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Activation of low K_m hexokinases in purified hepatocytes by binding to mitochondria

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Hepatocytes were purified on a Percoll gradient. The cell membrane of these hepatocytes was disrupted by digitonin in the presence of albumin, glucose and physiological concentrations of monovalent and divalent cations. This treatment led to a separation between free and loosely structure-bound cytosolic enzymes which is not achieved by conventional subfractionation techniques. According to kinetic and immunological analyses, the free extractable cytosolic fraction contained high K_m, hexokinase (glucokinase) and less than 10% of low K_m hexokinases, while the hexokinase activity bound to the cell structures represented exclusively low K_m isozymes. The total activity of the bound hexokinases was comparable to that observed in the supernate (approx. 1.0 U per g fresh weight). This activity decreased more than 10-fold upon desorption at higher digitonin concentrations. Such activation by binding, as well as inactivation by desorption, could also be demonstrated in intact hepatocytes correlated to different metabolic states, and also in vitro with isolated mitochondria and purified isozyme I. The binding of low K_m hexokinases in hepatocytes was restricted to the mitochondrial fraction and there it was observed in the contact sites between the two mitochondrial boundary membranes. In view of these findings it appears that the binding-dissociation equilibrium of low K_m hexokinases plays an important role in metabolic regulation of glucose uptake and glycogen synthesis in the liver and presumably in muscle.

Introduction

The presence of different hexokinase isozymes and their intracellular distribution has a great influence on the regulation of glucose catabolism in various tissues. In insulin-insensitive kidney medulla and brain, where glucose is almost exclusively utilized in energy metabolism and little glycogen is formed, isozyme I, which is permanently bound to the mitochondria, is predominant [1,2]. In contrast, insulin-sensitive tissues like liver, muscle, and adipose tissue which metabolize fatty acids and as liver and muscle synthesize glycogen, contain high activity of isozyme II. The total hexokinase activity found in the mitochondrial fraction of the latter three tissues is considerably lower than that of brain and is sensitive to insulin and/or glucose [3–5]. These findings indicate the importance of mitochondrial binding in the regulation of hexokinase activity which is supported by our recent in vitro observation that binding to liver mitochondria increases isozyme II activity by 40-fold [6]. The very low hexokinase activity in the mitochondrial fraction of liver parenchymal cells seems to contradict the postulated regulatory im-

Abbreviation: DNP, 2,4-dinitrophenol.

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portance of hexokinase binding in carbohydrate metabolism. Furthermore, it is still in discussion whether the low K_m hexokinases derive exclusively from non-parenchymal cells in the liver [7–9]. However, if hexokinase binding in liver cells is regulated by hormones and metabolites, it is plausible that it may be sensitive to environmental changes. It is thus not astonishing that conventional techniques of tissue subfractionation lead to an almost complete loss of structure-bound activity due to dilution and changes in ionic environment. The regulatory changes of the intracellular distribution of hexokinase in hepatocytes should therefore, be investigated by less invasive techniques. It has recently been shown that digitonin-treated hepatocytes lose their plasma membrane but remain otherwise intact when prepared in 0.25 M sucrose containing 25 mM monovalent salt [10]. In the present investigation we treated hepatocytes in a similar way, and observed that up to 60% of the total cellular hexokinase activity remained bound in the sediment, while more than 90% of the lactate dehydrogenase activity could be extracted. The total activity of the structure-bound hexokinases can reach 1–2 U per g fresh weight and in agreement with our earlier observations depends on the metabolic status of the cells [4,11].

Materials and Methods

All chemicals were purchased from Boehringer Mannheim and E. Merck Darmstadt, F.R.G.

Experimental animals. Female rats of the Chbb-THOM strain (200–250 g body weight) were used. They were fed with a standard diet of Altromin in Lage, F.R.G. In some cases the rats were starved for 48 h.

Preparation of cells. Isolated hepatocytes were prepared by perfusion with collagenase of livers from fed rats essentially as described by Berry and Friend [12]. Gelatine (1.5%) was used instead of 3% of bovine serum albumin.

Purification of hepatocytes. The hepatocytes were further purified by centrifugation on a percoll gradient according to Kraemer et al. [13]. The purity of the cells was proved by the absence of pyruvate kinase K. The two isozymes of pyruvate kinase, K and L, were identified by column chromatography on DEAE-cellulose as described by Reyes and Cárdenas [9].

Digitonin treatment of the cells. The isolated hepatocytes were suspended in Krebs-Henseleit bicarbonate buffer equilibrated with 95% O_2/5% CO_2 containing 2% albumin, 10 μM $CaCl_2$, 5 mM MgCl, and either 15 mM glucose or 0.5 mM alanine, or 0.5 mM alanine and 2 mM oleate as indicated. The suspension was adjusted to contain 30 mg protein per ml. Aliquots of 0.2 ml were incubated at room temperature for 30 s with varying concentrations of digitonin ranging between 10 and 200 μg/mg protein, and subsequently centrifuged for 30 s in a tabletop centrifuge. The supernatant fraction was removed and the pellet was resuspended in 0.2 ml Krebs-Henseleit medium. For the determination of K_m, the hexokinase was extracted from the sediment by 0.5% triton X-100.

Assays. Hexokinase (EC 2.7.1.30), adenylate kinase (EC 2.7.4.3), and lactate dehydrogenase (EC 1.1.1.27) were determined photometrically according to Bücher et al. [14]. The structure-bound hexokinase in the cell sediment was determined after freezing and thawing of the samples. The activities in the photometric assays could be inhibited either by specific antibodies against hexokinase, or by *N*-acetyl-D-glucosamine. Glucose was removed from the extracts by chromatography on Sephadex G50, to determine K_m of hexokinase. The assays of monoamine oxidase (EC 1.4.3.4) and succinate dehydrogenase (EC 1.3.99.19) were carried out as described previously [15]. NADPH cytochrome-*c* reductase (EC 1.6.2.a) was assayed according to Sottocasa et al. [16].

Isolation of mitochondria, microsomes and particle-free supernatant. Mitochondria and microsomes were isolated from rat liver by differential centrifugation in a medium containing 0.25 M sucrose and 10 mM Hepes (pH 7.4), designated below as sucrose medium. The postmitochondrial supernate was centrifuged for 15 min at 15 000 × *g* and subsequently for 45 min at 200 000 × *g*. The pellet of the second centrifugation was resuspended in the isolation medium. A specific activity of NADPH-cytochrome-*c* reductase 10-times higher than the mitochondrial fraction indicated that this sediment contained microsomal membranes. The supernatant fraction was chromato-

graphed on Sephadex G50 to remove glucose, and subsequently concentrated by vacuum dialysis against sucrose medium.

Isolation of hexokinase type I. Hexokinase I was purified from rat brain according to Chou and Wilson [17].

Preparation of antibodies. Antibodies active against hexokinase I were raised in rabbits by injection of isolated hexokinase I as described recently [4]. The antibodies inhibited the activity of hexokinase I completely and 50% of the low K_m hexokinase activity of a liver supernate. No effect was found on the activity of glucokinase.

Electrophoresis and decoration by antibodies. After treatment of the hepatocytes with digitonin the supernatant fraction and a 3% SDS extract of the sediment were subjected to SDS polyacrylamide gel electrophoresis as described by laemmli [18]. Proteins were transferred from the slab gel to nitrocellulose filters as described by Rott and Nelson [19]. The transferred proteins were incubated with biotinylated antibodies [20] against SDS denatured hexokinase I, followed by a streptavidine-peroxidase conjugate. The peroxidase reaction was performed as described recently [21].

Binding of hexokinase to mitochondria and microsomes. Mitochondria and microsomes were suspended in a sucrose medium to a protein concentration of 30 mg/ml. Of this suspension 0.3 ml aliquots were incubated for 20 min in an ice-bath with increasing activity levels of hexokinase in the presence of 10 mM $MgCl_2$ and 5 mM glucose. The mitochondria and the microsomes were subsequently separated from the supernate by centrifugation. The pellet was resuspended in 0.3 ml of sucrose medium. In both fractions hexokinase activity was determined photometrically. For binding studies only those isozyme I preparations were used which were adsorbed completely to the mitochondrial fraction at low concentrations, i.e., completely bindable hexokinase I.

Treatment of isolated mitochondria with digitonin. Mitochondria were isolated from rat liver by differential centrifugation in a sucrose medium. The mitochondrial fraction contained a protein concentration of 30 mg/ml. Aliquots of 0.2 ml of this mitochondrial suspension were incubated for 30 s at room temperature with concentrations of digitonin ranging from 50 to 200 μg/ml protein. The suspension was subsequently centrifuged for 1 min in a tabletop centrifuge. The supernate was then removed and the sediment was resuspended in sucrose medium.

Results

Purification of hepatocytes

It has been described that hepatocytes are virtually free of low K_m hexokinases [7,8]. Therefore, we attempted to separate the hepatocytes from nonparenchymal cells by Percoll gradient centrifugation according to Kraemer et al. [13]. To prove the efficiency of the separation we looked for the isozymes of pyruvate kinase in the hepatocyte fraction [9]. Isozyme K which is specific for the nonparenchymal cells was almost absent in the purified hepatocytes (Fig. 1). In the crude hepatocyte preparation a 50% reduction of pyruvate kinase K compared to the liver homogenate was already achieved (results not shown).

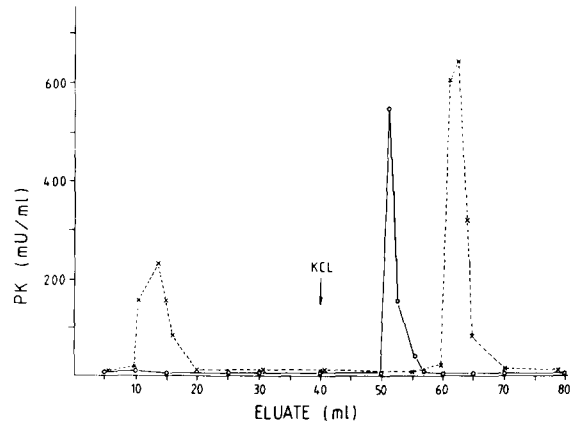

Fig. 1. Isozymes of pyruvate kinase (PK) in purified hepatocytes. The hepatocyte-specific isozyme of pyruvate kinase was identified by column chromatography. A DEAE-cellulose column was loaded with comparable amounts of pyruvate kinase activity of a liver supernate (broken line) or of an extract of hepatocytes (solid line) which had been purified on a percoll-gradient. Pyruvate kinase K (nonparenchymal cells) became eluted with equilibration buffer (10 mM potassium phosphate (pH 7.4)/2 mM dithioerythritol/0.5 mM sucrose), while pyruvate kinase L (hepatocyte specific) was extracted from the DEAE column by 1 M KCl. The fact that the two isozyme L peaks do not coincide is due to different column capacity in separate experiments. Isozyme K was not present in the purified hepatocytes.

Subcellular distribution of hexokinase in isolated hepatocytes

Hepatocytes from fed and starved rats were suspended in Krebs-Henseleit medium containing 2% albumin, 5 mM glucose, 10 μM $CaCl_2$, 0.5 mM $MgCl_2$ in a concentration of 30 mg protein/ml. The cells were treated with increasing digitonin concentrations for 30 s. Soluble and structure-bound enzymes were separated by subsequent centrifugation. The recovery of enzymes and protein (not shown) in the supernate plus sediment averaged 95% of the untreated cells. Most of the lactate dehydrogenase representing the cytosolic enzymes was extracted into the supernate at a concentration of 50 μg digitonin per mg cell protein (Fig. 2a) and less than 10% of the total activity remained in the sediment. The liberation of adenylate kinase, a marker enzyme for the mitochondrial intermembrane space, into the supernatant fraction at about 100 μg of digitonin per mg cell protein indicated the disruption of the mitochondrial outer membrane (Fig. 2a). In spite of the initial extraction of 90% of lactate dehydrogenase at digitonin concentrations of 50 μg/mg, 30–50% of the total hexokinase activity remained bound to the pellet in hepatocytes from fed and starved rats, respectively (Fig. 2b and c). Thus hexokinase did not behave as a free cytosolic enzyme. The large difference of total activity between fed and starved rats clearly resulted from a decrease in the free cytosolic hexokinase. Kinetic analysis (Table I) revealed that this hexokinase activity which could be extracted from the cells, like LDH, represented to 90% high K_m glucokinase, whereas the structure-bound hexokinase activity was exclusively low K_m. The high-affinity

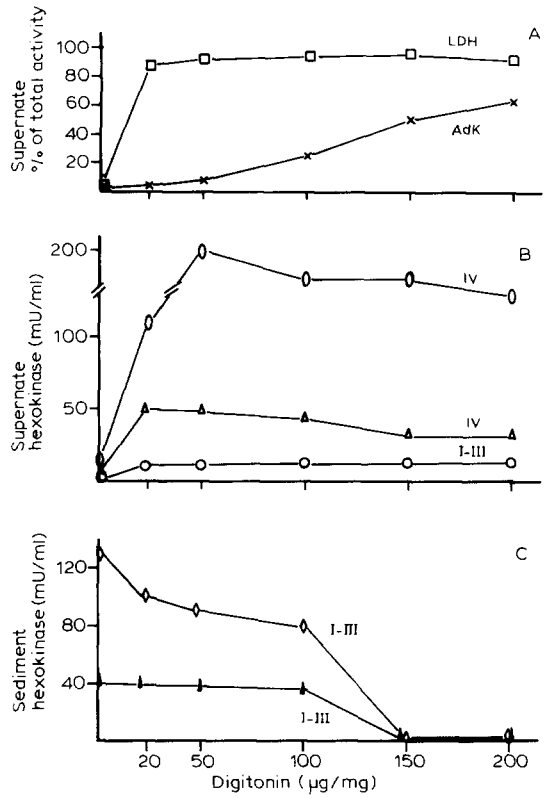

Fig. 2. Subcellular distribution of enzymes in isolated hepatocytes subjected to digitonin treatment. Isolated hepatocytes were suspended in Krebs-Henseleit bicarbonate buffer containing 2% albumin, 10 μM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM glucose. The suspension was adjusted to contain 20–30 mg of protein per ml. Aliquots of 0.2 ml were incubated at room temperature for 30 s with digitonin between 20 and 200 μg/mg protein and subsequently centrifugated for 30 s in a tabletop centrifuge. The supernate was removed and the pellet was resuspended in the same volume of Krebs-Henseleit medium. (A) Lactate dehydrogenase (LDH), and adenylate kinase (AdK) in the supernate are expressed as % of total cellular activity. (B) Hexokinase activity in the supernate (mainly glucokinase): upper curve, IV from fed rats; lower curve, IV from 48 h starved rats. Curve I–III shows activity of low K_m hexokinases in the supernate of starved and fed rats. (C) Represents activity of low K_m hexokinases in the sediment, upper curve I–III from fed rats, lower curve I–III from 48 h starved rats.

TABLE I

KINETIC ANALYSIS OF LOW AND HIGH K_m HEXOKINASES IN PURIFIED HEPATOCYTES

Hepatocytes purified by Percoll gradient centrifugation were treated with 60 μg/mg digitonin. Cytosolic and structure-bound enzymes were separated by centrifugation. The bound enzymes were extracted from the sediment by 0.5% Triton X-100, as described in Materials and Methods. K_m(glucose) and maximal activity were determined by an optical test system. Inhibition of the enzyme activity was studied with antibodies (anti HK I) raised against isozyme I.

Cell fraction	Activity (mU/ml)	K_m(glucose) (mM)	Inhibition by anti HK I (%)
Supernate			
low K_m	7.6	0.06	48
high K_m	200	10.0	5
Sediment			
low K_m	12	0.04	50
high K_m	0.0	–	–

hexokinase was additionally characterized by 50% inhibition with antibodies raised against isoenzyme I isolated from rat brain (Table I). The low K_m hexokinases were liberated from the cells at digitonin concentrations above 100 μg/mg coinciding with the disruption of the outer mitochondrial membrane. The decrease of structure-bound enzyme activity did, however, not result in a corresponding increase of supernatant activity (Fig. 2). This suggested an inactivation of the low K_m isozymes by desorption, since one would expect a corresponding activity increase in the supernate. Furthermore, this experiment shows that the total amount of the structure-bound hexokinase activity is comparable to that of the supernatant activity which represents mainly glucokinase. This quantitative amount suggests that the structure-bound hexokinase must have regulatory importance.

Regulation of low K_m hexokinase activity by binding and desorption in hepatocytes

To investigate the possible effects of metabolites on binding and desorption of hexokinase, livers from starved rats were perfused with 2 mM lactate during hepatocyte preparation. The cells were subsequently purified on a Percoll gradient and incubated for 30 min in Krebs-Henseleit bicarbonate buffer, 1.5% gelatine, 2% bovine serum albumin in the presence of different metabolites: either 0.5 mM alanine or 0.5 mM alanine and 2 mM oleate, or 15 mM glucose. After incubation the structure-bound and free hexokinases were separated by treatment of the cells with 60 μg/mg digitonin and subsequent centrifugation. The activity of glucokinase in the supernate of the three samples was 30% of that observed in fed rats and did not change by incubation with the different metabolites. The same was true for the activity of low K_m hexokinases in the supernate, although their activity increased 2-fold in the sediment of the glucose treated cells compared to the cells which were incubated with alanine (Table II). This suggests that the glucose-treated cells gained hexokinase activity presumably by binding to the mitochondrial fraction. The magnitude of these changes was low compared to the 40 fold in-vitro activation of isozyme II [6]; however, this effect was induced exclusively by different metabolites.

TABLE II

EFFECT OF DIFFERENT METABOLITES ON THE ACTIVITY OF STRUCTURE-BOUND HEXOKINASE IN HEPATOCYTES

Hepatocytes were isolated from 48 h starved rats and purified on a Percoll gradient. The cells were subsequently incubated for 30 min in Krebs-Henseleit medium, 1.5% gelatine, 2% bovine serum albumin, 5 mM $MgCl_2$ and 1.3 μM $CaCl_2$ in the presence of (a) 0.5 mM alanine and 2 mM oleate, (b) 15 mM glucose. After incubation the structure-bound and free hexokinases were separated by treatment of the cells with 60 μg/mg digitonin and subsequent centrifugation. The probability that the experimental groups (A, B) are different was calculated by the Munn-Whitney-U-test [41] and is expressed as P% (P% < 5% is considered as significantly different).

Hexokinase isozyme	Hexokinase activity (mU/ml)		
	Cells A: alanine oleate	Cells B: glucose	%
Supernate			
High K_m	77.0 ± 44.9	90.9 ± 62.2	$P\% = 38.6$
Low K_m	14.0 ± 8.0	14.6 ± 6.1	$P\% = 50.0$
Sediment			
Low K_m	22.6 ± 10.2	42.5 ± 9.3	$P\% = 2.1$

It may be assumed that hormonal action in addition to metabolite effects produces more pronounced activity changes.

Immunological identification of structure bound hexokinases

The hexokinase activity remaining in the sediment after treatment of purified hepatocytes with digitonin was extracted by 3% SDS. The polypeptides of this extract and of the supernate were separated on SDS polyacrylamide gel electrophoresis and compared to isolated isozyme I. The proteins on the gel were transferred to nitrocellulose sheets and the presence of low K_m hexokinases was visualized by decoration with antibodies against hexokinase I. The antibodies reacted with isozyme I at a molecular mass of 100 kDa and with a polypeptide of the same molecular weight in the soluble and structure-bound fraction of hepatocytes, suggesting the presence of low K_m hexokinases in both fractions. There was no reaction in the molecular-weight range of 45 kDa corresponding to the M_r of glucokinase (Fig. 3). This agreed with the observation that the antibod-

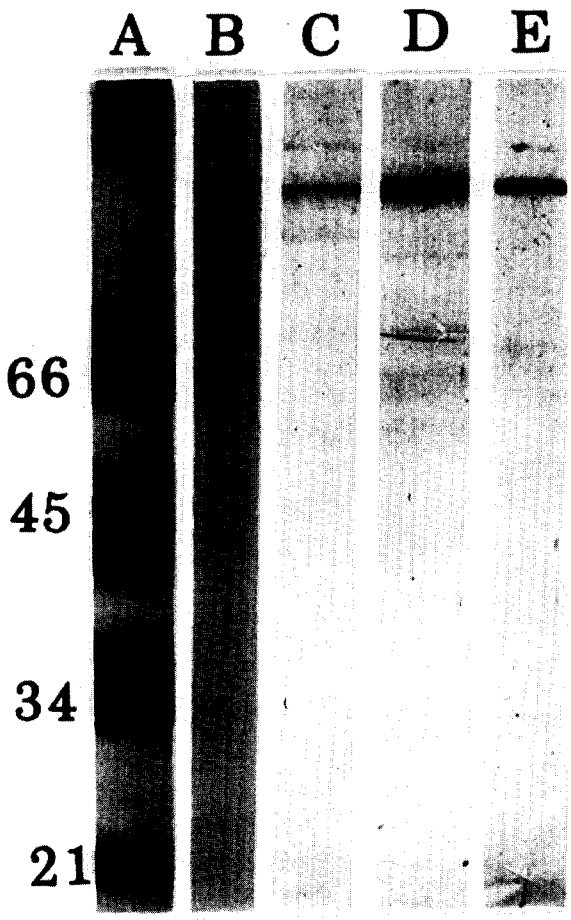

Fig. 3. Characterization of structure-bound hexokinases by electrophoresis and decoration with antibodies. Hepatocytes were treated with digitonin to liberate the soluble cytosolic enzymes. Hexokinase activity remaining in the cell sediment after centrifugation was solubilized by 3% SDS. The polypeptides in the extracts were separated on polyacrylamide gels, transferred to nitrocellulose sheets and immunodecorated with antibodies against hexokinase I. SDS gel electrophoresis (polypeptides stained with coomassie blue): lane A, molecular weight markers (the molecular mass is expressed in kDa); lane B, isolated isozyme I. Cellulose nitrate sheet decorated with antibodies against isozyme I; lane C, supernatant after digitonin treatment; lane D, extract of the sediment; lane E, isolated isozyme I.

ies inhibited the activity of low K_m hexokinases but had little effect on the activity of glucokinase (Table I).

Rebinding of hexokinase I to mitochondria and microsomes

It has been assumed that endoplasmic reticulum can bind hexokinase in the liver [22]. Therefore, isolated mitochondria and microsomes were incubated with increasing amounts of purified hexokinase I in the presence of Mg^{2+} and glucose. At a hexokinase concentration of about 16–20 mU per mg of mitochondrial protein, the mitochondrial fraction became saturated. Under the same conditions no activity of hexokinase could be bound to the microsomal fraction (Table III) although this fraction was contaminated by 1% with mitochondrial membranes (as calculated from succinate dehydrogenase activity). This can be explained by the fact that hexokinase binds to the outer surface of the outer membrane and at this surface preferentially to the regions of contact with the inner membrane. These structures represent approx. 0.3% of the mitochondrial membrane and therefore are neglectable in the mitochondrial contaminant of the microsomal fraction. Degradation of the enzyme by proteases in the microsomal fraction could be excluded because at higher con-

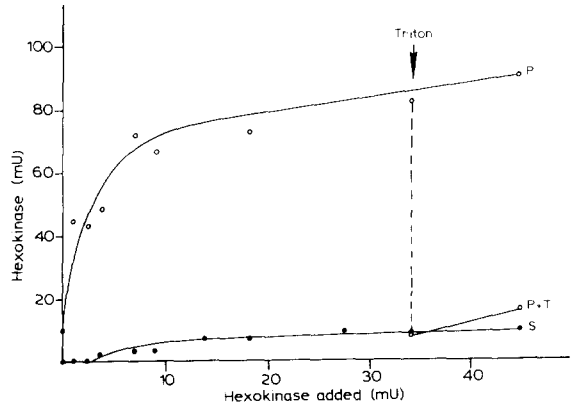

Fig. 4. Reversible activation of hexokinase type I by binding to isolated liver mitochondria. Mitochondria were suspended in sucrose medium to a protein concentration of 30 mg/ml. Of this suspension 0.3 ml was incubated for 20 min in ice-bath with increasing activity levels of hexokinase in the presence of 10 mM $MgCl_2$ and 5 mM glucose. The mitochondria were subsequently separated from the supernate by centrifugation. The pellet (P) was resuspended in 0.3 ml of sucrose medium. In both fractions, hexokinase activity was determined photometrically. The activity which was removed from the supernate by binding is shown in (S). P+T shows the activity in the sediment after solubilization of the mitochondrial membranes by addition of 1% Triton X-100.

centrations of the added enzyme, the same activity was present in the supernate. In agreement to that the addition of microsomes to the mitochondrial fraction did not reduce hexokinase binding to the mitochondrial surface. Glucose-6-phosphate phosphatase in the microsomal fraction did not interfere with the optical test system because this enzyme has a high K_m (5 mM) compared to the glucose-6-phosphate dehydrogenase in the assay system.

Effect of binding and desorption on activity of isozyme I

The observed activation by binding [6] as well as the inactivation by desorption of hexokinase in hepatocytes (Fig. 2B and C) could also be demonstrated in vitro with isolated isozyme I. When liver mitochondria were saturated with this purified isozyme I, a 5–6-fold activation of the enzyme by binding was observed (Fig. 4). This was calculated by comparison of the activity removed from the supernate with the activity gained in the sediment. On the other hand, liberation of the bound enzyme by solubilization of the membranes with Triton X-100 led to a significant decrease of activity by the same factor (Fig. 4).

Digitonin effect on mitochondrial bound hexokinase

Digitonin treatment of liver mitochondria fragments the outer membrane but leaves parts of the membrane attached at contact sites with the inner boundary membrane [23,24]. This technique was used to localize the hexokinase binding sites at the mitochondrial surface. Increasing concentrations of digitonin disrupted the outer membrane of isolated mitochondria and liberated adenylate kinase (not shown) and monoamine oxidase activity (Fig. 5 A and B). The activity of hexokinase remained in the sediment but increased at low (60 μg) concentration of digitonin, suggesting an activation of the enzyme because of removment of substrate barriers. At high (more than 100 μg) concentrations of digitonin we observed a decrease of hexokinase activity in the sediment which may be explained by partial desorption of the enzyme. Because of inactivation by desorption (Fig. 4) this debinding of the enzyme was not accompanied by an increase of activity in the supernate. In conclusion, although the recovery of hexokinase in the sediment (comparing the activity without digitonin and at 200 μg of digitonin) was about 100%, the results suggest that 50% of the total enzyme activity (referred to accessible and latent activity) was removed by 200 μg digitonin. This concentration of digitonin detaches most of the outer membrane beyond the contact sites as shown by a 90% decrease of monoamine oxidase activity in the sediment. In spite of that at least 50% of hexokinase remained bound to the significantly smaller fraction of the outer membrane in the contact regions [25]. A reassociation during digitonin treatment of the detached hexokinase to the free inner membrane can be excluded based on recent observations [25]. In the latter communication inner and outer membrane as well as contact sites were exposed to isolated isozyme I resulting in an exclusive binding to the contact fraction.

Discussion

The presence of low K_m hexokinases in hepatocytes

It has been described that hepatocytes are virtually free of low K_m hexokinases. However, these results were obtained either with hepatocyte extracts [7] or with cells of reduced integrity [8]. In the light of the above results, we conclude that the authors have not determined the structure-bound activity, and have inactivated most of the low K_m activity by desorption. Other investigators conclusively have shown the presence of all four isozymes in hepatocytes [9]. In agreement to this investigation we have observed low K_m hexokinases in purified hepatocytes by kinetic analysis and by immunological techniques (Table I, Figs. 1 and 3).

To which structure does hexokinase bind in the liver?

Treatment of isolated liver mitochondria with digitonin did not liberate hexokinase like monoamine oxidase (Fig. 5). Similar results were obtained by parry et al. [22], who assumed a binding of the enzyme to contaminating microsomes. However, this type of experiment only shows that hexokinase binds to a structure which is not disrupted by digitonin. Since it has been shown that digitonin does not remove the outer mitochondrial

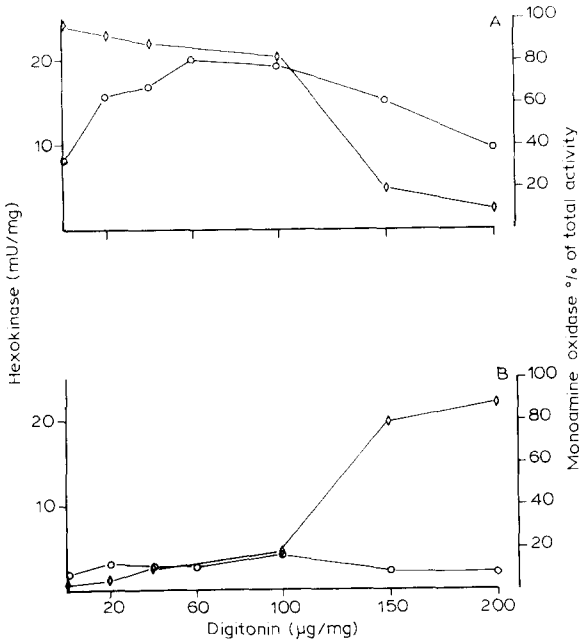

Fig. 5. Effect of digitonin on hexokinase bound to isolated mitochondria. The mitochondria were suspended in 0.25 M sucrose and 10 mM Hepes (pH 7.4) at a protein concentration of 30 mg/ml. Aliquots of 0.2 ml of this mitochondrial suspension were incubated for 30 s at room temperature with concentrations of digitonin ranging from 50 to 200 μg/mg of protein. The suspension was subsequently centrifuged for 1 min in a tabletop centrifuge. The supernate was removed and the sediment was resuspended in sucrose medium. (A) Activity in the sediment of monoamine oxidase (◇———◇) in % of total activity, and hexokinase (O———O) in mU/mg of protein. (B) Activity of the same enzymes in the supernate. Recovery: monoamine oxidase = 70–80%. Mean values of three experiments.

membrane completely [22,23], hexokinase may also bind to remnants of the outer membrane which remain attached in the contacts with the inner membrane. This has been suggested by Aubert-Foucher et al. [26] in heart mitochondria, and was recently confirmed by the observation of a high hexokinase binding capacity of the isolated contact site fraction from rat liver mitochondria [25]. In agreement with this interpretation, hexokinase could be desorbed from the mitochondrial surface when the contact sites were depressed by uncoupler DNP [25]. Additional binding of hexokinase to contaminating microsomes was not excluded by this experiment, since microsomal membranes can also be dissolved by digitonin. To exclude this possibility, the rebinding of hexokinase to the isolated mitochondrial fraction was compared to that of the microsomal fraction. Under the conditions used here, no binding of hexokinase to microsomes was observed (Table III), suggesting that a specific binding protein for the enzyme is required [27,28]. It has been shown that this binding protein (outer membrane pore protein) is missing in the microsomal fraction of the liver [29,30].

Physiological relevance and regulation of the structure-bound hexokinase activity in the liver

Felgner and Wilson [31] have described for isozyme I a repulsive interaction between negatively charged groups both on the enzyme and the mitochondrial outer membrane. We have shown in vitro that the binding of hexokinases, including isozyme II, in the liver is reduced by increasing the negative surface charge of this membrane [6,11]. This suggests that the charge of the mitochondrial surface might be involved in regulation of the binding. The surface of most intracellular membranes is negative due to intrinsic negative charges [32]. If counter-cations are lacking, as they are in sucrose isolation media, then the repelling effect of negative charges would increase and first desorb isozymes II and III. This explains why decreasing the space charge density by dilution, and reducing the ionic strength, causes liver mitochondria to lose almost all bound hexokinase activity during conventional isolation. By treating isolated hepatocytes with digitonin in Krebs-Henseleit medium substituted with 2% albumin, Ca^{2+} and Mg^{2+}, we avoided both strong dilution of intracellular structures and decrease of ionic strength, but allowed extraction of soluble proteins. Under these conditions, 60% of the total hexokinase activity remained structure bound, while 90% of the lactate dehydrogenase activity was extracted (Fig. 2B and C). According to kinetic and immunological characterization (Table I and Fig. 3) the soluble hexokinase represents glucokinase and less than 10% low K_m isozymes. In contrast, the structure-bound enzyme activity was exclusively of low K_m. A variation between 90 and 40 mU hexokinase per ml was determined in the sediment, depending on the metabolic state (Fig. 2C). Assuming a 10-fold dilution of the liver tissue (20–30 mg protein/ml of hepatocyte suspension

TABLE III

BINDING OF HEXOKINASE ISOZYME I TO ISOLATED MITOCHONDRIA AND MICROSOMES FROM RAT LIVER

The mitochondrial and microsomal fractions were prepared as described in materials and Methods and were characterized by the specific marker enzymes succinate dehydrogenase and NADPH-cytochrome *c* dehydrogenase, respectively. According to the marker enzymes the mitochondrial fraction was contaminated by 12% microsomal membranes, and the microsomal fraction by 1% of mitochondrial membranes. Mitochondria and microsomes (16 mg each) were incubated separately and as a 1:1 mixture in sucrose medium for 20 min at room temperature with increasing amounts of isolated hexokinase isozyme I in the presence of 5 mM glucose and 10 mM $MgCl_2$. The membranes were separated by centrifugation, the sediments were rinsed with sucrose medium, and then resuspended in the original volume. The activity of hexokinase in the sediment is based on the protein in the respective fraction and in the mixture of both membranes on the content of mitochondrial protein. Mean of two experiments. The differences between mitochondria and mitochondria + microsomes are not significant.

Addition of hexokinase I (mU)	Activity of hexokinase I bound to:		
	Mitochondria (mU/mg)	Microsomes (mU/mg)	Mitochondria + microsomes (mU/mg mitochondrial protein)
0	5.8	0.0	7.5
3.5	9.5	0.0	13.3
8.9	11.5	0.0	16.6
35.5	15.7	0.0	16.2
53.0	16.7	0.0	19.6

compared to 200–300 mg protein per gram fresh weight) this would result in 0.9–0.4 U of structure-bound hexokinase per gram of fresh weight. This value is comparable to the activity of glucokinase which, as calculated from the supernatant activity, varied between 2.8 and 0.7 U (Fig. 2B) in fed and starved rats, respectively.

The hexokinase activity in the hepatocyte sediment could be removed by applying high digitonin concentrations, without concomitant activity increase in the supernate (Fig. 2B and C). This points to a further dimension in the control of hexokinase activity by binding or debinding at the mitochondrial surface. It has already been shown in vitro that isozyme I and II become significantly activated by binding to the mitochondria [6]. In the present investigation (Fig. 2 and Table II) we show that the same mechanism functions inside intact cells regulated by different levels of metabolites: the binding, induced by glucose, of low K_m isozymes to the membrane resulted in a 88% increase in structure-bound hexokinase although no activity disappeared from the supernate. Furthermore, the activation by binding and inactivation by desorption could be demonstrated in vitro with isolated mitochondria and the purified isozyme I (Fig. 4).

The importance of mitochondrial hexokinase binding in metabolic regulation

It has been observed in liver [33] and brain [34] that the mitochondrial bound enzyme becomes functionally coupled to the inner mitochondrial compartment. As a consequence of this coupling [35,36], the activity of hexokinase is linked to the oxidation of pyruvate in the mitochondria and the activity of oxidative phosphorylation. By this way mitochondrial activity can regulate the glucose uptake, which in most tissues depends on the rate of glucose phosphorylation. Such type of regulation is realized in brain which generates its energy mainly from blood born glucose and therefore contains more than 80% of the hexokinase in the mitochondrial fraction. The soluble fraction of isozyme I in brain is regulated by the glucose-6-phosphate level which strongly inhibits the activity and at 5 times higher concentrations shifts the solubilization-rebinding equilibrium to the side of free enzyme [1]. However, insulin-sensitive tissues like muscle, and liver in contrast to brain metabolize fatty acids, and additionally synthesize glycogen. This implies that the activity of hexokinase should be regulated by the availability of glucose and independent of the energy demand of the tissue. If glucose is available, it is pertinent to

continue with the uptake by phosphorylation also at high glucose-6-phosphate concentration to activate glycogen synthesis; if carbohydrates are low glucose phosphorylation should become reduced in spite of low glucose-5-phosphate levels to suppress further uptake and oxidation of free glucose. Accordingly, isozyme II which is the predominant isozyme in liver, muscle, and adipose tissue is not desorbed by glucose 6-phosphate [6] but by free fatty acids [11]. Furthermore, in starved animals, the total cellular capacity for glucose phosphorylation is reduced because of the significant inactivation of hexokinase (mainly isozyme II) by desorption. In addition, the role of the low K_m isozymes in the liver cell might be a rapid regulation of hexokinase activity by hormones and metabolites via an effect on the association-dissociation equilibrium of the enzymes. The existence of such mechanism seems likely because the regulation of glucokinase is slow: the insulin-dependent induction of glucokinase takes hours [37]. The function of liver hexokinases during refeeding has come into new discussion, since in studies with perfused rat liver, glucose was poorly utilized and neither directly incorporated into glycogen nor into fatty acids (for a review, see Ref. 38). In the light of this so-called glucose paradox, the function of low K_m hexokinases in the liver may be to counteract glucose-6-phosphatase activity during α1 receptor-stimulated glycogenolysis. In agreement with this interpretation glucagon decreased the structure-bound hexokinase activity in hepatocytes [39] and incubation of the cells with glucose increased it (Table III). The interplay between low K_m hexokinases and glucose 6-phosphatase may be especially important in the periportal liver cells, which contain a larger mitochondrial fraction (i.e., more hexokinase binding sites) and two times more glucose-6-phosphatase activity, while glucokinase is mainly located in the perivenous cells [40].

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