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A MODIFIED FORM OF MITOCHONDRIAL HEXOKINASE PRODUCED BY ATP-INDUCED SOLUBILIZATION

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Summary

Mitochondrial hexokinases from several rat tissues were analyzed by DEAE-cellulose chromatography. Solubilization by glucose-6-P or Triton X-100 released hexokinases A and B. Solubilization by ATP resulted in a decrease of hexokinase A and the concomitant appearance of a new fraction of lower net charge (hexokinase A_m) which readily reverts to hexokinase A by dialysis or dilution. Treatment of homogeneous or partially purified hexokinase A with ATP did not generate hexokinase A_m. Hexokinases A_m and A were equally inhibited by an anti-hexokinase immune serum and displayed the same K_m values for glucose and ATP. Hexokinase A_m may represent a conformer or an oligomer produced during ATP-induced solubilization of hexokinase A from mitochondria.

Introduction

The loose association of hexokinase activity (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) to the outer mitochondrial membrane of mammalian tissues is a well established fact (1-6). Incubation of mitochondria with either ATP or glucose-6-P brings about the specific release of hexokinase whereas addition of magnesium or calcium ions results in rebinding (2,7). A few differences between the mitochondrial-bound and the solubilized enzyme activities have been documented (for a review see ref. 3), but a special mitochondrial hexokinase, distinct from the well characterized hexokinases A, B, C and D (8,9), has not been so far identified. The regulatory relevance of the reversible mitochondrial hexokinase association has been proposed by several investigators (3,7,10,11).

Solubilization of mitochondrial hexokinase by either ATP or glucose-6-P differs since temperature, inorganic phosphate, and divalent cations affect in a diverse mode the release by those agents (7). A further difference will

Abbreviations: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonylfluoride; DEAE-, diethylaminoethyl-; EDTA, ethylene diaminetetracetic acid.

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be documented by the results presented in this paper. We have found that a modified form of hexokinase A is released from mitochondria by incubation with ATP, but not with glucose-6-P or Triton X-100.

Methods

Materials. Adult and neonatal (3 to 5 days old) Sprague-Dawley rats were used. Buffalo rats bearing Morris hepatomas 5123D, 7794A and 7800, were a gift from Dr. H. P. Morris (Howard University, Washington D.C.). Novikoff ascites cells were grown by weekly transfers into Sprague-Dawley rats. An immune serum was prepared in rabbits by repeated injections of homogeneous brain hexokinase A purified according to Chou and Wilson (12). Reagents used (mostly Sigma Chemical Co.) were of the highest purity commercially available.

Isolation of mitochondria. Twenty per cent (w/v) homogenates from neonatal liver, adult brain and Morris hepatoma were prepared in a Potter-Elvehjem apparatus in a medium containing 250 mM sucrose, 10 mM Hepes, 1 mM EDTA, 1 mM DTT, pH 7.4. Minced heart tissue was homogenized with sand in a cold mortar using the same buffer. Novikoff extracts were prepared by sonication of cells during 20 s at 4° (repeated thrice at 1 min intervals) using a small probe at 70 per cent output of a Sonifier Cell Disruptor (Heat Systems-Ultrasonics, Inc.). Mitochondria were obtained by differential centrifugation (13).

Solubilization of mitochondrial hexokinase. Unless otherwise indicated, purified mitochondria were incubated at 30° during 20 min with either 10 mM ATP, or 2 mM glucose-6-P, or 0.15 per cent (v/v) Triton X-100 in the sucrose buffer described above. The mixtures were then centrifuged at 10,000 x *g* for 10 min at 4° in a Sorvall RC-2B centrifuge and the supernatant liquids used for chromatography. The concentrations of ATP and glucose-6-P used were able to solubilize about 80 % of the bound hexokinase activity.

Chromatographic separation of hexokinases. Chromatography was performed at 4° in 2-ml columns of DEAE-cellulose equilibrated with 10 mM Hepes, 1 mM EDTA, 1 mM DTT, pH 7 (14). Fractions of 0.3 ml were collected while eluting with a KCl gradient from 0 to 0.5 M in the same buffer.

Measurement of hexokinase activity. Glucose phosphorylation was determined at 30° following the formation of NADPH under the conditions previously described (14). Assay systems with ATP omitted were used as blanks. One unit of hexokinase activity is defined as the amount of enzyme catalyzing the phosphorylation of 1 μmol of glucose in 1 min at 30°. Hexokinase isozymes were identified by their differential activities at a low (0.5 mM) or a high (100 mM) glucose concentration in the assay (14). High to low activity ratios are 1, 1.3 and 0.5 for hexokinases A, B and C, respectively.

Results

Hexokinase isozymes of mitochondria from neonatal rat liver. DEAE-cellulose chromatography of mitochondrial hexokinase solubilized with either Triton X-100 or with glucose-6-P showed the presence of hexokinase A and a small peak of hexokinase B (Fig. 1, *left*). Hexokinase C, which is present in the cytosolic fraction (15), was not observed. On the other hand, incubation of mitochondria with ATP resulted in the resolution of three peaks of hexokinase activity (Fig. 1, *left*). The most retained peak corresponds to hexokinase B. The other two forms were identified as hexokinase A because using the criteria specified in **Methods**, the ratios of their activities measured with high and low glucose was 1. On the basis of elution position, "normal"

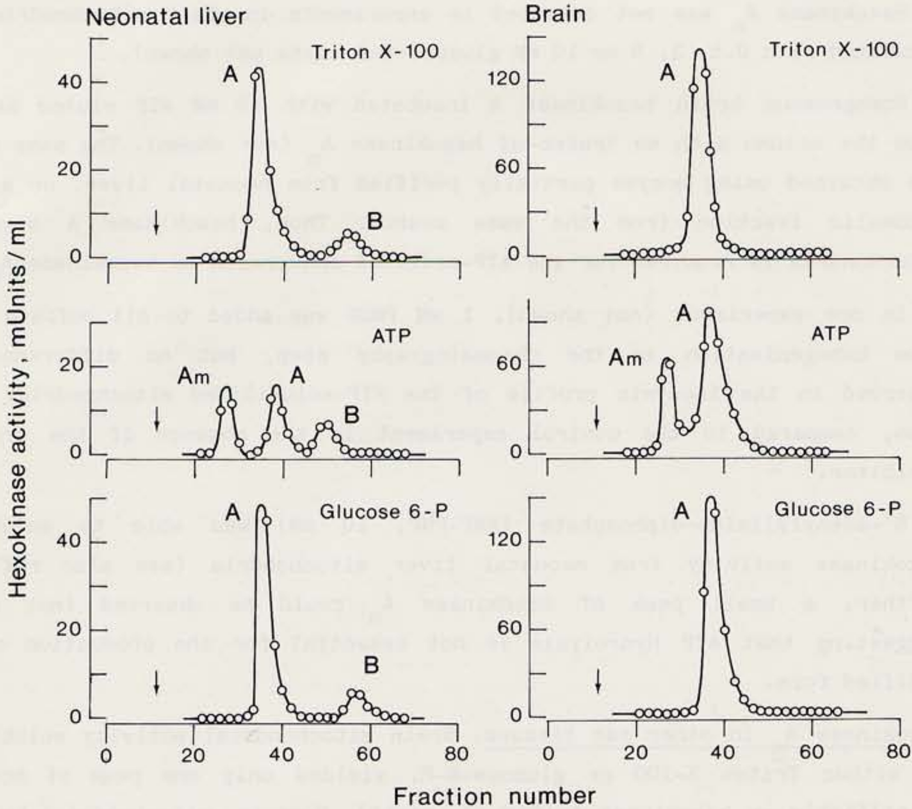

Fig. 1. Hexokinase isozymes in mitochondria from neonatal rat liver and adult brain. **Left. Neonatal rat liver.** The washed mitochondrial pellet from the livers of sixteen 3-day old rats was resuspended in 3 ml of buffered 0.25 M sucrose and divided into three portions of 1 ml each (containing 10 mg of mitochondrial protein) which were simultaneously treated either with 0.15 % (v/v) Triton X-100, 10 mM ATP, or 2 mM glucose 6-P. After 20 min at 30º the mixtures were centrifuged at 10,000 x g for 10 min at 4º and the supernatant liquids charged into DEAE-cellulose columns which were simultaneously operated. Fractions were assayed at two glucose concentrations (see METHODS). For simplicity, only the activities measured at 0.5 mM glucose are represented. Top: Triton X-100-treated mitochondria; Middle: ATP-treated mitochondria; Bottom: Glucose 6-P-treated mitochondria. The arrow (↓) indicates the beginning of KCl gradient elution. **Right. Adult rat brain.** The experimental protocol was exactly as described above, except that the brain of one adult rat was used as starting material.

isozyme A was identified as the second peak. Since the first fraction appeared to be a modified form of mitochondrial hexokinase A, it will be hereafter referred to as hexokinase A_m. Note that the activity of hexokinase A in the detergent- or glucose-6-P-treated mitochondrial fractions is approximately equal to the sum of hexokinases A and A_m, i.e., solubilization by ATP seems to split isozyme A in two activity peaks differing in chromatographic behavior.

Hexokinase A_m was not detected in experiments in which mitochondria were incubated with 0.5, 2, 5 or 10 mM glucose-6-P (data not shown).

Homogeneous brain hexokinase A incubated with 10 mM ATP eluted as such from the column with no traces of hexokinase A_m (not shown). The same result was obtained using enzyme partially purified from neonatal liver, or a crude cytosolic fraction from the same source. Thus, hexokinase A bound to mitochondria is required for its ATP-mediated conversion to hexokinase A_m.

In one experiment (not shown), 1 mM PMSF was added to all buffers used, from homogenization to the chromatography step, but no difference was observed in the isozymic profile of the ATP-solubilized mitochondrial fraction, compared to the control experiment in the absence of the protease inhibitor.

5'-adenylylimido-diphosphate (AMP-PNP, 10 mM) was able to solubilize hexokinase activity from neonatal liver mitochondria (see also ref. 11). Further, a small peak of hexokinase A_m could be observed (not shown) suggesting that ATP hydrolysis is not essential for the production of the modified form.

Hexokinase A_m in other rat tissues. Brain mitochondrial activity solubilized by either Triton X-100 or glucose-6-P, yielded only one peak of activity identifiable as hexokinase A (Fig. 1, right). However, mitochondrial hexokinase solubilized by ATP was found to present hexokinase A_m in addition to isozyme A.

Hexokinase A_m was also observed in ATP-solubilized mitochondrial fractions from heart, Morris hepatomas 5123D, 7794A and 7800, or Novikoff ascites cells (not shown).

Antigenic identity of hexokinase A_m and hexokinase A. Glucose phosphorylating activities of hexokinases A_m and A isolated from neonatal liver mitochondria were equally inhibited by an antibody generated against homogeneous rat brain hexokinase A (Fig. 2). Hexokinases B, C, and D (the so-called glucokinase) from rat liver cytosol, were not inhibited at all by such antibody (Fig. 2). Thus, hexokinase A_m seems to expose the same antigenic determinants as hexokinase A.

Stability of hexokinase A_m. The level of hexokinase A_m, relative to hexokinase A, after incubation with 10 mM ATP was found to be proportional to the amount of mitochondria used in the solubilization mixture. Thus, using 7.3, 14.6, and 29.2 mg of mitochondrial protein in a fixed final volume of 1 ml,

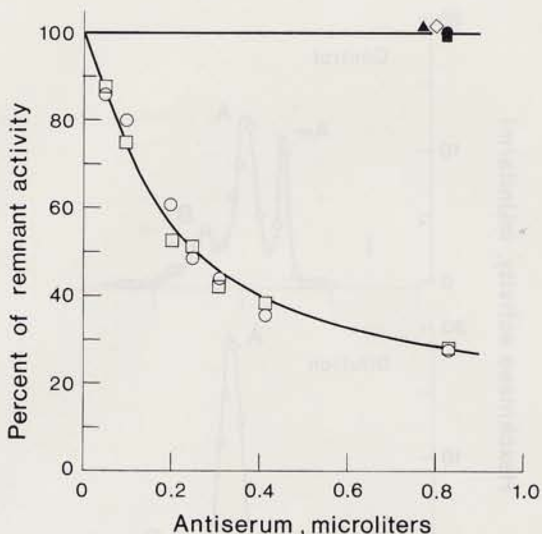

Fig. 2. Immunological identity between hexokinases A and A_m. Hexokinases A and A_m were obtained from neonatal rat liver in experiments such as described in Fig. 1, left. Hexokinases B and C were obtained by DEAE-cellulose chromatography of neonatal rat liver supernatant liquids. Hexokinase D was a highly purified preparation kindly donated by C. Toro and H. Niemeyer. Mixtures of approximately equal fixed amounts of the enzymes with either anti-hexokinase A serum or uninjected rabbit serum were incubated at room temperature for 60 min and afterwards at 4° overnight. After centrifugation, the supernatant liquids were assayed for remnant glucose phosphorylating activity. Controls include measurements at zero time and at 24 hr in mixtures containing preimmune serum. ○: hexokinase A; □: hexokinase A_m; ●: hexokinase B; ■: hexokinase C; ▲: hexokinase D; ◇: hexokinase A or A_m incubated with preimmune serum.

the proportion of hexokinase A_m was 0.14, 0.36, and 0.57, respectively ($1 = A + A_m$). The point was not further explored but the values obtained may be related to the extreme lability of hexokinase A_m to dilution (see below).

Chromatography of an ATP-solubilized fraction after 10-fold dilution resulted in the appearance of hexokinase A only (Fig. 3). Dilution in buffer containing 10 mM ATP resulted in a very distorted chromatographic pattern presumably because of anionic overcharge of the column. Short term dialysis (2 hr) against the equilibration buffer, also prevented the appearance of hexokinase A_m (data not shown). Rechromatography of the isolated hexokinase A_m, a procedure implying prior dialysis, resulted in the appearance of only one peak eluting as hexokinase A, with full recovery of the activity (not shown).

Michaelis constants for glucose and ATP of hexokinase A_m. No differences in K_m values for glucose or ATP between hexokinases A and A_m were observed.

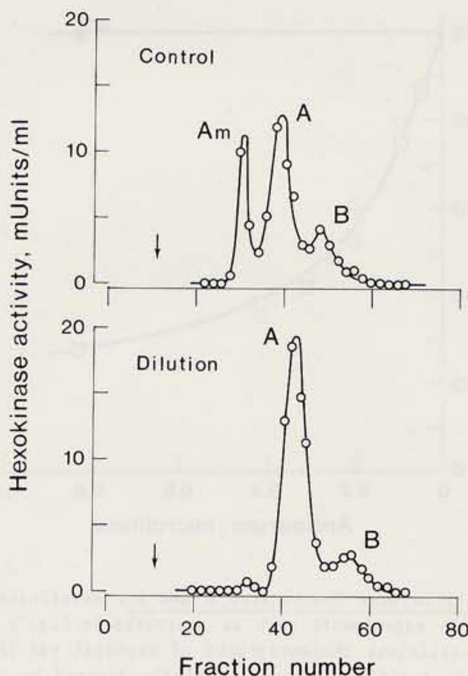

Fig. 3. The effect of dilution on hexokinase A_m. Mitochondria (containing about 20 mg/ml of protein) isolated from neonatal rat liver were incubated with 10 mM ATP during 20 min. After centrifugation, the supernatant liquid was divided in two equal portions. One of them was immediately chromatographed (top). The second portion was diluted 10-fold with equilibration buffer and the whole volume was immediately charged in the column and chromatographed (bottom). DEAE-cellulose chromatography was performed exactly as described in the legend to Fig. 1. Other details as in the legend to Fig. 1.

Apparent K_m values for glucose, at 10 mM ATP, were 0.052 ± 0.008 mM (n = 5) and 0.048 ± 0.010 mM (n = 7) for hexokinases A and A_m, respectively. For ATP, apparent K_m values, at 0.5 mM glucose, were 0.46 ± 0.06 mM (n = 4) and 0.50 ± 0.06 mM (n = 6), respectively. Since hexokinase A_m readily reverts to hexokinase A it is quite possible that the values reported correspond to the latter. Thus, these findings should be regarded as tentative until conditions to stabilize hexokinase A_m have been worked out.

Discussion

In all rat tissues so far examined, solubilization of mitochondrial hexokinase by ATP results in the splitting of the hexokinase A fraction in two chromatographic peaks, the less-retained of which corresponds to a modified form which we have called hexokinase A_m. Attempts to define the

nature of the modification have been unsuccesful. It is not an ATP-mediated proteolysis since hexokinase A_m reverts to hexokinase A and solubilization by ATP in the presence of PMSF did not prevent the appearance of the modified form. Preliminary experiments in which [γ-^{32}P]ATP was used for solubilization did not show labeling of hexokinase A_m.

The possibility that hexokinase A_m may correspond to a dimer (or oligomer) of hexokinase A is supported by experiments (Fig. 3) which suggest that conditions favoring protein dissociation prevent the appearance of hexokinase A_m. Preliminary experiments using sucrose gradient centrifugation show that the molecular weight of the modified form is about 100,000, i.e., not different to that of hexokinase A, but the result may be the consequence of dilution. Cross-linking reagents might be useful to solve this problem and will be employed in the near future.

To summarize: it has been found that a modified form of hexokinase A is solubilized by ATP from mitochondria of several rat tissues. The modified form reverts readily to hexokinase A and may correspond to a conformer or, more likely, to an oligomer of hexokinase A. Solubilization of mitochondrial hexokinase by glucose-6-P did not result in the generation of hexokinase A_m. We will pursue the characterization of the structural basis of the new form and of its kinetic properties. Also, we will set out to falsify the hypothesis that hexokinase A_m is the form of hexokinase actually bound to mitochondria and that ATP, but not glucose-6-P, is able to maintain the modified state of the enzyme during solubilization.

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