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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

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Abstract

Complexes between hexokinase, outer membrane porin, and the adenylate translocator (ANT) were recently found to establish properties of the mitochondrial permeability transition pore in a reconstituted system. The complex was extracted by 0.5% Triton X-100 from rat brain membranes and separated by anion exchanger chromatography. The molecular weight was approximately 400 kDa suggesting tetramers of hexokinase (monomer 100 kDa). By the same method a porin, creatine kinase octamer, ANT complex was isolated and reconstituted in liposomes. Vesicles containing the reconstituted complexes both retained ATP that could be used by either kinase to phosphorylate external creatine or glucose. Atractyloside inhibited this activity indicating that the ANT was involved in this process and was functionally reconstituted [1]. Exclusively from the *hexokinase complex* containing liposome internal malate or ATP was released by addition of Ca^{2+} in a *N*-methylVal-4-cyclosporin sensitive way, suggesting that the hexokinase porin ANT complex might include the permeability transition pore (PTP). The Ca^{2+} dependent opening of the PTP-like structure was inhibited by ADP (apparent I_{50}, 8 μM) and ATP (apparent I_{50}, 84 μM). Also glucose inhibited the PTP-like activity, while glucose-6-phosphate abolished this effect. Although porin and ANT were functionally active in vesicles containing the *creatine kinase octamer complex*, Ca^{2+} did not induce a release of internal substrates. However, after dissociation of the creatine kinase octamer, the complex exhibited PTP-like properties and the vesicles liberated internal metabolites upon addition of Ca^{2+}. The latter process was also inhibited by *N*-methylVal-4-cyclosporin. The activity of peptidyl-prolyl-*cis-trans*-isomerase (representing cyclophilin) was followed during complex isolation. Cyp D was co-purified with the hexokinase complex, while it was absent in the creatine kinase complex. The inhibitory effect of *N*-methylVal-4-cyclosporin on the creatine kinase complex may be explained by direct interaction with the creatine kinase dimer that appeared to support octamer formation. © 1998 Elsevier Science B.V.

Keywords: Kinase complex; Porin; Hexokinase; Creatine kinase; Adenylate translocator; Permeability transition pore

1. Introduction

Mitochondria from a variety of sources exhibit a Ca^{2+} dependent increase of inner membrane permeability to molecules with $M_r < 1500$ Daltons, the ''permeability transition''.

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Responsible for this behaviour is a regulated inner membrane channel, the permeability transition pore (PTP). Pore opening is dependent on both the transmembrane potential difference and on matrix pH and is modulated by a variety of effectors acting on multiple sites (for reviews see [2–4]). Cyclosporin A (CsA) [5] is a very potent (apparent I_{50} in the nanomolar range) selective inhibitor of the PTP. Because of inhibition by CsA the nature of the mitochondrial ''permeability transition'' was recognised as being due to opening of a channel, as first proposed by Hunter and Haworth [6], rather than to a permeability change of the membrane lipid phase.

In recent publication's evidence was presented indicating that mitochondrial permeability transition is an important step in the apoptotic process. In a cell free system containing isolated mitochondria and nuclei, opening of the PTP was necessary to induce DNA fragmentation [7,8].

So far the proteins constituting the channel were not known. The observation by Halestrap et al. [9,10] that atractyloside opened the PTP, while bongkrekate and ADP closed it, led to the assumption that the 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 PTP [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 PTP-like channels [15].

From kinetic studies with hexokinase and creatine kinase it appeared that the kinase product ADP was directly channelled to the oxidative phosphorylation [16,17]. Furthermore, hexokinase and creatine kinase were localised in contact sites at the surface and between the two mitochondrial membranes by electron microscopic analysis and isolation of the contact site fraction [18,19]. On the whole, the kinetic behaviour and organisation of the kinases in contact sites pointed to a possible interaction between kinase porin and ANT.

We were able to generate complexes in vitro from isolated porin and dimeric (M_r 80 000) mitochondrial creatine kinase [20] as well as with hexokinase monomers (M_r 100 000) [21,22]. The complexes had a M_r of 400–430 kDa indicating that the octamer of creatine kinase and a tetramer of hexokinase was associated with porin. We recently succeeded to isolate hexokinase and creatine kinase complexes with a similar molecular weight of about 400 kDa from washed kidney and brain membrane fractions by mild detergent extraction [1,22]. This suggested the physiological existence of porin kinase oligomers at the mitochondrial surface. Immunological analysis of the composition revealed the ANT as a further constituent. The kinase complexes were reconstituted in phospholipid vesicles that retained ATP. Both kinases could however utilise the internal ATP for phosphorylation of external glucose or creatine.

The hexokinase complex, when reconstituted into liposomes or black lipid membranes, conferred the system with permeability and conductance properties that resembled those of the mitochondrial PTP [1]. Furthermore, when the proteoliposomes were preloaded with malate or ATP, a gradual release of the trapped compound by increasing Ca^{2+} could be demonstrated. As is the case for the PTP, flux was inhibited by CsA or by *N*-methylVal-4-cyclosporin [1], a CsA derivative that binds to and inhibits mitochondrial cyclophilin (CyP) but not calcineurin [23,24].

Considering that the ANT might be a component of the PTP we were interested to study the effect of kinase activity and kinase structure in the reconstituted complexes on the regulation of the PTP-like activity. It is known that only octameric mitochondrial creatine kinase is able to interact with the two boundary membranes [25–27]. In the complex, containing mitochondrial creatine kinase, exclusively octamer was present interacting with porin [20] and presumably the ANT. It was interesting to analyse the effect of octamer association–dissociation on the PTP-like properties. Besides a possible effect of kinase structure on the regulation of the presumptive

PTP, an additional regulation by the kinase activity through the ADP production was expected [9,10]. Thus, the effects on the PTP-like activity of substrates and metabolic kinase regulators were investigated.

Rat liver mitochondria possess a matrix CyP, an enzyme with peptidyl-prolyl-*cis*-*trans*-isomerase activity, which can be also inhibited by CsA [10,28]. cDNA sequencing of rat mitochondrial CyP has revealed that it is the homologue of human CyP D [29]. It has been proposed that CyP D regulates the PTP 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 because of a direct effect on the pore [23,30]. 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.

2. Materials and methods

2.1. Chemicals

If not otherwise indicated, were all bought from Boehringer-Mannheim and Merck-Darmstadt, Germany. *N*-methylVal-4-cyclosporin was generous gift of Dr. Roland Wenger of Sandoz Pharma AG, Basel.

2.2. Phospholipids

Asolectin (a mixture of soybean phospholipids) and cholesterol was obtained from Avanti Biochemicals, Birmingham, AL. Turkey egg yolk lecithin was purchased from Sigma-Aldrich Chemie, Germany.

2.3. Enzyme assays

Hexokinase (EC 2.7.1.1) and creatine kinase (EC 2.7.3.2) activity was measured in agreement with Bücher et al. [31]. Both enzymes were determined in a coupled optical enzyme assay with either glucose or creatine and ATP as substrates. For hexokinase glucose 6-phosphate dehydrogenase and NADP were used as indicator reaction, while for creatine kinase the activity was indicated through pyruvate kinase and lactate dehydrogenase in the presence of phosphoenol pyruvate and NADH. Peptidyl-prolyl-*cis*-*trans* isomerase (EC 5.2.1.8) activity was determined according to Schutkowski et al. [32].

2.4. Substrate tests

Malate and ATP concentrations were determined according to [33].

2.5. Isolation of mitochondria

Mitochondria from rat brain were isolated by a method described previously [19].

2.6. Respiration measurements

The respiratory rates were measured in isolation medium at 25°C in a closed vessel by a custom built rate meter equipped with a Clark-type oxygen electrode. The oxygen content of the incubation medium was assumed to be 229 nmol O_2/ml [34].

2.7. Reconstitution of the complexes in liposomes

The hexokinase complex fraction was reconstituted in Asolectin phospholipids and 2% cholesterol, while turkey egg yolk lecithin and 2% cholesterol was used for the creatine kinase complex. The phospholipid complex mixture was dialysed over night against 125 mM sucrose, 10 mM Hepes pH 7.4. The vesicles were loaded with 10 mM KCl and 5 mM ATP or 5 mM malate by sonication and subsequent chromatography on Sephadex G50 in 125 mM sucrose, 10 mM Hepes. After chromatography the permeability for ATP was tested. This was performed by centrifugation of the vesicles for 30 min at 400 000 × *g*, followed by determination of ATP and malate in the supernate and sediment fraction.

2.8. Measuring the permeability transition pore

Malate loaded liposomes containing reconstituted hexokinase- or creatine kinase complex and dissociated creatine kinase complex were suspended in 125 mM sucrose, 10 mM Hepes pH 7.4. The vesicles were analysed for malate release by 15 min incubation at room temperature with Ca^{2+} between 0 and 500 μM. Malate release was inhibited by pre-incubation of the vesicles with 0.1 μM *N*-methylVal-4-cyclosporin. After incubation the liposomes were cen-

trifuged for 30 min at 400 000 × *g* and the liberated malate was determined in the supernate.

2.9. Dissociation of the creatine kinase in the complex

The mitochondrial creatine kinase complex containing fractions from the DEAE column were reconstituted in turkey egg yolk lecithin and 2% cholesterol as described above. The dissociation of the octamer in the creatine kinase complex was performed according to Gross et al. [35,36] in the liposomes and in aliquots of the DEAE column fractions. The samples were incubated for 20 min at room temperature with 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3 and 4 mM ADP. It is possible to stop the dissociation by addition of 25 mM EDTA. This method was used during the analysis of CsA effects on the dissociated enzyme. To confirm that the malate loaded vesicles remain sealed during dissociation of creatine kinase, they were centrifuged and the supernate was tested for malate. The dissociation of the creatine kinase octamer was verified by Superdex 200 chromatography.

3. Results

3.1. Reconstitution of the hexokinase complex in vesicles

3.1.1. Inhibition of PTP-like properties by ADP and ATP

It has been postulated by several authors [10,11,13,15] that the mitochondrial permeability transition pore might be formed by porin and the ANT. A direct interaction between porin and ANT can be assumed in the hexokinase complex. To investigate whether the hexokinase complex may resemble the permeability transition pore, the complex was reconstituted in vesicles that were loaded with 10 mM KCl and 5 mM malate or 5 mM ATP. By centrifugation the vesicles proved to be sealed for the substrates. It was shown recently that malate or ATP was liberated successively by addition of Ca^{2+} concentrations between 100 and 600 μM. The Ca^{2+} dependent substrate release was inhibited by pre-incubation with 0.1 μM *N*-methylVal-4-cyclosporin [1]. In the experiment shown in Fig. 1(A,B) the release of malate by

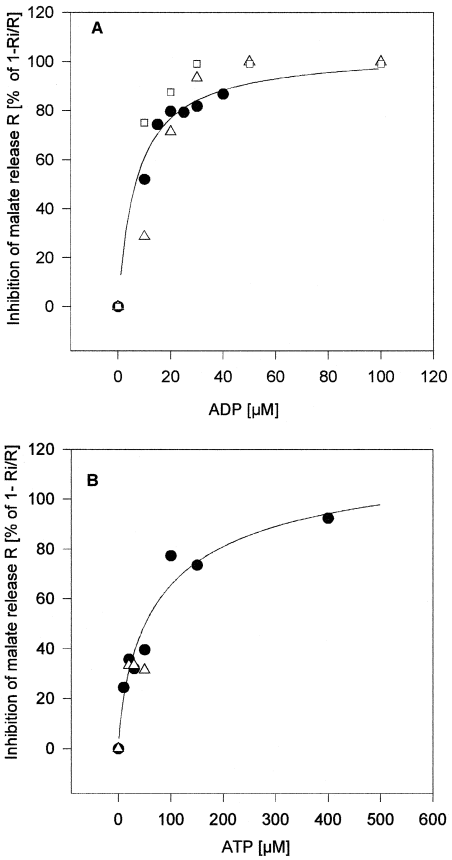

Fig. 1. ATP and ADP effect on PTP-like properties in the reconstituted hexokinase complex. The hexokinase complex was reconstituted in Asolectin liposomes and the vesicles were loaded with malate as described in methods. After addition of different concentrations of ADP (panel A) or ATP (panel B) as indicated, the malate release was induced by 500 μM Ca^{2+}. The vesicles were sedimented by centrifugation for 30 min at 400 000 × *g* and malate was determined in the supernates. About 50% of the internal malate could be released in the control (R) whereas smaller amounts were liberated in the presence of the adenine nucleotides (Ri). The inhibition of the malate release by different ADP or ATP concentrations is shown as percentage of 1- the quotient of Ri/R. The I_{50} for ADP was calculated separately in 3 experiments depicted with different symbols in panel A. The mean value of 8 μM was used to generate the shown curve. The I_{50} of 84 μM for ATP was calculated from two experiments shown by different symbols in panel B. To eliminate any ADP contamination in the ATP, the ATP stock solution was preincubated with phosphoenol pyruvate and pyruvate kinase.

500 μM Ca^{2+} could be inhibited by increasing concentrations of ADP or ATP. The apparent I_{50} of ADP was 8 μM while that for ATP was 84 μM. The ADP concentration was in the range of the K_d for ATP

and ADP of the ANT [37] whereas that of ATP was closer to the binding constant of hexokinase between 100 and 400 μM. To eliminate any ADP contamination in the ATP, the ATP stock solution was preincubated with phosphoenol pyruvate and pyruvate kinase.

3.1.2. Regulation of the PTP-like activity by hexokinase substrates

From the results above it appeared that the inhibition of the PTP-like activity by ATP was an indirect effect transmitted from hexokinase by the outer membrane porin. We, therefore, investigated whether substrates and inhibitors of hexokinase such as glucose and glucose-6-phosphate (G-6-P) would have regulatory functions as well. As shown in Fig. 2(A), 5 mM glucose like 200 μM ATP inhibited malate release from the vesicles partially (column 2 and 4 Fig. 2(A)), while both together exerted a complete inhibition (column 5). The inhibition of the PTP-like activity by glucose/ATP was partially abolished by 5 mM G-6-P (column 7). This G-6-P effect was studied in more detail (Fig. 2(B)). It was observed that G-6-P alone did not inhibit the PTP-like activity (column 2 Fig. 2(B)). In contrast to the experiment shown in column A7, G-6-P was able to completely suppress the inhibition of the PTP-like channel by glucose and ATP (column 5). To achieve this effect the sequence of addition was important, as G-6-P had to be added before ATP and glucose. When it was added after the two substrates, some inhibitory effect of ATP and glucose on the PTP-like structure remained (column A7).

3.1.3. Regulation of the permeability transition pore by atractyloside

It was observed by Halestrap [9] that the PTP could be opened by atractyloside, while bongkrekate had the opposite effect. The PTP-like structure in the reconstituted hexokinase complex was also sensitive to atractyloside. In the presence of 100 μM Ca^{2+}, that per se did not open the PTP, atractyloside released malate in a concentration between 5 and 12 μM (Fig. 3(A)). Higher concentrations of 15 and 20 μM atractyloside had no effect. When the octamer in the reconstituted creatine kinase complex was dissociated as will be shown ahead (Fig. 4) it was possible to

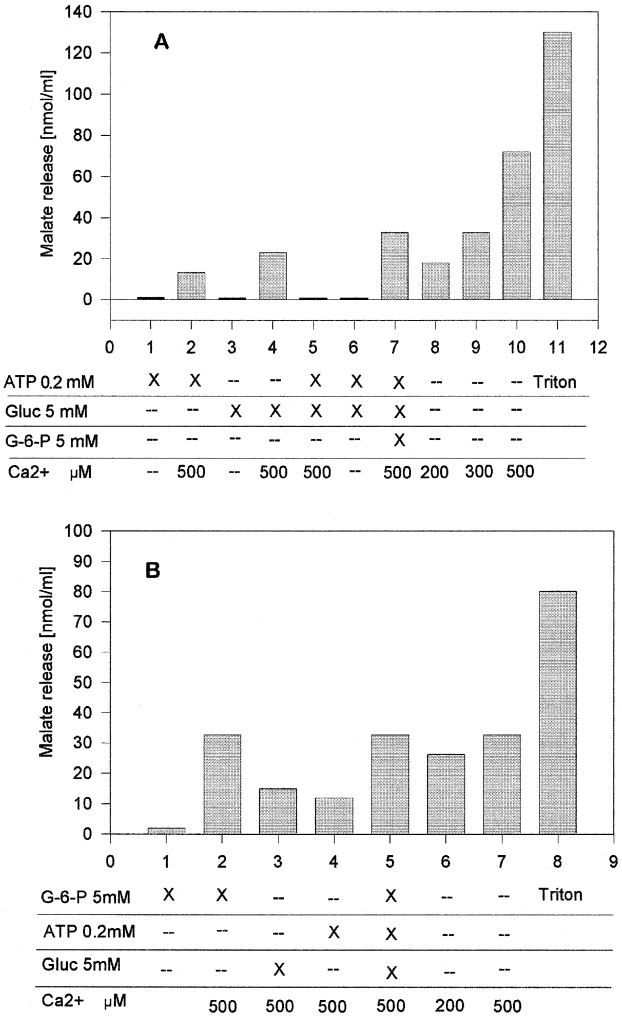

Fig. 2. Effect of hexokinase ligands on PTP-like behaviour in the reconstituted hexokinase complex. The hexokinase complex was reconstituted in liposomes and loaded with malate as described in methods. Approximately 50% of the malate were released by addition of 500 μM Ca^{2+}. The effect of hexokinase ligands on the malate release by 500 μM Ca^{2+} was studied in the same way as described in Fig. 1(A): Regulation of the Ca^{2+} dependent malate release by ATP, glucose (Gluc) and glucose-6-phosphate (G-6-P). (B): Regulation of Ca^{2+} dependent opening of the PTP-like channel by G-6-P.

open a PTP-like channel by 400 μM Ca^{2+}. In these vesicles containing the creatine kinase dimer, malate could be also released by atractyloside in the presence of low (100 μM) Ca^{2+} (Fig. 3(B)). The sensitivity for atractyloside was in the same concentration range as observed for the reconstituted hexokinase complex.

3.2. Analysis of the permeability transition in intact brain mitochondria

3.2.1. Effect of atractyloside and Ca^{2+} on respiration

Opening of the PTP causes a decrease of the inner membrane potential and as a consequence leads to increased oxidation rates. We measured the respiration of isolated brain mitochondria in the presence of succinate (Fig. 3(C)). Addition of 40 μM Ca^{2+} stimulated the respiration to 75 nmol O_2/min × mg. Subsequent addition of atractyloside caused a further increase up to 110 nmol O_2/min that could be inhibited by 100 nM CsA (not shown). The inhibition by CsA although not complete suggested that at least part of the observed respiration resulted from opening of the PTP. Atractyloside was stimulating the respiration only in a concentration range between 5 and 15 μM (Fig. 3(C)). This concentration range agreed with the atractyloside dependent opening of a PTP-like channel in the reconstituted hexokinase and creatine kinase dimer complexes (Fig. 3(A,B)).

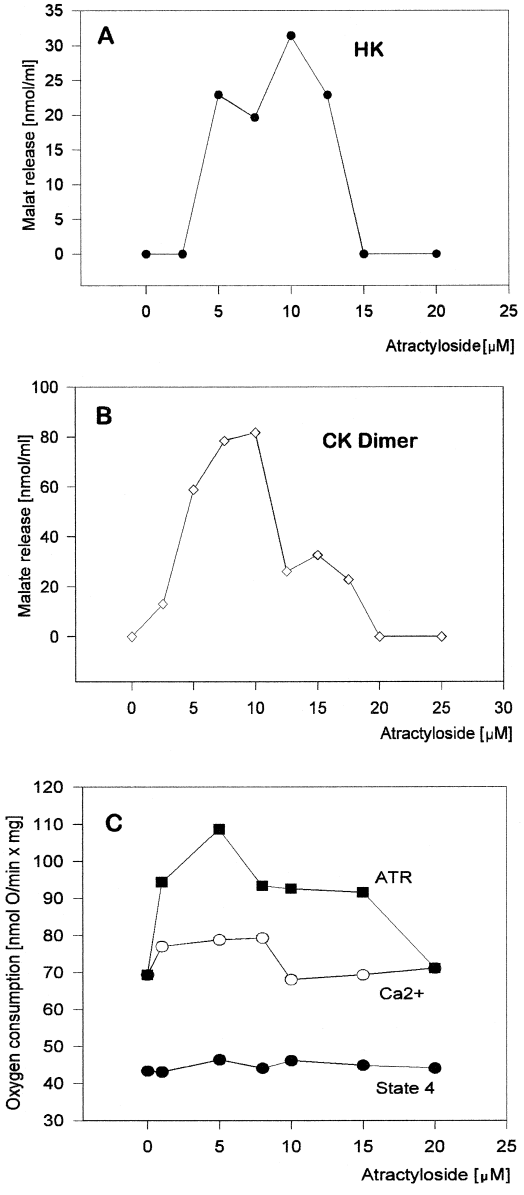

Fig. 3. Effect of atractyloside and Ca^{2+} on PTP-like properties of the reconstituted complexes and in mitochondria. (A) The hexokinase complex was reconstituted in liposomes and loaded with malate as described in methods. Upon addition of 100 μM Ca^{2+} the malate was not released. Atractyloside was added to the vesicles between 0 and 20 μM in the presence of 100 μM Ca^{2+}. The samples were incubated for 15 min at room temperature and subsequently centrifuged for 30 min at 400 000× *g*. The released malate was determined in the supernate. (B) The creatine kinase complex was reconstituted in liposomes and loaded with malate as described in methods. The creatine kinase octamer was dissociated by incubating the vesicles for 20 min at room temperature with 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3 and 4 mM ADP. The vesicles were centrifuged as in (A) and resuspended in 125 mM sucrose, 10 mM Hepes. Treatment of the proteoliposomes with atractyloside was performed as in (A). (C) Regulation of the PT by Ca^{2+} and atractyloside in mitochondria monitored by oxygen consumption. Respiration of rat brain mitochondria (0.83 mg/ml) in 250 mM sucrose, 10 mM Hepes (pH 7.5), 5 mM $MgCl_2$, 5 mM Na_2HPO_4, 5 mM succinate and 10% dextran 15 was monitored by oxygen electrode. The active rate of respiration with succinate (state 4) was increased by 40 μM Ca^{2+}. Subsequent addition of atractyloside (ATR) between 0 and 20 μM resulted in further stimulation of respiration.

3.3. Reconstitution of the creatine kinase complex in liposomes

A porin creatine kinase ANT complex was isolated and reconstituted in vesicles such as described for hexokinase. It was analysed whether the vesicles were sealed and internal ATP was accessible to creatine kinase in a way that was inhibited by atractyloside such as published recently [1]. This analysis (not shown) 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 (Fig. 4(B), octamer). In difference to the hexokinase,

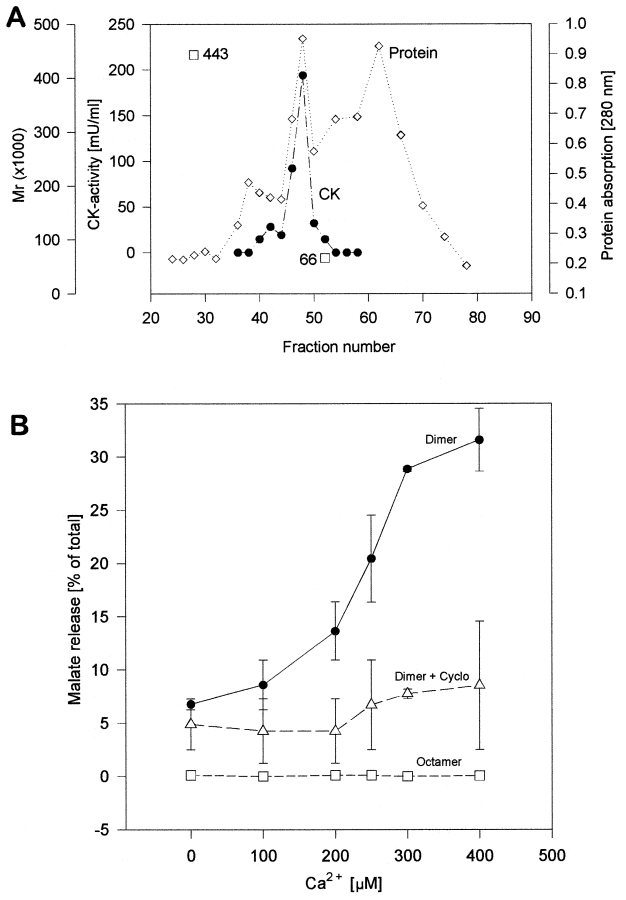

Fig. 4. Reconstitution of the creatine kinase complex in liposomes. (A) MW analysis of the complex after octamer dissociation. The fractions containing mitochondrial creatine kinase from the DEAE column were incubated for 20 min at room temperature with 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3 and 4 mM ADP. Subsequently the sample was loaded on a Superdex 200 column. The creatine kinase activity was eluted by 1.5 mM Na_2HPO_4, 1.5 mM K_2HPO_4 pH 8.0, 1.0 mM dithioerythrit, 100 mM glucose. The column was calibrated by apoferritin, and bovine albumin as molecular mass standards corresponding to 443, and 66 kDa respectively. (B) Regulation of PTP-like activity by the octamer dimer dissociation. The mitochondrial creatine kinase complex was reconstituted in turkey egg yolk liposomes and the vesicles were loaded with malate as described in methods. An aliquot of the liposomes was incubated for 20 min at room temperature with 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3 and 4 mM ADP. After incubation with different concentrations of Ca^{2+} the vesicles were sedimented by centrifugation for 30 min at 400 000 × *g*. As was determined in the remaining supernate, about 50% of the internal malate could be released by 400 µM Ca^{2+} only from the preincubated vesicles (dimer), while the untreated liposomes (octamer) retained the malate. The Ca^{2+} dependent malate release was inhibited by pre-incubation with 100 nM *N*-methylVal-4-cyclosporin (dimer + cyclo). The data are means of 3 experiments.

creatine kinase interacts directly with the ANT. Creatine kinase in the isolated contact sites [25] and in the reconstituted complex was always present as octamer, while the dimer after dissociation did loosely attach to membranes [26] and presumably to the ANT. We, therefore, dissociated the octamer directly in the isolated complex and after reconstitution in liposomes by the method described by Gross and Wallimann [35,36]. The dissociation was confirmed by gel permeation chromatography on Superdex 200 (Fig. 4(A)). After incubation with creatine, KNO_3, Mg^{2+} and ADP the isolated complex appeared to be completely dissociated.

3.3.1. The PTP-like properties are inhibited by the creatine kinase octamer

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 the dissociated or un-dissociated octamer were then incubated with increasing concentrations of Ca^{2+} between 0 and 400 µM (Fig. 4(B)). Malate was liberated by this treatment exclusively from the vesicles with the dissociated octamer (Fig. 4(B) dimer). The malate release could be inhibited by pre-incubation of the vesicles with 100 nM *N*-methylVal-4-cyclosporin (Fig. 4(B) dimer + cyclo).

3.4. Distribution of CyP D in the hexokinase and creatine kinase complexes

It has been proposed that CyP D regulates the PTP by reversible association from the matrix side to the inner membrane and that the inhibition of the PTP by CsA is due to CyP D desorption from the membrane [23,30]. Thus the activity of peptidyl-prolyl-*cis-trans*-isomerase activity was determined in the different fractions during the complex preparation (Table 1). It was observed that the CyP D activity was removed during the creatine kinase complex isolation, whereas it was co-purified with the hexokinase complex preparation. The specific activity of CyP D in the hexokinase fraction increased 8 fold, while the two kinases were purified through complex isolation by a factor of 19 (hexokinase) or 9 (creatine kinase). Of the creatine kinase a cytosolic isoenzyme is exist-

ing that is removed upon membrane washing. Therefore, the purification data were calculated based on the membrane extract that contains the mitochondrial isoenzyme.

3.5. Direct effects of cyclosporin on the creatine kinase oligomerisation

Upon dissociation of the creatine kinase octamer, Ca^{2+} induced release of substrate from the vesicles as shown in Fig. 4. This release was inhibited by *N*-methylVal-4-cyclosporin although no CyP D activity was found in the creatine kinase complex. We, therefore, investigated whether *N*-methylVal-4-cyclosporin might exert a direct effect on the creatine kinase. As the octamer (M_r 350 kDa) can be separated from the dimer (M_r 80 kDa) by centrifugation [20] we used this method to analyse a possible effect of *N*-methylVal-4-cyclosporin on the re-association of completely dissociated kinase. The dissociation process was blocked after completion by addition of 25 mM EDTA and the samples were incubated for 20 min with 0–25 nM *N*-methylVal-4-cyclosporin. Without cyclosporin no activity sedimented under these conditions. However up to 7.5% of the total activity was found in the sediment indicating that *N*-methylVal-4-cyclosporin might support the re-association of creatine kinase dimers (Table 2).

Table 1
Co-purification of CyP D with the hexokinase–porin–ANT complex

Cyclophilin D (CyP D)				
	Total act (Units)	Yield (% of Hom)	Specific act (mU/mg)	Purification
Homogenate	6.60	100.00	7.62	1.00
Membr extr	2.92	44.31	10.35	1.40
HK-DE	0.47	7.15	29.02	3.60
HK-Sup	0.47	7.17	68.15	8.35
CK-DE	0.03	0.45	0.003	0.0
CK-Sup	0.0	0.0	0.0	0.0
Hexokinase				
Homogenate	82.55	100.00	122.07	1.00
Membr extr	25.94	31.42	79.90	0.71
HK-DE	11.16	13.52	1036.44	9.30
HK-Sup	1.80	2.18	2104.01	18.82
CK-DE	0	–	–	–
CK-Sup	0	–	–	–
Mitochondrial creatine kinase				
Homogenate	232.65	0	258.53	–
Membr extr	45.1	100.00	289.90	1.00
HK-DE	0	–	–	–
HK-Sup	0	–	–	–
CK-DE	8.66	19.20	805.91	2.78
CK-Sup	0.535	1.19	2610.3	9.00

The activity of CyP D, hexokinase and creatine kinase was followed by optical tests in the different steps of the complex preparation as described in Section 2. A rat brain homogenate was centrifuged to yield the first supernatant and sediment. The sediment was washed 3 times by centrifugation, and the resulting membrane fraction was then extracted with 0.5% Triton X-100 (Membr extr). Hexokinase (HK-DE) in this extract was separated from mitochondrial creatine kinase (CK-DE) and cytosolic creatine kinase by chromatography on a DE 52-cellulose column. The hexokinase and mitochondrial creatine kinase peak fractions were collected and run on a gel-permeation Superose 6B column. This led to high molecular weight fractions of either hexokinase (HK-Sup) or creatine kinase (CK-Sup) that contained oligomers of the kinases, porin and ANT [1, 22]. Data are means of 5–9 experiments.

Table 2
Effect of cyclosporin on dimeric mitochondrial creatine kinase

Cyclosporin addition (nM)	Total activity (mU)	Sedimented activity (% of total)
0.0	360	0.0
5	333	1.7
10	400	7.5
25	320	6.2

The fractions containing mitochondrial creatine kinase from the DEAE column were incubated for 15 min at room temperature with 5 mM $MgCl_2$, 20 mM creatine, 50 mM KNO_3 and 4 mM ADP to dissociate the octamer. Subsequently 25 mM EDTA (to stop dissociation) and *N*-methylVal-4-cyclosporin between 0–25 nM was added. After a further incubation for 20 min the samples were centrifuged for 1.5 h at 436 000 × *g* in a Beckman Rotor TLA 100.2. Creatine kinase activity was determined in supernatant and sediment. The data are means of two experiments.

4. Discussion

4.1. Structure and function of the kinase porin ANT complexes

Previous investigations in isolated mitochondria suggested that oligomers of hexokinase and creatine kinase were predominantly found in boundary membrane contact sites as complexes with a contact spe-

cific porin structure [18–22,38]. Furthermore the results in this investigation and preceding publications [1,22] showed that such oligomers of hexokinase I and mitochondrial creatine kinase exist physiologically in brain, as they could be extracted as such from brain membranes. The isolated kinase complexes were composed of functionally active porin and ANT. Taken together the results indicated that an interaction between kinases, porin and ANT shaped the structure of contact sites between the two mitochondrial envelope membranes [39]. In the hexokinase complex, porin and ANT presumably interacted directly as has been assumed for the benzodiazepine receptor [14,15]. In difference to that, in the creatine kinase complex, the octamer was interacting directly with the two membrane components. A large number of experiments suggested that porin and the ANT was required for the transient oligomerisation of the kinases [18–22,38,39]. By demonstration of functional interaction between the constituents in the reconstituted system it was excluded that the two membrane components were contaminants of the isolated complexes. That both kinases were able to utilise the ATP trapped inside the liposomes suggested a direct interaction between the kinases, porin, and the ANT [1].

It appears conceivable, however, that the PTP is a supra-molecular structure involving components from both the outer and inner membranes, and from the intermembrane and matrix spaces. It appears likely that the ANT and porin are part of the transmembrane complex, or that they substantially affect its function, but a precise definition of the molecular nature of the pore remains matter for future work. Clearly, however, the present results are a step forward in the purification and molecular dissection of the PTP components. In further support of this view are the regulatory effects of kinase substrates and kinase structure on the PTP-like behaviour discussed below.

4.2. Effect of adenine nucleotides, atractyloside and hexokinase substrates on the PTP-like functions

Ligands either of the ANT (ATP, ADP) or of the hexokinase (glucose, ATP, G-6-P) were regulating the PTP-like properties of the reconstituted complexes (Fig. 1, and Fig. 2(A,B)). ADP suppressed the opening of the PTP at concentrations of 8 μM in the range of the ANT K_d of 20 μM [37], whereas the apparent I_{50} for ATP was 10 times higher. Halestrap et al. [10] in rat liver mitochondria determined two PTP K_i values of ADP of 1 and 25 μM and a much higher K_i for ATP of 544 μM. The same authors observed that carboxyatractyloside opens the PTP at concentrations of 10 μM [9]. Our data shown here with atractyloside (Fig. 3(A,B,C)) are comparable. Besides these effects of ANT ligands we additionally found that hexokinase substrates such as glucose inhibited the PTP-like behaviour, while glucose 6-phosphate abolished the inhibition by glucose and ATP completely (Fig. 2(B)). Glucose 6-phosphate is known to inhibit the free hexokinase in a concentration range of 1–4 mM but also causes desorption of hexokinase from the mitochondrial surface above 5 mM (see review [40]). That ligands of hexokinase can regulate the PTP-like structure, indicated that porin in the complex might transmit structural changes of hexokinase to the ANT. This would support the assumption of a complex between porin and the ANT to which hexokinase is bound. The structural interaction between kinases and the ANT is even more evident from the results obtained with the creatine kinase complex.

4.3. Effect of creatine kinase structure in the regulation of the PTP-like functions

Judged from the molecular weight of approximately 400 kDa, the creatine kinase complex was composed of an octamer of mitochondrial creatine kinase besides porin and ANT. In a preceding publication it was shown that the internal ATP in the liposomes with reconstituted creatine kinase complex was available to creatine kinase. The creatine phosphate production of the enzyme from external creatine and internal ATP was inhibited by atractyloside suggesting functional reconstitution of the ANT [1]. However the permeability transition could not be induced in these vesicles by Ca^{2+}. It is known that only octameric mitochondrial creatine kinase is able to interact with the two boundary membranes [25–27]. Thus, we assumed that the octamer of mitochondrial creatine kinase between the outer membrane porin and ANT may hinder the appearance of the PTP-like functions. As already described [35,36,41], a fast dissociation of the octamer can be induced by keep-

ing the enzyme in a catalytic transition-state with ADP, Mg^{2+}, creatine and KNO_3. When the octamer in the reconstituted complex was dissociated in that way, permeability transition could be induced by Ca^{2+} that was also sensitive to *N*-methylVal-4-cyclosporin such as observed for the hexokinase complex (Fig. 4). Supposing that the ANT causes permeability transition, this finding indicated that the octamer interacted with the ANT in a way that a structure specific for the function as exchange carrier was maintained. The octamer was, therefore, able to suppress the structural transition of the ANT into a channel that leads to permeability transition.

4.4. Regulation of the PTP by cyclophilin ?

Since PTP inhibition by CsA in mitochondria is mediated by CyP D [23,28], we suspected the presence of this protein in the purified kinase complexes. A finding of the present paper is indeed the demonstration by enzymatic methods that a fraction of mitochondrial CyP D co-purifies with, and is enriched in, hexokinase–porin–ANT but not in porin–creatine–kinase–ANT complexes (Table 1). Since only the former complexes made functional, PTP-like structures these data suggested that CyP D is essential for high-conductance channel formation by the hexokinase complex, and that the latter might correspond to the PTP [1]. However as shown here also the reconstituted creatine kinase complex showed PTP-like properties (release of internal substrates by atractyloside Fig. 3(B), and by Ca^{2+} Fig. 4) after dissociation of the octamer. Because of the absence of CyP D in the creatine kinase complex it was completely unexpected to observe the same inhibition of the PTP-like properties by *N*-methylVal-4-cyclosporin as was reported for the hexokinase complex (Fig. 4). However, the inhibition of *N*-methylVal-4-cyclosporin without CyP D may be explained by direct interaction of this substance with creatine kinase. As indicated from the sedimentation of creatine kinase activity after complete dissociation, 5–25 nM *N*-methylVal-4-cyclosporin might have induced octamer generation of creatine kinase (Table 2). This induction of creatine kinase oligomerisation may have been responsible for the inhibition of the PTP-like properties in the reconstituted creatine kinase complex. Although the results are preliminary they suggested that *N*-methylVal-4-cyclosporin might directly interact with the mitochondrial creatin kinase. It is a matter of future work to analyse whether this effect indeed shifts the oligomerisation equilibrium of creatine kinase to the side of octamer formation. Binding of CsA with high affinity to other proteins besides CyP D (K_i 3.6 nM [28]) is known (for a review see [42]). Thus, in Jurkat cells, CsA binds to G-Actin (K_d 60 nM) and heat shock protein hsp70 (K_d 53 nM) [43]. The findings indicate that PTP-like structures in the hexokinase complex might be regulated by CyP D, whereas in the creatine kinase complex this regulatory role may be played by the association–dissociation equilibrium of the creatine kinase octamer.

4.5. Regulation of apoptosis through energy metabolism

4.5.1. The role of kinase complexes

By using a cell free system composed of isolated mitochondria and nuclei, evidence was presented indicating that mitochondrial permeability transition constitutes an important step in the apoptotic process. Zamzami et al. [7] and Susin et al. [8] showed that opening of the PTP was essential to induce apoptotic DNA fragmentation in the nuclei. Besides other substances such as Bcl-2, ligands of the ANT (atractyloside, bongkrekate) were regulating the permeability transition suggesting that the ANT was at least one important component of the PTP. This agrees with findings of Halestrap [9,10] in liver mitochondria, Brustovetsky and Klingenberg with reconstituted ANT [11] and our results with the reconstituted complexes containing the ANT [1].

Dierks et al. were able to reversibly modify the aspartate/glutamate carrier and the ANT from obligate counter-exchange to uni-directional transport by blocking SH-groups with mercury reagents [44,45]. In view of the authors' results, the intrinsic channel appeared to be a structural requirement of the exchange carrier-mediated transport. Thus, it may be concluded that principally all exchange carriers can cause permeability transition. In support of this idea Petronilli et al. [46] reported that the permeability transition is tuned by the oxidation–reduction state of thiol groups.

Supposing that in principal several exchange carriers can cause permeability transition, it appears plausible that the ANT is a very important candidate because of its central role in energy metabolism. The effects of kinase substrates (glucose, creatine, ATP) and of the locally produced ADP on the regulation of the PTP-like activity described above support this view. Thus transformation of the ANT from the adenylate exchanger structure into a channel structure, uncoupling oxidative phosphorylation, is most effectively inhibited by local ADP. This would preserve the membrane potential as the driving force for ATP generation.

4.5.2. Regulation of the kinase complexes

The observed inhibition of the PTP-like structure by ATP and glucose led to the question of how the PTP could ever open under physio-logical (approx. 5 mM) concentrations of the hexokinase substrates. This may be answered by considering that complexes constituting the contacts sites are dynamic structures that are subjected to metabolic and hormonal regulation [39]. In addition binding of hexokinase is a question of isozyme composition of the tissue. While brain specific isozyme I binds permanently to mitochondria, the binding of muscle and liver specific isozyme II is strongly controlled by hormones (insulin, glucagon) and metabolites (glucose, free fatty acids) (for reviews see Wilson [40], and Brdiczka [47]). Thus contact sites in liver of starved rats were found to be depressed [48] and hexokinase was desorbed by free fatty acids [49]. It was observed that free fatty acids open the PTP [2]. Thus, closing of the PTP by ATP and glucose may be counter-acted by high levels of free fatty acids.

PTP opening is involved in myocardial cells injury during ischemia and reperfusion. The creatine analogs cyclo-creatine and guanidino propionic acid such as CsA have been shown to be potent inhibitors of such damage in cardiac tissue [50,51]. As cyclocreatine and guanidino propionic acid turnover by creatine kinase is very slow it may lock the creatine kinase ANT complex in the exchange transporter conformation. On the other hand this role of creatine kinase is further emphasised by recent observations that the enzyme is the main target of reactive oxygen species in cardiac myofibrils [52]. In general the findings support the view that creatine kinase structure in muscle and brain tissue may play an important role in PTP regulation.

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