Complexes between kinases, mitochondrial porin and adenylate translocator in rat brain resemble the permeability transition pore

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Abstract **In vitro incubation of isolated hexokinase isozyme I or isolated dimer of mitochondrial creatine kinase with the outer mitochondrial membrane pore led to high molecular weight complexes of enzyme oligomers. Similar complexes of hexokinase and mitochondrial creatine kinase could be extracted by 0.5% Triton X-100 from homogenates of rat brain. Hexokinase and creatine kinase complexes could be separated by subsequent chromatography on DEAE anion exchanger. The molecular weight, as determined by gel-permeation chromatography, was approximately 400 kDa for both complexes. The M_r suggested tetramers of hexokinase (monomer 100 kDa) and creatine kinase (active enzyme is a dimer of 80 kDa). The composition of the complexes was further characterised by specific antibodies. Besides either hexokinase or creatine kinase molecules the complexes contained porin and adenylate translocator. It was possible to incorporate the complexes into artificial bilayer membranes and to measure conductance in 1 M KCl. The incorporating channels had a high conductance of 6 nS that was asymmetrically voltage dependent. The complexes were also reconstituted in phospholipid vesicles that were loaded with ATP. Complex containing vesicles retained ATP while vesicles reconstituted with pure porin were leaky. The internal ATP could be used by creatine kinase and hexokinase in the complex to phosphorylate external creatine or glucose. This process was inhibited by atractyloside. The hexokinase complex containing vesicles were furthermore loaded with malate or ATP that was gradually released by addition of Ca^{2+} between 100 and 600 μM. The liberation of malate or ATP by Ca^{2+} could be inhibited by *N*-methylVal-4-cyclosporin, suggesting that the porin translocator complex constitutes the permeability transition pore. The results show the physiological existence of kinase porin translocator complexes at the mitochondrial surface. It is assumed that such complexes between inner and outer membrane components are the molecular basis of contact sites observed by electron microscopy. Kinase complex formation may serve three regulatory functions, firstly regulation of the kinase activity, secondly stimulation of oxidative phosphorylation and thirdly regulation of the permeability transition pore.**

Key words: Kinase; Mitochondrial porin; Adenylate translocator

1. Introduction

Hexokinase distribution at the mitochondrial surface studied by electron microscopic techniques, and by removing unattached outer membrane with digitonin, led to preferential localisation of the enzyme in the contact sites [1–3].

Similarly mitochondrial creatine kinase in brain [4] and retina [5] was localised by electron microscopy along the cristae and at the periphery between the two envelope membranes. The distribution of peripheral mitochondrial creatine kinase was analysed by immunological [4] and histochemical [6] electron microscopic techniques, and by removing unattached outer membrane with digitonin. Clusters of the creatine kinase, such as described for hexokinase, were found in the attachment points [4,6].

This non-random distribution of the two kinases agreed with the analysis of isolated mitochondrial contact sites. In contact fractions enriched from osmotically disrupted liver, brain and kidney mitochondria, hexokinase and mitochondrial creatine kinase (except in liver) were concentrated [2,7,8]. Hexokinase had a significantly higher affinity to this membrane fraction compared to isolated outer membrane [7,9]. It was observed by freeze fracture analysis that contacts were dynamic structures. They were dependent on the functional state of mitochondria [10,11] but also on the preservation of the physiological structure of the outer compartment [12].

Hexokinase binding to contact-rich mitochondria showed sigmoidal binding curves and led to activation of the enzyme. In contrast hexokinase binding curves to mitochondria without contacts were hyperbolic and resembled those with isolated outer membrane [12]. The ADP produced by bound hexokinase and creatine kinase, under conditions favouring contact sites, was less available for external added pyruvate kinase [13–15]. Thus the organisation of hexokinase and creatine kinase in the contacts appeared to be important for ADP channelling to the oxidative phosphorylation. The kinetic studies suggested a direct interaction of hexokinase and mitochondrial creatine kinase with porin and adenylate translocator.

Indeed complexes were generated in vitro from isolated, dimeric, mitochondrial creatine kinase and porin [16]. The complexes had a M_r of 400 kDa, indicating that the octamer of creatine kinase was associated with porin. Hexokinase and porin in vitro also formed complexes of 400–430 kDa as analysed by gel-permeation chromatography [17]. Thus in both cases a kinase fraction with a M_r above 400 kDa would indicate the presence of porin enzyme complexes. The complexes would be clearly distinct from monomers of free hexokinase of 100 kDa or mitochondrial creatine kinase dimer of 80 kDa.

Considering these differences it appeared possible to directly identify kinase porin complexes in tissue homogenates and demonstrate their physiological existence. In addition, the isolation of the complexes would serve to analyse whether the adenylate translocator besides kinase and porin is the inner membrane component that constitutes the contact sites.

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2. Materials and methods

2.1. Chemicals

If not otherwise indicated, all chemicals were bought from Boehringer-Mannheim and Merck-Darmstadt, Germany.

2.2. Antibodies

The monoclonal antiserum against the N-terminus of human type I porin was a gift of Dr. F. Thinnes, MPI for Experimental Medicine, Göttingen. Preparation and characterisation of the antibodies are described in Thinnes et al. [18]. The polyclonal antibodies against the adenylate translocator from chicken heart were obtained from Dr. T. Wallimann, ETH, Zürich.

2.3. Enzyme assays

Hexokinase (EC 2.7.1.1) and creatine kinase (EC 2.7.3.2) activity was measured according to Bücher et al. [19]. 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 reactions, while for creatine kinase the activity was indicated through pyruvate kinase and lactate dehydrogenase in the presence of phospho-enol pyruvate and NADH.

2.4. Protein electrophoresis

2.4.1. Denaturing gel electrophoresis. Tricine sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) of the column fractions was performed in a gradient gel ranging between 5 and 12% polyacrylamide.

2.4.2. Analysis of the complexes by specific antibodies. The polypeptides from the PAGE were transferred to nitrocellulose sheets and the presence of porin and adenylate translocator was analysed by immuno-decoration of the sheets with specific antibodies.

3. Results and discussion

3.1. Extraction of kinase-porin complexes from rat brain membranes

Rat brain, stored frozen, was homogenised in a Teflon potter and washed several times in isolation medium by centrifugation. This process removed only 10% of total hexokinase and about 30–40% of the creatine kinase activity (cytosolic isozyme) from rat brain sediment. The membranes were then resuspended in the washing medium containing 0.5% Triton X-100 and were incubated for 45 min at room temperature. The undissolved membrane material was removed by centrifugation and the supernatant was incubated with DE 52 cellulose that had been equilibrated with 1.5 mM Na^+-,K^+-phosphate, 1 mM dithioerythritol and 0.1 M glucose, pH 8.0. The DE 52 cellulose, loaded with the extract, was packed as a column, from which hexokinase and creatine kinase activities were eluted by a KCl gradient between 50 and 500 mM. Hexokinase (HK) and creatine kinase (CK) were determined in the fractions. The elution profile of rat brain extract (Fig. 1A) shows a single peak of hexokinase activity between two creatine kinase activity peaks.

3.2. Co-migration of adenylate translocator (ANT) and porin with the kinase fractions

The distribution of porin and adenylate translocator (ANT) in the fractions of the DE 52 column was studied by immuno methods (Fig. 1A). The kinase containing fractions from the column and fractions before and after the activity peaks were concentrated and subjected to SDS-PAGE. The polypeptides were transferred to nitrocellulose sheets. The cellulose sheets were decorated with specific antibodies against human type I porin [18] and ANT from chicken heart. Both antibodies cross-react with the corresponding rat proteins. A strongly positive reaction was observed in the first creatine kinase and the hexokinase fractions after decoration with either anti-porin or anti-ANT antibodies, whereas only weak or almost no reaction was seen in the second creatine kinase peak and fractions without kinase activity (Fig. 1A). As characterised by isozyme electrophoresis the second creatine kinase activity fraction contained the cytosolic isozyme while the first activity maximum resulted from the mitochondrial isozyme. The fact that porin and ANT were mainly present in the mitochondrial creatine kinase and hexokinase activity peaks from the DEAE column suggested that both proteins might be components of the kinase complexes.

3.3. Characterisation of the kinase fractions by gel-permeation chromatography

The peak fractions of hexokinase and mitochondrial creatine kinase from rat brain were concentrated and loaded on a Bio-Sil SEC 400 column (Bio-Rad). The enzyme activity was eluted with 5 mM Na_2HPO_4 pH 6.8, 100 mM glucose, 150 mM NaCl. Hexokinase activity migrated as one main peak corresponding to a molecular weight of 400 000 (Fig. 1C). The

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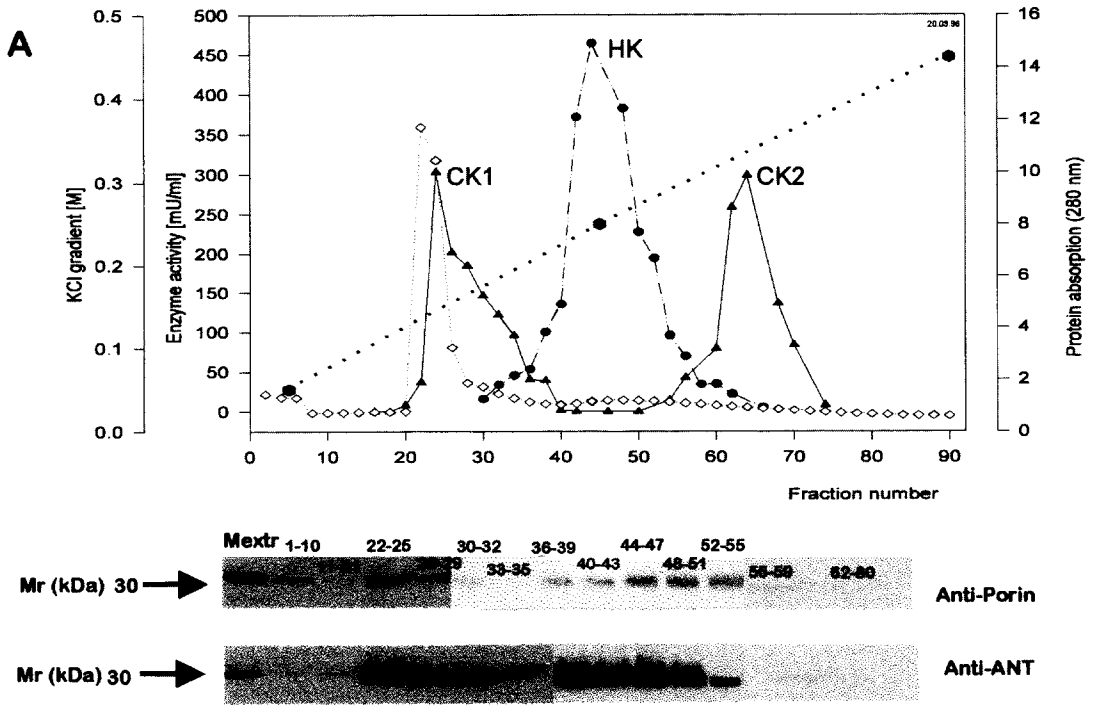
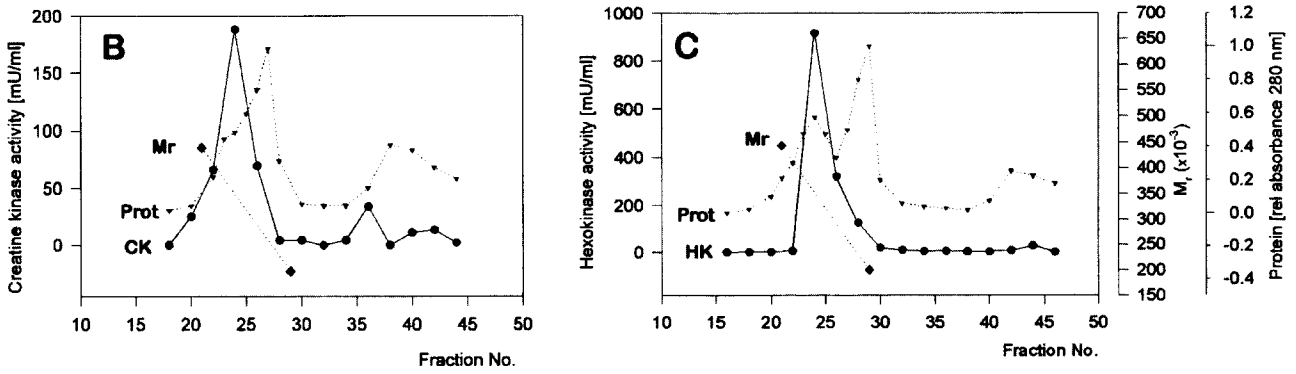
Fig. 1. Isolation and characterisation of hexokinase and creatine kinase complexes from rat brain. A: Anion exchange chromatography of Triton X-100 extracts from on DE 52 cellulose. Rat brains, stored frozen, were homogenised with a Teflon potter in a medium composed of 10 mM glucose, 10 mM monothioglycerol, pH 8. The membranes were washed 3 times by centrifugation for 15 min at $12000 \times g$. The complex was extracted from the final pellet by re-suspension in isolation medium containing 0.5% Triton X-100 and incubation for 45 min at room temperature. The undissolved membrane material was removed by centrifugation for 45 min at $40000 \times g$ in a Beckman Ti 50 rotor. The supernatant was stirred for 30 min at room temperature with DE 52 (Whatman) cellulose that had been equilibrated with 1.5 mM Na_2HPO_4, 1.5 mM K_2HPO_4 pH 8.0, 1.0 mM dithioerythritol, 0.1 M glucose. The amount of DEAE cellulose used was 3 g per U of hexokinase. Upper panel: After incubation a column was packed and the complexes were eluted with a linear KCl gradient between 0.05 and 0.5 M. Hexokinase (HK) and creatine kinase (CK1, CK2) were determined in 96 fractions of 2.5 ml. Lower panel: The fractions were collected and run on SDS-PAGE. The gels were blotted on nitrocellulose and the sheets were decorated with antibodies against porin (Anti-Porin) and adenylate translocator (Anti-ANT). The 30 kDa region of the gels is shown. Mextr. = membrane extract loaded to the column. B, C: Gel-permeation chromatography. D: Electrophoresis. E: Immuno-decoration. The hexokinase activity or mitochondrial creatine kinase peak from the DEAE column (A) was loaded on a Bio-Sil SEC 400 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 activity of creatine kinase (B) or hexokinase (C) was monitored by optical test. The Bio-Sil column was calibrated by separate runs under the same conditions as above using thyreoglobulin, apoferritin, IgG and ovalbumin as molecular mass standards corresponding to 669, 443, 158 and 44 kDa respectively. Peak determination in the calibration runs was performed by OD measurement at 280 nm wavelength. Fractions containing creatine kinase or hexokinase activity from the DEAE column (1.HK, 1.CK) and Bio-Sil column (2.HK, 2.CK) were concentrated by TCA precipitation and were subjected to tricine SDS-PAGE (D). The polypeptides from the PAGE were transferred to nitrocellulose sheets and the presence of adenylate translocator and porin was analysed by immuno-decoration of the sheets with specific antibodies (E). Mextr. = membrane (Triton) extract that was loaded to the DEAE column. kDa = molecular weight marker.


A
HK
CK1
CK2
KCl gradient [M]
Enzyme activity [mU/ml]
Protein absorption (280 nm)
Fraction number
Mextr 1-10 22-25 30-32 36-39 44-47 52-55
33-35 40-43 48-51 56-59 62-80
Mr (kDa) 30
Anti-Porin
Mr (kDa) 30
Anti-ANT


B
Mr
Prot
CK
Creatine kinase activity [mU/ml]
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C
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HK
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Mr (x10-3)
Protein [rel absorbance 280 nm]
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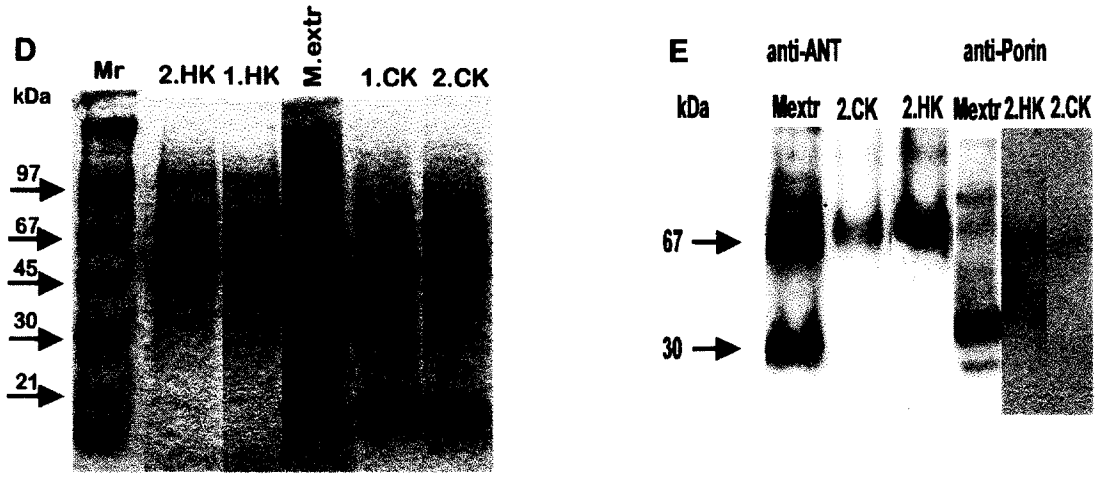
D
kDa
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activity profile of creatine kinase also showed a peak in the region of 400 000 and in addition a second smaller activity peak in the 100 000 M_r region (Fig. 1B). The results suggested that the fractions of approximately 400 kDa contained a complex of either 4 hexokinases (M_r of the monomer 100 kDa) or of 4 active creatine kinase dimers (M_r of the dimer 80 kDa).

3.3.1. Co-purification of adenylate translocator and porin with the complexes. The gel-permeation chromatography led to further purification of the two kinase complexes. This can be seen from the protein elution profile. Only one protein peak correlated with the enzyme activity, while two additional protein peaks without kinase activity were separated by the chromatography step (Fig. 1B,C). Compared to the specific activity of the membrane extract, the specific activity of the hexokinase complex fraction increased 28-fold after gel-permeation chromatography and that of creatine kinase 10-fold. The SDS-PAGE of the hexokinase and creatine kinase activity peaks (Fig. 1D) from the DEAE (1.HK, 1.CK) and gel-permeation chromatography (2.HK, 2.CK) showed an increase of a 100 kDa band representing hexokinase and a 45 kDa polypeptide representing creatine kinase while, compared to the membrane extract, other polypeptide bands disappeared or were reduced. In contrast polypeptide bands in the region of 30 kDa, where porin and ANT were to be expected, appeared not to be enriched during complex purification. Also specific antibodies against porin or ANT did not decorate in the 30 kDa region. A binding of the antibodies was observed in the region of 67 kDa, suggesting the presence of porin or ANT dimers (Fig. 1E: 2.HK, 2.CK). However, when the hexokinase and mitochondrial creatine kinase peak from gel-permeation chromatography were reconstituted in bilayer membranes or vesicles, the presence of porin and adenylate translocator in the complexes could be demonstrated functionally.

3.4. Functional analysis of the kinase-porin-translocator complexes

3.4.1. Reconstitution in artificial bilayer membranes. Diphytanoyl phosphatidylcholine membranes were formed across a circular hole (surface area about 1 mm^2) in the thin wall of a Teflon cell that separates two aqueous compartments filled with 1 M KCl solution [20]. Of the complex fraction isolated from rat brain 20 μg was added to one side of a bilayer membrane (cis-side) and the conductance was recorded when a voltage of 25 mV was applied (Fig. 2A). About 1–2 conductance steps of 6 nS were observed. When the polarity was positive on the cis-side, frequent changes between two conductance sub-states of the channel of 5 and 4 nS were recorded. When the polarity was negative (−25 mV) on the cis-side the maximal conductance was 6 nS and the channel switched to a sub-state of 4 nS conductance. Addition of 0.1 mM atractyloside arrested the conductance at 6 nS. Also the voltage sensitivity was reduced mainly at positive polarity on the cis-side.

The open time of the 6 nS conductance channels was voltage dependent and significantly longer at positive polarity. At negative polarity on the cis-side the open time of 6 nS conductance channels was already significantly reduced at −15 mV, whereas with positive polarity on the cis-side the open time decreased above +25 mV (Fig. 2B). Addition of 0.1 mM atractyloside reduced the voltage sensitivity. The 6 nS channel remained in the open state up to +45 mV when the polarity of the applied voltage was positive, while it changed to the 4 nS conductance state at negative polarity above −30 mV (Fig. 2B). The observed conductance is comparable to that of a mitochondrial channel described by Kinally [21] and Zoratti [22] that appeared to be identical [22] with the cyclosporin A sensitive permeability transition pore [23,24]. In agreement with this thought was the arresting of the channel in the open state and the reduction of voltage sensitivity of pore conductance by atractyloside. This effect was not seen with pure porin pores (results not shown). Atractyloside as inhibitor of the ANT has been described to open the permeability transition pore [24,25]. The reconstituted ANT accomplished a high conductance pore in the presence of Ca^{2+}. This was shown recently in patch-clamping experiments by Brustovetsky and Klingenberg [26]. The effect of atractyloside in the experiments above showed that the translocator was a component of the incorporated channels. However, the pore was open without Ca^{2+} indicating a different behaviour of the ANT in a complex with porin.

3.5. Reconstitution in vesicles

3.5.1. Function of the adenylate translocator. Free porin and the hexokinase complex or the mitochondrial creatine kinase complex was reconstituted in Asolectin/cholesterol vesicles. The vesicles were loaded with 5 mM ATP by sonification and the external ATP was removed by a subsequent run through a Sephadex G50 column. Pure phospholipid vesicles were retaining internal ATP during this procedure, whereas vesicles containing free porin (85 μg/ml vesicle suspension) were losing it completely. However, the vesicles with reconstituted hexokinase or creatine kinase complex retained ATP inside, although, as calculated, 25–50 μg/ml porin was present in the complexes. This suggested that the permeability through the pore in the complex was controlled. In addition we investigated the vesicle permeability for ATP by centrifugation for 30 min at 400 000 × *g*. The majority of the ATP was sedimented. When the creatine kinase complex was reconstituted less than 1% was observed in the supernatant, whereas 25–30% of the ATP leaked out from the vesicles containing

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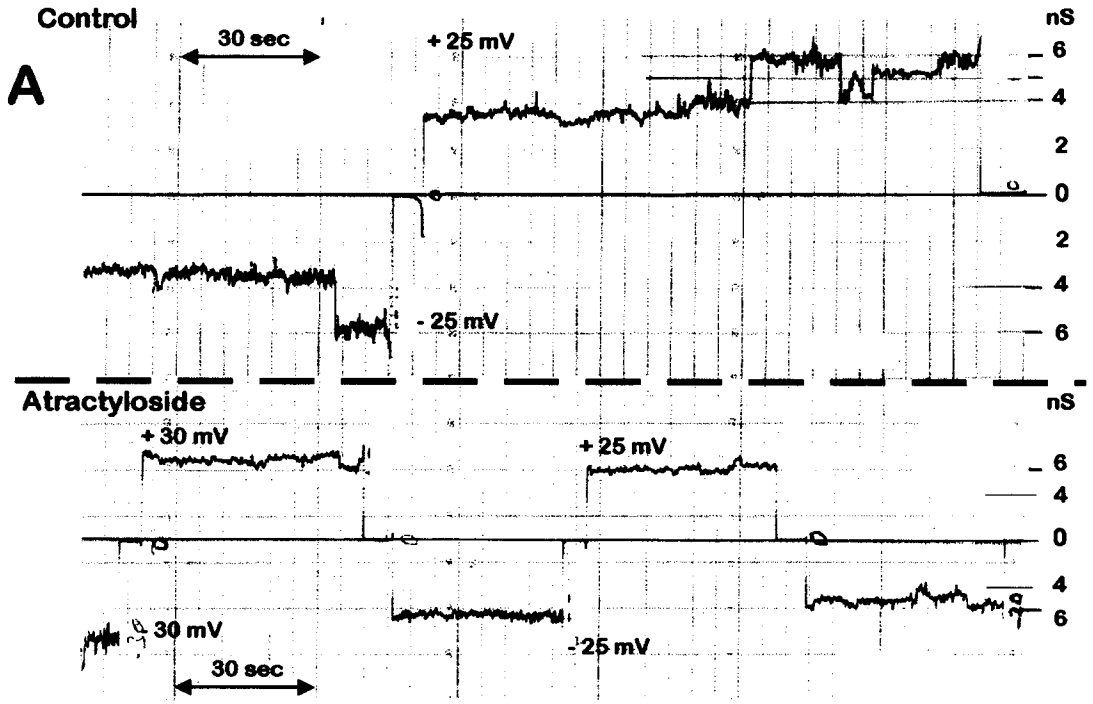
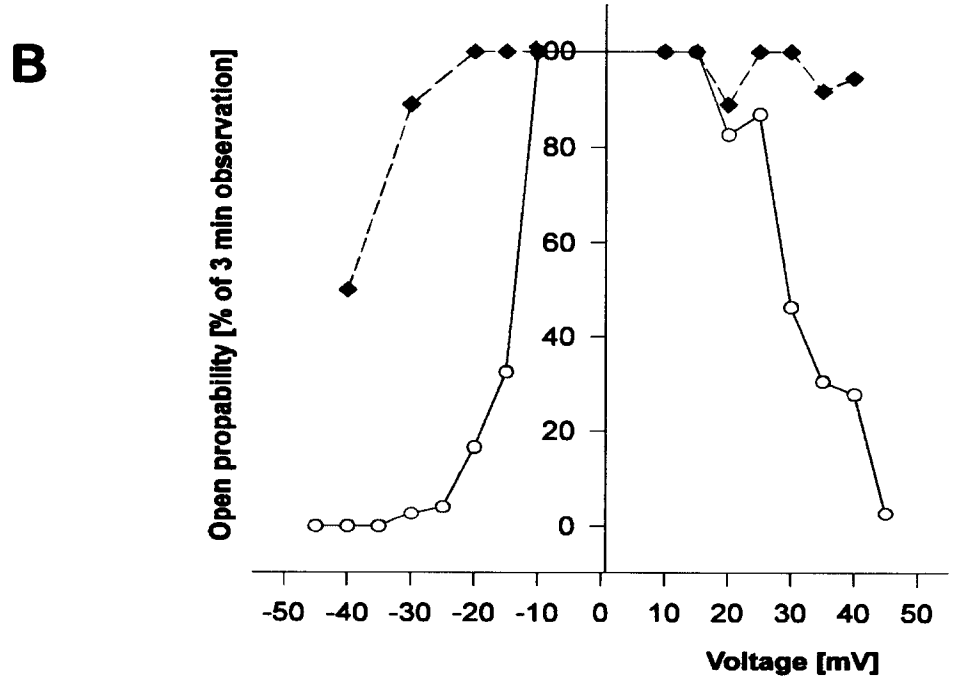
Fig. 2. Reconstitution of the hexokinase complex in planar bilayer membranes. A: Chart recording of single-channel conductance after addition of hexokinase-porin-adenylate translocator complex to a lipid bilayer membrane. The fractions 25 and 26 from gel-permeation chromatography of the hexokinase complex shown in Fig. 1C were combined and were used for the 'black' lipid bilayer experiments previously described by Benz et al. [20]. Of the sample, 20 μl was added to one side (cis-side) of an artificial membrane formed of diphytanoyl-phosphatidyl-choline/*n*-decane in 1 M KCl solution. 25 mV was applied and the current across the membrane was recorded. The conductance increased in discrete steps of 6 nS (depending on the polarity) representing incorporation of a single complex. Upper trace: The incorporated channels in the complex with hexokinase appeared to switch frequently between lower conductance states of 5 nS and 4 nS. Lower trace: In the presence of 0.1 mM atractyloside, the frequent changes of conductance were suppressed. At ±25 mV the channel stayed in the open state of 6 nS. B: Open probability of conductance sub-states of the channel formed by the hexokinase-porin complex. Hexokinase-porin complex was incorporated into artificial membranes as described above. The conductance of the channel was studied under increasing voltage with different polarity. Chart recordings as shown above were analysed for the open time of the 6 nS conductance states during several time periods in the presence and absence of 0.1 mM atractyloside. The data are shown as % of open time during 3 min at different applied voltages.

the hexokinase complex. Creatine kinase in the complex produced creatine phosphate from 5 mM external creatine and internal ATP as shown by the time course in Fig. 3A. The amount of creatine phosphate formation depended on the ATP concentration in the vesicle fraction. The ANT appeared to be involved in this process because creatine phosphate production was inhibited completely by atractyloside, indicating that only internal ATP was used by creatine kinase in the complex. Similar results were obtained with the hexokinase complex reconstitution. Glucose-6-P was formed from external glucose and internal ATP. However, because of some leak of the vesicles, atractyloside addition did not completely abol-





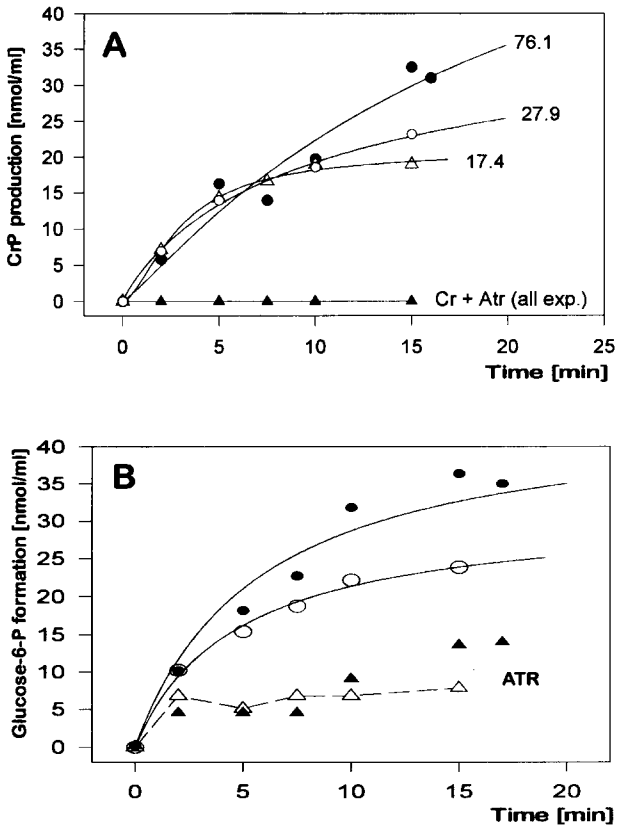


Fig. 3. Reconstitution of creatine kinase and hexokinase complexes in phospholipid vesicles: function of adenylate translocator. The hexokinase or creatine kinase complex containing, ATP loaded vesicles were incubated for 1–20 min at room temperature with 5 mM glucose or 5 mM creatine, 10 mM $MgCl_2$, and 0.1 mM ADP in the presence or absence of 0.1 mM atractyloside. The reaction was terminated by addition of perchloric acid and glucose-6-P or creatine-phosphate was determined in the supernatant. A: Time dependence of creatine phosphate formation by complex creatine kinase from external creatine. Three experiments with different internal ATP concentrations. Creatine phosphate production was completely inhibited in all experiments by the presence of 0.1 mM atractyloside, represented by one curve (filled triangles). B: Time dependence of glucose-6-P (G-6-P) formation by complex hexokinase from external glucose. Two experiments, filled and open circles, with different internal ATP concentrations. G-6-P production was significantly inhibited by 0.1 mM atractyloside (filled and open triangles).

ish the reaction (Fig. 3B). On the whole the experiments showed that the ANT was a component of both kinase complexes. The specific hexokinase binding in the hexokinase complex suggested that porin was an additional component.

3.5.2. Function of the permeability transition pore. It has been postulated by several authors [24–28] 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 (to generate a diffusion potential across the membrane) and 5 mM malate or 5 mM ATP. Upon centrifugation the vesicles proved to be sealed for the substrates. 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 preincubation with 0.5 μM *N*-methylVal-4-cyclosporin (Fig. 4). In one experiment (Fig. 4A) the release of malate by 500 μM Ca^{2+} could be inhibited through the activity of hexokinase with 5 mM glucose and 0.2 mM ATP. *N*-MethylVal-cyclosporin is a derivative of cyclosporin A that has been characterised as a specific inhibitor of the permeability transition pore [22–30]. *N*-MethylVal-cyclosporin binds to the mitochon-

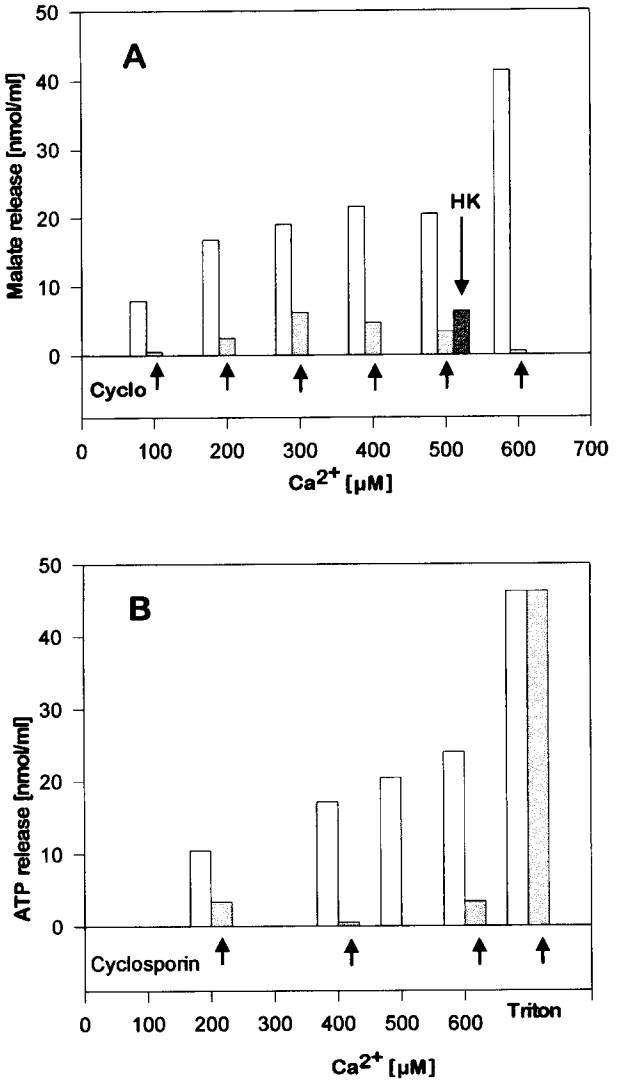


Fig. 4. Reconstitution of the hexokinase complex in phospholipid vesicles: function of permeability transition pore. A: The hexokinase complex from the DE 52 column (Fig. 1A) was reconstituted in vesicles. The vesicles were loaded with 10 mM KCl and 5 mM malate and were incubated for 15 min in 250 mM sucrose, 10 mM HEPES pH 7.4 with different concentrations of Ca^{2+} between 50 and 600 μM. The same experiment was performed with vesicles that had been preincubated with 0.5 μM *N*-methylVal-4-cyclosporin. In the case of hexokinase (HK) the release of malate was reduced by ADP produced by activity of hexokinase from 5 mM external glucose and 0.2 mM ATP. B: The hexokinase complex, further purified by gel-permeation chromatography (Bio-Sil column, Fig. 1C), was reconstituted in vesicles. The vesicles were loaded with 10 mM KCl and 5 mM ATP and were incubated for 15 min in 250 mM sucrose, 10 mM HEPES pH 7.4 with increasing concentrations of Ca^{2+} between 50 and 600 μM. The same experiment was performed with vesicles that had been preincubated with 0.5 μM *N*-methylVal-4-cyclosporin. After 15 min incubation the vesicles were removed by centrifugation for 45 min at 100 000×*g* and malate or ATP was determined in the supernatant.

drial cyclophilin but not to calcineurin [29,30]. Furthermore, 30–100 μM ADP inhibits the pore [23,28] while ATP has this effect in mM concentrations [31]. The inhibition of ATP release by *N*-methylVal-cyclosporin indicated that the hexokinase complex might resemble the permeability transition pore. The inhibitory effect of external ATP and glucose on malate release pointed to a possible physiological regulation of the permeability transition pore by hexokinase through the ADP produced by the enzyme reaction.

4. Conclusion

The results show that hexokinase I and mitochondrial creatine kinase in brain exist physiologically as complexes with porin and the adenine nucleotide carrier. The interaction between kinases, porin and ANT would provide the structural basis of contact sites between the two mitochondrial envelope membranes [32]. In the hexokinase complex, porin and ANT interacted directly as has been assumed for the benzodiazepine receptor [33]. In contrast in the creatine kinase complex, the kinase performs the interaction with the two membrane components. In both cases a structure was generated that coupled the enzyme directly to internal ATP (Fig. 3). This coupling explains the recent observation of Laterveer et al. [34] and Gellerich et al. [15] in isolated mitochondria with contact sites that were increased by the presence of dextran [12]. On induction of contact sites by macromolecules, the ADP produced by hexokinase or creatine kinase was channelled into the matrix and was less accessible for external pyruvate kinase. The interaction between porin and ANT in the hexokinase complex, in the absence of glucose and hexokinase activity, formed a pore that caused permeability transition of the vesicles. So far we have not been able to induce permeability transition with the reconstituted creatine kinase complex. Thus, mitochondrial creatine kinase between the two membrane components may hinder the formation of permeability transition pores. Recent investigations showed that opening of the permeability transition pore induces apoptosis through release of a molecule > 10 kDa from the mitochondria [35]. Related to this observation it is interesting to note that mitochondria in highly glycolytic HT29 tumour cells were lacking contact sites [32,36]. This might explain why these tumour cells did not undergo apoptosis as the mitochondria could not form permeability transition pores.

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