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Trace amounts of Triton X-100 modify the inhibitor sensitivity of the mitochondrial porin

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Abstract

Transport properties of mitochondrial porin were investigated on the basis of changes in the activity of hexokinase utilizing external ATP. Production of glucose 6-phosphate is inhibited by polyanion both in intact brain mitochondria and in contact point vesicles. Hexokinase activity is restored by solubilization of the enzyme by high ionic strength or 0.5–1% Triton X-100. In very low concentrations (0.001–0.005%) Triton does not mobilize hexokinase from its binding sites but it is able to release polyanion-inhibition completely. This finding provides an explanation for the discrepancy observed in the transport properties of porin when studied 'in situ' or in artificial lipid membranes.

Keywords: Porin; VDAC; Hexokinase; Triton; Polyanion; Contact point

1. Introduction

Mitochondrial porin is the most abundant protein in the outer membrane of these particles. In planar lipid membranes the isolated protein forms wide channels, whose conductance is significantly decreased by application of a voltage difference of either polarity across the membrane. In basal state porin exhibits a slight preference for anions, it is therefore often referred to as voltage-dependent anion channel (VDAC) [1–3]. Conductance through porin channels is also significantly impaired by König's polyanion (1:2:3 copolymer of methacrylate/maleate/styrene) [4]. In the 'closed' state (induced either by voltage or by polyanion) the conductance is decreased by a factor 2 and the channel diameter is reduced from 2 nm to 0.9 nm [1,4]. In accordance with these data, in proteoliposomes reconstituted with isolated porin, the transport of phosphate or ATP is only slightly inhibited by polyanion [5].

In intact mitochondria the transport properties of porin have been assessed on the basis of the function of enzymes located in the intermembrane space that obtain their substrates partly from the extra- and partly from the intramitochondrial compartment. The activity of both adenylate kinase and creatine kinase has been almost completely blocked by polyanion but it could be restored by solubilizing the outer membrane either by digitonin or by Triton X-100 [6,7]. These data indicate that in contrast to the reconstituted system, in intact mitochondria the transport of low molecular weight substrates (ATP, AMP, creatine) through porin can be fully inhibited by polyanion. The discrepancy between the results of experiments carried out on intact mitochondria or with isolated porin in artificial membranes was explained by suggesting the existence of additional factors modifying the transport properties of porin residing in its natural environment [8,9].

Porin situated in the outer mitochondrial membrane presents a binding site for hexokinase and it is enriched in the contact sites, i.e., the regions where the outer and inner membranes are in close connection [25,10]. Hexokinase activity was shown to be higher when ATP is produced in the course of oxidative phosphorylation than when provided in the extramitochondrial space [11]. These observations suggest that the active site of the enzyme is probably facing the intermembrane space and it is only limitedly accessible for external ATP but freely accessible for ATP synthesized in and released from the matrix. This arrangement of the proteins could provide another model to test

Abbreviations: VDAC, voltage-dependent anion channel; HK, hexokinase; CK, creatine kinase; DCCD, *N,N'*-dicyclohexylcarbodiimide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Triton, Triton X-100.

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the transport capacity of porin in its natural location. One single previous report investigated the effect of polyanion on exogenous brain hexokinase rebound to liver mitochondria. Benz et al. [7] found that polyanion did not inhibit hexokinase when ATP was supplied from the external space whereas it did inhibit when ATP was produced by oxidative phosphorylation.

The aim of the present study is to promote understanding of the different behavior of porin in artificial lipid membranes and 'in situ' by investigating the effect of polyanion on the endogenous hexokinase activity of brain mitochondria. Part of the results have been presented as an abstract [12].

2. Materials and methods

2.1. Chemicals

Glucose-6-phosphate dehydrogenase, NADP, ATP, cytochrome *c*, NADH, creatine, phospho*enol*pyruvate, oligomycin, pyruvate kinase/lactate dehydrogenase were purchased from Sigma, Nagarse and DCCD from Serva, Ficoll from Pharmacia. All other reagents were of analytical grade. Polyanion (1:2:3 copolymer of methacrylate/maleate/styrene) was a generous gift of Dr. Tamás König.

2.2. Preparation of brain mitochondria

Mitochondria from rat brain were prepared by differential centrifugation according to Clark and Nicklas [13] with modifications according to Rehncrona et al. [14] in a medium containing 225 mM mannitol, 75 mM sucrose and 0.1 mM EGTA. Inclusion of Nagarse in the initial medium did not improve the yield significantly and therefore it was omitted in the majority of the experiments. The final purification step was the removal of the synaptosomes by discontinuous Ficoll gradient (6% lower and 3% upper phase) centrifugation. Ficoll was removed from the mitochondrial fraction by washing with the isolation medium. Fatty acid free bovine serum albumin was added to the medium in a concentration of 0.1% in all the experiments where hexokinase was separated from mitochondria.

2.3. Preparation of contact point vesicles

Contact point vesicles were prepared according to Sandri et al. [15]. The last pellet of mitochondrial preparation was suspended in 3 ml hyposmotic medium (10 mM Tris-HCl pH 7.8). After 15 min incubation at 4° C 3 ml of 52% (w/w) sucrose was added and the suspension was kept on ice for further 15 min to allow the vesicles to reseal. The mitochondria were further fractionated by short (5–8 s) ultrasonic treatment (probe type Branson sonifier 250). The resulting vesicles were separated into outer membrane, contact point and inner membrane fractions by reverse discontinuous sucrose gradient centrifugation [15]. The fractions were characterized on the basis of relative specific activity of the following marker enzymes: rotenone insensitive NADH–cytochrome-*c* reductase, hexokinase and succinate dehydrogenase.

2.4. NADH–cytochrome-c reductase (rotenone-insensitive) assay

NADH–cytochrome-*c* reductase (EC 1.6.99.3) activity was determined in the presence of 1.5 μM rotenone, 0.1 mM NADH, 0.3 mM KCN and 0.1 mM cytochrome *c* in 50 mM potassium phosphate buffer (pH 7.5). Reduction of cytochrome *c* was followed spectrophotometrically at 550 nm. Vesicle fraction with the highest relative specific activity (as compared to sonicated mitochondria) was designated as outer membrane vesicles.

2.5. Hexokinase assay

Hexokinase (EC 2.7.1.1) activity was measured in the presence of 6.5 mM ATP, 10 mM glucose and 1.5 μM rotenone in 10 mM $MgCl_2$, 25 mM K-Hepes pH 7.4 by means of a coupled enzyme assay using 1 mU glucose-6-phosphate dehydrogenase and 3 mM NADP. Reduction of NADP was monitored spectrophotometrically at 340 nm. The glucose 6-phosphate production was calculated on the basis of NADPH-formation. The hexokinase activity was measured both as marker enzyme for the contact point fraction and as an indirect assay for transport through porin.

2.6. Succinate dehydrogenase assay

Succinate dehydrogenase (E.C. 1.3.99.1) was measured in the presence of 3 mM succinate, 0.3 mM KCN, 1.5 μM rotenone and 0.1 mM cytochrome *c* in 50 mM potassium phosphate pH 7.5. The reduction of cytochrome *c* was followed spectrophotometrically at 550 nm. The vesicle fraction with the highest relative specific activity was designated as inner membrane fraction.

2.7. Creatine kinase assay

Creatine kinase (EC 2.7.3.2) activity was determined in the presence of 4 mM ATP and 25 mM creatine in a coupled enzyme assay using pyruvate kinase/lactate dehydrogenase assay as indicator reaction for ADP production. The medium consisted of 7.5 mM EDTA, 20 mM Hepes, 12 mM $MgSO_4$, 4.5 mM phospho*enol*pyruvate, 1.4 μM rotenone, 14 μg/ml oligomycin, 1 mU/ml pyruvate kinase, 0.7 mU/ml lactate dehydrogenase and 0.4 mM NADH. Decrease of NADH concentration was monitored spectrophotometrically at 340 nm.

2.8. Solubilization of the bound hexokinase

Mitochondria were incubated in 50 mM potassium phosphate pH 7.5 in the presence of the respective solubilizing agent (1.5 mM glucose 6-phosphate or 250 mM KCl or 1 mM DCCD) for 10 min at 37° C. Thereafter mitochondria were sedimented by centrifugation and the pellet was resuspended in the original volume. Hexokinase activity was measured both in the pellet and in the supernatant in the absence or in the presence of 1% Triton-X 100. In control experiments mitochondria were incubated similarly as samples but without any additional agent.

2.9. Protein determination

Protein concentrations were determined according to Lowry et al. [16].

3. Results

3.1. Inhibition of hexokinase by polyanion in intact mitochondria

Endogenous hexokinase activity of intact brain mitochondria was measured in the presence of glucose and ATP in the medium, i.e., under conditions where the enzyme utilized extramitochondrial ATP as substrate. Typical results are shown in Fig. 1. Addition of polyanion resulted in complete inhibition of the enzyme activity. Similarly to adenylate kinase and creatine kinase [6,7], hexokinase activity could also be fully restored by solubilization of the membrane by 0.5–1% of Triton (Fig. 1A). Reappearance of the enzyme activity suggests that inhibition by polyanion could be due to a limitation of substrate supply rather than due to a direct inhibition of hexokinase. However, the enzyme activity obtained following Triton addition was consequently higher than the basal activity

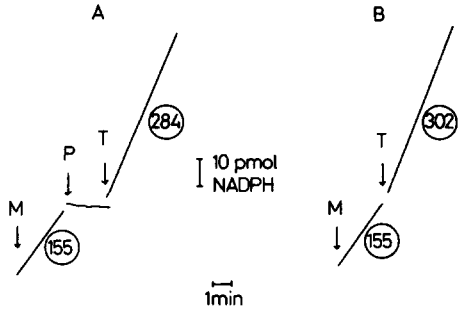

Fig. 1. Effect of polyanion treatment on the hexokinase activity in intact mitochondria. Original recording of the absorbance trace at 340 nm. The following additions were made where indicated by the arrows: M, mitochondria, 31 μg protein/ml; P, polyanion 0.3 μg/ml; T, Triton 0.5%. The numbers represent the rate of enzyme activity expressed in mU/mg mitochondrial protein. One representative experiment out of eight similar ones.

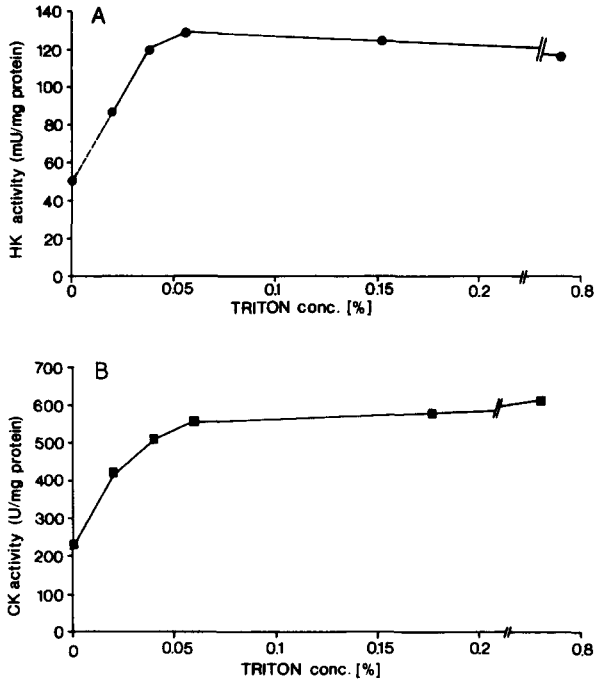

Fig. 2. Effect of Triton on the hexokinase (A) and creatine kinase (B) activity of intact mitochondria. Results of one representative experiment out of three similar ones are shown.

and this difference was clearly observable also in control samples which have not been treated with polyanion (Fig. 1B). Apparently, a fraction of the hexokinase molecules became accessible for glucose and/or ATP only in the presence of the solubilizing agent. In order to study the possible interaction of various agents with porin, conditions had to be found where the effect of Triton on polyanion-inhibited and latent activity could be clearly separated. Therefore, the latent enzyme activities of our mitochondrial preparation were investigated.

3.2. The Triton sensitive pool of hexokinase and creatine kinase

Earlier publications reported that both enzymes have a latent pool that could be revealed only in the presence of Triton, therefore it is designated 'Triton-sensitive' pool [17,18]. This latent activity was attributed to enzyme molecules located in contact sites or to mitochondria within synaptosomes [19,20]. In both cases diffusion of substrate and/or product molecules could be limited and the effect of Triton consisted of disruption of the strict organization of contact sites or solubilization of the synaptosomal plasma membrane.

A Triton-sensitive pool of both hexokinase and creatine kinase was present also in our preparation of brain mitochondria as both enzyme activities were increased by Triton (Fig. 2). The enhancement of the enzyme activity was proportional to the applied Triton concentration in the range of 0.02–0.06% and in accordance with previous

findings [7], the maximal activity observed was about 2.5-fold higher than that measured in the absence of the detergent. Thus, in the case of both enzymes, about 40% of the total activity was freely accessible for external substrate and about 60% of the total activity represented the Triton-sensitive pool.

The location of the latent hexokinase activity was investigated in more detail. It has been shown earlier that hexokinase could be liberated from mitochondria by glucose 6-phosphate or high ionic strength [17,21]. In our experiments, after incubation of brain mitochondria either with 1.5 mM glucose 6-phosphate or with 250 mM KCl, 64% of the total hexokinase activity was recovered in the supernatant and only 36% remained in the separated pellet (not shown). Thus, both glucose 6-phosphate and KCl were able to mobilize a part of the Triton-sensitive pool. Hexokinase activity detected in the supernatant was not increased by the addition of Triton at all. The Triton-sensitive pool that remained attached to the mitochondrial pellet after treatment with KCl represented only 6.8% of the total hexokinase activity. Following glucose 6-phosphate treatment, the same value was 23.7% (not shown). All these data indicate that the latent, Triton-sensitive fraction of hexokinase activity is attached to the outside of mitochondria and not entrapped in synaptosomes. The experimental data are consistent with the suggestion that the latent pool could be located at contact points.

In a previous report it has been shown that sonication of brain mitochondria liberates the latent activity of creatine kinase [18]. In the next experiments we investigated whether the same procedure could be applied for hexokinase. Ultrasonic treatment of brain mitochondria for 10 s resulted in complete disappearance of the Triton-sensitive pool, hexokinase activity in the absence and presence of 1% Triton being 158.6 ± 28.6 and 139.8 ± 21.8 mU/mg mitochondrial protein ($n = 6$), respectively (not shown).

3.3. Inhibition of hexokinase by polyanion in contact point vesicles

As the preparation of contact point vesicles involved ultrasonic irradiation, we next investigated the existence of a Triton-sensitive hexokinase pool in the contact point vesicle fraction. In agreement with our presumption, hexokinase activity was only slightly influenced by Triton (the rate was 160 ± 36.6 and 181.4 ± 38.4 mU/mg mitochondrial protein in the absence and presence of 1% Triton, respectively, $n = 5$). Contact point vesicles contain only one pool of hexokinase and thus provide a suitable model for the investigation of the effect of polyanion on hexokinase activity.

The results obtained on contact point vesicles are summarized in Fig. 3. Similarly to intact mitochondria, hexokinase activity was inhibited by polyanion also in the contact point vesicles (upper trace in Fig. 3), the inhibition was $96.8 \pm 6.5\%$ ($n = 28$). Addition of 1% Triton restored the

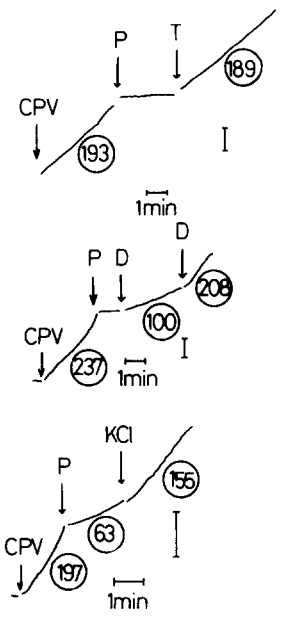

Fig. 3. Effect of polyanion treatment on the hexokinase activity of contact point vesicles. Original recording of the absorbance trace at 340 nm. The following additions were made where indicated by the arrows: CPV, contact point vesicles, 45 μg protein/ml; P, polyanion, 1 μg/ml; T, Triton, 1%; D, DCCD, 0.5 mM final concentration after the first and 1 mM after the second addition; KCl, 150 mM. The numbers represent enzyme activity expressed as mU/mg mitochondrial protein and the vertical bars show the absorbance change due to production of 10 pmol NADPH. Results of one representative experiment out of 20 similar ones are shown.

hexokinase activity to $90.6 \pm 23.1\%$ of the original value. Similar rates were attained after addition of DCCD or KCl, (middle and lower trace of Fig. 3) agents known to prevent binding of hexokinase to its mitochondrial binding sites [26,17]. Apparently, polyanion does not interfere directly with the enzyme but it limits the accessibility of the enzyme for its substrate(s).

3.4. Release of polyanion inhibition by low concentration of Triton

The experiments carried out on contact point vesicles clearly showed that detachment of hexokinase from its binding site on porin eliminates the inhibitory effect of polyanion. Triton, applied in a concentration of 0.5–1% acts most probably in a similar way. On the other hand Triton was shown to influence the activity of several enzymes in submicellar concentrations [22]. In view of these data the effect of very low concentrations of Triton has been tested on intact mitochondria. When applied in a concentration of 0.0025%, in the absence of polyanion Triton did not liberate any latent hexokinase activity (the rate was 167 ± 37 and 167 ± 30 mU/mg mitochondrial protein in its absence and presence, $n = 5$, not shown) yet it was fully active in restoration of glucose 6-phosphate production inhibited by polyanion (Fig. 4A). The differ-

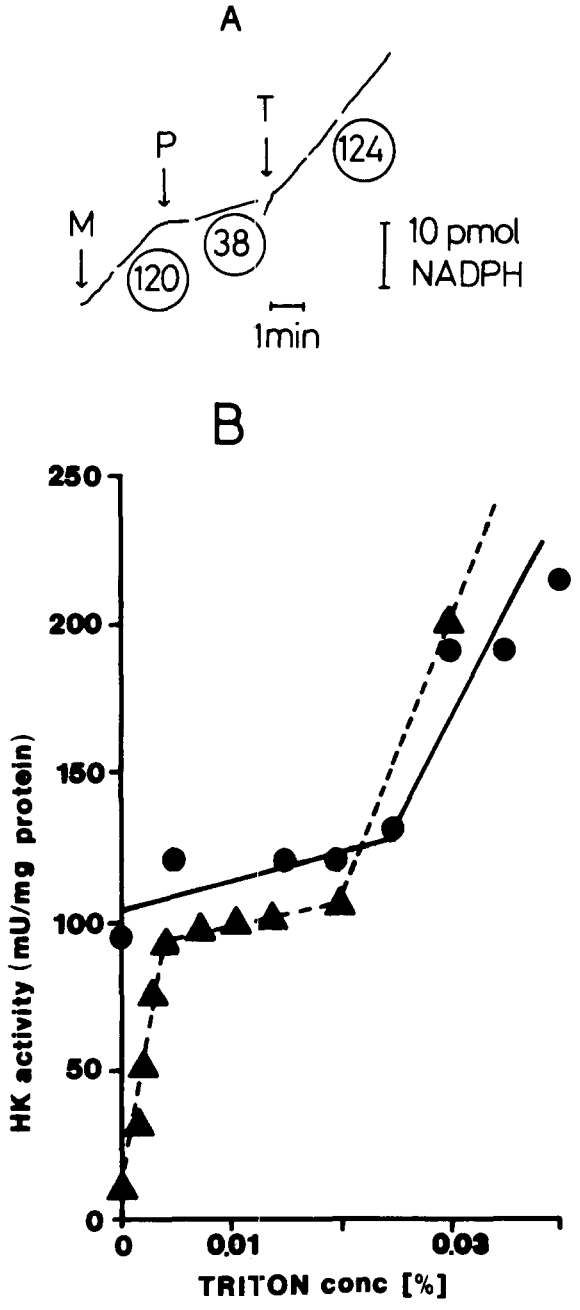

Fig. 4. Release of polyanion inhibition by low concentration of Triton in intact mitochondria. (A) Original recording of the absorbance trace at 340 nm. The following additions were made where indicated by the arrows: M, mitochondria, 30 μg protein/ml; P, polyanion, 9 μg/ml; T, Triton, 0.0025%. The numbers represent enzyme activity expressed as mU/mg mitochondrial protein. (B) Effect of various Triton concentrations on the hexokinase activity of polyanion-treated (▲) and non-treated (●) mitochondria. One representative experiment out of seven similar ones.

ences in the effects of the various Triton concentrations are summarized in Fig 4B. Below 0.01%, Triton had no effect on the basal activity of non-inhibited mitochondria whereas it gradually augmented the polyanion-inhibited hexokinase activity. Liberation of the latent hexokinase activity both in the absence and presence of polyanion occurred only upon addition of Triton in about tenfold higher concentration (above 0.025%). Thus extremely low concentrations of Triton are sufficient to prevent inhibition of porin by polyanion. It is important to note that treatment with Triton in concentrations below 0.01% did not mobilize the enzyme from its mitochondrial binding sites as no hexokinase activity could be detected in the supernatant after separation of the mitochondrial suspension by centrifugation.

4. Discussion

The described experiments carried out on rat brain mitochondria clearly show that the activity of hexokinase utilizing external ATP is significantly increased in the presence of 0.02–0.06% Triton. Thus, in the basal state, i.e., in the absence of any detergent, only a part of the total enzyme capacity is measurable, indicating a limited accessibility of a part or the totality of the enzyme molecules for external ATP. This observation could be explained by suggesting heterogeneity in the location of mitochondrial bound hexokinase molecules or the existence of multiple functional states of the enzyme.

In contact point vesicles, where the latent (Triton-sensitive) fraction of the hexokinase activity was absent, it could be demonstrated that the basal hexokinase activity utilizing external ATP is completely blocked by polyanion, the known inhibitor of porin. This observation suggests that extramitochondrial ATP reaches the active site of hexokinase via the porin channel. Inhibition of hexokinase can be fully reverted by allowing free access to ATP after solubilization of the enzyme. These data are similar to those reported earlier for adenylate kinase and creatine kinase [6,7] but they are different from those obtained by Benz et al. for hexokinase [7]. In the latter study hexokinase isolated from brain mitochondria has been reattached to liver mitochondria. This experimental procedure does not exclude the possibility that rebinding occurs to unspecific or to latent sites, in which cases the effect of polyanion would be undetectable.

Our most important finding is that inhibition of hexokinase, i.e., transfer of ATP via porin can also be released by extremely low concentrations (0.001–0.005%) of Triton when the enzyme remains attached to the mitochondrial membrane. As all the isolation procedures of porin involve solubilization of mitochondria by Triton applied in a concentration of 1–3%, trace amounts of the detergent could be present in all the functional tests carried out with purified porin preparations or membrane vesicles either on proteoliposomes or in BLM experiments [3]. Prevention of the inhibitory effect of polyanion by trace amounts of Triton provides a reasonable explanation for the discrepancy observed in the transport properties of porin when investigated in its natural environment in intact mitochondria or in artificial lipid membranes.

Interference with the effect of polyanion on porin recalls another recently described action of Triton. Porin was found to be one of the three subunits of the mitochondrial receptor-complex for benzodiazepines [23]. In this complex, Triton prevents the binding of the ligand to its binding sites on porin [24]. Interference with polyanion effect and benzodiazepine binding could have analogous mechanism.

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