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COMPARTMENTATION OF HEXOKINASE IN HUMAN BLOOD CELLS

CHARACTERIZATION OF SOLUBLE AND PARTICULATE ENZYMES

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The isozyme distribution, kinetic properties and intracellular localization of hexokinase (ADP: D-hexose-6-phosphotransferase, EC 2.7.1.1) were studied in erythrocytes, blood platelets, lymphocytes and granulocytes. Soluble and particulate fractions were separated by a rapid density centrifugation method after controlled digitonin-induced cell lysis. In lymphocytes and platelets the major part of total activity was particle-bound (78 and 88%, respectively). In granulocytes and erythrocytes most of the hexokinase activity was found in the cytosol. All cell types, except granulocytes, contain mainly the type I isozyme. Platelets contain only type I hexokinase, while in lyphocytes a minor amount of type III is present in the soluble fraction (less than 10% of total activity). The major constituent of granulocytes is type III hexokinase (70–80% of total activity), the remaining 20–30% is type I hexokinase. Erythrocytes contain a multibanded type I hexokinase. The substrate affinities of the type I hexokinase do not differ significantly between the different cell types or between soluble, bound and solubilized fractions. Only soluble hexokinase from lymphocytes shows a slightly decreased K_m apparent for glucose ($P < 0.05$).

Introduction

It is generally accepted that hexokinase (ADP: D-hexose-6-phosphotransferase, EC 2.7.1.1) is at least one of the enzymes which controls the utilization of glucose through the glycolytic pathway and hexose monophosphate shunt. In some tissues hexokinase is partly bound to the mitochondria and it was suggested that bound hexokinase plays a key role in an active glycolytic pathway [1]. Especially in malignant tissues an increased glycolytic activity was found to be associated with an increased hexokinase activity in the mitochondrial fraction [2]. Bound hexokinase is less inhibited by phosphorylated hexoses than the soluble form and could directly benefit from the ATP produced by the mitochondrial respiration for the phosphorylation of glucose [1]. All blood cells are strongly dependent on the energy supplied by glycolysis. In mature red cells no mitochondria are present anymore and the cell is entirely dependent on energy produced by glycolysis to maintain its shape and function. A partial deficiency of red cell hexokinase causes a severe hemolytic anemia [3,4], which illustrates the key role of hexokinase in red cell metabolism. Although in the other blood cells mitochondria and mitochondrial respiration are present, platelets [5], lymphocytes [6,7] and granulocytes [8] all depend more or less strongly on glycolytic energy supply to perform their functions properly. However, the mode of energy consumption is different per cell type. Platelets need

to respond to very urgent events, e.g. damage to blood vessel walls. They have a high glycolytic activity in order to meet the energy demands of aggregation and secretion [5] and it was shown that virtually all hexokinase is localized in the particulate fraction of platelets [9]. Lymphocytes are relatively dormant cells and transform rather slowly to metabolically active lymphoblasts when they are stimulated. Resting granulocytes have a relatively low level of metabolic activity too. However, during phagocytosis, they have to produce a lot of energy (the metabolic burst) and have to increase their glucose utilization through the hexose monophosphate shunt. In all these processes hexokinase is a likely candidate to perform a key role in the supply of energy.

The knowledge of isozyme distribution, kinetic features and intracellular localization of hexokinase in platelets, lymphocytes and granulocytes is rather incomplete. We, therefore, compared these properties of hexokinase in all blood cell types in order to gain more insight into the metabolic capacities of these cells.

Methods

Isolation of cells

Red cells. Freshly drawn human venous blood from healthy donors was collected in heparin (30 U/ml). Red cells were isolated by filtration through an α-cellulose-crystalline-cellulose mixture [10]. A pure erythrocyte preparation was obtained with less than 1% contamination of other cell types. For kinetic and electrophoretic experiments total red cell hexokinase was partially purified by batchwise treatment with DEAE-cellulose A50 (Pharmacia, Uppsala, Sweden) and concentrated by ammonium sulphate precipitation as described before [11]. The precipitate was dissolved in 0.2 M Tris-HCl, pH 7.8 containing 3 mM β-mercaptoethanol and extensively dialysed against the same buffer.

Platelets. Freshly drawn human venous blood from healthy donors was collected in acid/citrate/dextrose (final citrate concentration 13 mM) and centrifuged (10 min, $200 \times g$, 22°C). Platelets were isolated by gelfiltration at room temperature in Ca-free tyrode solution [9]. After centrifugation in the presence of 9 mM EDTA (10 min, $800 \times g$, 22°C) the platelets were suspended in Ca-free Tyrode solution to concentrations varying between 2 and 10×10^5 cells/μl. Contamination with other blood cells was less than 1%.

Lymphocytes. Lymphocytes were isolated from fresh human blood collected in either acid/citrate/dextrose or heparin. After density centrifugation (20 min, $1000 \times g$, 22°C) of the blood on Ficoll-Isopaque (Pharmacia, Uppsala, Sweden, density 1.077), the lymphocytes were carefully collected from the interface and treated with ammonium chloride (0.83%). Monocytes were removed by adherence to plastic Petri dishes. The resulting cell preparation consisted of more than 95% lymphocytes.

Granulocytes. The cell pellet remaining after the isolation of platelets and lymphocytes consisted of red cells and granulocytes. Red cells were removed by isotonic shock with 0.83% ammoniumchloride. The procedure was repeated until the cell preparation consisted of more than 95% granulocytes.

Isolation of cytosol and particulate fractions

Cytosolic and bound hexokinase were separated by discontinuous gradient centrifugation after controlled digitonin-induced lysis as described for human platelets by Akkerman et al. [9]. This procedure enables the isolation of cytosol and particulate fractions within 1 min after cell lysis. In an Eppendorf Microfuge centrifugation tube the following gradient was established: lower layer: 100 μl Ca-free Tyrode solution containing 10% (v/v) glycerol; middle layer: 250 μl (platelets) or 500 μl (other cell types) of a 3:2 (v/v) mixture of dibutylphthalate and dinonylphthalate (Merck, Darmstadt, F.R.G.) and upper layer: 500 μl cell suspension. The upper layer was mixed with 50 μl of a digitonin suspension (Merck, Darmstadt, F.R.G.) in various concentrations (as indicated in the Results). After incubation for 10 s (platelets), 20 s (lymphocytes, red cells) or 30 s (granulocytes) the suspension was centrifuged (45 s, $10000 \times g$) in an Eppendorf Microfuge equipped with a swingout rotor. Upper layer and lower layer were collected and analysed. To the lower layer 400 μl of Ca-free Tyrode was added and the pellet was suspended by mixing with a Vortex whirlmixer. The fractions were analysed for the activities of hexokinase, lactate-dehydrogenase (L-lactate:

NAD^+ oxidoreductase, EC 1.1.1.27) as a marker for the cytosol fraction and glutamate dehydrogenase (L-glutamate: NAD^+ oxidoreductase, EC 1.4.1.2) as a marker for the mitochondrial matrix. In erythrocyte fractions acetyl-cholinesterase (acetylcholine hydrolase, EC 3.1.1.7) was assayed as a marker for the plasma membrane. Percentages of hexokinase activity in soluble and particulate fractions were corrected for the percentage of non-cell lysis as based on the lactate dehydrogenase activity in the pellet fraction.

Enzyme assays and kinetic studies

Hexokinase and lactate dehydrogenase activities were determined according to Beutler [12], glutamate dehydrogenase according to Schmidt [13], acetylcholinesterase according to Ellman et al. [14]. Hexokinase activities are expressed in mU per 10^6 cells, in which 1 U is defined as the amount of enzyme which catalyzes the formation of 1 μmol of glucose 6-phosphate per min at 37°C.

Prior to the determination of enzyme activities in pellet fractions the enzymes were solubilized by the addition of 0.5% Triton X-100. The enzyme activities were unaffected by either digitonin or Triton X-100. Apparent K_m values of hexokinase for its substrates glucose and $MgATP^{2-}$ were determined at the concentrations indicated in the text.

Excess of free Mg^{2+} over $MgATP^{2-}$ was kept at 5.0 mM in all conditions. For the determination of kinetic parameters of bound hexokinase, the pellet fractions were suspended by mixing with a Vortex Whirlmixer (platelets) or by sonication 2 × 10 s (lymphocytes and granulocytes). Kinetic parameters of solublized hexokinase were determined after incubation of the particulate fractions with 0.5 M KCl for at least 10 min at 4°C. Solubilized hexokinase was separated from the cell debris by centrifugation (15 min, 40 000 × *g*, 4°C).

Electrophoresis

Hexokinase electrophoresis on cellulose acetate gels and subsequent staining for enzyme activity was performed as described before [15]. Prior to electrophoresis hexokinase from particulate fractions was solubilized by incubation with 0.5 M and subsequent centrifugation (15 min, 48 000 × *g*, 4°C).

Results

Digitonin-lysis technique

The optimal digitonin concentration enabling maximal liberation of cytosolic lactate dehydrogenase (> 90%) and minimal leakage of glutamate dehydrogenase was determined for each cell type by titration with increasing concentrations of digitonin (Fig. 1). This optimal concentration was also dependent on the cell concentrations (results not shown). The digitonin concentrations routinely used with corresponding amounts of lactate dehydrogenase and glutamate dehydrogenase liberation in the supernatant are summarized in Table I. At the optimal digitonin concentration for the lysis of erythrocytes (Table I) essentially all cholinesterase activity (> 90%) remained in the pellet fraction.

Activity and intracellular localization of hexokinase

The highest activity of hexokinase was found in lymphocytes and granulocytes as expressed per cell number, by far the lowest activity in erythrocytes (Table II). In platelets and lymphocytes most of the hexokinase activity was particulate bound,

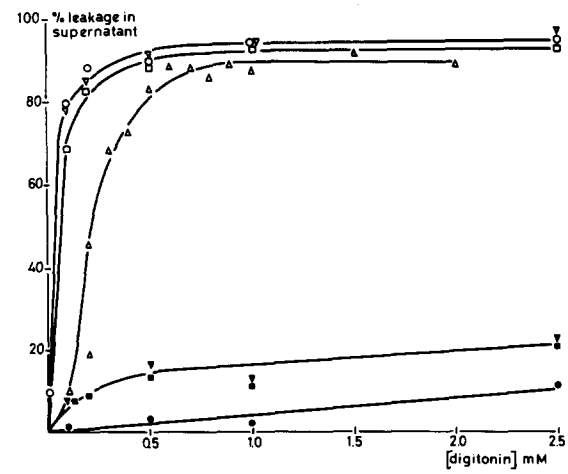

Fig. 1. Influence of digitonin concentration on lactate dehydrogenase liberation (open symbols) and glutamate dehydrogenase leakage (closed symbols) after gradient centrifugation of platelets, 200×10^6 cells/ml (○,●); lymphocytes, 20×10^6 cells/ml (□,■); granulocytes, 20×10^6 cells/ml (▽,▼) and erythrocytes, 600×10^6 cells/ml (△).

TABLE I

SEPARATION OF CYTOSOL AND PARTICULATE FRACTIONS BY GRADIENT CENTRIFUGATION AFTER DIGITONIN LYSIS

Recoveries of total activity in supernatant plus pellet were 90–110%. The values are mean ± S.D. with the number of experiments in parenthesis.

Cell type	Cell concentration ($\times 10^6$ cells/ml)	Optimal digitonin concentration (mM)	Lactate dehydrogenase liberation in supernatant (%)	Glutamate dehydrogenase leakage in supernatant (%)
Platelets	200–1000	0.4	94.5 ± 3.3 (19)	6.9 ± 4.0 (19)
Lymphocytes	20– 50	0.4	90.0 ± 5.2 (7)	8.6 ± 5.8 (7)
Granulocytes	20– 50	0.4	93.2 ± 5.4 (10)	10.7 ± 6.7 (4)
Erythrocytes	500–1000	0.65	84.1 ± 4.5 (6)	

while in granulocytes and erythrocytes the hexokinase activity was almost exclusively found in the cytosol. Digitonin itself did not influence the compartmentation of hexokinase. Reincubation of a pellet fraction from lymphocytes with the same concentration of digitonin did not result in any detectable digitonin-induced solubilization of bound hexokinase. The leakage of hexokinase activity from erythrocytes in the supernatant equaled the lactate dehydrogenase liberation in all circumstances.

Hexokinase isoenzyme distribution

The electrophoretic patterns of hexokinase from soluble and particulate fractions are shown in Fig. 2. A human liver extract is used as a reference. Three bands of hexokinase activity are present in liver, representing the 'low K_m' hexokinases type I, II and III. Hexokinase type IV, or glucokinase, is absent, at least in our conditions. As type III hexokinase is inhibited by high glucose concentrations [16], it could be identified in the electropherogram by adding 2 mM glucose and 100 mM glucose, respectively, in the staining mixture.

Partially purified hexokinase from human red cells showed a double-banded pattern in the 'type-I region' in these conditions, while in fresh preparations a minor type III band can be detected too [4]. In lymphocytes type I hexokinase is predominant. Bound lymphocyte hexokinase consists ex-

TABLE II

ACTIVITIES AND DISTRIBUTIONS BETWEEN SOLUBLE AND PARTICULATE FRACTIONS OF HEXOKINASE IN HUMAN BLOOD CELLS

The values are means ± S.D. with the number of determinations in parenthesis. Recoveries of total activity in supernatant plus pellet were 90–110%.

Cell type	Activity (mU/10^6 cells)	% Bound
Platelets	0.160 ± 0.039 (31)	88.5 ± 4.1 (13)
Lymphocytes	2.59 ± 0.80 (21)	78.1 ± 11.3 (12)
Granulocytes	2.59 ± 0.71 (18)	6.9 ± 4.9 (7)
Erythrocytes	0.040 ± 0.013 (41)	7.0 ± 7.6 (8)

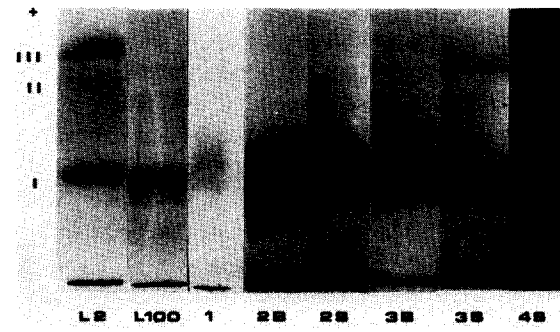

Fig. 2. Cellulose acetate electrophoresis of soluble hexokinase from erythrocytes (1S), platelets (2S), lymphocytes (3S) and granulocytes (4S); and bound hexokinase from platelets (2B) and lymphocytes (3B). Prior to electrophoresis bound hexokinase was solubilized by incubation with 0.5 M KCl. Erythrocyte hexokinase was partially purified and represents infact the total activity fraction, the major part (> 90%) of it being soluble. The percentage of bound hexokinase in erythrocytes and granulocytes is too low (Table II) to permit an electrophoretic evaluation.

clusively of the type I isoenzyme. In the cytosol, in which 20% of total activity is present, the activity is distributed between type I and type III hexokinase. Platelets contain only type I hexokinase. Solubilization of the particulate fraction of platelets sometimes resulted in a double-banded electrophoretic pattern. This phenomenon was not found consistently. Solubilized hexokinase from lymphocytes, however, always showed a single hexokinase I band. In granulocytes two types of hexokinase are present: type III being the major constituent, found exclusively in the cytosol, but also a considerable amount of type I hexokinase.

Kinetic properties

Most enzyme fractions containing hexokinase I as the major isoenzyme showed essentially the same affinity for the substrate glucose, as is shown in Table III. No significant differences were found either between soluble, bound and solubilized hexokinase or between the different cell types ($P > 0.10$ with Student's t-test). Only soluble hexokinase from lymphocytes showed a slightly decreased K_m apparent for glucose ($p < 0.05$). Furthermore, the soluble fraction from granulocytes, as well as the total activity fraction, showed non-linear Lineweaver-Burk plots, composed of a high affinity part and a low affinity part (results not shown). These non-linear kinetics are apparently the result of the presence of both hexokinase I and hexokinase III in the cytosol, as was also shown by electrophoresis.

Apparent Michaelis-Menten constants for the substrate $MgATP^{2-}$ showed nonsignificant differences between the individual cell fractions ($P > 0.10$). Determination of kinetic properties of bound platelet hexokinase was seriously hampered by the occurrence of a considerable degree of spontaneous solubilization. This phenomenon appeared to be specific for platelets. Reliable results could only be obtained by removing the spontaneously solubilized hexokinase by centrifugation immediately prior to the assay.

TABLE III

ENZYME PROPERTIES

The total activity fractions were obtained either by the addition of 0.5% Trition X-100 to the Ca-free Tyrode solution, or by sonication (3 × 10 s). Solubilization of particulate hexokinase was achieved by incubation with 0.5 M KCl (30 min) and subsequent centrifugation (15 min, 48000 × g, 4°C). The apparent K_m for $MgATP^{2-}$ was determined at a concentration of 10 mM glucose, the $K_{m,app}$ for glucose at 5 mM $MgATP^{2-}$. The numbers in parentheses indicate the number of determinations on different samples. When more than two determinations are performed, the values represent the means ± S.D.

	$K_{m,app}$ MgATP (mM)	$K_{m,app}$ glucose (mM)
Platelets		
total	0.43 ± 0.06 (3)	0.065–0.066 (2)
bound	0.52 ± 0.22 (3)	0.061 ± 0.019 (3)
soluble	0.68 ± 0.19 (5)	0.065 ± 0.011 (3)
solubilized	0.50–0.99 (2)	0.046–0.067 (2)
Lymphocytes		
total	0.72 ± 0.30 (5)	0.063 ± 0.012 (3)
bound	0.73 ± 0.10 (5)	0.045–0.065 (2)
soluble	1.09 ± 0.39 (6)	0.034 ± 0.004 (4)
solubilized	1.25–1.54 (2)	0.071–0.077 (2)
Granulocytes		
total	0.71 ± 0.14 (7)	non-linear kinetics
bound	1.17 ± 0.32 (4)	0.052 ± 0.022 (3)
soluble	0.81 ± 0.11 (6)	non-linear kinetics
Erythrocytes		
total	0.71 ± 0.09 (5)	0.062 ± 0.014 (5)

Discussion

The intracellular localization of hexokinase was studied by controlled digitonin induced cell lysis followed by discontinuous gradient centrifugation as described by Akkerman et al. [9] for platelets. In this technique disturbances in metabolism after cell lysis are minimized by separating soluble and particulate fractions within 1 min. We adapted this procedure for the fractionation of erythrocytes, lymphocytes and granulocytes. As hexokinase is presumed to be attached to the surface of the outer mitochondrial membrane [17], it might be more susceptible to digitonin than the intramitochondrial matrix marker glutamate dehydrogenase. However, even prolonged incubation of pellet fractions with digitonin gave no solubilization of bound hexokinase.

Summarized, the results show that the digitonin-lysis technique works equally well for erythrocytes, lymphocytes and granulocytes as was documented for platelets [9].

Almost all hexokinase activity in lymphocytes

and platelets was shown to be localized in the particulate fraction, presumably bound to the outer membrane of the mitochondria, which may provide a basis for a high glycolytic capacity in these cells [1]. Platelets contain only type I hexokinase, while lymphocytes contain a minor amount (less than 10%) of type III hexokinase in the cytosol fraction. On the contrary, hexokinase from granulocytes is almost entirely situated in the cytosol and the major part of it can be identified as type III hexokinase, an isoenzyme with relatively high affinity for glucose [16] and which is inhibited by high concentrations of glucose as was shown in the electrophoretic stains. A considerable amount (20–30% of total activity) of type I hexokinase is present too. How these findings fit into the metabolic requirements of the individual cell types is not clear.

Digitonin-induced lysis of mature human red cells showed that all hexokinase activity was liberated in the supernatant fraction together with the lactate dehydrogenase activity, while acetylcholinesterase activity remained in the pellet fraction.

In reticulocytes a part of the hexokinase activity is particulate bound [18,19]. Partially purified hexokinase from human red cells shows a two-banded pattern upon cellulose-acetate electrophoresis, presumably products of post-translational modification reactions. After partial purification and isolation of subtypes by phosphocellulose chromatography even four subtypes could be discerned, all belonging to the 'type I complex' as was shown by kinetic analysis [20]. The possibility, that one or more of these subtypes represent hexokinase bound to some minor fragments, left over after the destruction of the mitochondria in the reticulocyte, deserves serious attention. Our results cannot be conclusive in this respect. However, it seems clear that the adherence of hexokinase activity to the plasma membrane as reported by some authors [21,22] can be excluded. A minor fraction of type III hexokinase is present too in fresh preparations of erythrocyte hexokinase [4].

The kinetic properties of the hexokinase type I from the different cell types and different cell fractions showed no significant differences in substrate affinities, only soluble hexokinase from lymphocytes has a decreased affinity for glucose. Solubilization of bound hexokinase by KCl resulted usually in an electrophoretic mobility identical to soluble hexokinase type I, suggesting that the binding of hexokinase I to the mitochondria is not reserved to a particular subtype of hexokinase I. To the contrary, solubilized hexokinase from platelet pellets showed in some experiments a double-banded type I hexokinase upon electrophoresis. However, this phenomenon was not consistently found. So we might conclude that the heterogeneity of hexokinase I in erythrocytes is hardly found in other blood cell types. This presumably relates to the absence of protein renewal in erythrocytes together with a relatively long lifetime and consequently to a greater impact of post-translational modification processes in these cells. The underlying mechanism of the formation of hexokinase I subtypes, however, remains unclear.

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