Microheterogeneity of cytosolic and membrane-bound hexokinase II in Morris hepatoma 3924A

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Phosphorylation of glucose by hexokinase is the key step in glucose and energy metabolism of the cell. In the Morris hepatoma 3924A, hexokinase II is the predominant hexokinase isoenzyme and occurs in the cytosol as well as bound to membranes. Hexokinase II was isolated by DEAE-cellulose chromatography from both the cytosolic and the mitochondria-enriched fractions and further resolved by hydrophobic-interaction chromatography on phenyl-Sepharose into two components designated hexokinase IIa and IIb. In both the soluble and the mitochondria-enriched fractions, type IIb was the predominant form, but the IIb/IIa ratio was higher in the particulate (6–8) as compared with the cytosolic fraction (1.5–2.0). Binding of the isolated forms of the enzyme to rat liver mitochondria resulted in a 2–10-fold activation of both subtypes. Biochemical characterization showed that both subtypes are closely related to the isoenzyme commonly referred to as hexokinase II, and that the microheterogeneity was not a

consequence of contamination with hexokinase I or III. Both subtypes had a molecular mass of 110 kDa, they were inhibited by P_i at concentrations higher than 5 mM, and activated by the detergent CHAPS. The two subtypes differed in electrophoretic mobility (IIa > IIb), in K_m values for glucose (IIa, 0.109 mM; IIb, 0.216 mM), in K_i values for glucose 6-phosphate (IIa, 25 μ M; IIb, 0.106 mM), and in K_i values for glucose 1,6-bisphosphate (IIa, 12.2 μ M; IIb, 5.5 μ M). An artificial proteolytic cleavage as cause of the hexokinase II microheterogeneity can be excluded, since both subtypes show the same molecular mass and the ability to bind to mitochondria and phenyl-Sepharose. In addition, the relative proportions of the two subtypes did not vary markedly between several enzyme preparations. Northern-blot analysis with a hexokinase II-specific cDNA probe revealed two distinct mRNA transcripts of 5.2 and 6.3 kb in length, which offers the possibility that hexokinase II microheterogeneity is due to differential RNA transcription and/or processing.

INTRODUCTION

Hexokinase (HK), which catalyses the phosphorylation of glucose to glucose 6-phosphate (Glc6P) represents an important regulatory enzyme in glycolysis. High amounts of HK activity are usually found in tissues showing high rates of glucose utilization, including brain and tumour cells (Rijksen and Staal, 1985; Wilson, 1985). The reaction product, Glc6P, is also a substrate or modulator for many other anabolic and catabolic pathways. The HK reaction is thus involved in a wide variety of metabolic processes.

In mammalian tissues HK is present in four isoenzymic forms with distinct kinetic properties, designated as types I, II, III and IV according to their electrophoretic mobility (Wilson, 1985). In normal tissues the ratio of the four isoenzymes seems to be correlated with the relative rate of glucose consumption. Brain and erythrocytes, which depend strictly on blood-borne glucose, show high levels of HK I. In contrast, HK II is the predominant type in insulin-sensitive tissues, such as skeletal muscle and diaphragm. HK II is also the predominant isoenzyme in many tumours, including hepatocellular carcinomas (Hammond and Balinsky, 1978; Rijksen and Staal, 1985). In many organs HKs I and II are found intracellularly distributed between a cytosolic and a membrane-bound, most likely mitochondria-associated, form [for references, see Wilson (1985)]. The equilibrium between the soluble and bound HK has been regarded as a control mechanism for the intracellular glucose metabolism. Studies predominantly carried out on HK I revealed that the cytosolic

enzyme shows different kinetic properties as compared with the mitochondria-associated form (Knull et al., 1973; Wilson, 1985), and that the distribution of the cytosolic and soluble enzymes is influenced by the metabolic state of the tissue (Weber and Pette, 1990; Adams et al., 1991a).

The four isoenzymes are coded for by separate genes (Wigley and Nakashima, 1992). All isoenzymes, however, seem to exist in multiple molecular forms. Numerous reports provide evidence for a HK I microheterogeneity [Easterby, 1975; Ueda et al., 1975; Vowles and Easterby, 1979; Needels and Wilson, 1983; Magnani et al., 1988; for further references see Wilson (1985)]. Reports indicating multiple forms of the isoenzyme II are rare. A type II microheterogeneity was observed by Katzen et al. (1970) in rat fad pad, heart, diaphragm and small intestine, and by Balinsky and co-workers in human fetal tissues (Cayanis and Balinsky, 1975) and hepatomas (Hammond and Balinsky, 1978). The exact mechanisms underlying the microheterogeneity of HKs are not clear. In most cases the methods used for demonstrating subtypes were based on charge differences (electrophoresis, ion-exchange chromatography), but subtypes differing in molecular mass were also described (Easterby, 1975; Magnani et al., 1988). In addition, the existence of a tumour-specific HK is under discussion (Nakashima et al., 1988; Garkusha and Goncharova, 1990).

A variety of aberrations in carbohydrate metabolism, including changes in the activity of HK, have been observed in transplantable hepatomas (Weber, 1977) and in successive stages of hepatocarcinogenesis (Bannasch et al., 1991; Klimek and Ban-

Abbreviations used: anti-HK-IgGs, anti-hexokinase immunoglobulins; Glc6P, Glucose 6-phosphate; Glc(1,6)P₂, Glucose 1,6-bisphosphate; HK, hexokinase; MH, Morris hepatoma; MPMS, 1-methoxyphenazine methosulphate; NBT, Nitroblue Tetrazolium Chloride; PA, polyacrylamide; PMSF, phenylmethanesulphonyl fluoride; DTT, dithiothreitol; poly(A)⁺, polyadenylated.

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nasch, 1993). In order to gain further information on glucose utilization in hepatocellular tumours, we focused our investigations on HK, especially isoenzyme II isolated from the Morris hepatoma (MH) 3924A. In the present study we report on a HK II microheterogeneity in cytosolic and particulate fractions of this tumour, which is based on different hydrophobic properties rather than on charge differences. The two isolated subtypes show the same molecular mass, but differ in kinetic as well as electrophoretic properties. Part of this study has previously been published in abstract form (Rempel et al., 1993).

MATERIALS AND METHODS

Materials

CHAPS was from Boehringer Mannheim (Mannheim, Germany), DEAE-cellulose from Whatman Biosystems (Maidstone, Kent, U.K.), phenyl-Sepharose CL-4B and PhastSystem media from Pharmacia (Freiburg, Germany), phenylmethanesulphonyl fluoride (PMSF) from Sigma (München, Germany). Poly(A) Quick mRNA Purification Kit and Random Primer Kit were from Stratagene (Heidelberg, Germany), and Hybond-N nylon membranes and [α - 32 P]dCTP (sp. radioactivity 5000 Ci/mmol) from Amersham (Braunschweig, Germany). All other chemicals used were of analytical grade.

MH 3924A grew as a solid tumour in ACI-rats (Harlan Industries, Indianapolis, IN, U.S.A.) after subcutaneous transplantation. Rat brain HK I was isolated from Sprague-Dawley rats (Zentralinstitut für Versuchstierzucht, Hannover, Germany) as described by Chou and Wilson (1972). Antibodies against HK I were raised in chinchilla rabbits (Ivanovas, Kiblegg, Germany) and detected by the Ouchterlony assay (Friemel, 1980). Anti-HK immunoglobulins (anti-HK-IgGs) were isolated from the antiserum and from the serum of a non-immunized rabbit by chromatography on Protein A-Sepharose (Hjelm et al., 1972).

Isolation of HK isoenzymes from MH 3924A, skeletal muscle and liver

Cytosolic HKs were isolated from the 85000 g supernatants of the tissue homogenates [in 5 mM phosphate buffer (pH 7.6)/100 mM glucose/1 mM PMSF/1 mM dithiothreitol (DTT)/0.1 mM EGTA]. A mitochondrially-enriched fraction of MH 3924A was prepared as described by Chou and Wilson (1972) for brain mitochondria in 0.25 M sucrose/1 mM PMSF/1 mM DTT/10 mM glucose. Bound HK was solubilized by incubation at 25 °C for 1 h in the presence of 5 mM ATP, 1.5 mM Glc6P and 10 mM EDTA. After centrifugation (1 h at 85000 g) glucose and EGTA (100 mM and 0.1 mM final concns.) were added to the supernatant. The supernatants from cytosolic and particulate fractions were loaded on DEAE-cellulose columns equilibrated with phosphate buffer (see above), and HK was eluted by a linear gradient from 0–0.4 M KCl. The pooled active fractions of HK II from MH 3924A were applied to a phenyl-Sepharose column equilibrated with 50 mM phosphate buffer (pH 7.6)/100 mM glucose/1 mM PMSF/1 mM DTT/50 mM KCl. The column was resolved by a linear gradient from 0 to 2% (w/v) CHAPS in buffer (pH 8.0) without KCl.

The long-term influence of CHAPS on enzyme activity was studied in aliquots of HK I and II after DEAE-cellulose chromatography and of the separated subtypes IIa and IIb (after dialysis against 50 mM phosphate buffer (pH 8.0)/100 mM glucose/1 mM DTT), which were incubated with various amounts of CHAPS for 4–5 h at 4 °C.

Enzyme assays and protein determination

HK activity was measured spectrophotometrically at 334 nm and 30 °C, monitoring either the reduction of NADP⁺, as described by Bücher et al. (1964) or, if the influence of phosphate and Glc6P was to be tested, the NADH oxidation as described by Grossbard and Schimke (1966). The standard assay mixture contained 2 mM ATP, 8 mM MgCl₂ and 4 mM glucose. A unit of HK activity is defined as the amount of enzyme which catalyses the formation of 1 μ mol of Glc6P/min. Protein was determined by the Amido Schwarz assay (Heinzl, 1960), with BSA as standard.

Immunological methods

Immunoprecipitation studies

Samples of isolated HK dialysed against 5 mM phosphate (pH 7.6)/100 mM glucose/1 mM DTT/0.1 mM EGTA were added to PBS (36 mM Na₂HPO₄/11 mM KH₂PO₄/145 mM NaCl, pH 7.6) containing increasing amounts of anti-HK-IgGs or IgGs from a non-immunized rabbit in a final volume of 300 μ l. After incubation for 30 min at room temperature and 3.5 h at 4 °C, the supernatants (3000 g, 10 min) were assayed for HK activity.

Immunoinhibition studies

Aliquots of the isolated HKs were incubated with various amounts of anti-HK-IgGs or control IgGs in the enzyme assay mix for 10 min at room temperature. The HK reaction was then started by the addition of ATP.

Kinetic measurements

Enzyme solutions were dialysed against 0.21 M mannitol/0.07 M sucrose/5 mM Hepes, pH 7.4. The K_m value for glucose was determined as described by Eadie and Hofstee (Lehninger, 1977), the glucose concentration being varied between 0.005 and 6 mM. K_i values and K_{ci} for Glc6P versus glucose (0.07, 0.15 and 1.5 mM for HK IIa; 0.08, 0.21 and 2 mM for HK IIb) at 2 mM ATP and 8 mM MgCl₂, and K_i values for glucose 1,6-bisphosphate [Glc(1,6)P₂] versus ATP (0.5, 1, 3 and 5 mM) at 2 mM glucose were determined as described by Dixon (1953) and Cornish-Bowden (1973). MgCl₂ concentrations were kept 0.5 mM higher than the respective ATP concentrations. The computer program GraphPAD (ISI software) and the statistics package ADAM available from the central computer of the Deutsches Krebsforschungszentrum were used for regression analysis, determination of the intercepts and slopes, and calculation of S.D. values.

Electrophoretic procedures

Non-denaturing electrophoresis on 12.5% homogeneous polyacrylamide gels was performed in a PhastSystem apparatus (Pharmacia, 1986). HK activity in the gels was demonstrated by a Nitroblue Tetrazolium Chloride (NBT)-staining technique. The polyacrylamide (PA) gels were placed on the surface of a gel containing 16% gelatin, 0.46 M Mops (pH 7.3), 0.76 mM NADP⁺, 8 mM MgCl₂, 5 mM ATP, 4 mM glucose, 12 mM NBT, 0.8 mM 1-methoxyphenazine methosulphate (MPMS), and which was covered with a thin layer of Glc6P dehydrogenase solution (50 units/ml, 0.02% BSA and 0.1 M Mops, pH 7.3). The gels were incubated for at least 3 h at room temperature and then destained with 5% acetic acid.

Binding of HK to mitochondria

Rat liver mitochondria were isolated by differential centrifugation at 650 *g* and 5800 *g* and repeated washing, and resuspended in H-medium (0.21 M mannitol/0.07 M sucrose/5 mM Hepes, pH 7.4) to a final concentration of 10–15 mg of protein/ml. Aliquots (30–40 μ l) of the enzyme solutions dialysed against a medium containing 10 mM glucose, 1 mM EDTA and 1 mM PMSF were incubated for 45 min at 4 °C with 1–2 mg of freshly isolated liver mitochondria in the presence of 10 mM MgCl₂. Mitochondria were then separated by centrifugation, and the pellet was resuspended in the originally used volume of the same medium containing 10 mM MgCl₂. Pellet and supernatant were assayed for HK activity. The activity found in the mitochondrial pellet was corrected for the endogenously bound HK activity, and the activity obtained in the supernatant for the activity found in corresponding control assays which contained buffer instead of the enzyme solution.

Northern-blot analysis

Total RNA was isolated according to the method described by Chomczynski and Sacchi (1987). Polyadenylated [Poly(A)⁺] RNA was separated on oligo(dT)-cellulose using the Poly(A) Quick mRNA Purification Kit from Stratagene; 5–10 μ g of the poly(A)⁺ RNA were submitted to electrophoresis on 1.2% denaturing agarose gels in the presence of 2.2 M formaldehyde (Lehrach et al., 1977) and transferred to a nylon membrane (Hybond-N, Amersham). The blots were u.v.-cross-linked for 3 min, baked in vacuum at 80 °C (2 h) and hybridized at 45 °C in the presence of 40% formamide under standard conditions (Sambrook et al., 1989). An endonuclease-*Sph*I/*Kpn*I 1.37 kbp fragment of the full-length rat skeletal-muscle HK II cDNA (Thelen and Wilson, 1991), labelled by random priming, was used as probe. The probe corresponds to the C-terminal part of the cDNA, starting with nt 1706. After hybridization the filters were washed at high stringency and exposed to Kodak X-AR films.

RESULTS

Isolation and characterization of two subtypes IIa and IIb from the cytosolic fraction of MH 3924A

HK II was isolated from the cytosolic fraction of the MH 3924A by DEAE-cellulose chromatography (Figure 1a). A further purification step of the isoenzyme II on phenyl-Sepharose columns permitted resolution of two glucose-phosphorylating activities designated as HK type IIa and IIb (Figure 1b). The result shown in Figure 1(b) was reproducibly observed in four different preparations, whereby the proportion of IIa to IIb, based on their total activity, was in relative good agreement, ranging between 1:1.5 and 1:2. HK II solubilized from mitochondria was resolved in a similar way (Figure 1c).

Electrophoretic properties

In SDS-containing gels the two subtypes showed the same migration properties. This indicates little if any difference in molecular mass. A value of 110 kDa was estimated for both subtypes. In contrast, electrophoresis performed under non-denaturing conditions revealed slight differences between the two subtypes (Figure 2). Both subtypes had a higher mobility than rat brain HK I and migrated to approximately the same distance as rat skeletal-muscle HK II. The subtype IIa displayed a somewhat higher mobility towards the anode as compared with

the subtype IIb. Although rather small, these differences in electrophoretic mobility were consistently obtained with several enzyme preparations, and hence can be considered as true small differences.

Immunological properties

Immunoinhibition and immunoprecipitation studies of HK I, IIa and IIb with anti-HK I-IgG demonstrated only a reaction of HK I with the antibody. The two subtypes IIa and IIb did not cross-react.

Figure 1 Separation of HK II into two subtypes IIa and IIb

(a) Anion-exchange chromatography of the cytosolic HK isolated from the MH 3924A. Extracts prepared from the tumour tissue were loaded on to DEAE-cellulose columns. The columns were developed with a linear gradient (---) from 0 to 0.4 M KCl. Fractions (1 ml) were collected and assayed for HK activity at 0.5 mM (Δ) and 100 mM ($\circ$) glucose. (b) Hydrophobic-interaction chromatography of isolated HK II. HK II was applied to a phenyl-Sepharose column (1.6 cm $\times$ 10 cm) and eluted with a linear gradient of CHAPS (2 $\times$ 50 ml) up to 2%. (c) Resolution of membrane-bound HK II into HK IIa and IIb. Enzymes were detached from mitochondria with 1.5 mM Glc6P/5 mM ATP/10 mM EDTA. After separation of HK I and II on DEAE-cellulose, resolution of subtypes IIa and IIb was performed by hydrophobic-interaction chromatography on phenyl-Sepharose columns.

Figure 2 Electrophoretic separation of HK isoenzymes

Native electrophoresis was carried out on 12.5% homogeneous polyacrylamide gels, followed by activity staining of HK. Lane a, rat skeletal-muscle HK II; lane b, subtype IIb; lane c, subtype IIa isolated from MH 3924A; lane d, rat brain HK I.

Table 1 Kinetic parameters of HK IIa and IIb

K_m values were determined as described by Eadie and Hofstee (Lehninger, 1977), and K_i and K_{ci} values were determined as described by Dixon (1953) and Cornish-Bowden (1973). For regression analysis and calculation of slopes and intercepts, a computer program was used (see the Materials and methods section). Results are means $\pm$ S.D. from at least three preparations with multiple determinations each. Inhibition by Glc6P versus glucose was of mixed type for IIa and of the non-competitive type for IIb. Inhibition by Glc(1,6) P_2 versus ATP was of the competitive type for IIa and IIb.

HK	$K_{m, \text{glucose}}$ (mM)	$K_{i, \text{Glc6P}}$ (mM)	$K_{ci, \text{Glc6P}}$ (mM)	$K_{i, \text{Glc(1,6)P}_2}$ (μ M)
IIa	0.109 ± 0.009	0.025 ± 0.001	0.055 ± 0.0005	12.2 ± 1.4
IIb	0.216 ± 0.029	0.106 ± 0.008	0.112 ± 0.014	5.5 ± 0.5

Kinetic properties

HK subtype IIa showed a two-fold lower Michaelis constant for glucose compared with subtype IIb (0.109 mM compared with 0.216 mM; Table 1). Neither IIa nor IIb were inhibited by their substrate glucose. This indicates that the resolution of HK II is not due to a contamination with HK III, which is inhibited by glucose concentrations above approx. 0.3 mM (Wilson, 1985).

The subtypes differed also in their susceptibility to product inhibition. The K_i (dissociation constant of the enzyme-inhibitor complex) and K_{ci} values (dissociation constant of the enzyme-substrate-inhibitor complex) for Glc6P versus glucose are summarized in Table 1. The K_i value (0.106 mM Glc6P) and K_{ci} value (0.112 mM Glc6P) of IIb exceeded the corresponding values for the type IIa (K_i , 0.025 mM; K_{ci} , 0.055 mM) by a factor of 4 and 2 respectively. The data obtained from the Dixon and Cornish-Bowden plots indicated that inhibition kinetics relative to glucose is of mixed type for IIa, and non-competitive for type IIb. Inhibition studies with Glc(1,6) P_2 resulted in a K_i of 12.2 μ M for IIa, and a K_i of 5.5 μ M for IIb (Table 1). Glc(1,6) P_2 was competitive with ATP. The K_m value for ATP measured at 2 mM glucose was $\sim$ 0.6 mM for both subtypes.

P_i did not show any effect on both subtypes up to concentrations of 5 mM. At higher concentrations (10–50 mM) a marked, maximally 40% inhibition was observed. Similar results were obtained regarding the effect of P_i in the presence of Glc6P (0.05 mM Glc6P for IIa; 0.1 mM for IIb). P_i concentrations

Figure 3 Binding of HK IIa and IIb to mitochondria

Aliquots of the enzymes were incubated with freshly isolated rat liver mitochondria (for details, see the Materials and methods section). The activation of HK upon binding to mitochondria was calculated from the activity measured in the pellet (P) and from the activity which was removed from the supernatant (S).

higher than 5 mM inhibited both enzymes also under these conditions. A relieving effect of P_i on Glc6P inhibition was not seen. Since the two subtypes do not differ markedly in their response to P_i , either in presence or in absence of Glc6P at physiologically reasonable levels, it may be concluded that intracellular concentrations of P_i do not play a role in a potentially different regulation of the two subtypes. CHAPS showed a slight stimulatory effect on HK I and II obtained after DEAE-cellulose chromatography. A more detailed study revealed that CHAPS activated both HK II subtypes to a similar extent. At a concentration of 1% CHAPS a maximal stimulatory effect of 1.6–1.8 over the basal value was reached. This agrees with findings by Teichgräber et al. (1973), who observed an activation of soluble HK with Triton X-100. The activation is probably due to conformational changes in the enzyme, but a separation of bound lipid or polysaccharide complexes must also be considered.

Binding of the HK II subtypes to mitochondria

The binding of HK IIa and IIb to mitochondria and their activation upon binding were studied by incubation of the enzymes with freshly prepared rat liver mitochondria (Figure 3). Bound activity was calculated either from the activity measured in the pellet or from the activity which was removed from the supernatant by binding to mitochondria (difference between added activity and activity remaining in the supernatant after separation of mitochondria). For both subtypes the activity measured in the pellet was always markedly higher as compared with the activity removed from the corresponding supernatant. The large difference between the low amounts of enzyme activity removed from the supernatant by binding and the rather high activity found in the mitochondrial fraction indicates a marked activation of the enzymes upon binding. For both subtypes the activation factor varied between 2 and 10 in three or four studies.

Isoenzyme pattern of membrane-bound HK

A mitochondria-enriched fraction was isolated from MH 3924A and HK was solubilized. The recovery in the solute was 70%. After separation on DEAE-cellulose, about 50% of this total activity corresponded to HK II. By hydrophobic-interaction chromatography the HK II could be resolved into the subtypes IIa and IIb (Figure 1c). Similar to the findings on the cytosolic enzyme, the subtype IIb was the major enzyme. However, the

Figure 4 Analysis of HK II expression in MH 3924A

mRNA (5 μ g/lane) isolated from MH 3924A was hybridized with a 1.37 kbp *SphI/Kpn* fragment of a full-length rat skeletal-muscle cDNA as probe. The positions of 28 S and 18 S ribosomal RNA are indicated.

preponderance of this subtype was much more pronounced in the membrane fraction. The proportion of IIa:IIb in the cytosol was 1:1.5–1:2 compared with 1:6–1:8 in the membrane fraction.

Northern-blot analysis

In order to examine whether the two subtypes may be encoded by different mRNAs, a Northern-blot-hybridization analysis was performed using poly(A)⁺ RNA isolated from MH 3924A. The probe used showed only a very weak cross-reaction with mRNA of HK I from brain and MH 3924A, but gave a strong signal with mRNA from rat skeletal-muscle HK II (result not shown). Because of the different sizes of the two HK mRNAs, 4.3 kb for HK I (Paggi et al., 1991; Griffin et al. 1992) and 5.2 kb for HK II (Thelen and Wilson, 1991) this cross-hybridization did not affect HK II expression studies. As shown in Figure 4, two distinct mRNA-species of 6.3 and 5.2 kb length as calculated from the relative positions of the 18 S and 28 S rRNA were detected with this probe. The size of the smaller transcript is consistent with results obtained for HK II from rat skeletal muscle and Novikoff ascites tumour (Thelen and Wilson, 1991). However, the 6.3 kb transcript was by far the more abundant one.

DISCUSSION

HK isoenzymes from different organs have been reported to exist in multiple molecular forms differing either in molecular charge (Cayanis and Balinsky, 1975; Easterby, 1975; Ueda et al., 1975; Hammond and Balinsky, 1978; Vowles and Easterby, 1979; Magnani et al., 1982; Needels and Wilson, 1983) or, more rarely, in molecular mass (Magnani et al., 1988). In some cases microheterogeneity has been considered to be due to a dimerization or polymerization process (Easterby, 1975; Vowles and Easterby, 1979; Ureta et al., 1983).

The present data provide evidence for a microheterogeneity based on hydrophobic properties of HK II isoenzyme from MH 3924A. The chromatographic (Figure 1), electrophoretic (Figure 2) and immunological behaviour of the two subtypes indicate a

close relationship with rat skeletal-muscle HK II. Furthermore, the kinetic parameters determined agree well with values reported for the isoenzyme II from rat skeletal muscle and liver (Gonzalez et al., 1964; Grossbard and Schimke, 1966; Holroyde and Trayer, 1976; Easterby and Qadri, 1981, 1982). While Glc6P inhibition is competitive with ATP (Easterby and O'Brien, 1973), the inhibition of homogeneous HKs I and II from various organs by Glc6P versus glucose has been shown to be of the non-competitive type (Purich et al., 1973; Lueck and Fromm, 1974; Easterby and Qadri, 1982; Garkusha and Goncharova, 1990). This agrees with our findings on HK IIb. However, for HK IIa the data obtained rather point to a mixed-type inhibition. Interestingly, this type of inhibition versus glucose was published previously by Garkusha and Goncharova (1990) for their rat sarcoma M1 HK. HKs I, II and III are inhibited by Glc(1,6)P₂, inhibition is competitive with ATP. The sensitivity shows a large variation, K_i values reaching from 3 μ M for homogeneous rat skeletal-muscle HK II (Easterby and Qadri, 1982) and 7–20 μ M for HK II from an ascites (Rose and Warms, 1975), over 50 μ M for HK III (Rose et al., 1974) to 90–125 μ M for HK I (Rose et al., 1974). Thus Glc(1,6)P₂ is a good discriminator for HKs. Our data obtained for IIa and IIb subtypes agree best with those published for HK II.

The identical molecular mass, together with the fact that the relative proportions of the two subtypes did not vary markedly between several preparations, strongly argue against an artificial proteolytic cleavage as a reason for obtaining two subtypes. Moreover, a cleavage of the hydrophobic N-terminus, which is highly susceptible to proteolysis (Polakis and Wilson, 1985), would result in a complete loss of binding ability to the hydrophobic phenyl-Sepharose and mitochondria (Kurokawa et al., 1983; Polakis and Wilson, 1985). Katzen et al. (1970) observed a microheterogeneity of HK II from rat fat pad, heart, diaphragm and small intestine only in the absence of mercaptoethanol. In our study a similar mechanism involving thiol groups can be excluded, since all buffers contained DTT. A separation in the presence of thiols of HK II from human fetal tissues and tumours into two electrophoretically distinguishable forms was also described by Balinsky and co-workers (Cayanis and Balinsky, 1975; Hammond and Balinsky, 1978). However, their K_m values for glucose showed marked differences (0.07 mM and 0.14 mM) compared with those found in the present study.

The small differences in charge and hydrophobicity of HKs IIa and IIb may account for the different proportions of IIa and IIb in cytosolic and particulate fractions of MH 3924A. Both forces are considered to be involved in mitochondrial binding (Polakis and Wilson, 1985; Wilson, 1985), whereby a reduction of net negative charge and enhancement of hydrophobicity is thought to increase binding. The greater hydrophobicity and lower negative charge of the type IIb in comparison with IIa may therefore explain the pronounced preponderance of this subtype in the particulate fraction.

The molecular nature leading to the observed HK II microheterogeneity has still to be elucidated. The difference in hydrophobicity indicates structural differences at the N-terminal region, which has been shown to include the only hydrophobic peptide sequence, at least in brain HK (Xie and Wilson, 1988). A phosphorylation or deamidation of amino acid residues localized in this region would explain both the different behaviour in hydrophobic-interaction chromatography and the charge differences. *In vitro*, phosphorylation of glucokinase (Ekman and Nilsson, 1988), rat brain HK (Adams et al., 1991b) and tumour HK isolated from the c37 mouse hepatoma cell line (Arora and Pedersen, 1993), including autophosphorylation of the last two enzymes, has been described recently. It is of interest that

putative tyrosine phosphorylation motifs within tumour HK are not only localized close to regions which are considered to be important either for catalytic activity and for the enzyme structure, but also found in the N-terminal region, which is assumed to be involved in binding to membranes. Thus, if a phosphorylation mechanism accounts for the two different subtypes, it would also be of significance under *in vivo* conditions. A post-translational mechanism has also been proposed for the HK I microheterogeneity observed in human placenta (Magnani et al., 1991). It is worth mentioning that phosphofructokinase and pyruvate kinase are subject to phosphorylation (Hers & Hue 1983). Thus phosphorylation might be a common regulatory mechanism for glycolytic enzymes.

A 5.2 kbp full-length cDNA for HK II from rat skeletal muscle has recently been sequenced (Thelen and Wilson, 1991). The two distinct HK II transcripts observed in MH 3924A raise the possibility that two different mRNA species exist encoding the biochemically detected subtypes of HK II. Two mRNA-species were also detected by a HK II-specific probe in AH 130 hepatoma cells, rat skeletal muscle and rat heart (Shinohara et al., 1991) and very recently by Printz et al. (1993) in normal rat adipose tissue. Printz et al. (1993) concluded from their detailed analysis of the HK II gene, that the two transcripts are synthesized from one primary transcription initiation site in combination with two alternative termination sites. Therefore both mRNA species include the same open reading frame for HK II. If this holds true also for the two transcripts observed in the present study, this would suggest that the HK II microheterogeneity is most likely due to post-translational modification. Further, it cannot be ruled out that the larger of the two transcripts represents only a pre-mRNA for HK II.

The physiological significance of the two HK II subtypes is not yet clear. HK II microheterogeneity was also found in normal rat liver with the same methodological approach as described for MH 3924A. Hence it may be a common phenomenon and not restricted to tumour HK. The differences in kinetic parameters alone are presumably not large enough to be of significant effect for metabolic regulation. However, the distribution between the cytosolic and mitochondrial compartments is considered to be an important factor in the regulation of HK activity (Wilson, 1985). Therefore the different hydrophobic properties of HK IIa and IIb as well as the different proportions found in the mitochondria-enriched and the cytosolic fractions suggest that the differences in binding properties may play a crucial role in the fine regulation of glucose metabolism.

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