Adenine nucleotide translocator isoforms 1 and 2 are differently distributed in the mitochondrial inner membrane and have distinct affinities to cyclophilin D

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Different isoforms of the adenine nucleotide translocase (ANT) are expressed in a tissue-specific manner. It was assumed that ANT-1 and ANT-2 co-exist in every single mitochondrion and might be differently distributed within the membrane structures that constitute the peripheral inner membrane or the crista membrane. To discriminate between ANT originating from peripheral or from cristal inner membranes we made use of the fact that complexes between porin, the outer-membrane pore protein, and the ANT can be generated. Such complexes between porin and the ANT in the peripheral inner membrane were induced in rat heart mitochondria and isolated from rat brain and kidney. Using ANT-isotype-specific antibodies and sequence analysis of the N-terminal end, it was discovered that the peripheral inner membrane contained ANT-1 and ANT-2, whereas the cristal membrane contained exclusively ANT-2. Cyclophilin was co-purified with the porin–ANT complexes, whereas it was absent in the crista-derived ANT. This suggested that ANT-1 might have a higher affinity for cyclophilin. This specific intramitochondrial distribution of the two ANT isotypes and cyclophilin D suggests specific functions of the peripheral and crista-forming parts of the inner membrane and the two ANT isotypes therein.

Key words: contact sites, creatine kinase, hexokinase, myopathy, permeability transition pore.

INTRODUCTION

Expression of different isoforms of the adenine nucleotide translocase (ANT) shows tissue-specific patterns. In rat and mouse, ANT-1 is mainly found in brain, kidney, skeletal muscle and heart, whereas isotype ANT-2 is the predominant form in other tissues [1,2]. However, it is unclear whether the different ANT isotypes reside in different mitochondrial populations or co-exist in every single mitochondrion. If so, the question arises of whether the isoforms have different functions within the mitochondrion.

Besides its operation as an ATP/ADP antiporter a second function as a non-specific uniporter has been assigned to the ANT [3], that as hypothesized may cause permeability transition (PT) [4–9]. This increase in the inner membrane permeability leads to a collapse of mitochondrial membrane potential, mitochondrial swelling and the release of several factors, such as AIF (apoptosis-inducing factor) [10], cytochrome *c* [11–13] and Smac/Diabolo [14,15], which induce apoptosis.

In a recent publication Bauer et al. [16] described how expression of ANT-1 but not ANT-2 induced apoptosis in several cell lines even if the activity as a ATP/ADP antiporter was impaired by point mutations.

A further specific feature by which the two ANT isotypes may differ is the formation of contact sites through interaction with the outer membrane pore. The involvement of porin–ANT complexes in contact-site induction was postulated based on the observation that atractyloside, a specific inhibitor of the ANT, and ADP led to formation of contact sites [17]. Indeed, ANT–porin complexes could be generated from isolated porin and ANT *in vitro* [18]. Moreover, a complex of hexokinase with porin and ANT was isolated. Such complexes reconstituted in liposomes or black lipid membranes conferred the system with permeability and conductance properties reminiscent of the mitochondrial PT pore [8,19]. The Ca^{2+}-dependent release of proteoliposome-enclosed substrates was inhibited by cylosporin A, a PT-pore-specific inhibitor [5,20]. It was observed that cyclophilin was co-purified with the hexokinase–porin–ANT complex, but it was also present in variable amounts in a porin–creatine kinase (CK)–ANT complex [19]. This suggested that an ANT–cyclophilin D association might represent the regulated PT pore, as was postulated in [4,21].

It has been proposed that mitochondrial cyclophilin D regulates the Ca^{2+}-dependent PT pore open/closed transitions by reversible association from the matrix side with the ANT in the inner membrane, and that cyclosporin A inhibits the pore through its effects on cyclophilin rather than by a direct effect on the pore [4,21,22].

The presence of the cytosolic enzyme hexokinase together with porin in a complex with the ANT indicated that this ANT must have been derived from the peripheral inner membrane. Crompton et al. [23] analysed binding to immobilized cyclophilin D of proteins from a mitochondrial membrane extract, and ANT–porin complexes were found to be an exclusive docking site for cyclophilin. It was assumed that the affinity of these complexes to cyclophilin correlates with the occurrence of a specific isotype of the ANT located especially in the peripheral inner membrane. In fact, there is a set of data demonstrating that the peripheral inner membrane and cristal membranes can be considered as separate membrane structures. By electron-micro-

Abbreviations used: ANT, adenine nucleotide translocase; PT, permeability transition; CK, creatine kinase; mt-CK, mitochondrial CK; SDH, succinate dehydrogenase.

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scopic study of ultra-thin sections of heart and liver mitochondria in [24] we confirmed earlier observations, obtained by Deams and Wisse [25], that mitochondrial cristae are connected to the peripheral inner membrane through small tubules. Thus the inner mitochondrial membrane cannot be considered as a membrane continuum. Such different views on the mitochondrial structure were confirmed recently by electron-microscopic tomography of liver [26] and brain [27] mitochondria. The morphological difference between peripheral and crista-forming inner membranes was supplemented by functional differences. Whereas the cristal membrane contained cytochrome oxidase and ATP synthase [28,29], a specific organization was observed at the peripheral inner membrane involving energy-transferring contact sites.

Isolation and biochemical characterization of the contacts [30–32] revealed binding of kinases in these sites that directly utilized mitochondrial ATP such as hexokinase and mitochondrial CK (mt-CK). However, by immuno-electron microscopy mt-CK was also localized in the cristae, presumably forming complexes with a different isotype of ANT [33].

In this study two different methods of ANT-isotype identification in the peripheral inner membrane were followed that were based on the fact that, exclusively, the ANT located in this membrane can form complexes with porin in the contact sites. We first induced ANT–porin complexes by atractyloside and ADP and analysed the isotype composition and secondly we used the porin-associated kinases (such as hexokinase) as a handle with which to search for the porin-attached ANT. These complexes were compared with the central crista-derived ANT complex that is generated with mt-CK. Besides the ANT-isotype analysis of the complexes, the cyclophilin content was determined.

MATERIALS AND METHODS

Chemicals and antibodies

All materials were bought from Roche (Mannheim, Germany) and Merck (Darmstadt, Germany) if not indicated otherwise.

The monoclonal antiserum against the N-terminus of human type I porin was obtained from Calbiochem (catalogue no. 529538).

ANT-isoform-specific peptides (ANT-1, GDQALSFLKDF; ANT-2, TDAAVSFAKDF) were synthesized by BioScience (Göttingen, Germany). These amino acid sequences are located at the N-terminus of the respective ANT isoform proteins of rat and mouse. An additional cysteine was added to the N-terminus of the peptides for the coupling to keyhole-limpet haemocyanin. Antibodies were produced by the immunization of rabbits with the keyhole-limpet-haemocyanin-coupled peptides using a standard procedure.

ANT antibody specificity

Solutions of ANT-specific peptides (20–80 ng) were dotted on to two nitrocellulose membranes. The membranes were air-dried and incubated for 1 h at room temperature in blocking solution [PBS with 1 % (v/v) Tween 20], followed by an incubation step with antiserum directed against ANT-1- (1:40) and ANT-2-specific peptides (1:80), respectively. Membranes were washed intensively four times with blocking solution and then incubated for 2 h in PBS/1 % Tween 20 containing 10 ng/ml ^{125}I-Protein A. The filters were washed in blocking solution three times for a period of 20 min. The membranes were air-dried and the dots were cut out of the membrane. Bound ^{125}I-labelled Protein A was measured using a gamma counter. Under these conditions the antibodies were very specific and no cross-reaction was observed.

Isolation of contact sites

Contact sites were isolated by sucrose density-gradient centrifugation from osmotically shocked kidney mitochondria according to the method described by Adams et al. [32]. The method was modified slightly by omitting the sonification step after osmotic shock, and CK activity was measured in the presence of 0.2 % Triton X-100.

Isolation of hexokinase and CK complexes from brain membranes

Rat brain, stored frozen, was homogenized in a Teflon homogenizer in isolation medium (10 mM Hepes/NaOH, pH 7.4/100 mM glucose/1 mM monothioglycerol) at approx. 10 ml/g of wet tissue. The homogenate was centrifuged for 10 min at 4 °C and 10000 ***g*** in a Sorvall centrifuge with an SS-34 rotor. The pellet was re-suspended in the same volume of isolation medium as before and the centrifugation was repeated. This process removed only 10 % of total hexokinase and about 30–40 % of the CK activity (cytosolic isozyme) from rat brain sediment. The last pellet was re-suspended in the washing medium containing 1 % Triton X-100 and was incubated for 45 min at room temperature with gentle stirring. The insoluble membrane material was removed by centrifugation at 45000 ***g*** in a Beckman L8-M ultracentrifuge, with a TI-50 rotor, for 45 min at 4 °C. The supernatant was incubated with DE 52 cellulose that had been equilibrated with 5 mM potassium phosphate, pH 8.0/100 mM glucose/1 mM dithiothreitol. The DE 52 cellulose, loaded with the extract, was packed as a column from which hexokinase and CK activities were eluted by a KCl gradient between 150 and 500 mM and determined spectrophotometrically in the fractions.

Isolation of the ANT from mitochondria of rat heart muscle and liver

The isolation was performed according to Rück et al. [9]. All steps were carried out at 0–4 °C. Heart mitochondria were prepared from three rat hearts by homogenization in a Waring blender 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. Liver mitochondria were prepared in 0.25 M sucrose/10 mM Hepes by differential centrifugation. Isolated heart or liver mitochondria (20 mg) within 1 ml of isolation medium were incubated for 5 min with an equal volume of extraction buffer (1 ml), 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 24000 ***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 equilibration buffer (20 mM Mes/0.2 mM EDTA/0.5 % Triton X-100, pH 6.0). This sample was applied to a 1 ml HiTrap SP cation-exchange column (Pharmacia), connected to an FPLC system and eluted by an NaCl gradient (0–1 M NaCl in column buffer). Fractions of 2 ml were collected with a flow rate of 1 ml/min. The fractions were analysed by SDS/PAGE (12 % gel), Western blotting and immune decoration with specific antibodies against ANT, ANT-1, ANT-2 and porin after transfer on to nitrocellulose membranes.

For the induction of porin–ANT complexes in contact sites, 1 mM atractyloside or 10 mM ADP was added to freshly isolated and respiring mitochondria and incubated for 20 min at room

temperature. Extraction buffer and column buffer also contained 1 mM atractyloside or 10 mM ADP in this case.

Enzyme assays

Hexokinase (EC 2.7.1.1) and CK (EC 2.7.3.2) activities were measured in agreement with Bücher et al. [34]. Both enzymes were determined in a coupled optical enzyme assay with either glucose and ATP or phosphocreatine and ADP as substrates. For hexokinase, glucose-6-phosphate dehydrogenase and NADP were used for the indicator reaction, whereas for CK the activity was indicated using yeast hexokinase and glucose-6-phosphate dehydrogenase.

Peptidylprolyl *cis-trans* isomerase (EC 5.2.1.8) activity was determined according to Schutkowski et al. [35]. The determination of succinate dehydrogenase (SDH, EC 1.3.99.1) activity was performed as described in Brdiczka et al. [29]. Rotenone-insensitive NADH–cytochrome *c* reductase (EC 1.6.2.2) was determined according to Sottocasa et al. [36].

Isolation of kidney mitochondria

Mitochondria from rat kidney were isolated using a method described previously [32] by differential centrifugation in mannitol/sucrose/Hepes medium (0.25 M mannitol/0.075 M sucrose/1 mM EGTA/5 mM Hepes, pH 7.4). The last sediment was re-suspended in sucrose/Hepes medium (0.25 M sucrose/10 mM Hepes, pH 7.4).

Protein assay, electrophoresis, trans-blot and immune decoration

Protein was determined by the method of Lowry et al. [37]. PAGE was performed according to Laemmli [38]. Electrotransfer and immune decoration were performed as described by Rott and Nelson [39].

N-terminal sequence analysis of ANT

Fractions of the eluate from a DEAE-cellulose column corresponding to the different peaks of mt-CK activity were collected and concentrated by dry dialysis against poly(ethylene glycol) overnight at 4 °C. Protein was determined and 200 μg from each mt-CK peak was run on SDS/PAGE. After separation by electrophoresis the ANT band was determined by molecular mass and immune decoration. The ANT band was excised from the gel. The polypeptide was extracted, de-salted by a Sephadex PG-10 column (Pharmacia) and concentrated as described above. Preparation of methyl-isothiocyanate derivatives of the N-terminal end of the protein and subsequent determination of the products by GC were performed according to the method described by Pisano et al. [40].

RESULTS

ANT antibody specificity

The antibody specificity was very important in the following investigations. It was analysed by the dot-blot technique. Dilutions of ANT-specific peptides (20–80 ng) was dotted on to two nitrocellulose membranes. After drying and incubation with blocking buffer as described in the Materials and methods section, the cellulose sheets were incubated with antiserum directed against ANT-1-specific (1:40) and ANT-2-specific (1:80) peptides, respectively. Membranes were washed intensively with blocking solution and then incubated for 2 h in PBS/1 % Tween 20 containing 10 ng/ml ^{125}I-Protein A. Subsequently the filters were again washed intensively in blocking solution. The dots were cut out of the air-dried membranes and bound ^{125}I-labelled Protein A was measured using a gamma counter. Figure 1 shows bound radioactivity representing the antibodies against ANT-1 (Figure 1A) and ANT-2 (Figure 1B) versus antigen concentration. Under these conditions the antibodies were very specific and no cross-reaction was observed. Further proof of the antibody specificity will be provided in Figure 5 (see below), where the ANT in liver mitochondria, which do not contain isotype 1, was investigated. No reaction with ANT-1-specific antibodies was observed in contrast with antibodies against isotype 2.

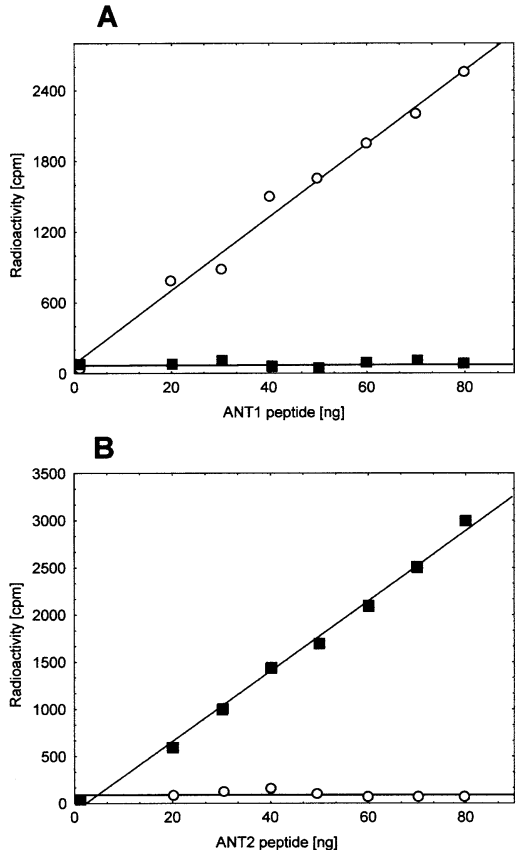

Figure 1 Antibody specificity

Dilutions (20–80 ng) of ANT-1- (**A**) and ANT-2- (**B**) specific peptides were dotted on to nitrocellulose membranes and incubated with ANT-1- (○) and ANT-2- (■) specific antisera. After incubation in a ^{125}I-labelled Protein A solution, dots were cut out of the membrane and bound ^{125}I-labelled Protein A was measured using a gamma counter. The radioactivity representing bound antibodies was plotted against the applied peptide amount. No cross-reactivity was observed, proving that the antibodies used were highly specific.

Isolation and characterization of the hexokinase and CK complexes from brain and kidney

The hexokinase complex was isolated from brain and kidney membranes as described in [8,19] by anion-exchange chromatography. Figure 2(A) depicts a representative experiment. It shows the elution profiles of hexokinase, CK and cyclophilin (peptidylprolyl *cis-trans* isomerase) activities extracted from brain membranes. The activity of hexokinase formed one peak (fractions 31–43) that was separated from two CK peaks. The first two CK fractions (22–26 and 27–28) were identified by isoenzyme electrophoresis as mt-CK. Cyclophilin activity was

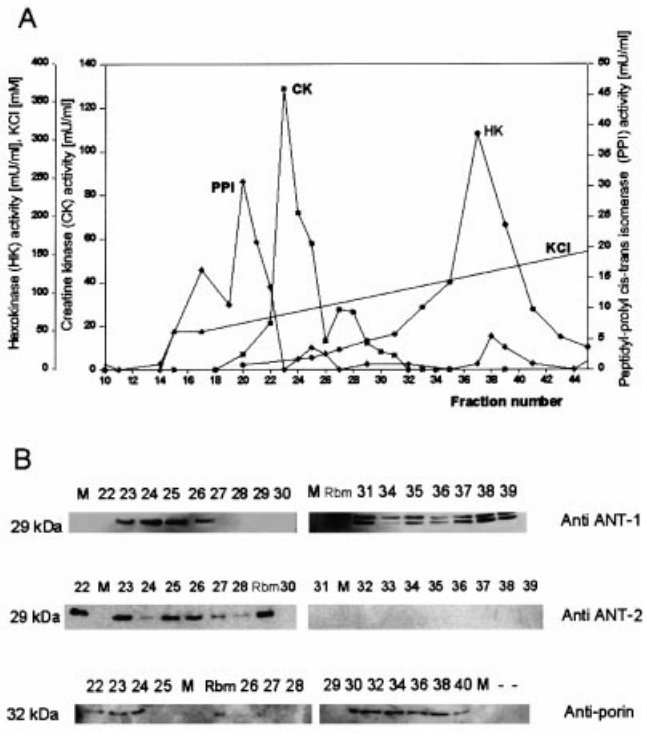

Figure 2 Isolation and characterization of the hexokinase complex from total brain membranes

Upper panel: elution profile of hexokinase (HK, ●), peptidylprolyl *cis-trans* isomerase (PPI; ◆) and CK (■) activities from a DEAE-cellulose column. The extraction and chromatography procedures were performed as described in the Materials and methods section. The activity was eluted by a salt gradient ranging from 150 to 500 mM KCl. Lower panel: fractions corresponding to the hexokinase (31–39) and CK (22–30) activity peaks were run on PAGE. The separated polypeptides were blotted on to nitrocellulose membranes. The cellulose sheets were treated with monoclonal antibodies against porin and ANT-1 and ANT-2. M = molecular-mass marker lane; Rbm, rat brain mitochondria.

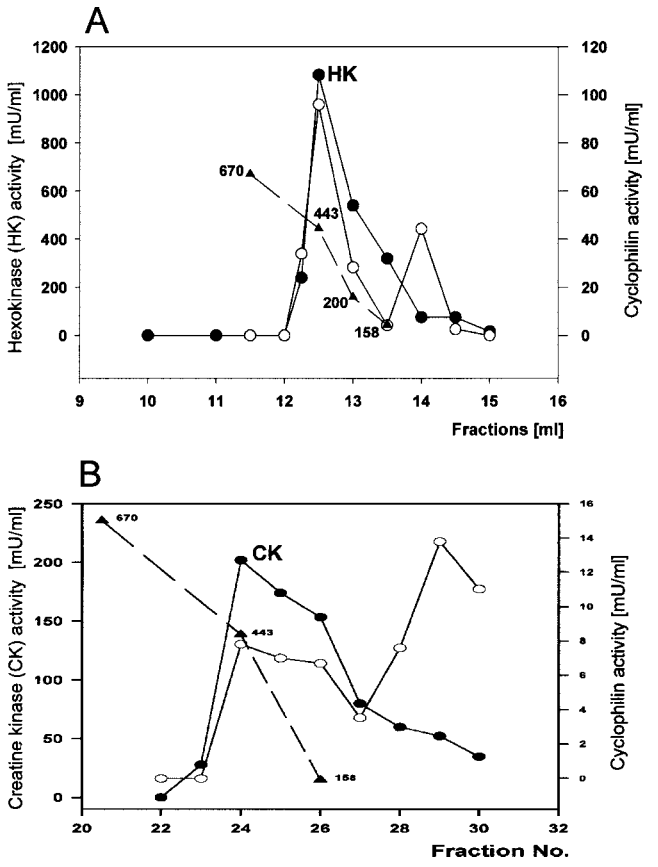

Figure 3 Gel-permeation chromatography of the hexokinase and CK complex

(**A**) The hexokinase activity peak from the DEAE-cellulose column (Figure 2) was loaded on to a Bio-Sil SEC 400 gel-filtration column (Bio-Rad) and was eluted with 5 mM Na_2HPO_4, pH 6.8/100 mM glucose/150 mM NaCl. The eluate was collected in 120 fractions of 250 μl in which the activities of hexokinase (HK, ●) and cyclophilin (peptidylprolyl *cis-trans* isomerase, ○) were monitored by optical testing. The Bio-Sil column was calibrated by separate runs under the same conditions as above using thyroglobulin, apoferritin, catalase and IgG as molecular-mass standards corresponding to 670, 443, 200 and 158 kDa (▲) respectively. Peak determination in the calibration runs was performed by absorbance measurement at 280 nm. (**B**) The first CK activity peak from the DEAE-cellulose column (fractions 22–24, Figure 2) was loaded on to a Bio-Sil SEC 400 gel-filtration column and treated in the same way as described for (**A**). CK (●) and cyclophilin (○) activities were determined in the eluate.

distributed among different peaks. The highest activity was eluted in the first fractions (15–19) and may represent cytosolic cyclophilin. Two other cyclophilin activity peaks (24–26 and 37–41) correlated with the first mt-CK and the hexokinase activity peak.

Fractions 31–39, containing hexokinase activity, were run on PAGE and transferred on to nitrocellulose membranes. The cellulose sheets were decorated with antibodies against porin and ANT. Porin, the hexokinase-binding protein, was present in all hexokinase fractions. Porin was also observed in fractions 22–24, which contained the first mt-CK peak, indicating that this CK may be derived from the mitochondrial periphery. The immunological identification of ANT isotypes clearly showed the presence of ANT-1 in fractions 31–39 correlating to the hexokinase complex, whereas antibodies against ANT-2 did not bind to these fractions. In contrast, antibodies against ANT-2 and ANT-1 were bound to fractions 22–28 containing the two mt-CK peaks (Figure 2B).

The double band seen upon decoration with anti-ANT-1 in Figure 2 was also observed in Figure 9 (see below) after interaction with antibodies against ANT-2. It thus appears not to be isotype- or antibody-specific. The nature of the ANT modification causing slightly faster migration is, as yet, not known; however, we did not observe any phosphorylation.

Identification of cyclophilin in the kinase–porin–ANT complexes

The mitochondrial cyclophilin D has been characterized as an ANT-binding protein [23,41]. It was therefore suggested that the cyclophilin activity found in the porin-containing fractions (Figure 2) might be bound to the ANT in the first mt-CK and the hexokinase complex. To test this assumption, fractions containing hexokinase activity or the first mt-CK activity peak of the anion-exchange chromatography were separated by size-exclusion chromatography. As shown in Figure 3(A), hexokinase was confined to a fraction corresponding to a molecular mass of 440 kDa, indicating that in this fraction hexokinase existed in the form of a tetramer (400 kDa), bound to porin (30 kDa) and ANT (30 kDa), as was shown by Beutner et al. [8]. Essential cyclophilin activity was co-migrating with the hexokinase activity. However, a second activity peak of cyclophilin was observed that was corresponding to free cyclophilin oligomers and also monomers of 20 kDa. The data demonstrated that part

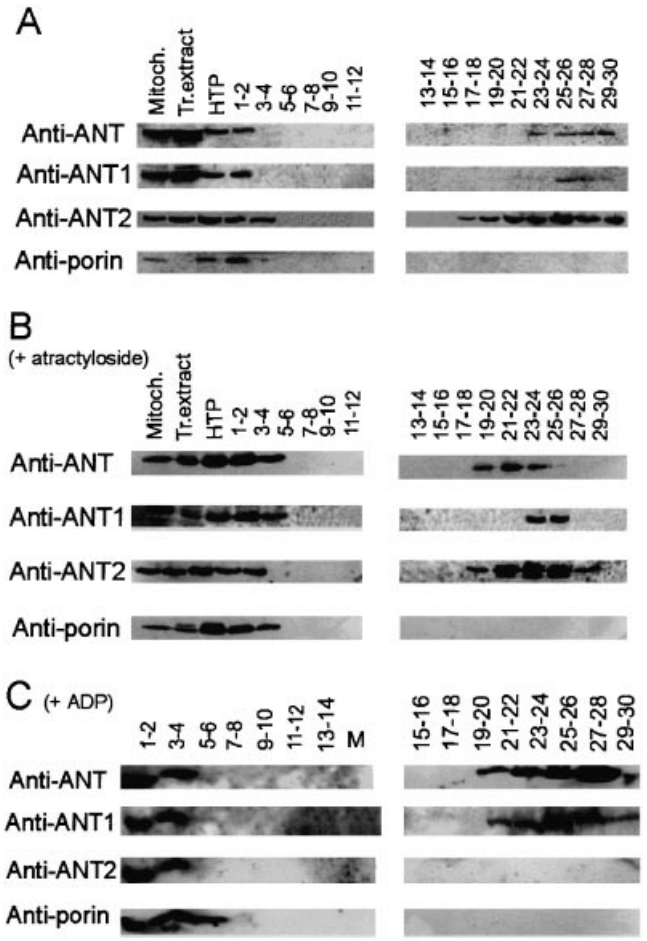

Figure 4 Isolation of porin–ANT complexes from heart muscle mitochondria

ANT was prepared as described in the Materials and methods section. PAGE and immunological identification on blots of the ANT was performed in the different preparation steps: mitochondria (Mitoch.), Triton extract (Tr.extract), eluate from the hydroxyapatite column (HTP) and fractions 1–30 of the cation-exchange chromatography (HiTrap). The ANT was characterized by an isotype-unspecific antibody (Anti-ANT) and specific antibodies against ANT-1 or ANT-2. In addition, immune decoration by porin-specific antibodies was performed. (**A**) Preparation of ANT in the absence of atractyloside. (**B**) Preparation of ANT in the presence of 1 mM atractyloside. (**C**) Preparation of ANT in the presence of 10 mM ADP.

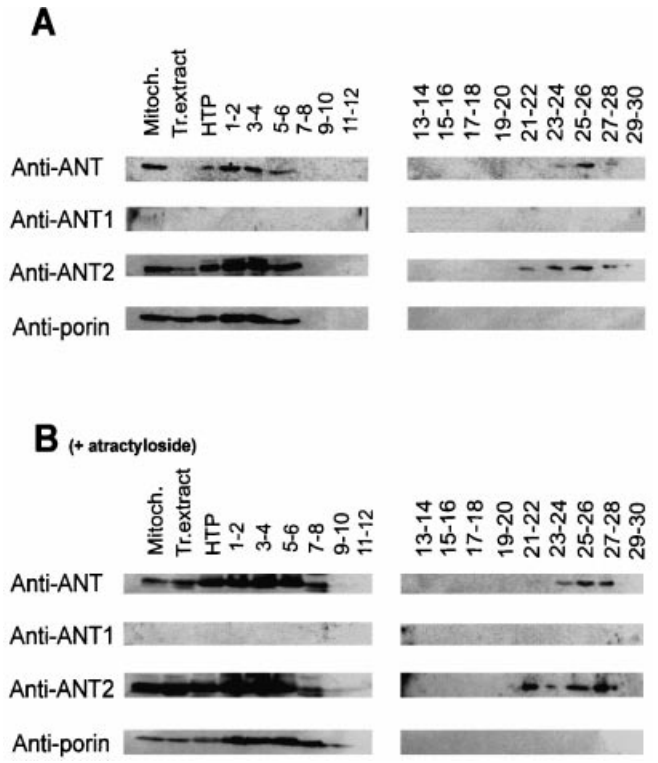

Figure 5 Isolation of porin–ANT complexes from liver mitochondria

The same procedure as described in Figure 4 was applied to isolated liver mitochondria. (**A**) Preparation of ANT in the absence of atractyloside. (**B**) Preparation of ANT in the presence of 1 mM atractyloside.

of the cyclophilin remained bound to the hexokinase–porin–ANT complex. Comparable results were obtained when the first mt-CK fractions of the anion-exchange chromatography (Figure 2) were analysed by size-exclusion chromatography. The CK activity was distributed in fractions corresponding to a molecular-mass range of between 440 and 150 kDa (Figure 3B). This indicated that most of the mt-CK in the complex was associated as an octamer of 350 kDa. As shown previously the high-molecular-mass fractions of mt-CK contain porin and ANT [8]. In addition, significant activity of cyclophilin was bound to the mt-CK complex, as it was eluted in the high-molecular-mass fractions. However, higher cyclophilin activity was found in fractions that corresponded to a lower molecular mass, demonstrating free cyclophilin.

Isolation of porin–ANT complexes from heart mitochondria

Based on the observation that atractyloside or ADP increased the frequency of contact sites, we investigated whether atractyloside might induce ANT–porin complex formation where a specific ANT isotype is presented. The ANT was extracted by Triton X-100 from heart or liver mitochondria after treatment with atractyloside or ADP. It was purified by a run through a hydroxyapatite column [42]. The ANT eluate still contained porin. To analyse an interaction between specific ANT isoforms and porin, the ANT eluate was applied to a cation-exchange (HiTrap) column [9]. Whereas free recombinant porin was not bound, the porin–ANT complex was eluted from this column in fractions 4 and 5 at low NaCl concentrations. Porin-free ANT was found in fractions 20–28 at high NaCl concentrations. Figure 4(A) shows different steps of the ANT isolation from heart mitochondria analysed by PAGE followed by antibody decoration using various ANT-specific antibodies. Both ANT isotypes were present in the mitochondria, Triton extract and hydroxyapatite eluate. ANT-1 appeared to be present in the hydroxyapatite eluate. Also, a smaller amount of ANT-1 was eluted from the cation exchanger in fractions 1 and 2 together with porin and in fractions 25–30 without any porin. In contrast with this, in the presence of atractyloside (Figure 4B), ANT-1 was also present in significantly higher amounts in the first four fractions that were eluted from the cation-exchange column. This correlated with higher concentrations of porin in the same fractions. In addition, higher amounts of ANT-1 were found in the presence of atractyloside in fractions 23–26, where more ANT-2 was also eluted. We also applied the method of ANT preparation to heart mitochondria in the presence of 10 mM ADP. Figure 4(C) depicts the immune analysis of only the NaCl

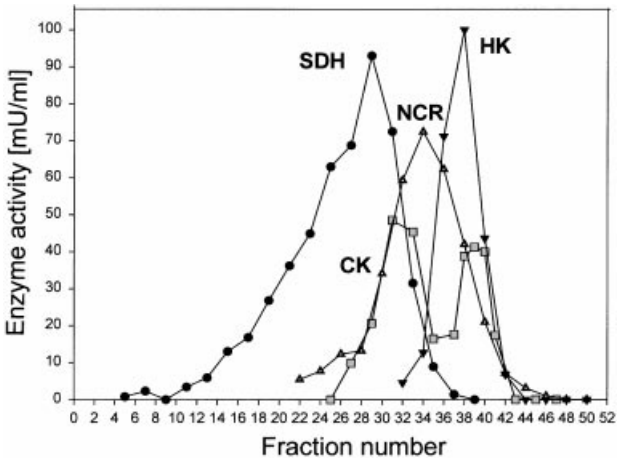

Figure 6 Isolation of contact sites from kidney mitochondria

Contact sites were isolated from kidney mitochondria disrupted by osmotic shock and separated by sucrose density gradient by centrifugation as described in the Materials and methods section. To characterize the membrane subfractions in the gradient, specific marker enzymes were used: hexokinase (HK, ▼) representing contact sites, activity of rotenone-insensitive NADH oxidase (NCR, △) as marker for outer membrane and SDH (●) as a marker for inner membrane and unbroken mitochondria. Activity of CK (□) is distributed in the contact-site fractions but also in the inner-membrane fractions. Each fraction corresponds to 0.46 ml. Sucrose density changes between 53% in fraction 1 and 30.8% in fraction 50.

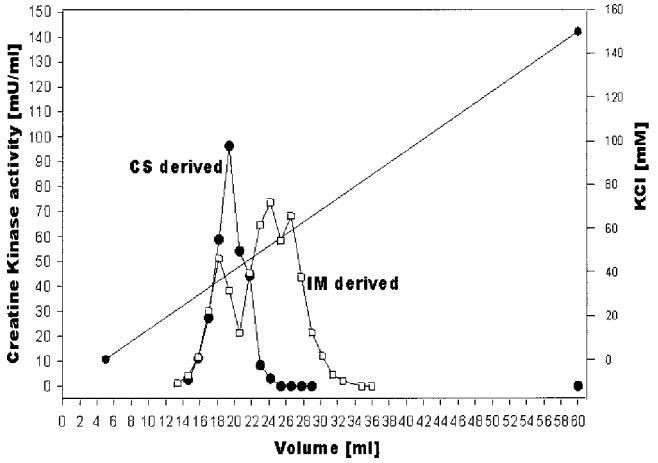

Figure 7 Characterization of contact-site- and crista-derived CK fractions by anion-exchange chromatography

Fractions 25–33 (inner-membrane-derived, IM derived) and 37–43 (contact-site-derived, CS derived) from the density gradient in Figure 6 were collected and the mt-CK complex was extracted by 1% Triton X-100 as described in the Materials and methods section. The extracts were bound to an anion-exchange column of 28 g of pre-swollen DEAE-cellulose equilibrated with 1.5 mM phosphate buffer, pH 7.4/1 mM dithiothreitol. The elution profiles (0–150 mM KCl) of mt-CK from contact-site-derived extract (CS derived, ●) and inner-membrane-derived extract (IM derived, □) are shown.

eluates from the cation-exchange column. In the presence of ADP porin complexes were formed with ANT-1 and ANT-2 in fractions 1–4. Interestingly, mainly free ANT-1 and almost no free ANT-2 was bound to the column and was eluted in fractions 21–29 (compare with Figures 4A and 4B)

The results showed that both ANT isotypes were co-eluted with porin so they can both form a complex with porin. However, ANT-1 appears to form complexes with porin preferentially in the cytosol-oriented c-conformation, which was induced by atractyloside, whereas porin interaction with ANT-2 was independent of its conformation.

Isolation of porin–ANT complexes from liver mitochondria

In order to analyse the atractyloside influence on the ANT-2 porin interaction more closely we isolated ANT–porin complexes from liver mitochondria. According to Stepien et al. [1] and Dörner et al. [2], liver mitochondria are almost lacking ANT-1. When the same method of ANT preparation was applied to liver mitochondria no reaction with antibodies against ANT-1 in the ANT preparation was observed (Figure 5) whether atractyloside was present or not. However, the concentration of ANT-2 porin complexes appeared to be somewhat increased (Figure 5B). This means that ANT-2 might also have a higher affinity for porin in the c-conformation.

Separation of mt-CK complexes derived from contact sites or cristal membranes

The hexokinase–porin–ANT complex could be attributed explicitly to the contact sites formed by the peripheral inner membrane [30–32]. mt-CK, however, was found to be distributed in the periphery, but was also located along the cristae [33]. In this study, the contact sites were isolated from osmotically shocked kidney mitochondria by sucrose density centrifugation. Figure 6 shows the distribution of specific marker enzymes: hexokinase I, which specifically binds in the contact sites, was separated from SDH, representing the inner membrane. However, rotenone-insensitive NADH oxidase, a marker for the outer membrane, was distributed in the hexokinase- and SDH-containing fractions of the gradient. This means that contact sites could be separated by this method, but the fractions with high SDH activity, besides cristal membranes, contained contact sites as well as unbroken mitochondria. The CK activity was distributed in two peaks. One CK peak coincided with that of hexokinase, whereas a second CK activity maximum was present in the fractions with high SDH activity. It was observed that CK in the latter fractions was less accessible to substrates and thus activity was determined in the presence of Triton X-100. The fractions containing hexokinase activity (representing contacts) and those with high activity of SDH (representing cristal membranes) were collected, and the CK complexes were extracted with 1% Triton X-100 from the membrane sediments. The extracts were bound to DEAE-cellulose and the complexes were subsequently eluted from the anion exchanger by a KCl gradient at pH 9. Figure 7 shows that two mt-CK fractions could be eluted from the anion exchanger. The contact-site-derived mt-CK was eluted at lower KCl concentrations and formed mainly one activity peak, whereas the extract of the inner membrane fraction contained two mt-CK activity peaks. The first peak coincided with the contact-derived mt-CK, whereas the second, larger activity peak was eluted at higher KCl concentrations. The results suggested that the density gradient centrifugation resulted in separation of contact sites from the rest (unbroken mitochondria, contacts and inner membrane fragments) rather than pure inner membrane. In addition, we found that contact-site-derived and central mt-CK complexes differed in net electrical charge. Therefore, it was assumed that it is possible to directly isolate the two complexes from a total membrane extract.

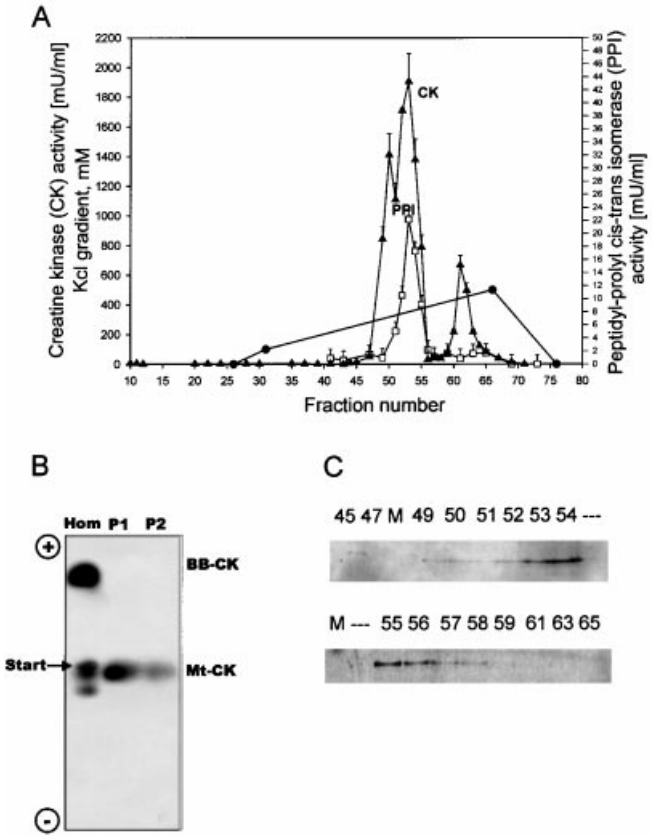

Figure 8 Isolation and characterization of two different mt-CK complexes from total brain membranes

(**A**) Activity profiles of CK (▲) and cyclophilin (peptidylproly *cis-trans* isomerase, PPI, □) upon elution from anion-exchange column. The preparation of the extract and column chromatography were performed as described in the Materials and methods section. The enzyme activities were eluted by a gradient between 150 and 350 mM KCl (●). The data are means ± S.D. from three experiments. (**B**) Isoenzyme electrophoresis of the first (P1) and second (P2) CK activity peaks separated in (**A**). In the left-hand lane an extract of brain homogenate (Hom) was run containing cytosolic BB isoform and mt-CK. About 20 μ-units of enzyme activity were loaded in each lane. (**C**) Fractions corresponding to the first (51–54) and second (61–64) CK activity peaks shown in (**A**) were run on SDS/PAGE, blotted on to nitrocellulose membranes and decorated by specific antibodies against porin as a marker protein for the outer mitochondrial membrane. M, molecular-mass marker lane.

Direct isolation of peripheral and central mt-CK ANT complexes from total brain membranes

As observed in previous investigations and Figure 2, it was possible to isolate porin–mt-CK–ANT complexes directly from rat brain membranes [19]. We therefore attempted to separate the peripheral porin–mt-CK–ANT complex and a central mt-CK–ANT complex from their mixture in the same extract with 1% Triton X-100. The extract was bound to an anion exchanger and two different CK activity peaks were eluted by KCl gradient (Figure 8A). Hexokinase activity was not detected in the CK fractions (results not shown). By applying isoenzyme electrophoresis, the two different CK fractions could be characterized as having mt-CK activity only (Figure 8B). This suggested that two mt-CK fractions exposing different net electrical charges could be separated. Considering the behaviour of the contact-site-derived mt-CK described above, it appeared that the first mt-CK fraction could be assigned to the periphery. To prove this, the presence of porin and cyclophilin in the mt-CK complexes was investigated.

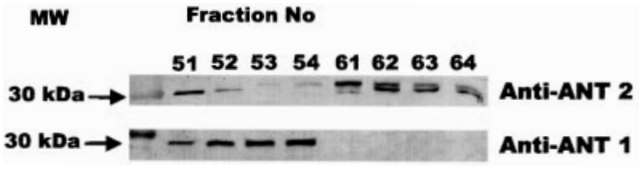

Figure 9 Immunological identification of ANT isotypes

The fractions containing the peripheral (lanes 51–54) and the central (lanes 61–64) mt-CK complexes separated by DEAE-cellulose column chromatography (Figure 8A) were run on PAGE. The polypeptides were blotted on to nitrocellulose membranes. The nitrocellulose sheets were incubated with specific antibodies against the N-terminal ends of the ANT-1 and ANT-2 isoforms. Each lane contained 25 μg of total protein. ANT-1 appears to be a component of the peripheral mt-CK complex, whereas ANT-2 is present in the peripheral and crista-derived complexes.

Table 1 Determination of the N-terminal amino acid sequences of ANT isotypes in the two mt-CK complexes from rat brain

The amino acids that are different in the two isotypes are underlined; those that are identical to either one or the other isotype are shown in italics; bold residues indicate mismatches between the published sequence and that found in the two complexes.

Source	Sequence
ANT isotype 1 (rat liver and heart)[a]	MGDQALSF
Peripheral mt-CK complex	*MGDQA***I***SF*
Sequence for generating type I antibodies	GDQALSFLKDF
ANT isotype 2 (rat liver)[a]	MTDAAVSF
Central mt-CK complex	*MTDAA***L***SF*
Sequence for generating type II antibodies	TDAAVSFAKDF

[a] From the Expasy data bank.

Distribution of cyclophilin and porin in the two mt-CK complexes

It was observed that most of the cyclophilin activity was concentrated in the first CK activity fractions (Figure 8A). Immunological analysis, using porin-specific antibodies, showed that porin distribution also correlated with the first cyclophilin-containing mt-CK peak (Figure 8C).

Characterization of ANT isotypes in peripheral and central mt-CK complexes

Samples of the two mt-CK activity peaks were run on PAGE and the separated polypeptides were transferred on to nitrocellulose sheets that were decorated with specific antibodies against ANT isotypes 1 and 2 (Figure 9). It was observed that isotype 1 was present exclusively in the peripheral complex, whereas isotype 2 was distributed in both complexes. This agreed with the analysis of the hexokinase complex from brain and kidney mitochondria that also contained mostly ANT-1. Taken together, both results showed that the peripheral inner membrane contains most if not all of the ANT-1 present in a mitochondrion and also ANT-2, whereas the crista-forming part is composed mainly of ANT-2.

In addition to the immunological identification, the two ANT isotypes from the different mt-CK complexes were characterized by sequencing their N-terminal ends. It is known that the two isotypes differ by three of the eight amino acids at the N-terminus. The ANT that was isolated from the two rat brain mt-CK complexes was subjected to Edman amino acid degradation of the N-terminal end. The results were identical with the

immunological analysis showing again two different ANT isotypes in the two mt-CK complexes (Table 1). When compared with the known sequences of ANT-1 and ANT-2, the ANT sequence from the peripheral complex was identical with that of isotype 1 and that from the central complex was almost equal to isotype 2.

DISCUSSION

Of all mitochondrial inner membrane proteins, ANTs are the most abundant components. They are encoded by three different but related genes earlier referred to as T1, T2 and T3 [43,44]. The roles of the different ANT isoforms still remain to be understood, although attention has been drawn to them in the last few years because of the interest in mitochondria due to their central role in programmed cell death. The critical role of the ANT in apoptosis is rather controversial. Based on data from ANT-deficient yeast it has been concluded that the ANT is not required for Bax-dependent cytochrome *c* release and $\Delta\Psi$ loss [45], thus indicating that ANT is not involved in apoptotic mitochondrial changes. At the same time, the demonstration that ANT-1 over-expression in cells resulted in apoptotic degradation, while ANT-2 over-expression did not do this, gives strong evidence for ANT-1 involvement in programmed cell death [16]. Besides this, it has been shown that bongkrekic acid prevents the oxygen radical burst concomitant with PT, suggesting an apparent role for ANT in the net generation of reactive oxygen species by mitochondria and activation of the apoptotic cascade [46].

The peripheral inner membrane contains both ANT isotypes

This study has focused on the analysis of ANT complexes derived from the peripheral inner membrane. The strategy for the elucidation of ANT localization was based, first, on an appropriate use of hexokinase and the peripheral mt-CK as instruments to identify the outer-membrane and peripheral inner membrane complexes with porin and ANT, and second on the induction of the complexes between ANT and the outer-membrane pore by atractyloside or ADP followed by isotype analysis. Identification of the peripheral inner membrane was performed through the presence of porin in the hexokinase complex (Figure 2B) and the peripheral mt-CK complex (Figure 8C). In agreement with recent observations [8,19], the ANT was a constituent of the isolated hexokinase and mt-CK complexes. Immuno-decoration by isotype-specific ANT antibodies and sequencing of the N-terminal ends of the different ANT peptides indicated that preferentially isotype 1 was present in the hexokinase and mt-CK complexes that were derived from the peripheral inner membrane (Figures 2, 8 and 9, and Table 1). However, in both complexes ANT-2 was also found as a minor constituent.

ANT–porin complexes were isolated directly from extracts of isolated heart and liver mitochondria that were pre-treated with either atractyloside or ADP. It was observed that atractyloside and ADP induced porin complexes mainly with ANT-1 (Figure 4), whereas porin complexes with ANT-2 were observed also in the absence of atractyloside. This showed that both ANT isotypes co-exist in the peripheral inner membrane, but behave differently in forming complexes with porin.

The mt-CK complex derived from cristal membranes contains mainly ANT-2

In order to obtain ANT derived from cristal membranes we again used the isolation of a kinase complex. Compared with the attempt to isolate pure mitochondrial cristal membranes this method has the advantage of higher purity of the isolated complex components. However, it might have the disadvantage that not all ANT in the cristae forms complexes with mt-CK. Kusnetsov and Saks [47] calculated molar ANT/CK ratios of 1:1 in heart muscle. It was thus expected that all mt-CK could interact with the ANT in the cristae. After separation of the peripheral and central mt-CK complexes by chromatography (Figures 7 and 8) the central complex was characterized by the absence of porin. In the mt-CK complex derived from the cristae, ANT isotype 2 was present exclusively and no ANT-1 could be detected (Figure 9 and Table 1).

ANT-1 isotype may have a higher affinity for cyclophilin

Besides porin, the two peripheral kinase complexes were characterized by the presence of cyclophilin (Figures 2 and 8, [19]), whereas cyclophilin activity was absent from the crista-derived CK complex. The peripheral CK complex contained both ANTs, whereas the central complex only contained ANT-2. Thus the absence of cyclophilin from the crista-derived complex leads to the assumption that ANT-1 has a higher affinity for cyclophilin than ANT-2. Immobilized cyclophilin D was shown recently to have a specific affinity for the ANT [23,41]. The ANT fractions that were used for these binding studies were different. Woodfield et al. [41] prepared the ANT-containing extract from enriched inner membrane particles devoid of outer membrane, whereas Crompton et al. [23] extracted the ANT from total membranes of isolated heart muscle mitochondria in the presence of 0.1 mM ADP. Thus the first extract contained mainly crista-derived ANT-2, whereas the latter extract contained both ANT isotypes and, in addition, porin–ANT complexes were induced by ADP. Although the crista-derived ANT-2 in the latter case was certainly the major component, mainly ANT–porin complexes were bound to cyclophilin. Considering our results that ADP induced porin complexes with the ANT-1, this indicates that the peripheral ANT isotype 1 had a higher affinity for cyclophilin.

Different functions of ANT isotypes?

The ANT-1 and ANT-2 isotypes are present in tissues with a high energy turnover, such as heart muscle, skeletal muscle, brain and kidney [1,2], whereas liver appears to have only ANT-2 (Figure 5; [1]). Our data suggest that both ANT isotypes were presumably existing in every single mitochondrion. Otherwise one would have to assume mitochondria with no ANT in the cristae. ANT-2 was distributed in the peripheral and crista-forming part of the inner membrane, whereas ANT-1 was observed mainly in the peripheral inner membrane. Thus this isotype must have a special property that optimizes common functions of both ANTs according to the specific location. Comparing the two ANTs, two functions were found to be different in the present investigation: first, ANT-1 tended to form porin complexes upon induction by either atractyloside or ADP, and secondly ANT-1 might have a higher affinity for cyclophilin. Both properties might be important in the constitution [8,23] and regulation of the mitochondrial PT [8,23,41]. It is still controversial as to whether the ANT forms the PT pore. In case the ANT would open as a PT pore, it would be ANT-1 that is involved preferentially in this process, whereas the central ANT-2 might be more engaged in energy supply.

A similar conclusion was drawn in a recent publication of Bauer et al. [16]. The authors identified the ANT-1 gene as a dominant apoptosis-inducing gene, whereas expression of the ANT-2 gene did not have this effect. In addition, they observed

that co-expression of cyclophilin led to a suppression of ANT-1-induced apoptosis.

The physiologically different functions of the two ANT isotypes may explain why it was possible to generate mice with a deficiency in ANT-1 only. The mice survived, but exhibited biochemical, histological and metabolic characteristics of mitochondrial myopathy and cardiomyopathy [48]. This means that the two isotypes have specific physiological functions and cannot replace each other. It is thus not surprising that a change in the relation of peripheral versus central ANT isoforms can cause pathological effects. In dilated cardiomyopathy ANT-1 was significantly increased with a simultaneous reduction of ANT-2 [49], resulting in induction of apoptosis [16].

In general, the specific localization of the two ANT isotypes points to the little-recognized fact of different functions and structures of the peripheral inner membrane compared with the crista-forming part. This may be especially important in tissues with high and rapidly fluctuating energy turnover.

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REFERENCES

1 Stepien, G., Torroni, A., Chung, A. B., Hodge, J. A. and Wallace, D. C. (1992) Differential expression of adenine nucleotide translocator isoforms in mammalian tissues and during muscle cell differentiation. J. Biol. Chem. **267**, 14592–14597

2 Dörner, A., Pauschinger, M., Badorff, A., Noutsias, M., Giessen, S., Schulze, K., Bilger, J., Rauch, U. and Schultheiss, H. P. (1997) Tissue-specific transcription pattern of the adenine nucleotide translocase isoforms in humans. FEBS Lett. **414**, 258–262

3 Dierks, T., Salentin, A., Heberger, C. and Krämer, R. (1990) The mitochondrial aspartate/glutamate and ADP/ATP carrier switch from obligate counterexchange to unidirectional transport after modification by SH-reagents. Biochim. Biophys. Acta **1028**, 268–280

4 Halestrap, A. P. and Davidson, A. M. (1990) Inhibition of Ca^{2+} induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl *cis-trans* isomerase and preventing it interacting with the adenine nucleotide translocase. Biochem. J. **268**, 153–160

5 Crompton, M., Ellinger, H. and Costi, A. (1988) Inhibition by cyclosporin A of a Ca^{2+}-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. Biochem. J. **255**, 357–360

6 Brustovetsky, N. and Klingenberg, M. (1996) The mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by Ca^{2+}. Biochemistry **35**, 8483–8488

7 Tikhonova, I. M., Andreyev, A. Yu., Antonenko, Yu. N., Kaulen, A. D., Komrakov, A.Yu. and Skulachev, V. P. (1994) Ion permeability induced in artificial membranes by the ATP/ADP antiporter. FEBS Lett. **337**, 231–234

8 Beutner, G., Rück, A., Riede, B., Welte, W. and Brdiczka, D. (1996) Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore. FEBS Lett. **396**, 189–195

9 Rück, A., Dolder, M., Wallimann, T. and Brdiczka, D. (1998) Reconstituted adenine nucleotide translocase forms a channel for small molecules comparable to the mitochondrial permeability transition pore. FEBS Lett. **426**, 97–101

10 Susin, S. A., Lorenzo, H. K., Zamzami, N., Marzo, I., Snow, B. E., Brothers, G. M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M. et al. (1999) Molecular characterisation of mitochondrial apoptosis-inducing factor. Nature (London) **397**, 441–446

11 Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P. and Wang, X. (1997) Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science **275**, 1129–1132

12 Liu, X., Kim, C. N., Yang, J., Jemmerson, R. and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell **86**, 147–157

13 Kluck, R. M., Bossy-Wetzel, E., Green, D. R. and Newmeyer, D. D. (1997) The release of cytochrome *c* from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science **275**, 1132–1136

14 Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome *c*-dependent caspase activation by eliminating IAP inhibition. Cell **102**, 33–42

15 Verhagen, A., Ekert, P. G., Pakusch, M., Silke, J., Conolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J. and Vaux, D. L. (2000) C Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell **102**, 43–53

16 Bauer, M. K., Schubert, A., Rocks, O. and Grimm, S. (1999) Adenine nucleotide translocase-1, a component of the permeability transition pore, can dominantly induce apoptosis. J. Cell Biol. **147**, 1493–1502

17 Bücheler, K., Adams, V. and Brdiczka, D. (1991) Localisation of the ATP/ADP translocator in the inner membrane and regulation of contact sites between mitochondrial envelope membranes by ADP. A study on freeze fractured isolated liver mitochondria. Biochim. Biophys. Acta **1061**, 215–225

18 Bühler, S., Michels, J., Wendt, S., Rück, A., Brdiczka, D., Welte, W. and Przybylski, M. (1998) Mass spectrometric mapping of ion channel proteins (porins) and identification of their supramolecular membrane assembly. Proteins **2**, 63–73

19 Beutner, G., Rück, A., Riede, B. and Brdiczka, D. (1998) Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. Biochim. Biophys. Acta **1368**, 7–18

20 Broekemeier, K. M., Dempsey, M. E. and Pfeiffer, D. R. (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. J. Biol. Chem. **264**, 7829–7830

21 McGuinnes, O. M., Yafei, N., Costi, A. and Crompton, M. (1990) The presence of two classes of high-affinity cyclosporin A binding sites in mitochondria. Evidence that the minor component is involved in the opening of an inner-membrane Ca(2+)-dependent pore. Eur. J. Biochem. **194**, 671–679

22 Nicolli, A., Basso, E., Petronelli, V., Wenger, M. and Bernardi, P. (1996) Interactions of cyclophilin with the mitochondrial inner membrane and regulation of the permeability transition pore, and cyclosporin A-sensitive channel. J. Biol. Chem. **271**, 2185–2192

23 Crompton, M., Virji, S. and Ward, J. M. (1998) Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. Eur. J. Biochem. **258**, 729–735

24 Brdiczka, D. and Reith, A. (1987) Functional and structural heterogeneity of the inner mitochondrial membrane. In The Organisation of Cell Metabolism (Welch, G. R. and Clegg, J. S., eds.), pp. 277–287, Plenum Publishing, New York

25 Deams, W. T. and Wisse, E. (1966) Shape and attachment of the cristae mitochondriales in mouse hepatic cell mitochondria. J. Ultrastruct. Res. **16**, 123–140

26 Mannella, C., Marko, M. and Buttle, K. (1997) Reconsidering mitochondrial structure: new views of an old organelle. Trends Biochem. Sci. **22**, 37–38

27 Perkins, G., Renken, C., Martone, M. E., Young, S. J., Ellisman, M. and Frey, T. (1997) Electron tomography of neural mitochondria: three-dimensional structure and organisation of cristae and membrane contacts. J. Struct. Biol. **119**, 260–272

28 Fernández-Morán, H., Oda, T., Blair, P. V. and Green, D. E. (1964) Negative staining of mitochondrial membranes. J. Cell Biol. **22**, 63–70

29 Brdiczka, D., Dölken, G., Krebs, W. and Hofman, D. (1974) The inner boundary membrane of mitochondria; localisation and biochemical characterisation, possible functions in biogenesis and metabolism. Hoppe Seyler's Z. Physiol. Chem. **335**, 731–743

30 Ohlendieck, K., Riesinger, I., Adams, V., Krause, J. and Brdiczka, D. (1986) Enrichment and biochemical characterization of boundary membrane contact sites in rat-liver mitochondria. Biochim. Biophys. Acta **860**, 672–689

31 Kottke, M., Adams, V., Riesinger, I., Bremm, G., Bosch, W., Brdiczka, D., Sandri, G. and Panfili, E. (1988) Mitochondrial boundary membrane contact sites in brain: points of hexokinase and creatine kinase location and of control of Ca^{2+} transport. Biochim. Biophys. Acta **395**, 807–832

32 Adams, V., Bosch, W., Schlegel, J., Wallimann, T. and Brdiczka, D. (1989) Further characterization of contact sites from mitochondria of different tissues: topology of peripheral kinases. Biochim. Biophys. Acta **981**, 213–225

33 Kottke, M., Wallimann, T. and Brdiczka, D. (1994) Dual localisation of mitochondrial creatine kinase in brain mitochondria. Biochem. Med. Metabol. Biol. **51**, 105–117

34 Bücher, T., Luh, E. and Pette, D. (1964) Einfache und zusammengesetzte optische Tests mit Pyridininucleotiden. In Hoppe-Seyler/Thierfelder, Handbuch der physiologisch- und pathologisch-chemischen Analyse, vol. VI/A (Lang, K. and Lehnartz, E., eds.), pp. 292–339, Springer, Berlin

35 Schutkowski, M., Wöllner, S. and Fischer, G. (1995) Inhibition of peptidyl-prolyl *cis/trans* isomerase activity by substrate analogue structures: thioxotetrapeptide-4-nitroanilides. Biochemistry **34**, 13016–13026

36 Sottocasa, G. L., Kuylenstierna, B., Ernster, L. and Bergstrand, A. (1967) An electron-transport system associated with the outer membrane of liver mitochondria. J. Cell Biol. **32**, 415–438

37 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275

38 Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**, 680–685

39 Rott, R. and Nelson, N. (1981) Purification and immunological properties of proton-ATPase complexes from yeast and rat liver mitochondria. J. Biol. Chem. **256**, 9224–9228
40 Pisano, J. J., Bronzert, T. and Brewer, H. B. (1972) Advances in the gas chromatographic analysis of amino acid phenyl- and methylthiohydantoins. Anal. Biochem. **45**, 43–59
41 Woodfield, K., Rück, A., Brdiczka, D. and Halestrap, A. P. (1998) Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. Biochem. J **336**, 287–290
42 Riccio, P., Aquila, H. and Klingenberg, M. (1975) Purification of the carboxy-atractylate binding protein from mitochondria. FEBS Lett. **56**, 133–138
43 Houldsworth, J. and Attardi, G. (1988) Two distinct genes for ADP/ATP translocase are expressed at the mRNA level in adult human liver. Proc. Natl. Acad. Sci. U.S.A. **85**, 377–381
44 Cozens, A. L., Runswick, M. J. and Walker, J. E. (1989) DNA sequences of two expressed nuclear genes for human mitochondrial ADP/ATP translocase. J. Mol. Biol. **206**, 261–280
45 Shimizu, S., Sinohara, Y. and Tsujimoto, Y. (2000) Bax and Bcl-xl independently regulate apoptotic changes of yeast mitochondria that require VDAC but not adenine nucleotide translocator. Oncogene **19**, 4309–4318
46 Zorov, D. B., Filburn, C. R., Klotz, L. O., Zweier, J. L. and Sollott, S. J. J. (2000) ''ROS-induced ROS release'': a new phenomenon accompanying induction of the mitochondrial permeability transitions in cardiac myocytes. Exp. Med. **192**, 1001–1014
47 Kusnetsov, A. V. and Saks, V. A. (1986) Affinity modification of creatine kinase and ATP-ADP translocase in heart mitochondria: determination of their molar stoichiometry. Biochem. Biophys. Res. Commun. **134**, 359–366
48 Graham, B. H., Waymire, K. G., Cottrell, B., Trounce, I. A., MacGregor, G. R. and Wallace, D. C. (1997) A mouse model for mitochondrial myopathy and cardiomyopathy resulting from a deficiency in heart/muscle isoform of the adenine nucleotide translocator. Nat. Genet. **16**, 226–234
49 Doerner, A., Schulze, K., Rauch, U. and Schultheiss, H. P. (1997) Adenine nucleotide translocator in dilated cardiomyopathy: pathophysiological alterations in expression and function. Mol. Cell. Biochem. **174**, 261–269

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