BioFactors 8 (1998) 235–242
IOS Press

The molecular structure of mitochondrial contact sites. Their role in regulation of energy metabolism and permeability transition

Dieter Brdiczka [a,*], Gisela Beutner [a], Alexander Rück [a], Max Dolder [b] and Theo Wallimann [b]
[a] *Faculty of Biology, University of Konstanz, D-78457 Konstanz, Germany*
[b] *Institute for Cell Biology, ETH Zürich, CH-8093 Zürich, Switzerland*

Received 15 April 1998

Abstract. Contact sites between the outer and peripheral inner membrane of mitochondria are involved in protein precursor uptake and energy transfer. Hexokinase and mitochondrial creatine kinase could be attributed by different techniques to the energy transfer contacts. Kinetic analyses suggested a functional interaction between the kinases, outer membrane pore protein, and inner membrane adenylate translocator (ANT). This suggestion was strongly supported by isolation of hexokinase and creatine kinase complexes that were constituted of kinase oligomers, porin and ANT. Phospholipid vesicles carrying reconstituted kinase–porin–ANT complexes enclosed internal ATP in contrast to vesicles containing free porin only. This indicated that unspecific transport through porin was regulated by its interaction with a specific antiporter, ANT. A direct interaction between porin and ANT in the hexokinase complex conferred the reconstituted system with permeability properties reminiscent of the mitochondrial permeability transition (PT) pore. In the creatine kinase complex this interaction between porin and ANT was replaced by contact of both with the kinase octamer. Thus PT-pore-like functions were not observed unless the creatine kinase octamer was dissociated, suggesting that the ANT was locked in the antiporter state by interaction with the octamer. Indeed, reconstituted pure ANT showed PT-pore-like properties concerning Ca^{2+} sensitivity. However, as cyclophilin was missing, sensitivity against cyclosporin was not observed.

1. Introduction

Mitochondria from a variety of sources exhibit a Ca^{2+}-dependent increase of the inner membrane permeability to ions and solutes, the permeability transition (PT). Responsible for this behaviour are regulated inner membrane channels, the PT pores. Pore opening is dependent on the transmembrane potential difference [1], matrix pH [2], SH group reactants [3] and is modulated by a variety of effectors acting on multiple sites (for a review, see [4,5]). Cyclosporin A (CsA) is a very potent, selective inhibitor of the PT (the apparent I_{50} is in the nanomolar range) [6–8]. The size of the pore was found to be variable. In most cases the pore was permeable to molecules up to 1500 Da, however, Ichas et al. [9] recently reported impermeability for sucrose (Mr 340). To account for the different permeabilities, the existence of either one pore forming molecule with variable structure or different molecules must be assumed

*Corresponding author: Dieter Brdiczka, Faculty of Biology, University of Konstanz, D-78457 Konstanz, Germany. Tel.: +49 7531 88 4112; Fax: +49 7531 88 4257; E-mail: Dieter.Brdiczka@uni-konstanz.de.

to accomplish PT. So far, the protein(s) constituting the channel were not known. The observation by Halestrap et al. [8,10] that atractyloside opened the PT pore, while bongkrekate and ADP closed it, led to the assumption that the adenylate translocator (ANT) could be at least one component of the channel. In agreement to this, Brustovetsky and Klingenberg [11] 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 [12]. The observed channels had some mitochondrial porin specific properties (e.g., asymmetric voltage dependence), suggesting that porin and an inner membrane protein might constitute the PT pore [13]. It was discussed that the patches, in which the investigated channels were studied, might have evolved from the contact sites. Indeed, complexes between porin and ANT were isolated as components of the mitochondrial benzodiazepine receptor [14] and ligands of the receptor inhibited the conductance of the PT-pore-like channels [15].

2. Molecular structure of mitochondrial contact sites

It has been noticed that the contact sites between inner and outer boundary membranes were dynamic structures [16]. The contact site frequency was variable and increased, for example, by addition of ADP [17] or by the presence of mitochondrial precursor proteins [18,19]. Based on these results, it was assumed that the contacts may be formed by specific interaction of inner and outer membrane components such as the outer membrane pore or TOM's (precursor Translocase Outer Membrane) and the ANT or TIM's (precursor Translocase Inner Membrane) in the inner membrane [16,20]. It is likely that the so-called energy contact sites, where high energy phosphates are shuttled out off the mitochondria, are different compared to the contacts that are involved in mitochondrial precursor protein import.

2.1. Organisation of kinases at the mitochondrial periphery

A specific organisation at the mitochondrial periphery in contact sites of hexokinase (HK) and mitochondrial creatine kinase (Mi-CK), respectively, was observed by electron microscopy [21,22] as well as by biochemical isolation of the contacts [21,23,24]. This location together with kinetic studies suggested the formation of functional complexes between the kinases, porin in the outer membrane, and the ANT in the inner membrane. *In vitro* both kinases formed tetrameric (HK) or octameric (Mi-CK) complexes with porin that had a molecular mass of about 400 kDa [25,26].

2.2. Oligomeric complexes between porin, kinases, and ANT

Such high molecular weight complexes of either Mi-CK or HK were extracted from rat brain or kidney homogenates with 0.5% Triton X-100, separated by DEAE chromatography and subsequently analysed by Superose-6B chromatography. The molecular mass between 400 and 450 kDa suggested the presence of an HK tetramer and a Mi-CK octamer. As analysed immunologically, the complexes were composed, besides of the kinases, of porin and ANT. This indicated a structure of the observed contacts between the boundary membranes formed by either a direct interaction of porin and ANT, or of mitochondrial creatine kinase with porin and ANT (Fig. 1) [27,28]. The existence of these complexes explained the observation of molecular channelling of adenine nucleotides between HK or Mi-CK and the inner mitochondrial compartment [29,30]. This was additionally supported by the experiments with the reconstituted complexes as described below.

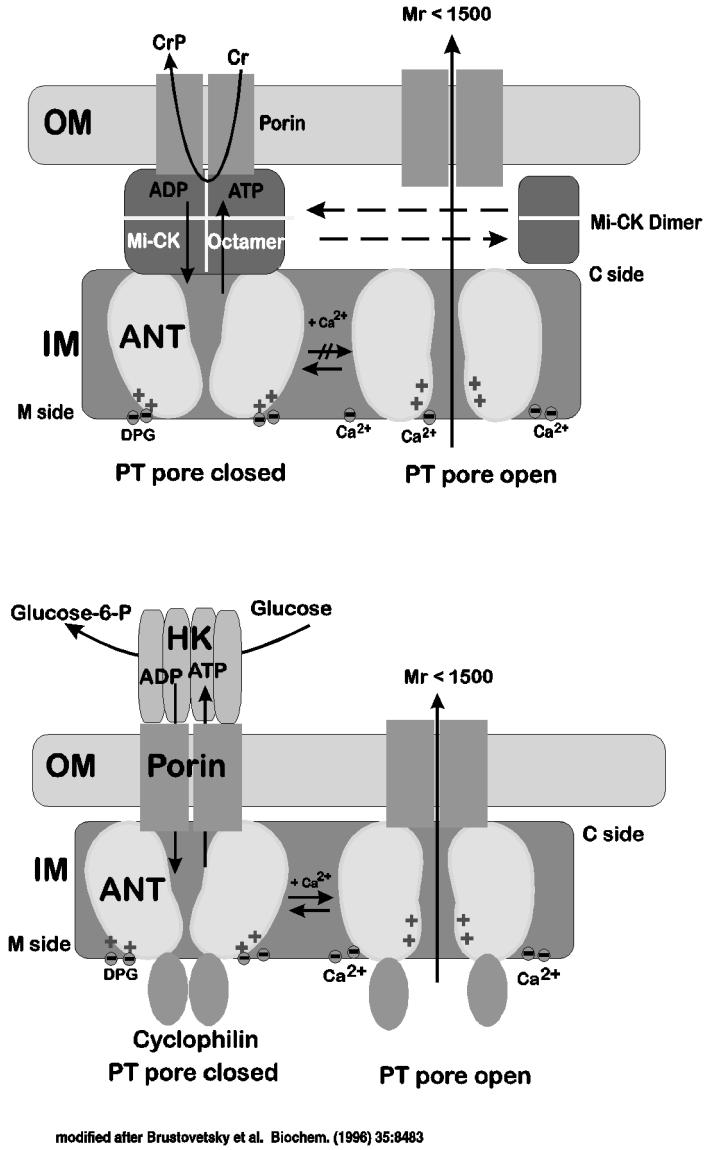

Fig. 1. Model of protein complexes between mitochondrial boundary membranes. Two kinase complexes that could be extracted from brain membranes by 0.5% Triton X-100. These complexes are composed of the outer membrane pore protein, the active kinases, either tetrameric hexokinase (HK) or octameric creatine kinase (Mi-CK), as well as the adenylate translocator (ANT) from the inner membrane. Although this overall composition appears to be similar in both complexes, the interaction between single components in the two complexes is very different. While the adenylate translocator in the HK complex interacts with porin and through this indirectly with the kinase (lower), it is directly linked to the kinase in the Mi-CK complex (upper). This direct interaction with Mi-CK locks the translocator in the antiporter state. The interaction between creatine kinase and the adenylate translocator is regulated by the association–dissociation equilibrium of the Mi-CK octamer as only the latter structure has a high affinity to the membrane and the translocator. Upon dissociation of Mi-CK from the membrane or from ANT by dimerisation the ANT is no longer locked in the antiporter sate. According to Brustovetsky et al., the structural transition of the translocator into the uniporter state is facilitated by Ca^{2+}. This divalent cation displaces the translocator from its interaction with cardiolipin (DPG) and by doing so allows structural changes. The sensitivity for Ca^{2+} of the adenylate translocator may be regulated by the bound cyclophilin that is enriched in the hexokinase complex but absent in the creatine kinase complex.

3. Reconstitution of kinase complexes

3.1. Functional interaction between kinases and ANT

Phospholipid vesicles carrying reconstituted kinase–porin–ANT complexes enclosed internal ATP in contrast to vesicles containing free porin only. However, the trapped ATP could be utilised by Mi-CK or HK to form creatine-phosphate with external creatine or glucose 6-phosphate from added glucose, respectively. In both cases, creatine-phosphate or glucose 6-phosphate production was inhibited by 100 μM atractyloside. The results suggested that the ANT in the reconstituted system was functionally active as an exchange carrier and that internal ATP was released upon external production of ADP by the kinases [27,28].

3.2. PT-pore-like behaviour of the reconstituted HK porin ANT complex

Rat liver mitochondria possess a matrix cyclophilin (CyP), an enzyme with peptidyl-prolyl-*cis-trans*-isomerase activity, which can be specifically inhibited by cyclosporin A (CsA) [8,32]. cDNA sequencing of rat mitochondrial CyP has revealed that it is the homologue of human CyP D [32]. It has been proposed that CyP D regulates the Ca^{2+}-dependent PT pore open–closed transitions by reversible association from the matrix side of the inner membrane, and that CsA inhibits the pore through its effects on CyP D rather than by a direct effect on the pore [33,34]. The isolated kinase complexes were analysed for the CyP D content. It was observed that CyP D co-purifies with the hexokinase complex but was absent in the creatine kinase complex [28] (Fig. 1).

When reconstituted into liposomes or black lipid membranes, the isolated hexokinase, porin, ANT complex conferred the system with permeability and conductance properties reminiscent of the mitochondrial PT pore [27,28]. Indeed, when the proteoliposomes were pre-loaded with malate or ATP, a gradual release of the trapped compound by increasing Ca^{2+} could be demonstrated. As is the case for the PT pore, flux was inhibited by CsA or by N-methylVal-4-cyclosporin dissolved in ethanol [27,28].

3.3. Different complexes of the mitochondrial creatine kinase

Mitochondrial creatine kinase (Mi-CK) differs from its cytosolic counterparts in that it can form a highly ordered octameric oligomer from four dimers, whereas the cytosolic enzymes have a dimeric structure only [35]. The Mi-CK octamers display a cube-like structure with identical top and bottom faces that are suited to interact with the two mitochondrial boundary membranes (for a review, see [36]). Indeed, Rojo et al. [37] demonstrated *in vitro* that the octameric form of Mi-CK was able to connect two artificial membranes. In agreement with this, Kottke et al. [38] observed that mainly the octameric form of the enzyme was present in isolated contact site fractions of brain mitochondria. Furthermore, it was shown by electron microscopy that the enzyme was indeed frequently located *in situ* between adhering outer membrane vesicles and the inner envelope membrane but a second location between the cristae membranes was additionally recognised [22]. Considering this dual localisation of Mi-CK octamers and assuming an interaction with the ANT, the existence of two different types of Mi-CK octamer–ANT complexes must be expected: a peripheral complex constituted of porin, Mi-CK, and ANT (Fig. 2, left) and a central complex composed of only Mi-CK and ANT (Fig. 2, right). So far, a Mi-CK complex fraction with a Mr of 400 kDa was isolated from the Triton extract of brain membranes that contained presumably both types of complexes, as porin and ANT was found by immunological methods in this fraction [28,39].

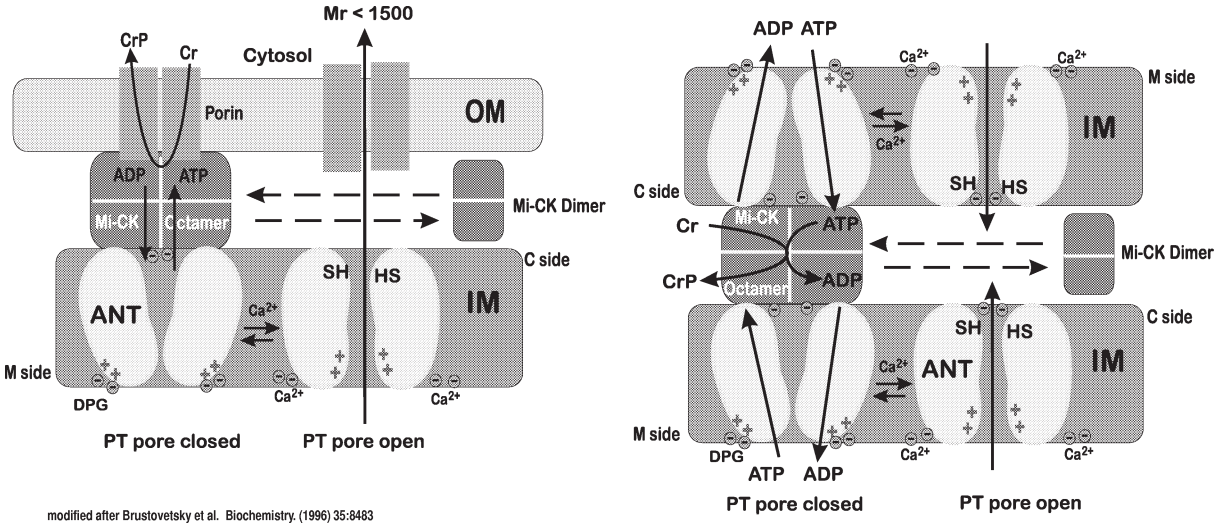

Fig. 2. Model of the two complexes that the creatine kinase octamer can form with inner and outer mitochondrial membrane components. The catalytically active enzyme of the mitochondrial creatine kinase (Mi-CK) is a dimer. Four dimers associate to an octamer that has an equal top and bottom face. The octamer can link two membranes and is known to interact with porin dimers in the outer membrane and to be functionally coupled to adenylate translocator (ANT) tetramers in the inner membrane. Thus, a peripheral creatine kinase complex is build by interaction with porin and the adenylate translocator (left), whereas the central complex involves two adenylate translocators and one creatine kinase octamer (right). Interaction with the creatine kinase octamer keeps the adenylate translocator in the antiporter state. The dissociated octamer has a low affinity to the membranes and the adenylate translocator may switch to the uniporter state (right). According to Brustovetsky et al., this structural transition is facilitated by Ca^{2+}. This ion displaces the translocator from its interaction with cardiolipin (DPG) and by that allows structural changes into the uniporter state. The uniporter state can as well be induced by $HgCl_2$ reaction with an SH group in each monomer that appears to be important for this conformational switch. As long as the Mi-CK connects two cristae membranes (IM) and interacts with adenylate translocators, functional coupling of the two will result in vectorial transport of ATP from the matrix space to give phospho-creatine (PCr) in the intra-cristae space that has to diffuse towards the periphery (left side of the right model). If Mi-CK is dissociated from the membrane or dimerised, this tight functional coupling is lost (right side of the right model) and the ANT may now be in a state to be converted, e.g., by high Ca^{2+}, into a permeability transition pore.

The mixture of assumed peripheral and central Mi-CK–ANT complex was isolated and reconstituted in vesicles such as described for hexokinase. Analysis of the proteoliposomes revealed that the vesicles were sealed and internal ATP was accessible to creatine kinase in a way that was inhibited by atractyloside such as published recently [28]. This proved that the ANT was correctly reconstituted. However, internal ATP and malate were not released by increasing Ca^{2+} concentrations as was observed with the reconstituted hexokinase complex described above. In contrast to hexokinase, creatine kinase interacts directly with the ANT (Fig. 1). Creatine kinase in the isolated contact sites [38] and in the reconstituted complex was always present as octamer, while the dimer after dissociation did only loosely attach to membranes [37] and presumably to the ANT. We therefore dissociated the Mi-CK octamer after reconstitution of isolated complexes in liposomes by the method described by Gross and Wallimann [40, 41] using KNO_3, creatine, Mg^{2+} and ADP. The detachment of the Mi-CK octamer was confirmed by gel-permeation chromatography on Superdex 200.

3.4. Regulation of the PT pore by the mitochondrial creatine kinase

The complex containing the octamer of creatine kinase was reconstituted in liposomes that were loaded with malate. An aliquot of the proteoliposomes was used to dissociate the octamer as described above.

After this treatment, the liposomes were collected by centrifugation and were found to retain the malate. The vesicles containing either dissociated or un-dissociated Mi-CK octamers were then incubated with increasing concentrations of Ca^{2+} between 0 and 400 μM. Under these conditions, malate was liberated exclusively from those vesicles with the dissociated octamer. Although no cyclophilin was found in the Mi-CK–ANT complexes, the Ca^{2+}-dependent malate release could be inhibited by pre-incubation of the vesicles with 500 nM N-methylVal-4-cyclosporin. This observation might be an artefact, produced by DMSO that in this case was used as solvent of the CsA.

After reconstitution of the protein complex in vesicles, Mi-CK therein was able to generate phosphocreatine from internal ATP and external creatine. The fact that this reaction was inhibited by atractyloside suggested that the enzyme in the complex was coupled to the ANT. It was furthermore assumed that dissociation of the Mi-CK octamer might result in a detachment between Mi-CK and ANT. It appears plausible that the free ANT would structurally be more flexible than in a complex with Mi-CK and thus would readily form channels in the presence of Ca^{2+}. This assumption would agree with the idea of Halestrap [8,10] and Brustovetsky [11] that the ANT might be able to form the PT pore.

3.5. Is the adenylate translocator responsible for PT-pore-like behaviour?

To answer this question, we recently purified the ANT from rat heart and proved immunologically that it was free of porin, Bcl-2, Bax, and cyclophilin. The ANT was functionally reconstituted in asolectin/cardiolipin vesicles and vesicles were loaded with malate. The trapped malate could be released from the vesicles by addition of Ca^{2+} concentrations ranging between 50 and 500 μM, thus demonstrating a Ca^{2+}-dependent transformation of the pure ANT from antiporter into uniporter function. The opening of the reconstituted ANT by 500 μM Ca^{2+} was not inhibited by N-methylVal-4-cyclosporin in a concentration range between 10 and 150 nM [42]. In contrast, N-methylVal-4-cyclosporin in the same concentrations inhibited ($I_{50} = 20$ nM) the permeation through the ANT in the reconstituted hexokinase complex. In this complex that was isolated as described recently [27], the ANT is coupled to porin and cyclophilin [28] and thus may have a different structure.

The Ca^{2+}-dependent liberation of malate from the ANT proteoliposomes was influenced by ANT ligands in the same way as already observed by Halestrap et al. [8] in isolated mitochondria. ADP inhibited the Ca^{2+}-dependent liberation of malate between 50 and 100 μM, while 30 μM atractyloside was able to open the channel in the presence of low Ca^{2+} [42].

It has been already described that the free ANT can reversibly change its functional state from antiporter to uniporter in the presence of SH-group reagents such as $HgCl_2$ [42–44]. The Ca^{2+}-dependent functional change of the ANT may be related. It appears that the ANT cannot perform this transformation in a complex with Mi-CK. This suggestion is supported by our investigations concerning the PT pore regulation in a transgenic mouse model expressing Mi-CK in liver [39]. PT pore was opened in liver mitochondria of control mice by atractyloside in the presence of low Ca^{2+}. In contrast, in liver mitochondria from transgenic mice containing Mi-CK, PT could be suppressed by Mi-CK substrates such as creatine or cyclocreatine.

4. Conclusion

The coupling between the kinases and the ANT seems to have at least two functions: molecular channelling of ATP and ADP through the mitochondrial boundary membranes and regulation of the permeability transition.

The molecular channelling of ADP into the mitochondria via the bound kinases allows a high activity of oxidative-phosphorylation in the presence of high ATP/ADP quotients in the cytosol. Furthermore, such a process avoids equilibration of ADP and ATP with the free phosphate when CrP is formed from mitochondrial ATP in a Mi-CK–ANT complex. It is advantageous thermodynamically to avoid equilibration with the phosphate, as this would cause a loss of free energy.

Regulation of PT may result from a change of the antiporter structure of the ANT to a uniporter structure. This structural change appears to be regulated by the association–dissociation equilibrium of Mi-CK. The octamer of Mi-CK exclusively interacts with the ANT and may lock it in the exchange carrier structure. The same conformation of the ANT is adopted in the presence of the specific ligand ADP that is produced locally by the kinases. ADP is thus a very strong inhibitor of the PT pore. The regulation of PT would therefore depend on the level of creatine or glucose through the action of the kinases.

Although our data strongly favour a direct involvement of the ANT together with porin and the respective kinase in PT regulation, it is entirely conceivable that other related inner membrane antiporters may also display similar characteristics as the ANT and may regulate the PT in a slightly different way.

References

[1] V. Petronilli, C. Cola, S. Massari, R. Colonna and P. Bernardi, *J. Biol. Chem.* **268** (1993), 21 939–21 945.
[2] A. Nicolli, V. Petronilli and P. Bernardi, *Biochemistry* **32** (1993), 4461–4465.
[3] V. Petronilli, P. Costantini, L. Scorrano, R.Colonna, S. Passamonti and P. Bernardi, *J. Biol. Chem.* **269** (1994), 16 638–16 642.
[4] M. Zoratti and I. Szabo, *Biochim. Biophys. Acta* **1241** (1995), 139–176.
[5] P. Bernardi, K.M. Broekemeier and D.R. Pfeiffer, *J. Bioenerg. Biomembr.* **26** (1994), 509–517.
[6] M. Crompton, H. Ellinger and A. Costi, *Biochem. J.* **255** (1988), 357–360.
[7] K.M. Broekemeier, M.E. Dempsey and D.R. Pfeiffer, *J. Biol. Chem.* **264** (1989), 7829–7830.
[8] A.P Halestrap and A.M. Davidson, *Biochem. J.* **268** (1990), 153–160.
[9] F. Ichas, L.S. Jouaville and J.P. Mazat, *Cell* **89** (1997), 1145–1153.
[10] A.P Halestrap, K.Y. Woodfield and C.P. Connern, *J. Biol. Chem.* **272** (1997), 3346–3354.
[11] N. Brustovetsky and M. Klingenberg, *Biochemistry* **35** (1996), 8483–8488.
[12] I. Szabo and M. Zoratti, *J. Biol. Chem.* **266** (1991), 3376–3379.
[13] K.W. Kinnally, M.L. Campo and H. Tedeschi, *J. Bioenerg. Biomembr.* **21** (1989), 497–506.
[14] M.W. McEnery, A.M. Snowman, R.R. Trifiletti and H. Snyder, *Proc. Natl. Acad. Sci. USA* **89** (1992), 3170–3174.
[15] K.W. Kinnally, D.B. Zorov, Y.N. Antonenko, S.H. Snyder, M.W. McEnery and H. Tedeschi, *Proc. Natl. Acad. Sci. USA* **90** (1993), 1374–1378.
[16] D. Brdiczka, *Biochim. Biophys. Acta* **1071** (1991), 291–312.
[17] K. Bücheler, V. Adams and D. Brdiczka, *Biochim. Biophys. Acta* **1061** (1991), 215–225.
[18] L. Pon, T. Moll, D. Vestweber, B. Marshallsay and G. Schatz, *J. Cell Biol.* **109** (1989), 2603–2616.
[19] H. Ono and S. Tuboi, *Eur. J. Biochem.* **168** (1987), 509–514,
[20] W. Neupert, *Ann. Rev. Biochem.* **66** (1997), 863–917.
[21] M. Kottke, V. Adams, I. Riesinger, G. Bremm, W. Bosch, D. Brdiczka, G. Sandri and E. Panfili, *Biochim. Biophys. Acta* **395** (1988), 807–832.
[22] M. Kottke, Th. Wallimann and D. Brdiczka, *Biochem. Med. Metabol. Biol.* **51** (1994), 105–117.
[23] K. Ohlendieck, I. Riesinger, V. Adams, J. Krause and D. Brdiczka, *Biochim. Biophys. Acta* **860** (1986), 672–689.
[24] V. Adams, W. Bosch, J. Schlegel, T. Wallimann and D. Brdiczka, *Biochim. Biophys. Acta* **981** (1989), 213–225.
[25] D. Brdiczka, Ph. Kaldis and Th. Wallimann, *J. Biol. Chem.* **269** (1994), 27 640–27 644.
[26] D. Brdiczka, U. Wicker and F. Gellerich, in: *Molecular Biology of Mitochondrial Transport Systems*, M. Forte and M. Colombini, eds, NATO ASI Series, Vol. H83, Springer, Berlin, 1994, pp. 297–311.
[27] G. Beutner, A. Rück, B. Riede, W. Welte and D. Brdiczka, *FEBS Lett.* **396** (1996), 189–195.
[28] G. Beutner, A. Rück, B. Riede and D. Brdiczka, *Biochim. Biophys. Acta* **1368** (1998), 7–18.
[29] F.D. Laterveer, F.N. Gellerich and K. Nicolay, *Eur. J. Biochem.* **232** (1995), 569–577.
[30] F.N. Gellerich, M. Kapischke, W. Kunz, W. Neumann, A. Kusnetsov, D. Brdiczka and K. Nicolay, *Mol. Cell. Biochem.* **133/134** (1994), 85–104.

[31] C.P. Connern and A.P. Halestrap, *Biochem. J.* **284** (1992), 381–385.
[32] K.-Y. Woodfield, N.T. Price and A.P. Halestrap, *Biochim. Biophys. Acta* **1351** (1997), 27–30.
[33] A. Nicolli, E. Basso, V. Petronilli, R.M. Wenger and P. Bernardi, *J. Biol. Chem.* **271** (1996), 2185–2192.
[34] C.P. Connern and A.P. Halestrap, *Biochemistry* **35** (1996), 8172–8180.
[35] J. Schlegel, B. Zurbriggen, B. Wegmann, M. Wyss, H.M. Eppenberger and T. Wallimann, *J. Biol. Chem.* **263** (1988), 16 942–16 953.
[36] Th. Wallimann, M. Wyss, D. Brdiczka, K. Nicolay and H.M. Eppenberger, *Biochem. J.* **281** (1992), 21–40.
[37] M. Rojo, R. Hovius, R.A. Demel, K. Nicolay and T. Wallimann, *J. Biol. Chem.* **266** (1991), 20 290–20 295.
[38] M. Kottke, V. Adams, T. Wallimann, V.K. Nalam and D. Brdiczka, *Biochim. Biophys. Acta* **1061** (1991), 215–225.
[39] E. O'Gorman, G. Beutner, M. Dolder, A.P. Koretsky, D. Brdiczka and T. Wallimann, *FEBS Lett.* **414** (1997), 253–257.
[40] M. Gross and T. Wallimann, *Biochemistry* **32** (1993), 13 933–13 940.
[41] M. Gross and T. Wallimann, *Biochemistry* **34** (1995), 6660–6667.
[42] A. Rück, M. Dolder, T. Wallimann and D. Brdiczka, Reconstituted adenine nucleotide translocase forms a channel for small molecules comparable to the mitochondrial permeability transition pore, *FEBS Lett.* (1998), in press.
[43] T. Dierks, A. Salentin, C. Heberger and R. Krämer, *Biochim. Biophys. Acta* **1028** (1990), 268–280.
[44] T. Dierks, A. Salentin and R. Krämer, *Biochim. Biophys. Acta* **1028** (1990), 281–288.