Complexes between hexokinase, mitochondrial porin and adenylate translocator in brain: regulation of hexokinase, oxidative phosphorylation and permeability transition pore

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Introduction
Hexokinase I binds to the mitochondrial surface of brain [1], muscle [2], fat [3], kidney [4] and liver cells [5]. Responsibility for this association lies with a specific binding protein [6], which has been identified as mitochondrial porin in the outer membrane [7,8] and is also known as voltage-dependent anion channel (VDAC) [9]. The isolated outer membrane pore, when reconstituted in liposomes, specifically binds hexokinase [6,7]. However, the distribution of hexokinase and porin at the mitochondrial surface of intact liver and brain mitochondria do not coincide. The localization of hexokinase was analysed by electron-microscopic techniques and by removing unattached outer membrane with digitonin. By the use of these techniques, the enzyme was found to be concentrated in contact sites between the mitochondrial envelope membranes in liver [10] and brain mitochondria [11,12]. In contrast, porin appeared to be randomly distributed in the outer membrane [13,14].

This discrepancy was explained later by analysis of isolated mitochondrial contact sites. In contact-site fractions enriched from osmotically disrupted liver, brain and kidney mitochondria, hexokinase was concentrated [11,14,15] and had a significantly higher affinity for this membrane fraction than for isolated outer membrane [14,16]. By freeze–fracture analysis it was observed that contact sites were dynamic structures that were dependent not only on the functional state of the mitochondria [17,18] but also on the preservation of the physiological structure of the outer compartment during isolation [19]. In freeze–fractured mitochondria, fracture planes ran through the contact sites, pointing to a hydrophobic interaction between components of the two boundary membranes [17].

Because of the higher affinity of hexokinase for contact sites, the binding of the enzyme to mitochondria was dependent on the frequency of these contact sites. Thus hexokinase binding could be used to indicate contact-site frequency.

This was demonstrated when mitochondria with intact outer compartment and high frequency of contact sites were compared with those with separated boundary membranes and almost no contact sites. Hexokinase exhibited sigmoidal binding behaviour and was activated by binding to contact-site-rich mitochondria. In mitochondria without contact sites, however, the enzyme-binding curve was hyperbolic and resembled that of isolated outer membrane [19]. The activation of the enzyme and the sigmoidal binding to contact sites suggests its oligomerization. Indeed Xie and Wilson [20], by cross-linking experiments, observed the formation of hexokinase tetramers on binding to mitochondrial membranes.

Formation of complexes between hexokinase and porin in vitro
Encouraged by these results, we studied the interaction of porin (30 kDa) and hexokinase (100 kDa) in vitro. Isolated brain hexokinase I [21] was gradually activated up to 2.5-fold by the addition of increasing amounts of porin, which had been isolated with octyl glucoside [22]. The maximal activation was less than that observed during binding to mitochondria [10]. This could be explained by the fact that, under the experimental conditions used, only 27% of the total enzyme activity formed a complex with porin, which was sedimented on centrifugation for 1 h at 326,000 g. The sedimented hexokinase had an oligomeric structure. This was shown by gel-permeation chromatography of the resuspended pellet. The latter analysis was performed as described in Figure 2 for the hexokinase–porin complex from brain homogenate (see below) with the same result. A molecular mass of 440 kDa was determined, suggesting a tetramer of hexokinase.

Extraction of hexokinase–porin complexes from tissue homogenates
From freeze–fracture studies, it was assumed that contact sites were formed by hydrophobic interaction between components of the two boundary membranes [17,18]. In addition, hexokinase isoenzyme I interacts hydrophobically with

Abbreviation used: G6P, glucose 6-phosphate.
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phospholipid membranes [23]. It thus appeared possible, by employing detergent extraction, to isolate directly from tissue homogenates a hexokinase-containing complex consisting of the contact sites.

Rat brain, which had been stored frozen, was homogenized in a Teflon Potter–Elvehjem homogenizer. The membranes were washed by centrifugation and then extracted in medium containing 0.5% Triton X-100. After removal of the undissolved membrane material by centrifugation, the supernatant was bound to DEAE-cellulose DE-52 and packed into a column, from which hexokinase activity was eluted by a KCl gradient between 0.05 and 0.5 M. Hexokinase and creatine kinase were determined in the fractions. The elution profile of rat brain extract (Figure 1, top) shows a single peak of hexokinase activity separated from two creatine kinase peaks. The first creatine kinase peak represents mitochondrial creatine kinase, and the second was characterized as the cytosolic isoenzyme. The concentrated column fractions were subjected to SDS/PAGE. After polypeptides in the gels had been blotted on to nitrocellulose sheets, immunodecoration with specific antibodies against porin and adenylate translocator was performed. As shown in Figure 1(bottom), the distribution of porin and adenylate translocator coincided with the first creatine kinase peak and the hex-

Figure 1
Isolation of Triton X-100 extracts from rat brain on DEAE-cellulose DE-52

Rat brains, which had been stored frozen, were homogenized in a Teflon Potter–Elvehjem homogenizer in medium composed of 10 mM glucose and 10 mM monothioglycerol, pH 8. The membranes were washed three times by centrifugation for 15 min at 12,000 g. The complex was extracted from the final pellet by resuspension 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 40,000 g in a Beckman Ti 50 rotor. The supernatant was stirred for 30 min at room temperature with DEAE-cellulose DE-52 (Whatman) that had been equilibrated with buffer A (1.5 mM Na2HPO4, 1.5 mM KH2PO4, pH 8.0, 1.0 mM dithioerythritol, 0.1 M glucose). The amount of DEAE-cellulose used was 3 g per unit of hexokinase. Top: After incubation, a column was packed and the complexes were eluted with a linear KCl gradient between 0.05 and 0.5 M KCl (---). Hexokinase (HK) and creatine kinase (CK1, CK2) were determined in 96 fractions of volume 2.5 ml. ○, Protein; ▲, creatine kinase; ●, hexokinase. Bottom: 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 on the column.
okinase peak. The results suggest that hexokinase and mitochondrial creatine kinase might be present as complexes with porin and the adenylate translocator.

**Characterization of the hexokinase fraction by gel-permeation chromatography**

The fractions of the hexokinase peak from rat brain were concentrated and loaded on a Superose 6B column. The enzyme activity was eluted with 3 mM potassium phosphate/0.1 M glucose, pH 8.0. The result was essentially the same as that obtained for complex-formation in vitro. Hexokinase activity correlated as a single peak, which corresponded to a molecular mass of 443 kDa (Figure 2a). The enzyme activity correlated with the second protein peak that was eluted from the column. Two additional protein fractions without hexokinase activity were removed by this chromatography step, suggesting further purification of the complex. Indeed the specific activity of hexokinase after Superose chromatography increased 28-fold over the first membrane extract. Between 80 and 90% of hexokinase in rat brain homogenates was membrane-bound. As the extracted hexokinase activity originated from mitochondrial membranes disrupted by freezing the brains, it may be assumed that the physiological state of the bound hexokinase was not disrupted in the fragmented membrane on thawing. This would mean that hexokinase complexes represent approx. 80% of the total enzyme activity in the active rat brain.

**Dissociation of the complex with urea**

Free hexokinase purified from rat brain [21] and the hexokinase complex fractions were treated for 15 min on ice with urea concentrations from 0 to 6 M. In contrast with the free enzyme, hexokinase activity in the complex fraction decreased by 90% with increasing urea concentrations, indicating dissociation of the oligomer (Figure 2d). The latter assumption was confirmed by subsequent molecular-mass analysis of the complex treated with 1 or 4 M urea. Superose 6B chromatography (Figure 2) after treatment with 1 M urea led to a hexokinase activity maximum in the region of 443 kDa and a second smaller peak in the region of 100 kDa (Figure 2b). Treatment with 4 M urea, however, resulted in only one activity maximum in the 100 kDa region (Figure 2c). The fractions in the region of 30 kDa were proved immunologically to contain porin. In order to estimate the stoichiometry of hexokinase, porin and adenylate translocator, the 100 and 30 kDa peak fractions were isolated from the eluate and concentrated. Protein determination gave a stoichiometry of 4 mg of hexokinase to 1 mg of the 30 kDa polypeptide fraction composed of porin and adenylate translocator.

**Functional analysis of the hexokinase–porin–translocator complex**

**Reconstitution in artificial bilayer membranes**

Diphytanoylphosphatidylcholine membranes were formed across a circular hole (surface area about 1 mm²) in the thin wall of a Teflon cell that separates two aqueous compartments filled with 1 M KCl solution. Of the complex fraction isolated from rat brain, 10–20 µg was added to one side (cis side) of a bilayer membrane and the conductance was recorded when a voltage of 25 mV was applied. About one to two conductance steps of 6–8 nS were observed. When the polarity was positive at the cis side, frequent changes between two conductance substates of the channel of 6 and 7 nS were recorded. When the polarity was negative (−25 mV) at the cis side, the maximal conductance was 6 nS and the channel switched to a substate of 4 nS conductance. Addition of 0.1 mM atractylloside reduced the conductance to 6 nS (+) and 4 nS (−) respectively. Also the voltage sensitivity was decreased mainly at positive polarity on the cis side.

The length of time that the 6–8 nS conductance channels were open 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 at positive polarity on the cis side the open time started to decrease above +25 mV. Addition of 0.1 mM atractylloside 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, and it changed to the 4 nS conductance state at negative polarity above −30 mV. The pore conductance observed resembled that reported in patch-clamp experiments [24,25] for the cyclosporin A-sensitive mitochondrial permeability transition pore [26]. In agreement with this similarity was the reduction of voltage sensitivity of pore conductance caused by atractylloside. This inhibitor of the adenylate translocator has been reported to open the permeability transition pore [27]. Furthermore the effect of
Figure 2
Isolation and characterization of hexokinase complex by gel-permeation chromatography

(a) The fractions from the DEAE-cellulose DE-52 column (Figure 1) containing hexokinase activity were loaded on a 30 ml Superose 68 column and eluted with equilibrium buffer A (Figure 1); (b) part of the hexokinase complex was incubated for 15 min with 1 M urea at 0 °C before Superose chromatography; (c) a third aliquot of the complex was incubated with 4 M urea as above. The eluate was collected in 120 fractions of volume 250 μl in which the activity of hexokinase was determined by spectroscopy. Thyroglobulin, apoferritin, alcohol dehydrogenase and albumin were used as molecular-mass standards corresponding to 667, 443, 150 and 67 kDa respectively (indicated by vertical lines): O, A_{280}; ●, hexokinase activity. (d) Free hexokinase isolated from rat brain and hexokinase–porin complex from brain were treated with different concentrations of urea. The activity was determined after 15 min incubation on ice.
Reconstitution in vesicles
Isolated porin [22] and the hexokinase complex were reconstituted in asolectin/cholesterol liposomes. The vesicles were loaded with 5 mM ATP and 10 mM KCl by sonication, and external ATP was removed by a subsequent run through a Sephadex G-50 column. Pure phospholipid vesicles retained internal ATP during this procedure, whereas vesicles containing purified porin (85 μg/ml of vesicle suspension) lost it completely. However, the vesicles with reconstituted hexokinase complex retained ATP inside even though 25–50 μg/ml porin was present in the vesicles. This suggested that the permeability through the pore in the complex was controlled.

To investigate whether the vesicles leaked ATP, they were centrifuged for 30 min at 400 000 g. The majority (70–80%) of the ATP was sedimented; however, some (20–30%) leaked out and was observed in the supernatant. Hexokinase in the complex formed glucose 6-phosphate (G6P) in the presence of 5 mM glucose and 10 mM Mg2+ with internal ATP (Figure 3A). The adenylate translocator appeared to be involved in this process because up to 70% of the G6P production was inhibited by atractyloside, indicating that mainly internal ATP was used by hexokinase in the complex.

It has been postulated by several authors [27–30] that the mitochondrial permeability transition pore might be formed by porin and the adenylate translocator. A direct interaction between porin and adenylate translocator could be assumed to occur in the hexokinase complex. To investigate whether the hexokinase complex resembles the permeability transition pore, the complex was reconstituted in vesicles that were loaded with 10 mM KCl and 5 mM ATP or 5 mM malate, and ATP or malate was liberated by addition of increasing Ca2+ concentrations between 50 and 600 μM (Figure 3B).

(A) Functional test of the adenylate translocator. The hexokinase complex fraction from the DEAE-cellulose DE-52 column (Figure 1) or after gel-permeation chromatography (Figure 2d) was reconstituted in asolectin phospholipids +2% cholesterol. The mixture of phospholipid and complex was dialysed overnight against 0.25 M sucrose/10 mM Hepes, pH 7.4. The hexokinase-complex-containing vesicles were loaded with 5 mM ATP and 10 mM KCl and incubated for 1–15 min at room temperature with solution containing 5 mM glucose, 10 mM MgCl2, and 0.1 mM ADP in the presence or absence of 0.1 mM atractyloside. The reaction was terminated by the addition of perchloric acid, and G6P was determined in the supernatant. O, time-dependence of G6P formation by complex hexokinase from external glucose at two different internal ATP concentrations. △, G6P production in the presence of atractyloside at the two different internal ATP concentrations. (B) Functional test of the permeability transition pore. The hexokinase-complex-containing vesicles loaded with 10 mM KCl and 5 mM ATP or 5 mM malate were incubated for 15 min in 250 mM sucrose/10 mM Hepes, pH 7.4, with increasing concentrations of Ca2+ between 50 and 600 μM N-methylvaline-4-cyclosporin (Cyclosporin). In one case (HK), the vesicles were loaded with 5 mM malate, and incubation with 500 μM Ca2+ was performed in the presence of 5 mM glucose and 0.2 mM ATP. After 15 min incubation, the vesicles were centrifuged for 45 min at 100 000 g, and the liberated ATP or malate was determined in the supernatant. The ATP in the vesicles was completely released by the addition of Triton X-100.
cyclophilin but not to calcineurin [31]. Furthermore, 30–100 μM ADP inhibits the pore [27,30], whereas ATP has this effect at millimolar concentrations [28]. The inhibition of ATP release by N-methylvaline-cyclosporin indicated that the hexokinase complex may 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 via the ADP produced by the enzyme reaction.

Conclusions
The results show that some of the hexokinase I in brain exists physiologically in an activated state as an oligomer at the mitochondrial surface. Hexokinase oligomerization occurs in complexes with porin and the adenine nucleotide carrier. The latter interaction between porin and adenylate translocator provides the structural basis of contact sites between the two mitochondrial envelope membranes [32]. In the hexokinase complex, porin and the adenine nucleotide carrier form a channel, which couples the enzyme directly to internal ATP (Figure 3/4). This coupling process explains the recent observation of Laterveer et al. [33] in isolated mitochondria of increased contact sites because of the presence of dextran [19]. On induction of contact sites by macromolecules, the ADP produced by hexokinase is channelled into the matrix, making it less accessible to external pyruvate kinase. In the absence of glucose and hexokinase activity, however, the channel formed from porin and the adenine nucleotide carrier may open as a permeability transition pore to release a peptide that induces apoptosis [34]. Related to this observation is the interesting observation that mitochondria in highly glycolytic HT29 tumour cells lack contact sites [32,35]. This might explain why the tumour cells did not undergo apoptosis: the mitochondria were unable to form permeability transition pores.

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Role of Glc-6-P in the activation of hepatic glycogen synthase

The mechanism by which glycogen synthesis is activated in liver in response to increased circulating glucose levels is controversial. It was originally suggested that unmetabolized glucose can indirectly activate glycogen synthase, the key enzyme in the control of glycogen synthesis, by relieving the inhibitory effect of phosphorlase $\alpha$ on the protein phosphatases (1 and 2A) responsible for dephosphorylation, and hence activation, of glycogen synthase [1]. Several lines of evidence, however, argue that free glucose cannot be solely responsible for the activation of glycogen synthesis and suggest that glucose phosphorylation is a key step in the activation of glycogen synthesis [2–5]. There are several mechanisms by which Glc-6-P can regulate activation of glycogen formation. Increases in intracellular Glc-6-P may lead to the allosteric activation of glycogen synthase, this effect being reversible when Glc-6-P returns to basal levels [6]. In addition, Glc-6-P promotes the covalent activation of glycogen synthase, possibly inducing a conformational change that favours the dephosphorylation of the enzyme by phosphatases [7]. Finally, increases in Glc-6-P levels trigger translocation of glycogen synthase between a supernatant fraction and a $10,000g$ pellet fraction. This effect has been observed in cultured and isolated hepatocytes as well as $\textit{in vivo}$ [8–10].

Differential metabolic effects of hexokinase I (HKI) and glucokinase (GK) on hepatic glycogen synthesis

Glucose is phosphorylated to Glc-6-P in mammalian cells by members of the hexokinase gene family. Hepatocytes contain primarily GK, also called hexokinase IV, and small amounts of HKI [11]. These two enzymes differ in that GK is approximately half as large, is not allosterically inhibited by Glc-6-P, and has a lower affinity for glucose.

Different metabolic effects are observed when GK and HKI are overexpressed in rat hepatocytes by adenovirus-mediated gene transfer. GK-overexpressing hepatocytes exhibit a large increase in glycogen synthesis, whereas overexpression of HKI has no effect at any concentration of glucose [12]. Overexpression of GK in hepatocytes has potent effects on glucose storage (glycogen synthesis) and glycolytic flux. Overexpression of HKI, in contrast, has no effect on glycogen synthesis and its effect on the glycolytic pathway occurs mainly at low glucose levels and is less extensive than with GK overexpression. The proportion of HKI activity associated with mitochondria-enriched cellular fractions is the same in untreated and HKI-overexpressing cells, suggesting that the relatively weak metabolic effect induced by HKI overexpression is not due to lack of access to mitochondrial binding sites. In GK-overexpressing cells activation of glycolysis, an energy-producing pathway, occurs at glucose levels that are at or below the fasted range (1–5 mM), whereas the stimulation of glycogen synthesis occurs in the range of glucose.

Abbreviations used: GK, glucokinase; HKI, hexokinase I.