BBA 66249

STUDIES ON PARTICLE-BOUND HEXOKINASE IN RAT ASCITES HEPATOMA CELLS

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(Received September 11th, 1970)

SUMMARY

The subcellular distribution of hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) in cells of Yoshida ascites hepatoma AH7974 was studied. About 40% of the activity was localized in the 18 000 × *g* precipitate. The particulate hexokinase was found to be mainly bound to mitochondria by subfractionation of the 18 000 × *g* precipitate and by comparison of the distribution of the enzyme with those of marker enzymes of mitochondria and cytoplasmic membranes. Mitochondrial-bound hexokinase was released using nucleoside triphosphates, glucose 6-phosphate or detergent. The released hexokinase contained more type I than type II enzyme while the soluble fraction contained predominantly type II. The mechanism and physiological significance of the binding of hexokinase to mitochondria are discussed.

INTRODUCTION

There are four molecular species of hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) in rat liver[1,2]. The changes of the isozyme pattern of hexokinase in experimental rat hepatomas have been investigated in our laboratory[3,4] and elsewhere[5–8]. The hexokinase isozyme pattern of slowly growing hepatomas (Morris hepatomas) was very similar to that of normal liver[4,8]. However, fast growing hepatomas (Yoshida ascites hepatomas) showed only type I and II hexokinases, the latter predominating. In some strains of Yoshida ascites hepatomas which grow rather slowly, type III hexokinase appeared in addition to types I and II[4].

During studies of these isozyme patterns, we noticed that the hexokinase activity in Yoshida ascites hepatoma cells was partly bound to subcellular particles, whereas almost all the hexokinase activity in normal rat liver was recovered in the soluble fraction.

This paper reports the distribution of hexokinase activity and its release from

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cellular particles in some strains of Yoshida ascites hepatomas. The bound hexokinase in the particulate fractions of the cells was found to be almost exclusively localized in the mitochondria and was readily released by glucose 6-phosphate (Glc-6-*P*) or by various nucleoside triphosphates, as reported by other workers[9–12]. The hexokinase released from particles was compared with that in the soluble fraction.

MATERIALS AND METHODS

Chemicals

ATP, $NADP^+$, NADH, and *p*-nitrophenyl phosphate were purchased from Sigma Chemical Co., St. Louis. Glucose 6-phosphate dehydrogenase was obtained from Boehringer and Soehne GmbH, Mannheim, Western Germany. ^{32}P-labeled 5'-AMP was prepared from ^{32}P-labeled NAD^+ obtained in this laboratory.

Enzyme assay

Hexokinase was assayed by the method of SALAS *et al.*[13] with a slight modification. The reaction mixture contained in a final volume of 1.0 ml: 0.1 M glucose, 5 mM ATP, 5 mM $MgCl_2$, 0.4 I.U./ml glucose-6-phosphate dehydrogenase, 5 mM $NADP^+$, 5 mM 2-mercaptoethanol, 0.3 M Tris–HCl buffer (pH 7.4) and 0.1 ml of appropriately diluted enzyme preparation. The change in absorbance at 340 nm was measured at 30° with a Gilford multiple sample absorbance recorder, Model 2000.

Cytochrome *c* oxidase activity was assayed by the rate of oxidation of reduced cytochrome *c*, determined at 30° by measuring the decrease in absorbance at 550 nm after addition of the enzyme preparation. Appropriately diluted enzyme preparation (0.1 ml) was added to 0.9 ml of reaction mixture containing 90 μM cytochrome *c* and 0.1 M phosphate buffer (pH 7.4). This is a slight modification of the method of SMITH[14,15].

5'-Mononucleotidase activity was assayed in reaction mixture containing 0.1 M glycine–NaOH buffer (pH 8.7), 10 mM $MgCl_2$, 38.9 μM of ^{32}P-labeled AMP (specific activity $1.62 \cdot 10^3$ counts/min per μmole) and appropriately diluted enzyme preparation. Incubation was carried out at 37° for 15 min, and the reaction was stopped by adding 0.1 ml of 70% $HClO_4$ after addition of an appropriate amount of inorganic phosphate as carrier. A sample of 0.5 ml of the acid-soluble fraction was extracted with isobutanol and the inorganic ^{32}P radioactivity was measured in a gas-flow counter. A unit of activity corresponded to the liberation of 1 μmole of inorganic phosphate, and the specific activity of the enzyme was defined as units/mg of protein.

Protein assay

Protein was measured by the method of LOWRY *et al.*[16] with crystalline bovine serum albumin as standard.

Animals and tumor cells

Several million cells of Yoshida ascites hepatoma AH7974 were injected intraperitoneally into male Donryu strain rats weighing about 100 g. Tumor cells were harvested 7 days after transplantation and washed with cold physiological saline until no contaminating red blood cells could be detected under a light microscope.

Preparation of homogenate

Tumor cells were homogenized with 5 vol. of 0.1 M Tris–HCl buffer (pH 7.4) containing 5 mM 2-mercaptoethanol, 5 mM EDTA and 10 mM glucose with a Vir-Tis 45 homogenizer. During homogenization aliquots of the homogenate were taken at intervals and examined under a phase contrast microscope to check the degree of cell disruption. For homogenization, a Potter–Elvehjem type homogenizer and a sonicator were also tested and they gave similar results to those with a Vir-Tis 45 homogenizer.

Centrifugal fractionation of subcellular particles

The homogenate was centrifuged at 1000 × *g* for 20 min. The resulting precipitate was washed 3 times with the buffer used for homogenization and the washing fluids were combined with the supernatant. The washed precipitate was resuspended in 10 ml of the buffer used for homogenization. The supernatant was centrifuged at 18 000 × *g* for 20 min. The resulting precipitate was also washed and resuspended in 10 ml of the same buffer. The supernatant was centrifuged at 105 000 × *g* for 60 min and the precipitate was suspended in 5 ml of the same buffer.

Subfractionation of particles using a discontinuous sucrose density gradient

9 ml of the 18 000 × *g* precipitate suspension were gently layered on 15 ml of 0.34 M sucrose solution and centrifuged at 18 000 × *g* for 20 min. The precipitate was resuspended in 3.3 vol. of 63% sucrose solution to adjust its density to 1.22. 1 ml of this suspension was transferred to the bottom of a 5-ml tube and successively overlayed by 0.5 ml of 48% sucrose ($d = 1.22$), 2 ml of 45% sucrose ($d = 1.20$) and 1.5 ml of 37% sucrose ($d = 1.16$). This gradient was centrifuged at 40 000 rev./min for 120 min using an RPS 40 swinging bucket type rotor (Hitachi, Japan). A very small amount of precipitate at the bottom of the tube and two turbid bands at the interphases of the sucrose gradients were obtained. The tube was cut with a tube slicer to obtain five fractions. Subfractionation of the 1000 × *g* precipitate was carried out in the same way. The activities of hexokinase, cytochrome *c* oxidase and 5′-mononucleotidase in each fraction were assayed.

Identification of mitochondria

Mitochondria were identified by the method of GREENWALT *et al.*[17]. The 18 000 × *g* precipitate obtained from 5 g of cells was divided into two fractions. One was suspended in 10 ml of solution containing 10 mM Tris–HCl buffer (pH 7.4), 10 mM $MgCl_2$, 10 mM succinate, 2 mM ATP, 4 mM $CaCl_2$, 4 mM NaH_2PO_4 and 0.25 M mannitol. The other was suspended in 10 ml of sucrose. Both mixtures were incubated at 30° for 20 min and then centrifuged at 18 000 × *g* for 20 min. The resulting precipitate was gently washed with 0.25 M sucrose and resuspended in 1 ml of 0.25 M sucrose. A sample of 0.5 ml of this suspension was layered onto a discontinuous sucrose density gradient following the method of GREENWALT *et al.*[17]. After centrifugation at 39 000 rev./min for 180 min with an RPS 40 rotor, the gradient was fractionated by slicing the tube. The hexokinase and cytochrome *c* oxidase activities of each fraction were determined.

Release of hexokinase from the 18 000 × g precipitate

The 18 000 × *g* precipitate of tumor cells was resuspended in 0.25 M sucrose in 10 times the volume of the original cells and incubated with various nucleoside triphosphates, Glc-6-*P* and detergent. The ratio of hexokinase activity in the supernatant after centrifugation at 20 000 × *g* for 20 min to that in the incubated suspension before centrifugation, times 100 was expressed as the percentage release of hexokinase activity. The effects of the times of incubation with ATP and Glc-6-*P* and of the concentration of these compounds on release of hexokinase were also investigated.

Effect of heating on released and soluble hexokinases

The activities of hexokinases released from the 18 000 × *g* precipitate with ATP or Glc-6-*P* and that in the soluble fraction of AH cells incubated at 45° were measured at intervals. For reference, soluble hexokinase of normal rat brain was also incubated, and its activity was measured.

Electrophoresis of released and soluble hexokinases

Electrophoresis of the hexokinases released from the 18 000 × *g* precipitate and in the soluble fraction of AH cells was performed on a cellulose acetate membrane, and activities were located by staining as reported previously[4].

RESULTS

Distribution of hexokinase activity in subcellular fractions

Table I shows the percentage distribution of hexokinase activity in various fractions of tumor cells and normal rat liver. Much of the activity in the homogenate of Yoshida ascites hepatoma AH7974 cells was found to be associated with subcellular particles, especially those precipitated at 18 000 × *g*. Much of the remaining activity was found in the supernatant after centrifugation at 105 000 × *g*. A similar distribution of hexokinase activity was found in cells of other strains of Yoshida ascites hepatomas. Almost all the hexokinase activity in normal liver was recovered in the 105 000 × *g* supernatant.

TABLE I

PERCENTAGE DISTRIBUTION OF HEXOKINASE ACTIVITY IN SUBCELLULAR FRACTIONS OF AH 7974 CELLS AND NORMAL RAT LIVER

Homogenates of tumor cells and liver were fractionated by centrifugation and the hexokinase activity of each fraction was assayed. The percentage activity in each fraction is shown. Values are averages of 7 values for tumor cells and 2 values for liver.

Fraction	*AH 7974 cell (%)*	*Normal rat liver (%)*
1 000 × *g* ppt.	27.1	0
18 000 × *g* ppt.	37.0	0
105 000 × *g* ppt.	9.0	1.8
105 000 × *g* sup.	26.9	98.2

TABLE II

PERCENTAGE DISTRIBUTION OF HEXOKINASE AND CYTOCHROME *c* OXIDASE ACTIVITIES IN SUBFRACTIONS OF AH 7974 CELLS

A homogenate of tumor cells was fractionated and the activities of the two enzymes in each fraction were assayed.

Fraction	*Hexokinase (%)*	*Cytochrome c oxidase (%)*
1 000 × *g* ppt.	30.1	29.5
18 000 × *g* ppt.	37.1	62.2
105 000 × *g* ppt.	14.7	8.3
105 000 × *g* sup.	18.1	0

The subcellular distribution of cytochrome *c* oxidase, a marker enzyme of mitochondria is compared with that of hexokinase in Table II. This table shows that mitochondria are mainly present in the 18 000 × *g* precipitate to which much of the hexokinase activity was also bound.

Subfractionation of the 18 000 × g precipitate

The 18 000 × *g* precipitate was further fractionated by discontinuous sucrose density gradient centrifugation to obtain five fractions and the enzyme activities of these fractions were assayed as described in MATERIALS AND METHODS. Fractions 1–5 refer, respectively, to material in the 37% sucrose layer, the turbid band between the 37 and 45% sucrose layers, the 45% sucrose layer, the turbid band between the 45 and 48% sucrose layers and the 48% sucrose layer. As shown in Fig. 1 the activities of hexokinase, cytochrome *c* oxidase and 5′-mononucleotidase were high in Fractions 2, 4 and 5. The distribution of hexokinase in these fractions corresponded with that of cytochrome *c* oxidase but not with that of 5′-mononucleotidase, a marker enzyme of cytoplasmic membranes. Similar results were obtained on fractionation of the 1000 × *g* precipitate.

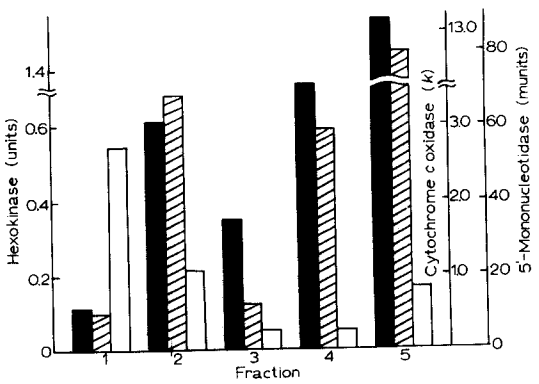

Fig. 1. The 18 000 × *g* precipitate was fractionated by discontinuous sucrose density gradient centrifugation to obtain five fractions as described in the text. The total activities of hexokinase (black bars), cytochrome *c* oxidase (shaded bars) and 5′-mononucleotidase (blank bars) in each fraction were assayed.

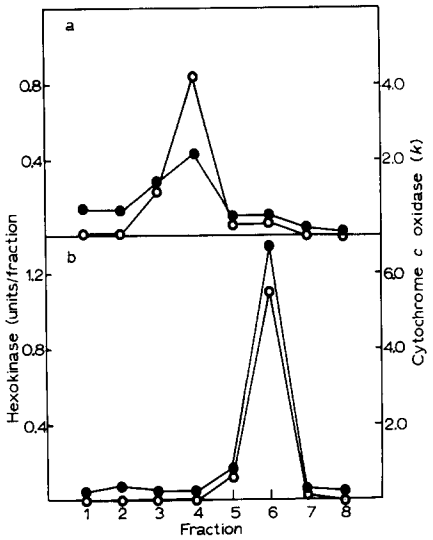

Fig. 2. After incubation with (b) and without (a) calcium phosphate, the 18 000 × *g* precipitate of tumor cells was layered on a discontinuous sucrose density gradient and centrifuged as described in the text. After centrifugation the gradient was cut into eight fractions of equal volume, and hexokinase (●—●) and cytochrome *c* oxidase (○—○) activities were assayed in each fraction. 1 and 8 represent the top and bottom fractions, respectively.

Identification of mitochondrial-bound hexokinase

To confirm that the hexokinase in the 18 000 × *g* precipitate of ascites tumor cells was actually bound to mitochondria, the method of GREENWALT *et al.*[17] was used. Fig. 2 shows that the fractions with the bulk of the turbidity contained hexokinase and cytochrome *c* oxidase and that these fractions shifted to the bottom after accumulation of Ca^{2+} and HPO_4^{2-}. This clearly indicates that the particles with hexokinase activity were predominantly mitochondria.

TABLE III

PERCENTAGE RELEASE OF HEXOKINASE ACTIVITY FROM THE 18 000 × *g* PRECIPITATE OF AH 7974 CELLS

The 18 000 × *g* precipitate of tumor cells was suspended in 0.25 M sucrose and incubated with various compounds. The percentage release of activity was determined as described in the text.

Addition	*Concn. (mM)*	*Release (%)*	*Addition*	*Concn. (mM)*	*Release (%)*
Control		21.5	Glc-6-*P*	0.5	62.2
AMP	5.0	21.0	Glc-1-*P*	0.5	21.9
ADP	5.0	31.8	Fru-1-*P*	0.5	21.4
ATP	1.0	55.7	Fru-1,6-P_2	0.5	22.0
	5.0	69.6	ADP-Rib	5.0	20.5
dATP	1.0	66.3	Citrate	5.0	21.0
GTP	1.0	38.8	Triton X-100	0.5%	38.5
	5.0	76.9	EDTA	5.0	21.7
CTP	1.0	48.5		10.0	20.3
UTP	1.0	50.8	$MgCl_2$	5.0	19.0
NAD+	5.0	22.0	P_i	5.0	18.2
NADP+	5.0	21.0	PP_i	5.0	22.5

Release of hexokinase from the 18 000 × g precipitate

As shown in Table III hexokinase was released and solubilized on incubating the 18 000 × *g* precipitate of AH7974 cells at 0° with various nucleoside triphosphates, Glc-6-*P* or detergent. Bound hexokinase was rapidly released with 0.5 mM Glc-6-*P*, reaching a maximal value within 10 min while with 2.5 mM ATP, the rate and the maximal amount of release of the enzyme were less (Fig. 3). Fig. 4 shows the effect of the concentrations of these compounds on release of hexokinase. The release of hexokinase activity was correlated with the concentration of ATP added. Release

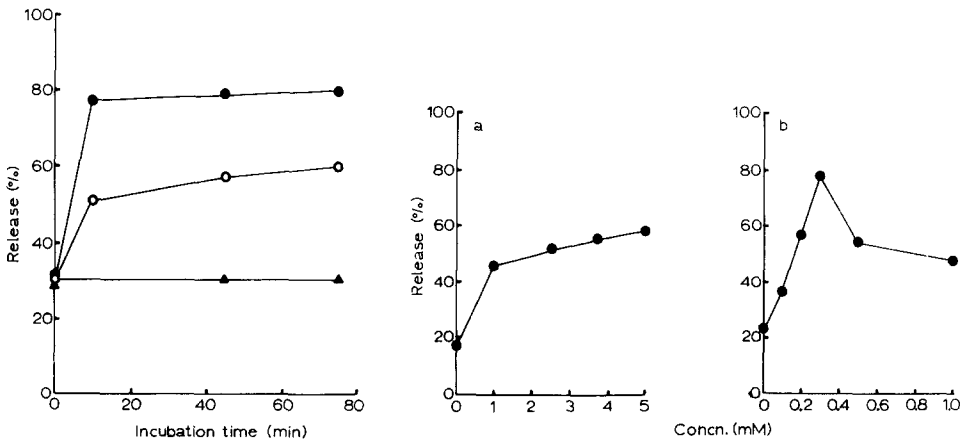

Fig. 3. The 18 000 × *g* precipitate suspended in 0.25 M sucrose was incubated with 2.5 mM ATP (●—●) or 0.5 mM Glc-6-*P* (○—○) or without additions (▲—▲) at 0° for various lengths of time and release of hexokinase activity was assayed .

Fig. 4. The 18 000 × *g* precipitate of tumor cells was incubated with various concentrations of ATP (a) or Glc-6-*P* (b) in 0.25 M sucrose at 0° for 30 min, and release of hexokinase activity was assayed.

of enzyme was correlated with the concentration of Glc-6-*P* up to a concentration of 0.3 mM, but the percentage release apparently decreased at higher concentrations. This apparent decrease in the release of hexokinase from the 18 000 × *g* precipitate may be due to inhibition of the enzyme by Glc-6-*P*.

Effect of heating on released and soluble hexokinases

Fig. 5 shows the effect of heating on the hexokinase released from the 18 000 × *g* precipitate, that in the soluble fraction of AH cells and that in the soluble fraction of normal rat brain. Soluble hexokinase of tumor cells, which is mainly type II, was 90% inactivated by heating at 40° for 90 min. Rat brain soluble hexokinase, which is mainly type I, was very stable on heating, as reported by other workers[2]. The hexokinase activity released from the 18 000 × *g* precipitate of tumor cells was about 50% inactivated under the same conditions. These results indicate that the hexokinase released from the 18 000 × *g* precipitate contained considerably more type I enzyme than the soluble fraction. This was confirmed by electrophoresis.

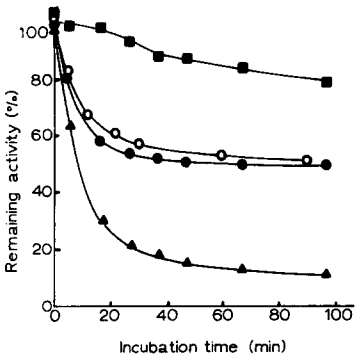

Fig. 5. Hexokinase released from the 18 000 × *g* precipitate with 10 mM ATP (○—○) or 0.5 mM Glc-6-*P* (●—●) and in the soluble fraction (▲—▲) of tumor cells, and hexokinase in the soluble fraction of normal rat brain (■—■) were heated at 45° for various times and remaining activity was assayed.

Electrophoretic patterns of soluble and released hexokinases

The isozyme patterns of soluble hexokinase and that released with ATP are shown in Fig. 6. The soluble hexokinase in AH 7974 cells formed two bands corresponding to type I and II enzymes, the latter predominating. The released hexokinase also contained types I and II, but type I predominated. The hexokinases

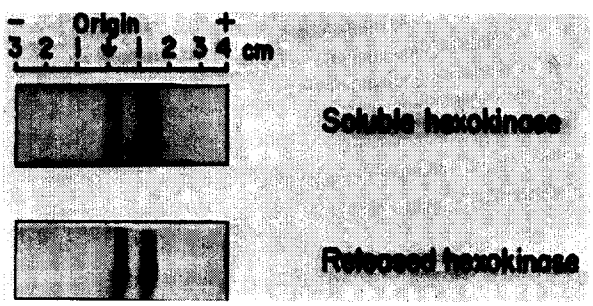

Fig. 6. Isozyme patterns on cellulose acetate membrane electrophoresis of hexokinase in the soluble fraction of tumor cells and that released from the 18 000 × *g* precipitate with 10 mM ATP.

released with Glc-6-*P* and Triton X-100 gave similar patterns to hexokinase released with ATP.

DISCUSSION

The present work showed that particulate hexokinase in cells of Yoshida ascites hepatoma AH7974 was mainly localized in the mitochondria. Slight activity was detected in other particulate fractions, possibly cytoplasmic membranes, as reported by EMMELOT AND BOS[18] and DAVIDOVA *et al.*[19] and microsomes of normal rat liver, as recently described by BERTILLIER *et al.*[20]. The presence of hexokinase bound to mitochondria has been reported in many tissues such as brain[21–26], retinal

rods[27], heart[9] and intestinal mucosa[28]. It has also been reported in some strains of ascites tumor cells[10,29–33].

The soluble fraction of Yoshida ascites hepatoma cells contains type I and II hexokinases, and the latter is predominant. However, the hexokinase released from mitochondria contains more type I enzyme than the soluble fraction, as demonstrated by experiments on heat stability and by electrophoresis. This is in contrast with the generally accepted concept that particle-bound hexokinase in malignant cells is mainly composed of type II enzyme and that this also predominates in the soluble fraction, as reported by KOSOW AND ROSE[30] and GUMAA AND GREENSLADE[7]. Recently, KROPP AND WILSON[26] reported that hexokinase released from particles in rat brain, which is mainly type I, can combine with the outer membrane of mitochondria prepared from rat liver. BAQUER AND MCLEAN[34] also detected changes in the ratio of type I to type II hexokinase in the particulate fraction but not in the soluble fraction of the uterus of a rat after estradiol administration. These findings, together with our results, suggest that the mechanisms of binding of hexokinase isozymes to particles may differ.

The nature of the binding of hexokinase to subcellular particles is unknown. Electrostatic and some hydrophobic forces have been suggested to be involved in the binding[10], and Glc-6-*P* or nucleotides may convert the enzyme to a form which weakens its hydrophobic interaction with mitochondria.

The physiological significance of mitochondrial-bound hexokinase is also unknown. CHANCE AND HESS[35] have suggested that the ADP formed by mitochondrial-bound hexokinase in some ascites tumor cells readily diffuses into the mitochondria and stimulates their respiration. Mitochondrial-bound hexokinase is widely distributed in normal tissues, such as brain or retina, and in tumor cells where the rate of glycolysis is high; so the binding of hexokinase to mitochondria may be common in cells with a high rate of glycolysis.

ACKNOWLEDGEMENTS

The authors wish to express their sincere thanks to Professor Toru Tsumita, the University of Tokyo, for his kind encouragement during this work.

This work was partly supported by a grant from Ministry of Education, Japan.

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