. In agreement to this, Brustovetsky and Klingenberg [9] recently provided evidence by patch-clamping that isolated ANT reconstituted in liposomes could form high-conductance channels in the presence of Ca^{2+}. Similar high conductance channels have been characterised in patch clamped mitochondrial membranes and were found to be CsA sensitive [10]. While investigating the association of kinases with porin at the mitochondrial contact sites [11,12], we recently isolated two complexes enriched either in a hexokinase tetramer, porin and ANT, or in a mitochondrial creatine kinase octamer, porin and ANT. When reconstituted into liposomes or black lipid membranes, the hexokinase/porin/ANT complex conferred to the system permeability and conductance properties that resembled those of the MTP [13]. Indeed, when the proteoliposomes were pre-loaded with malate or ATP, a gradual release by increasing Ca^{2+} of the trapped compound could be demonstrated. As is specific for the MTP, the flux through these protein complexes was inhibited by CsA or by *N*-methyl-Val-4-cyclosporin [13], a CsA derivative which binds to and inhibits mitochondrial cyclophilin (CyP D), but not calcineurin [14,15]. Proteoliposomes containing the creatine kinase/porin/ANT complex did not display MTP-like properties unless the octamer of the creatine kinase [16] was dissociated into dimers [17]. It was observed that ligands of creatine kinase, inducing the octamer, and of hexokinase, such as glucose and ATP, inhibited MTP-like behaviour [17,18]. As shown earlier, CyP D co-purifies with the hexokinase/porin/ANT complex, but was absent in the creatine kinase/porin/ANT complex [18]. We thus concluded that ANT is the channel forming unit that is regulated either by interaction with porin and cyclophilin or direct with the mitochondrial creatine kinase octamer. The results obtained here with purified and reconstituted ANT indicate that this ATP/ADP antiporter carrier protein can indeed form an unspecific uniporter for small molecules and thus, by itself, seems to constitute the key element of the MTP.

2. Materials and methods

2.1. Chemicals

If not otherwise indicated, chemicals were all bought from Boehringer-Mannheim and Merck-Darmstadt, Germany. *N*-Methyl-Val-4-cyclosporin was a generous gift of Dr. Roland Wenger of Sandoz Pharma AG, Basel, given to us by Prof. P. Bernardi, University of Padua.

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Abbreviations: ANT, adenine nucleotide translocase; MTP, mitochondrial permeability transition pore; CsA, cyclosporin A; CyP D, mitochondrial cyclophilin; PAGE, polyacrylamide gel electrophoresis

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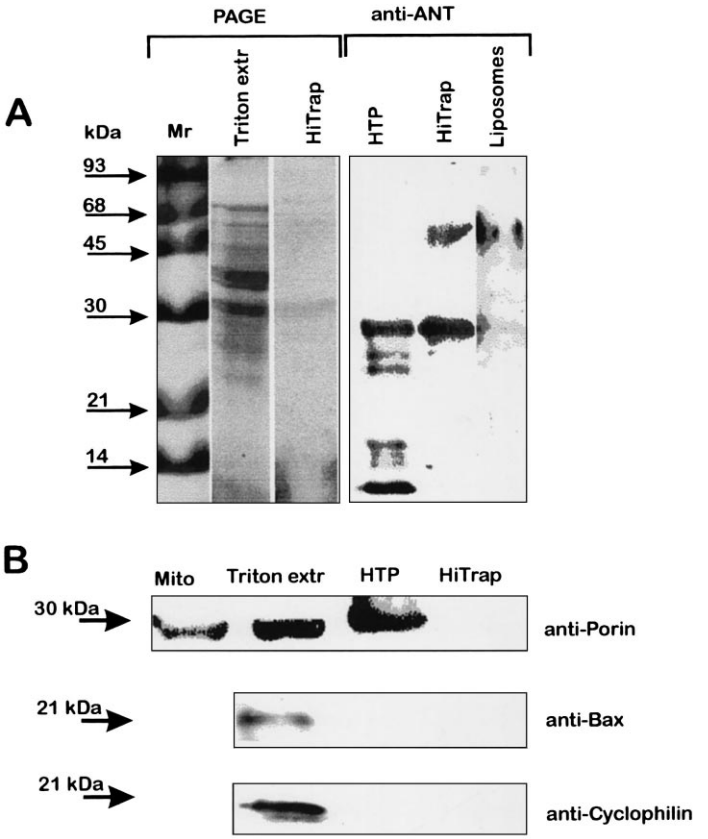

Fig. 1. Characterisation of the purified ANT by PAGE and specific antibodies. A: SDS-polyacrylamide gel electrophoresis (PAGE) stained with Coomassie Blue was performed with fractions representing different steps of the ANT isolation procedure described in Section 2: 3% Triton extract of mitochondrial membrane (Triton extr), the first HTP column (lane 1) and final ANT fraction after cation exchange chromatography (HiTrap, lane 2) and after reconstitution in liposomes (Liposomes). B: The polypeptides separated by PAGE (shown in A) were electrotransferred onto a nitrocellulose blotting membrane which was subsequently decorated by specific antibodies against ANT (anti-ANT; A), porin (anti-Porin), Bax (anti-Bax) or cyclophilin (anti-Cyclophilin).

2.2. Antibodies

The monoclonal antiserum against the N-terminus of human type I porin was bought from Calbiochem. The antibodies against Bax were bought from Santa Cruz Biotec. Specific antibodies against cyclophilin D were a gift from P. Bernardi, Padova. The polyclonal antibodies against adenylate translocase from chicken heart were prepared as described [19].

2.3. Enzyme assays

Peptidyl-prolyl-*cis-trans* isomerase (EC 5.2.1.8, cyclophilin) activity was determined by optical test according to Schutkowski et al. [20].

2.4. Substrate tests

Malate, AMP, NAD and ATP concentrations were also determined enzymatically according to Bergmeyer [21].

2.5. Isolation of adenylate translocase from rat heart muscle

A modified isolation procedure for the ATP/ADP carrier protein, resulting in highly enriched ANT (Dolder and Wallimann, unpublished), was used here. All steps were carried out at 0–4°C. In short, heart mitochondria were prepared from 5 rat hearts by Waring blender homogenisation and differential centrifugation in a medium containing 220 mM mannitol, 70 mM sucrose, 10 mM HEPES, 0.2 mM EDTA and 1 mM mercaptoethanol, pH 7.4. Isolated heart mitochondria (20 mg) within 1 ml of isolation medium were incubated for 5 min with an equal volume of extraction buffer, consisting of 40 mM KH_2PO_4, 40 mM KCl, 2 mM EDTA and 6% Triton X-100, pH 6.0. The suspension was centrifuged for 30 min at $24\,000\times g$ and the supernatant was loaded on a column filled with 1 g of dry hydroxyapatite (Bio-Rad). After elution with extraction buffer, the flow-through fraction was collected and diluted 1:1 with column buffer (20 mM MES, 0.2 mM EDTA, 0.5% Triton X-100, pH 6.0). This sample was applied to a 1-ml 'HiTrapSP' cation exchanger column (Pharmacia), connected to an FPLC system, and eluted by an NaCl gradient (0–1 M NaCl in column buffer). The fractions were analysed by sodium dodecyl sulphate (SDS), 12% polyacrylamide gel electrophoresis (PAGE) according to Laemmli [22] and by immuno decoration with specific antibodies against ANT, porin, CyP D, and Bax after transfer to nitrocellulose sheets.

2.6. Isolation and reconstitution of the hexokinase-porin-ANT complex

Isolation and reconstitution of the hexokinase-porin-ANT complex was performed as described recently [13].

2.7. Reconstitution of ANT in liposomes

Purified ANT was reconstituted in asolectin vesicles containing 2% cardiolipin. The ANT fractions were mixed with the liposomes and dialysed overnight against 125 mM sucrose, 10 mM HEPES, pH 7.4. The vesicles were loaded with 5 mM ATP or 5 mM metabolites (NAD, AMP, malate) plus 10 mM KCl by sonification and extracellular ATP or malate was subsequently removed by chromatography on Sephadex G50 in 125 mM sucrose, 10 mM HEPES. After chromatography, the permeability for ATP or malate was tested by centrifugation for 30 min at $400\,000\times g$ and subsequent determination of the substrates in the supernatant and sediment fraction.

2.8. Analysis of the adenylate translocase function

ATP plus KCl loaded liposomes containing reconstituted ANT were suspended in 125 mM sucrose, 10 mM HEPES, pH 7.4. The vesicles were analysed for ATP release by incubation for 30 min at room temperature with ADP in the range between 50 and 1000 μM. After incubation the vesicles were centrifuged as described above and the liberated ATP was determined enzymatically in the supernatant. ATP release was also measured in the presence of atractyloside in a concentration twice that of ADP.

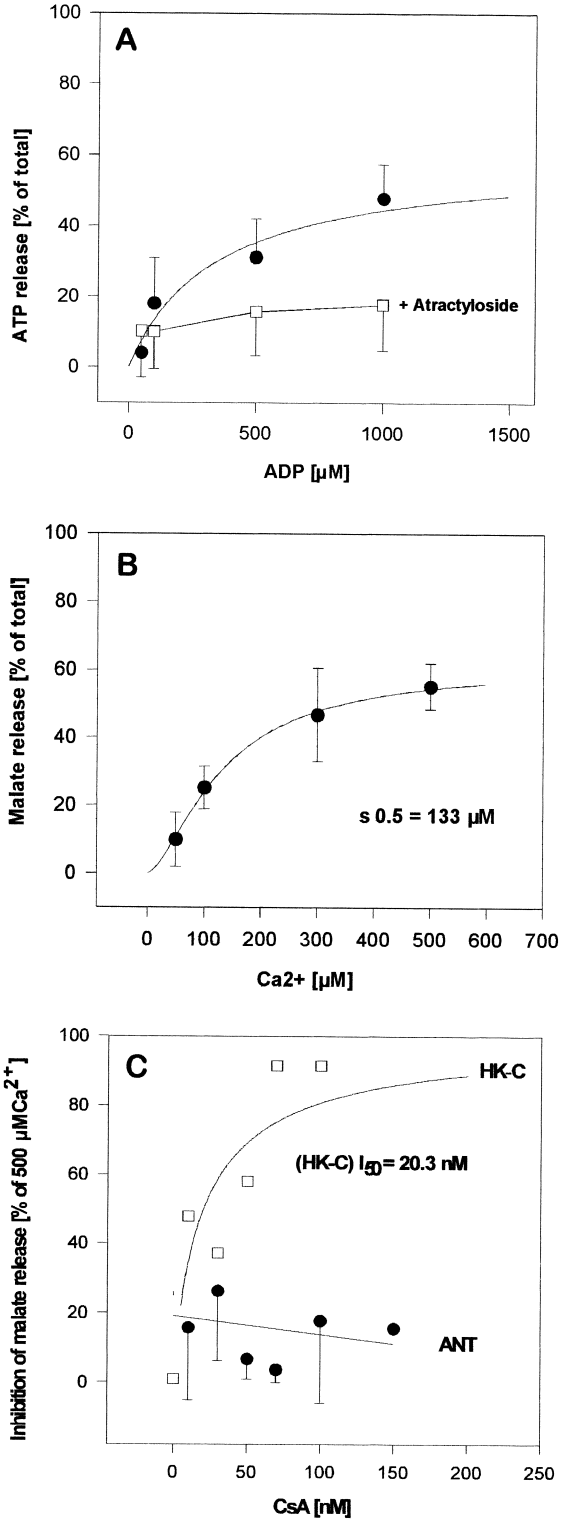

Fig. 2. Function of the reconstituted ANT as ATP/ADP-antiporter and as unspecific uniporter. A: Isolated ANT was reconstituted in liposomes as described in Section 2. The liposomes were loaded with 5 mM ATP plus 10 mM KCl. ATP was liberated from the vesicles by external addition of different concentrations of ADP as indicated (●). Specific inhibition of the antiport activity was measured with ADP plus atractyloside at 1:2 molar ratio (□). The results are means of four experiments. B: ANT containing proteoliposomes were generated and loaded with malate plus KCl as described in Section 2. The release of trapped malate by different Ca^{2+} concentrations is shown as % of total malate content in the vesicles as determined after solubilisation with Triton. Mean values and S.D. of 4 experiments. C: The hexokinase-porin-ANT complex that contained cyclophilin was isolated and reconstituted in vesicles as described recently [13]. These vesicles and in parallel proteoliposomes containing purified ANT only, as in B, were loaded with malate and preincubated with concentrations of methyl-Val-4-cyclosporin between 10 and 150 nM. Subsequently, malate was released by 500 μM Ca^{2+} that was adequate to liberate between 50 and 60% of the total malate (shown in B). The inhibition is expressed as 100-% of malate that was set free by 500 μM Ca^{2+}. Data are means of three experiments in case of ANT. Note: Ca^{2+}-dependent malate release from ANT liposomes is not inhibited by cyclosporin.

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2.9. Measuring the permeability transition pore

Metabolite (malate, NAD, AMP) and KCl loaded liposomes containing reconstituted ANT were suspended in 125 mM sucrose, 10 mM HEPES, pH 7.4. The vesicles were analysed for metabolite liberation by incubation for 30 min at room temperature in the presence of Ca^{2+} between 0 and 500 μM. The liberated substances were determined enzymatically in the supernatant after centrifugation as described above.

3. Results

3.1. Isolation and characterisation of the adenylate translocase

The purified ANT fraction used for reconstitution in liposomes was almost free of contaminating polypeptides as shown by PAGE in Fig. 1A (lane 2). The ANT fraction contained mainly two bands at 30 and 65 kDa that correspond to the monomer and dimer of ANT as shown by immuno decoration of purified ANT as well as reconstituted ANT in liposomes (Fig. 1A). Analysis of the PAGE transblots by specific antibodies, anti-porin, anti-cyclophilin, anti-Bax demonstrated the absence of porin, cyclophilin and Bax in the final ANT fraction (Fig. 1B). During the different steps of the preparation procedure, cyclophilin activity was also examined. Mitochondrial cyclophilin (0.2 mU/mg) was found in the extract of disrupted mitochondria but was removed during ANT preparation presumably by the high ionic strength that was used during column chromatography.

3.2. Reconstitution of the adenylate translocase in liposomes

3.2.1. Function of ANT as adenine nucleotide antiporter.

ANT was reconstituted in asolectin/cardiolipin liposomes. The vesicles were loaded with 5 mM ATP that remained inside, as was proven by centrifugation. After addition of external ADP, however, the internal ATP was gradually released (Fig. 2A). As expected for an obligate antiporter, the amount of ATP released correlated with the increasing concentrations of externally added ADP. This process was inhibited by atractyloside present in a 2:1 ratio to ADP. As atractyloside acts as a competitive inhibitor for ADP and ATP, the addition of double concentrations of inhibitor vs. nucleotide does not result in a complete inhibition. The S.D. of the mean values in the presence of atractyloside, shown in Fig. 2A, was always towards zero and never above the mean value. In summary, the results clearly suggest a functionally competent reconstitution of ANT as ATP/ADP exchange carrier. The release of ATP by external ADP did not exceed more than 60% of total ATP in the vesicle fraction. This agrees with the observation of Ca^{2+}-dependent malate liberation shown below. It may be

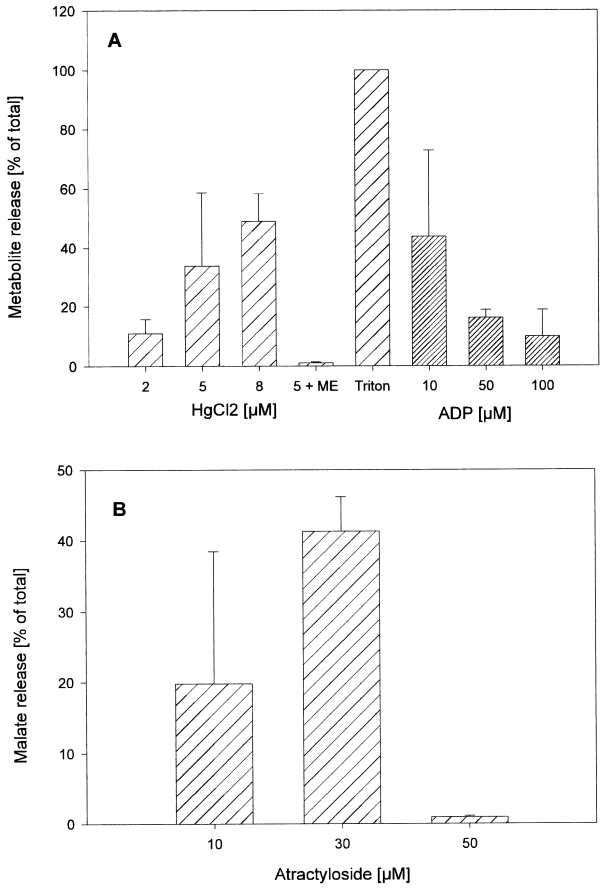

Fig. 3. Regulation of the uniporter activity of reconstituted ANT by specific ligands and SH-groups. A: Vesicles loaded with 5 mM malate, or AMP, or NAD and 10 mM KCl were incubated with different $HgCl_2$ concentrations as indicated. In one experiment 5 μM $HgCl_2$ was added in the presence of 2.5 mM mercaptoethanol (5+ME). The effect of ADP was studied by pre-incubation of the vesicles with different ADP concentrations as indicated. Subsequently, 500 μM Ca^{2+} was added. This concentration was sufficient to release 50–60% of the total malate in the absence of ADP (see Fig. 2B). B: The effect of different atractyloside concentrations on the malate loaded vesicles was studied in the presence of 50 μM Ca^{2+} that, by itself, was not adequate to release more than 10% malate (see Fig. 2B). The malate release is shown as % of total malate determined after solubilising the vesicles by Triton. A,B: mean values of 3 experiments.

explained by the assumption that approximately 50% of ANT has been functionally reconstituted.

3.2.2. Function of ANT as unspecific uniporter. To study the function of ANT as unspecific uniporter, the proteoliposomes were loaded with malate. Fig. 2B shows that the trapped malate could be released from the vesicles by the addition of Ca^{2+} concentrations ranging between 50 and 500 μM. The opening of the reconstituted ANT by 500 μM Ca^{2+} was not inhibited by *N*-methyl-Val-4-cyclosporin in a concentration range between 10 and 150 nM (Fig. 2C). In contrast *N*-methyl-Val-4-cyclosporin in the same concentrations inhibited ($I_{50} = 20$ nM) the permeation through ANT in the reconstituted hexokinase complex. In this complex that was isolated as described recently [13], ANT is coupled to porin and cyclophilin [18] and thus may have a different structure.

The Ca^{2+}-dependent liberation of malate from the proteoliposomes was influenced by ANT ligands in the same way as already observed by Halestrap et al. [7] in isolated mitochondria. Between 50 and 100 μM ADP inhibited the liberation of malate induced by 500 μM Ca^{2+} (Fig. 3A), while atractyloside was able to open the channel (Fig. 3B). As observed recently [17], the atractyloside action in the presence of low Ca^{2+} had a maximum effect at 15–20 μM, while higher or lower concentrations of the same compound were less effective in channel opening. In the present experiment, we observed a maximal release of malate at 30 μM atractyloside, in the presence of 50 μM Ca^{2+} (Fig. 3A), which on its own had no such effect (see Fig. 2B). Dierks et al. (1990) were able to reversibly modify the aspartate/glutamate carrier and ANT from obligate counter-exchange to uni-directional transport by blocking SH-groups with mercury reagents [23,24]. According to the authors' results, the intrinsic channel appeared to be a structural requirement of the exchange carrier-mediated transport. In support of this idea, Petronilli et al. [25] reported that the permeability transition is tuned by the oxidation-reduction state of thiol groups. As shown in Fig. 3A, addition of $HgCl_2$ in concentrations between 2 and 8 μM to our loaded ANT vesicles led to increasing release of the trapped AMP, or NAD or malate up to 60%. This effect was completely abolished by the presence of 2.5 mM mercaptoethanol.

4. Discussion

The results described above suggest that ANT can reversibly adopt two conformations that cause two functions of the protein to act either as a specific ATP/ADP exchange carrier or as an unspecific channel. As shown by Dierks et al. [23,24] and Schroers et al. [26], this reversible transformation into uniporter function appears to be specific for a number of mitochondrial exchange carriers such as ANT, aspartate/glutamate- and phosphate/OH^- exchanger. In all cases, dimers of the proteins which have 6 transmembrane helices in each monomer, form the active transport unit. The reversible antiport-uniport conversion of the phosphate carrier, for example, depends on the presence of one single cysteine (cysteine 28) in the monomer, that are two cysteines in the functionally active dimer [26]. Our results showing initiation of malate release through ANT by $HgCl_2$ fully corroborated those of Dierks et al. [23,24]. The results furthermore suggested that, generally, all mitochondrial exchange carriers may be able to be transformed into mitochondrial permeability transition pores. The question of whether high [Ca^{2+}] can induce antiport-uniport conversion of some or all of these mitochondrial exchange carriers, such as observed here for the reconstituted ANT (Fig. 2B), has not yet been answered.

Cyclophilin D, the target for cyclosporin, was not present in our reconstituted ANT. ANT conferred the vesicles with Ca^{2+}-induced permeability properties that resembled those of the MTP. In agreement with the results of Brustovetsky and Klingenberg [9], this suggested that the presence of cyclophilin was not required for MTP-like behaviour (Fig. 2B). However, ANT coupled to porin, as in the hexokinase complex, appeared to be different. It needed cyclophilin for Ca^{2+}-dependent opening. If the cyclophilin was removed by cyclosporin, MTP-like behaviour of the hexokinase-porin-ANT complex was suppressed (Fig. 2C).

It has been shown that ANT builds complexes with porin, hexokinase and/or creatine kinase [13,17]. ADP, the product of these kinases [18], in physiological concentrations of 20–50

μM, inhibited the Ca^{2+}-dependent conversion of ANT into a uniport channel (Fig. 3A). Therefore, physiologically, the uniport conversion of ANT would occur only at low ADP and substrate levels, e.g. glucose or creatine, for these kinases. However, at high substrate levels still other antiporters, such as the aspartate/glutamate carrier, may be ready to perform mitochondrial permeability transition.

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