Hexokinase isoenzymes from the Novikoff hepatoma

Purification, kinetic and structural characterization, with emphasis on hexokinase C

Jasna RADOJKOVIĆ* and Tito URETA†

Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile

The purification to homogeneity of hexokinases B and C from the cytosol of rat Novikoff hepatoma was achieved by a protocol using an initial chromatography on Blue 2-agarose to separate the isoenzymes from each other. After that step each hexokinase was subjected to chromatography on DEAE-cellulose, hydroxyapatite and Sephacryl S-300, followed by re-chromatography on hydroxyapatite. The final preparations of hexokinases B and C had specific activities of 86 and 23.5 units/mg of protein respectively, and gave single bands on electrophoresis under non-denaturing conditions or in SDS/polyacrylamide gels. M_r values of about 100000 were found for both isoenzymes either by Sephacryl S-300 chromatography or by SDS/polyacrylamide-gel electrophoresis. Values of apparent K_m for glucose and ATP of pure hexokinase B were similar to those reported for the enzyme from other sources. The apparent K_m value for glucose of hexokinase C was 0.025 mM. Marked inhibition of hexokinase C by glucose concentrations above 0.2 mM was found. The effect was partially relieved by ATP concentrations above 1 mM and was independent of pH. Glucose 6-phosphate was inhibitory, but the K_i value (0.18 mM) is higher than those reported for other animal hexokinases. The amino acid composition of hexokinase C was found to be similar to those reported for hexokinases B and D. Also, an immune serum directed against hexokinase A was able, at low dilutions, to bind hexokinases B and C. An immune serum directed against hexokinase C was able, at low dilutions, to bind hexokinase B and also, but weakly, hexokinase A.

INTRODUCTION

Since their recognition in 1964 [1] the hexokinase isoenzymes (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) from rat tissues have been the subject of several investigations. The availability of homogeneous preparations of three of the four isoenzymes, namely hexokinase A from brain [2], hexokinase B from muscle [3,4], and hexokinase D from liver [5] and from islets of Langerhans [6], is responsible for the present wealth of data on their kinetic, structural and regulatory properties (for reviews see [7–10]).

Conspicuously absent from the list above is hexokinase C, an isoenzyme which shows marked substrate inhibition by glucose concentrations in the physiological range. The rather limited tissue distribution of the enzyme and its very low amounts (when present) explain the relative lack of data regarding this isoenzyme. A few studies on hexokinase C have been restricted to partially purified preparations from rat liver [11,12], human spleen [13], bovine liver [14] and frog liver [15,16]. A procedure for the purification to homogeneity of hexokinase C from rat liver, by a combination of conventional ion-exchange and affinity chromatography on Sepharose-N-aminoacylglucosamine, was described by Wright *et al.* [17], but we are not aware of any study using that protocol to obtain an enzyme preparation amenable to structural or kinetic studies. Also, Stocchi *et al.* [18] reported a somewhat similar procedure for the purification of hexokinase C from pig erythrocytes.

It has been known for several years that Novikoff ascites-cell rat hepatoma contains high hexokinase activity, most of it represented by isoenzymes B and C,

although a minor peak of hexokinase A is also present [19]. Further, the low-affinity hexokinase D, formerly known as 'glucokinase' [20,21], was found to be absent from the ascites cell hepatoma [19,22]. For those reasons it was decided to assess the possibility of using tumour cells as a convenient source for the purification of hexokinase C. This paper reports procedures for the purification to homogeneity of hexokinase B and hexokinase C from the Novikoff hepatoma. A few properties of the enzymes, with emphasis on hexokinase C, will also be described. Parts of this work were previously reported and published in abstract form [23].

EXPERIMENTAL

Materials

ATP, ADP, NAD⁺, NADH, phosphoenolpyruvate, monothioglycerol, Hepes, Mes, Bicine, phosphoglucose isomerase, glucose-6-phosphate dehydrogenase from *Leuconostoc*, pyruvate kinase (containing lactate dehydrogenase), glucose 6-phosphate, bovine serum albumin, Sephacryl S-300 and Blue 2-agarose were purchased from Sigma Chemical Co. DEAE-cellulose (DE-52) and cellulose (CF1) were from Whatman. Hydroxyapatite (Bio-Gel HTP) and M_r standards for SDS/polyacrylamide-gel electrophoresis were products of Bio-Rad Laboratories. All other reagents were of the highest purity available.

Novikoff ascites-hepatoma cells were obtained through the courtesy of Cecilia Colarossi (Universidad Cayetano Heredia, Perú) and maintained by weekly transfers in Sprague-Dawley albino rats.

* Deceased 14 June 1986.

† To whom correspondence and reprint requests should be addressed.

Preparation of crude extracts

All operations were performed at 0–4 °C unless otherwise stated. After washing by low-speed centrifugation, with 115 mM-NaCl/5 mM-sodium phosphate, pH 7.4, to remove blood clots and red cells, the hepatoma cells were weighed and resuspended in 3 vol. of 10 mM-Tris/HCl (pH 7)/1 mM-EDTA/250 mM-sucrose. The suspension was sonicated for 20 s (repeated three times at 1 min intervals) in a cell disruptor (W185; Heat Systems–Ultrasonics) at 70% output. In a few experiments, crude mitochondria were obtained from these homogenates by differential centrifugation [24]. For enzyme purification, the homogenates were centrifuged at 105000 *g* for 60 min in the 50.2 Ti rotor of a Beckman L5-5B ultracentrifuge. The supernatants were used as starting material for the purification of hexokinases as described below.

Measurement of hexokinase activity

Glucose-phosphorylating activity was measured by coupling the formation of glucose 6-phosphate with NAD⁺ and glucose-6-phosphate dehydrogenase (Method A). NADH formation was measured spectrophotometrically at 340 nm in a standard assay medium containing, in a final volume of 0.5 ml: 100 mM-KCl, 100 mM-Hepes, pH 7.5, 12 mM-MgCl₂, 5 mM-ATP, 100 mM- or 0.5 mM-glucose, 1 mM-EDTA, 1 mM-NAD⁺, 0.15 unit of glucose-6-phosphate dehydrogenase and a suitably diluted sample of enzyme. When fructose was used as substrate, glucose 6-phosphate was measured by the addition of 1 unit of phosphoglucose isomerase. A Zeiss PMQ II spectrophotometer, thermostatically maintained at 30 °C, was used. Assay systems with ATP omitted were used as blanks. Modifications of this standard assay system are described in the legends to the Figures. To assess the effect of glucose 6-phosphate, hexokinase activity was measured by monitoring the rate of ADP formation by coupling the phosphotransferase reaction to pyruvate kinase and lactate dehydrogenase (Method B). NADH oxidation at 30 °C was followed spectrophotometrically at 340 nm in a medium containing (final concns.): 10 mM-KCl, 100 mM-Hepes (pH 7.5), 0.5 mM-NADH, 1 unit of lactate dehydrogenase (containing pyruvate kinase), 2.5 mM-phosphoenolpyruvate, substrates and enzyme in a final volume of 0.5 ml. A similar system with sugar omitted was used as a blank. One unit of enzyme activity is defined as the amount of enzyme catalysing the formation of 1 μ mol of glucose 6-phosphate in 1 min under the specified conditions. Specific activity is defined as units/mg of protein.

Protein determination

This was done by the method of Bradford [25], with bovine serum albumin as standard.

DEAE-cellulose column chromatography

For analytical purposes, hexokinase isoenzymes from high-speed supernatants or from mitochondria-solubilized material were separated on small (7.5 cm $\times$ 0.6 cm) DEAE-cellulose DE-52 columns, eluted with a linear gradient of 0–0.5 M-KCl in 10 mM-Hepes/1 mM-EDTA/1 mM-dithiothreitol, pH 7.5. Gradient volume was 34 ml, and 0.3 ml fractions were collected.

Solubilization of hexokinase activity from mitochondria

Crude mitochondria were incubated with 2 mM-glucose 6-phosphate essentially as described by Polakis & Wilson [26]. After centrifugation at 105000 *g* for 20 min, the supernatant was used for enzyme-activity determination and chromatographic fractionation to separate the isoenzymes in a small (2 ml) DEAE-cellulose column equilibrated and developed as described above.

Purification of hexokinase B and hexokinase C: general comments

The procedures for the isolation of homogeneous hexokinase B and hexokinase C are very similar. The key step is the initial separation of both isoenzymes in a column of Blue 2-agarose. Buffer 1 contained 10 mM-Tris (pH 7), 5% (v/v) glycerol, 1 mM-EDTA and 10 mM-monothioglycerol. Buffer 2 contained 1 mM-potassium phosphate (pH 6.9), 5% glycerol, 10 mM-monothioglycerol and 10 mM-glucose. Dialysis and concentration steps were simultaneously performed in an Amicon ultrafiltration apparatus with a PM 10 membrane under N₂. Salt gradients used for chromatographic elution were checked by conductimetry. In the homogeneous state both hexokinases are stable at –20 °C, but only in the presence of glucose, glycerol and thiol-reducing agents. For analysis, the protecting agents were removed from small volumes of the enzyme preparations by filtration on Centricon units (Amicon). The whole procedure, aimed at obtaining both enzymes simultaneously from the same starting batch of cells, can be completed in about 10 working days and gives reproducibly pure preparations. So far we have used the procedure with 30 batches of Novikoff cells, with essentially the same results in terms of specific activities and yields.

Purification of hexokinase B

Step 1: Reactive Blue 2-agarose. About 150 ml of high-speed supernatant was adsorbed on a column (5.5 cm $\times$ 7 cm) containing 160 ml of packed Blue-agarose in buffer 1. Hexokinase C was not bound under these conditions. Hexokinase B was quantitatively retained and could be eluted with 5 mM-ATP (without Mg²⁺) in the same buffer.

Step 2: chromatography on DEAE-cellulose. Enzyme-containing fractions from the Blue-agarose column (about 400 ml) were applied (with a peristaltic pump) to columns (1.7 cm $\times$ 22 cm) packed with DEAE-cellulose equilibrated in buffer 1 plus 10 mM-glucose. The columns were developed with a linear KCl gradient (0–0.4 M; total volume 720 ml) in buffer 1/glucose; 6 ml fractions were collected. A small peak of hexokinase A was eluted at about 65 mM-KCl and was discarded. Hexokinase B was eluted at about 100 mM-KCl. Fractions with specific activity higher than 7 units/mg were pooled, dialysed against the equilibrating buffer of the next step and concentrated to about 20 ml.

Step 3: hydroxyapatite chromatography. The fraction from the previous step was adsorbed on a column (1.7 cm $\times$ 22 cm) packed with hydroxyapatite mixed with an equal weight of cellulose and equilibrated with 25 mM-potassium phosphate buffer (pH 7)/5% glycerol/10 mM-monothioglycerol/10 mM-glucose. Elution was accomplished with a linear phosphate gradient from 25

to 100 mM (total volume 720 ml); 6 ml fractions were collected, and those with specific activities higher than 23 units/mg were pooled and concentrated to 4.5 ml.

Step 4: Sephacryl S-300 exclusion chromatography. The concentrated fraction from the previous step was carefully layered on top of a column (66 cm $\times$ 2.5 cm) containing Sephacryl S-300 equilibrated and eluted with buffer 1/glucose/100 mM-KCl; 3 ml fractions were collected. Enzyme fractions with specific activities higher than 25 units/mg were pooled, dialysed and concentrated.

Step 5: second chromatography on hydroxyapatite. The enzyme fraction from the previous step was adsorbed on a column (15.5 cm $\times$ 1 cm) containing hydroxyapatite plus cellulose. Buffers used and the procedure were exactly those described in Step 3. The fractions with the highest activities were pooled, dialysed and concentrated. The specific activity at this final step was 85 units/mg. For storage, the enzyme preparation, in 10 mM-phosphate (pH 7)/10 mM-glucose/10 mM-monothio-glycerol, was diluted with 1 vol. of glycerol.

Purification of hexokinase C

Step 1: Reactive Blue 2-agarose. As stated above, hexokinase C does not bind to the Blue-agarose column. The front fractions plus washings (about 300 ml) were pooled and used without further manipulation in the second step.

Step 2: DEAE-cellulose chromatography. The pooled enzyme fraction from the previous step was applied to a DEAE-cellulose column (1.7 cm $\times$ 22 cm) equilibrated with buffer 1 plus 10 mM-glucose. A KCl gradient (0–0.4 M; 720 ml) in the same buffer was used to elute hexokinase C at about 130 mM-KCl. Fractions with specific activities higher than 1 unit/mg were pooled, dialysed against buffer 2 and concentrated.

Step 3: hydroxyapatite chromatography. The enzyme fraction from the previous step was applied to a column (1.7 cm $\times$ 22 cm) packed with hydroxyapatite/cellulose and equilibrated with buffer 2. Elution was accomplished with a phosphate gradient from 1 to 50 mM; 6 ml fractions were collected, and those with specific activities higher than 4 units/mg were pooled and concentrated to about 4 ml.

Step 4: Sephacryl S-300 exclusion chromatography. The enzyme fraction from step 3 was layered on top of a Sephacryl S-300 column (66 cm $\times$ 2.5 cm) equilibrated and eluted with buffer 1/glucose/100 mM-KCl; 3 ml fractions were collected. Enzyme fractions with specific activities higher than 4 units/mg were pooled and concentrated. No increase in specific activity is accomplished at this step, but it is essential for the large increase in the next step to occur.

Step 5: second chromatography on hydroxyapatite. The enzyme fraction from step 4 was rechromatographed on hydroxyapatite as in step 3. Specific activity at this final step was 20–25 units/mg. Storage solution was the same as for hexokinase B.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was performed essentially as described by Davis [27], with Tris/glycine, pH 8.6. The separating gel contained 7% acrylamide. Electrophoresis was carried out at 10 °C in a Bio-Rad apparatus. Electrophoresis in the presence of SDS was performed as described by Weber & Osborn [28]. Protein bands were detected with Coomassie Blue. Bands with hexokinase activity were stained by using the standard assay medium described above, with the inclusion of 0.05 mg of phenazine methosulphate/ml and 0.5 mg of Nitro Blue Tetrazolium/ml.

Immunological studies

The immunological properties of the serum from a rabbit repeatedly injected with pure hexokinase A from rat brain have been previously described [29]. For the preparation of immune serum directed against Novikoff hexokinase C, three BALB/c mice were injected intraperitoneally with 0.1 mg of a pure enzyme preparation, followed 1 month later by an additional intravenous injection of a similar amount; 10 days later, whole blood was obtained from the retro-orbital cavities. After clotting, the sera were pooled and used without further manipulation. Sera from non-immunized mice were used as controls. Spleens from the mice were used for an unsuccessful attempt to prepare monoclonal antibodies.

For the assessment of immunological cross-reaction among hexokinase A, B, and C, an enzyme-linked immunoassay technique was used as described previously [30], except that pure hexokinase preparations were used for the coating of the micro-titre plate wells and that alkaline phosphatase-conjugated Protein A was used instead of anti-mouse IgG.

Determination of M_r

A column (66 cm $\times$ 2.5 cm) packed with Sephacryl S-300 equilibrated with buffer 1/10 mM-glucose/100 mM-KCl was calibrated with alcohol dehydrogenase, brain hexokinase A, ovalbumin and myoglobin. Void volume was established with Blue Dextran and internal volume with [14 C]lysine; 3 ml fractions were collected. M_r values of the proteins under denaturing conditions were estimated by SDS/polyacrylamide-gel electrophoresis. A mixture of standards was routinely applied to the gels: myosin, M_r 205 000; β -galactosidase, 116 200; phosphorylase *b*, 92 500; bovine albumin, 66 200; ovalbumin, 45 000.

Amino acid analyses

The amino acid composition of hexokinase C was determined as described by Bidlingmeyer *et al.* [31]. The protein was hydrolysed in vapour of 6 M-HCl for 22 h at 110 °C. The liberated amino acids were made to react with phenyl isothiocyanate and the resulting derivatives analysed by reversed-phase h.p.l.c. in a Waters 840 system equipped with a Waters Pico-Tag column.

RESULTS

Glucose-phosphorylating activity of Novikoff hepatoma

Total hexokinase activity of Novikoff hepatoma homogenates was 14 ± 1.3 (mean $\pm$ S.E.M.; $n = 6$) units/g wet wt. This value is about 4 times that of rat liver, which

Fig. 1. Hexokinase isoenzymes from Novikoff-hepatoma cytosol

A sample (0.4 ml; 1.7 mg of protein) of high-speed supernatant from sonicated ascites cells was applied to a small (2 ml) DEAE-cellulose column, and the hexokinases were eluted with a linear gradient (34 ml) of 0–0.5 M-KCl in 10 mM-Hepes (pH 7.5)/1 mM-EDTA/1 mM-dithiothreitol; 0.3 ml fractions were collected and assayed at (○) 100 mM- or (●) 0.5 mM-glucose as substrate in parallel assays. The arrow (↓) indicates the beginning of gradient elution.

Fig. 2. Hexokinase isoenzymes solubilized from a crude mitochondrial fraction from Novikoff ascites cells

Mitochondria prepared by differential centrifugation were incubated with a buffer solution containing 2 mM-glucose 6-phosphate. After 30 min incubation at 30 °C, the mitochondria were discarded by centrifugation, and a portion of the supernatant was applied to a small (2 ml) DEAE-cellulose column. Hexokinases were eluted as described in the legend to Fig. 1.

is 3 ± 0.3 units/g [32,33]. In liver, almost 90% of the total activity is accounted for by hexokinase D, which is absent (see below) from the Novikoff hepatoma. Therefore, low- K_m hexokinase activity in this tumour is almost 50 times that in liver cells. Hexokinase activity in high-speed supernatant was 7.5 ± 0.5 ($n = 20$) units/g, reflecting the partial location of the enzyme(s) in mitochondria [22].

Three hexokinases were separated by DEAE-cellulose column chromatography from high-speed supernatant

from Novikoff cells (Fig. 1) and identified as hexokinases A, B and C by their differential velocities at a low (0.5 mM) or a high (100 mM) glucose concentration in parallel assays. High-glucose/low-glucose activity ratios for semipurified or pure isoenzymes are typically 1, 1.3, 0.5 and > 10 for hexokinase A, B, C and D respectively. The first isoenzyme eluted, hexokinase A, was always a minor peak (about 10%) of the total activity. Hexokinases B and C were usually equivalent in their activities, although on occasions the latter was predominant. Hexokinase D, the low-affinity isoenzyme, was never observed. The hexokinase chromatographic pattern is similar to that described by Shatton *et al.* [19].

About 50% of the total glucose-phosphorylating activity could be recovered in the mitochondria-enriched fraction. Incubation of such a fraction with 2 mM-glucose 6-phosphate released 80–90% of the bound activity. Chromatographic analyses of the solubilized material showed the presence of two hexokinase peaks of equivalent activity (Fig. 2), identified as hexokinases A and B because of their velocities at high or low glucose concentrations.

Parry & Pedersen [22] identified the mitochondrial hexokinases of the Novikoff ascites tumour as hexokinases type I and III (equivalent to our hexokinases A and C respectively; see reference [20] for a nomenclatural discussion), on the basis of the chromatographic mobilities of the isoenzymes, assayed only at 0.5 mM-glucose, in a Cellex-D column. The experiment shown in Fig. 2 of the present paper was very similar to that reported by Parry & Pedersen [22]. However, the results of assays performed at 100 mM- and 0.5 mM-glucose clearly show that the hexokinases of Novikoff-cell mitochondria are in fact hexokinases A and B, i.e. hexokinases I and II in the nomenclature used by Parry & Pedersen [22].

Purification of hexokinase B and hexokinase C

The purification procedures for the major hexokinases from Novikoff ascites cells result in homogeneous protein preparations, as shown by electrophoresis in both the native (not shown) and the denatured states (Fig. 3). Tables 1 and 2 illustrate the results for both isoenzymes. Specific activities of 78–88 units/mg for hexokinase B and 20–25 units/mg for hexokinase C were usually obtained. The value of specific activity for pure hexokinase C is uncertain, since the velocity measured at 0.2 mM-glucose does not correspond to the maximal velocity, because of substrate inhibition.

Properties of hexokinase B

The isoenzyme from a number of sources has been extensively studied from the kinetic point of view (for a review see reference [10]). K_m values for glucose (0.16 mM) and ATP (0.4 mM) of hexokinase B from Novikoff hepatoma (not shown) were similar to those reported in previous papers [1,3,11,12]. The apparent M_r , measured by exclusion chromatography on Sephacryl S-300 (results not shown) was 97000 ± 3000 ($n = 3$). Under denaturing conditions, the value obtained by polyacrylamide-gel electrophoresis was 98000 ± 1100 ($n = 3$).

Properties of hexokinase C

Inhibition of glucose. Biphasic curves were obtained in a plot of velocity versus glucose concentrations (Fig. 4).

Fig. 3. SDS/polyacrylamide-gel electrophoresis of purified hexokinase B and hexokinase C from Novikoff hepatoma

(a) Lane 1, protein M_r markers as indicated; lane 2, hexokinase B, 11.4 μ g. (b) Lanes 1, 2 and 3, hexokinase C, 10, 3.5 and 3 μ g respectively; lane 4, protein M_r standards as indicated.

Table 1. Purification of hexokinase B from Novikoff ascites-tumour cells

The activity of hexokinase-B in the starting supernatant was calculated by assuming that one-half of the total activity corresponds to that isoenzyme (see Fig. 1).

Step	Activity (units)	Protein (mg)	Sp. activity (units/mg)	Yield (%)	Purification (fold)
Starting supernatant	713	4475	0.2	100	1
1. Reactive Blue-agarose	484	540	0.9	68	5.6
2. DEAE-cellulose	379	28	13.5	53	85
3. 1st hydroxyapatite	185	6.7	27.5	26	173
4. Sephacryl S-300	89	2.0	43	12	273
5. 2nd hydroxyapatite	25	0.3	86	3.6	539

Table 2. Purification of hexokinase C from Novikoff ascites-tumour cells

The activity of hexokinase C in the starting supernatant was calculated by assuming that one-half of the total activity corresponds to that isoenzyme (see Fig. 1).

Step	Activity (units)	Protein (mg)	Sp. activity (units/mg)	Yield (%)	Purification (fold)
Starting supernatant	740	7400	0.1	100	1
1. Reactive Blue-agarose	471	608	0.8	64	8
2. DEAE-cellulose	165	86	1.9	22	19
3. 1st hydroxyapatite	163	24.4	6.7	22	67
4. Sephacryl S-300	50	7.5	6.7	6.8	67
5. 2nd hydroxyapatite	47	2.0	23.5	6.4	235

The observed velocity was a maximum at 0.2 mM-glucose, a value which is similar to that observed with the enzymes from pig erythrocytes [18], rat [11] or frog [15] liver. K_m values calculated from the first four points of the data in Fig. 4 (and three other replicate experiments) were 0.025 ± 0.0015 mM-glucose. When fructose was used as substrate the inhibition was very slight or non-existent, and the K_m value was 2.4 mM.

The extent of inhibition by high glucose concentrations was affected by ATP (Fig. 5). At 0.2 mM-ATP a very

strong inhibition was observed at glucose concentrations above 0.1 mM, whereas at 10 mM-ATP inhibition was less severe and the observed velocity was a maximum at 0.2–0.5 mM-glucose. Similar behaviour was observed in a previous study with frog hexokinase C [15]. At low glucose concentration (0.01–0.1 mM), a slight, but always observed, inhibition by 10 mM-ATP occurs (Fig. 5).

When the velocity of hexokinase C was analysed as a function of ATP concentration at several fixed glucose concentrations, a very complex pattern of the double-

Fig. 4. Hexokinase C activity as a function of hexose concentration

The concentrations of ATP and Mg^{2+} were 5 and 6 mM respectively. When fructose was the substrate, 1 unit of phosphoglucose isomerase was added to the incubation mixture. With glucose as substrate, the addition of isomerase did not modify the velocities observed in its absence.

Fig. 5. Hexokinase C activity as a function of glucose concentration at several fixed values of ATP concentration

The incubation mixture was that indicated in the Experimental section. Mg^{2+} concentrations were 1 mM in excess over the indicated ATP concentrations.

reciprocal plots was observed (results not shown). 'Normal' curves were found at 0.2 mM-glucose or higher concentrations. Apparent K_m values for ATP at those glucose concentrations were dependent on the glucose concentration. At 0.2 mM-glucose a value of 0.35 ± 0.02 mM ($n = 3$) was calculated whereas at 10 mM-glucose a value of 0.89 ± 0.13 mM ($n = 4$) was found. On the other hand, with fructose as hexose acceptor a set of straight lines at all fructose concentrations used was always observed (results not shown), from which a K_m value for ATP of 0.6 mM was calculated.

Fig. 6. Hexokinase activity as a function of glucose concentration in the absence or presence of glucose 6-phosphate

Velocities were measured at pH 7.5 by assay method B described in the Experimental section. The concentrations of ATP and Mg^{2+} were 5 and 6 mM respectively.

Table 3. Amino acid compositions of rat hexokinase isoenzymes

The amino acid composition of hexokinase C was determined as described in the Experimental section. Other values were taken from the literature as follows: hexokinase A [2], hexokinase B [3], hexokinase D [5]. The matrix shows the values of the uncorrected $S\Delta n$ index [34] for all comparisons, assuming that hexokinases A, B and C are 'dimer-like' (i.e. case 3 as described in reference [34]): * satisfy the 'strong' test; † satisfy the 'weak' test [34].

		Content (residues/96 000 g)				
Residue	Hexokinase . . .	A	B	C	D	
	Tissue . . .	Brain	Muscle	Hepatoma	Liver	
Asp		98	92	60	92	
Thr		50	54	52	48	
Ser		48	52	65	62	
Glu		88	104	111	118	
Pro		36	26	31	28	
Gly		80	86	99	80	
Ala		48	66	74	60	
Val		64	66	72	64	
Met		30	30	16	36	
Ile		50	36	33	34	
Leu		84	92	105	82	
Tyr		8	16	16	16	
Phe		36	36	32	36	
His		18	23	21	22	
Lys		60	52	32	50	
Arg		54	56	54	56	
		Σ	426	443.5	436.5	442
	A	B	C	D		
A	—	151*	649	228†		
B		—	292†	70*		
C			—	375†

Fig. 7. Hexokinase C activity as a function of glucose concentration at three fixed pH values

ATP and Mg^{2+} concentrations were 5 and 6 mM respectively. Buffers used were 100 mM-Mes, pH 6.1, Hepes, pH 7.1, and Bicine, pH 8.9.

Inhibition by glucose 6-phosphate. As is the case for the low- K_m vertebrate hexokinases so far studied, glucose 6-phosphate was found to be a strong inhibitor of hexokinase C (Fig. 6). The inhibition was of the competitive type versus ATP (results not shown). The K_i value for glucose 6-phosphate (versus ATP) at 0.2 mM-glucose was 0.18 mM.

Effect of pH. The inhibition by glucose was studied at pH values in the range 6.1–8.9 (Fig. 7). Apart from higher velocities at pH values higher than 8, no differences in the shape of the curves were observed. This

behaviour is markedly different from that found for the frog enzyme [15,16], in which almost no inhibition by glucose was observed at alkaline pH values.

Apparent M_r . The M_r of hexokinase C estimated by Sephacryl S-300 chromatography (results not shown) was 97000 ± 4500 ($n = 3$). Under denaturing conditions, an M_r of 101000 ± 420 was found.

Amino acid composition

Duplicate analyses of the amino acid composition of hexokinase C gave the values listed in Table 3, which also includes the reported corresponding values for hexokinase A from rat brain [2], hexokinase B from rat muscle [3] and hexokinase D from rat liver [5]. A comparison of the amino acid compositions for these isoenzymes, using the index $S\Delta n$ developed by Cornish-Bowden [34], shows that extensive sequence homology among the four hexokinases must exist, since most values satisfy at least the 'weak' statistical test [34].

The amino acid composition of tumour hexokinase C differs rather markedly from that reported for the enzyme isolated from pig erythrocytes [18]. Although a species difference may account for this discrepancy, we think it unlikely in view of the postulated structural conservation of the hexokinase (see the Discussion section). A comparison of the data reported by Stocchi *et al.* [18] with those for the rat enzyme by means of the Cornish-Bowden [34] index was not possible because, for the pig enzyme, values for proline were not reported and the very high content of glycine (24% of the total) would result in a meaningless high value.

Immunological comparisons

Conventional antisera prepared against hexokinase A or hexokinase C (see the Experimental section) were used to study immunological cross-reactions of hexokinases A, B and C. The antisera only inhibited the activity of the

Fig. 8. Immunological cross-reactivities between hexokinases (Hex) A, B and C

An indirect enzyme-linked-immunoassay technique was used. Plate wells were coated with solutions containing $10 \mu\text{g}$ of the indicated purified hexokinases/ml. (a) Rabbit serum directed against rat brain hexokinase A was added to the wells at the indicated dilutions. (b) Mouse immune serum directed against hexokinase C was used. Alkaline phosphatase-conjugated protein A was used for the final colour reaction.

enzyme used as antigen (results not shown). However, by using an enzyme-linked-immunoassay technique, cross-reactions were indeed detected (Fig. 8), confirming and expanding results reported by Lawrence & Trayer [35], who used hexokinases A, B and D from rat and the corresponding antisera.

DISCUSSION

The finding in Novikoff ascites cells of high activity of hexokinase C has permitted the purification and partial characterization of this isoenzyme, which is present in small amounts in only a few mammalian tissues.

A strong motive to find a convenient source of hexokinase C and to devise a reliable protocol for its purification lies in our desire to answer the question 'why hexokinases?' as a test case of the more general question 'why isoenzymes?' [36]. We believe that the distinctive properties exhibited by each hexokinase may reflect the specific involvement of each isoenzyme in one and only one of the several pathways of glucose utilization in most organisms. A test of that hypothesis clearly requires a detailed knowledge of the structure and properties of this family of isoenzymes.

An interesting point in this respect concerns the nature of the inhibition of hexokinase C by glucose. A previous study on hexokinase C from frog liver suggested the existence of a second (inhibitory) site for glucose [15]. However, for the rat enzyme, as opposed to the frog enzyme, the inhibition by glucose is not pH-dependent (Fig. 7), and attempts to relieve it by photo-oxidation or treatment with thiol reagents have been so far unsuccessful (T. Ureta, unpublished work).

With respect to the structural relationships among the hexokinases, a few facts emerge from the results presented in this paper. Thus the immunological comparisons of hexokinases A, B and C (Fig. 8), using immune sera against hexokinase A or hexokinase C, show that the three hexokinases share at least a few common epitopes. These results and those obtained by Lawrence & Trayer [35] permit the conclusion that all four rat hexokinases are immunologically related. Also, the similarity of the amino acid compositions of the four isoenzymes (Table 3) permits the prediction of extensive sequence homology. Although the complete amino acid sequences of animal hexokinases have not so far been reported, the primary structure of seven tryptic peptides of hexokinase C could be aligned with portions of the derived amino acid sequences [37–39] of both yeast hexokinases A and B [40], suggesting that about 45% homology exists between the yeast hexokinases and hexokinase C from Novikoff rat hepatoma. It can be predicted that even more extensive homology will be found among the four vertebrate hexokinases. Therefore the primary structure of the hexokinases has been highly conserved during evolution (see also Fothergill-Gilmore [41]).

The properties of hexokinase C are peculiar enough for us to suspect an important role for it in either the operation or the regulation of glucose metabolism. Although the available information on hexokinase C is still very limited, we offer the following list of the features which, in our opinion, seem to be the most relevant for a discussion about the physiological role of this isoenzyme: (1) a K_m value for glucose which is the lowest of all the hexokinases so far studied; (2) inhibition

by glucose concentrations higher than 0.2 mM, which is relieved by ATP concentrations above 1 mM; (3) relative insensitivity to inhibition by glucose 6-phosphate, inasmuch as K_i values for this product are at least one order of magnitude higher than for hexokinases A or B (the inhibition of hexokinase D by glucose 6-phosphate is physiologically irrelevant); (4) like hexokinase D, it appears not to be associated with mitochondria, a localization that seems to be an important regulatory feature of hexokinases A and B [10]; (5) its very low activity in the few tissues in which its presence has been convincingly documented. So far, the only exceptions are the Novikoff hepatoma and pig erythrocytes. We are aware that this list does not answer the question of what role hexokinase C plays in glucose metabolism, but it is offered nevertheless as an attempt to pinpoint a few essential features which may prove relevant for future work.

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