Hexokinase Isozyme Distribution in Human Skeletal Muscle

Vladimir B. Ritov and David E. Kelley

Two isoforms of hexokinase (type I and type II) are expressed in skeletal muscle; however, the intracellular distribution of these hexokinase isoforms in human skeletal muscle is unclear. The current study was undertaken to assess this issue because binding of hexokinase to subcellular structures is considered to be an important mechanism in the regulation of glucose phosphorylation. Vastus lateralis muscle was obtained from healthy lean individuals. Muscle homogenate was separated at 45,000 g into particulate and cytosolic fractions. The activity and subcellular distribution of hexokinase isozymes in human skeletal muscle was determined using ion-exchange chromatography and a highly sensitive high-performance liquid chromatography–based hexokinase assay. This criterion method was used to validate a modified thermal inactivation method for distinguishing type I and type II isoforms. Mean hexokinase activity was 3.88 ± 0.65 U/g wet wt or 0.64 ± 0.11 U/mU creatine kinase (CrK) in the particulate fraction and 0.45 ± 0.22 U/g wet wt or 0.07 ± 0.03 U/mU CrK in the cytosolic fraction. Hexokinase I and II accounted for 70–75 and 25–30% of total hexokinase activity, respectively. Nearly all (95%) of hexokinase I activity (0.52 ± 0.09 U/mU CrK) was found in the particulate fraction, consistent with the known high affinity of hexokinase I for mitochondria. Hexokinase II activity was also largely bound to the particulate fraction (72%), but 28% was found within the cytosolic fraction. Thus, within the particulate fraction, the relative contributions of hexokinase I and hexokinase II were 81 and 19%, whereas within the cytosolic fraction, the relative contributions for hexokinase I and hexokinase II were 37 and 63%.

Hexokinase plays an essential role in glucose utilization by cells. Glucose 6-phosphate, the product of the hexokinase reaction, is a key intermediate of glucose conversion in metabolic pathways, including ATP and NADPH regeneration and glycogen synthesis. Hexokinase might also be considered part of the glucose transport system because it maintains a high transmembrane gradient for free glucose. Mammalian skeletal muscles contain two hexokinase isozymes: hexokinase type I and type II (1–3). For rat skeletal muscle, the predominant type is hexokinase II (1,4,5). The content of the hexokinase II isozyme in skeletal muscle can vary significantly depending on physical activity and hormonal status. For example, electrical stimulation of rat skeletal muscle increases hexokinase II activity >10-fold (6). Exercise, cold stress, and catecholamines also increase hexokinase activity and hexokinase II mRNA level in skeletal muscle and adipose tissue (7–10). The content of hexokinase II mRNA in rat skeletal muscle also increases after infusion of insulin (4,11,12). In contrast to these patterns with hexokinase II, the activity and expression of the hexokinase I isozyme are relatively insensitive to hormonal influence (13).

The hormonal regulation of hexokinase II expression suggests a specific role in glucose metabolism. Binding of hexokinase isozymes to mitochondria and other subcellular structures has been considered as a potential mechanism for the regulation of enzyme activity and is therefore potentially important for the regulation of glucose metabolism (3,14–18). Most studies on hexokinase in skeletal muscle have been performed using rodent models. Studies of hexokinase isozymes in human skeletal muscle are limited. The results that are available suggest that insulin can regulate hexokinase II expression (19,20). A recent study addressed hexokinase isozyme cellular distribution and found a high level of hexokinase II binding to mitochondria (21). However, these results are controversial (21,22). The estimation of activity of hexokinase isozymes in human skeletal muscle was questioned by Wilson (23). His studies indicated that methods previously used to estimate the content of hexokinase I and II isozymes in muscle (21,24), namely thermal inactivation and electrophoresis, may not be reliable (23) for distinguishing hexokinase I and II isozymes, especially in relation to mitochondrial binding. Therefore, the goal of the current study was to examine the cellular distribution and activities of hexokinase I and II in human skeletal muscle in healthy volunteers. To accomplish this aim, we explored the application of ion-exchange chromatography, thermal
inactivation, and a highly sensitive high-performance liquid chromatography (HPLC)-based hexokinase assay for measuring the activity and distribution of hexokinase isozymes among subcellular fractions in human skeletal muscle.

RESEARCH DESIGN AND METHODS

Materials. Glycerol, diethanol (DTT), and P1, P-Di(O-adenosine-5')-pentaphosphate were purchased from Boehringer Mannheim (Indianapolis, IN). Diethylamino ethyl-agarose was a product of Bio-Rad Laboratories (Hercules, CA). Glucose-6-phosphate dehydrogenase (Glc-6-P DH), creatine kinase (CrK), and other reagents were obtained from Sigma (St. Louis, MO).

Sampling. Volunteers (11 healthy lean individuals: 5 men and 6 women; BMI 23.5 ± 0.5 kg/m²) were recruited and medically screened. Written consent was obtained.

Tissue material from the vastus lateralis portion of quadriceps femoris muscle was obtained after an overnight fast using the percutaneous biopsy technique of Bergstrom (25). Samples were frozen in liquid nitrogen and kept at −75°C before analysis.

Preparation of homogenate and separation of soluble and particulate hexokinase.

Preparation of homogenate. The homogenization medium contained 100 mmol/l mannitol, 80 mmol/l potassium gluconate, 20 mmol/l KF, 10 mmol/l glucose, 1 mmol/l MgCl₂, 0.1 mmol/l deferoxamine, 0.1 mmol/l EGTA, 0.1 mmol/l MgCl₂, 0.2 mmol/l Triton X-100, and 4 mmol/l DTT to a final pH of 7.1. Columns were washed by 5–7 ml of buffer and then eluted with 25 ml of a linear gradient of KCl from 20 to 500 mmol/l (buffer A) or from 0 to 500 mmol/l (buffer B) at a flow rate of 0.25–0.5 ml/min. The gradient was formed using a fast protein, peptide, and polynucleotide liquid chromatography (FPLC) system equipped with two P-50 pumps, a LCC-500 controller, and a FRAC-100 fraction collector (Pharmacia). Fractions (0.5 ml) were collected starting from the time the sample was applied.

Hexokinase. Activity of hexokinase was determined by HPLC detection of NADPH generated in a coupled enzymatic reaction with Glc-6-P DH. A fluorescence detector at an excitation of 340 nm and an emission of 460 nm monitored fluorescence of NADPH in eluates. The reaction medium contained 100 mmol/l potassium gluconate, 50 mmol/l mannitol, 10 mmol/l glucose, 5 mmol/l ATP, 10 mmol/l phosphocreatine, 10 mmol/l MgCl₂, 0.2 mmol/l EGTA, 0.1 mmol/l deferoxamine, 10 mmol/l NaCl, 9-[3-(cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid (CHAPS), 0.5 mg/ml BSA, 20 mmol/l KCl, 3 mmol/l KF, 0.1 U/ml CrK, and 10 mmol/l TEA-MOPS buffer (pH 7.6 at 21°C).

Selective thermoinactivation of hexokinase isozymes. To an aliquot of the soluble fraction or extract from the sonicated particulate fraction (10 μl) were combined with 190 μl of the dilute mixture (100 mmol/l potassium gluconate, 50 mmol/l mannitol, 0.2 mmol/l EGTA, 0.1 mmol/l deferoxamine, and 10 mmol/l TEA-MOPS, pH 7.6). For analysis of chromatographic fractions, aliquots of chromatographic fractions (10 μl) were combined with 40 μl of the dilute mixture. Diluted samples were kept on ice before assay. The reaction was started by adding 50 μl diluted sample to 50 μl reaction mixture and was incubated at 30°C for 40 min. For a blank test, the diluted samples were heated at 65°C for 5 min before mixing with reagents. The reaction was terminated by the addition of 120 μl of 10 mmol/l hydrazine plus 30 mmol/l 2-amino-2-methyl-1-propanol in methanol. Samples were kept at −80°C before HPLC analysis. Freezing the samples facilitated aggregation of denatured protein. Thawed samples were centrifuged at 45,000g for 5 min in a Heraeus Stratos Biofuge. Aliquots of supernatant (10 μl) were injected onto a C-18 reverse-phase column (Hewlett Packard Hypersil, 5 μm, 100 × 4.6 mm). The column was eluted by a mobile phase composed of 0.2 mol/l KH₂PO₄ (pH 6.0), 2 mmol/l tetrabutylammonium-ÖH as an ion-paring reagent, and 14% methanol. The flow rate was 1 ml/min. A Shimadzu LC-10AT VP HPLC system equipped with a Shim-pack C-18 octyl column (model RF-10Axd) and an autosampler (model SIL-10AD vp) were used. In experiments on hexokinase latency, a Waters HPLC system (Shimadzu, Kyoto, Japan) equipped with an ultraviolet detector was used (Fig. 1). Chromatograms were processed and stored in digital form with Class-VP software. A calibration curve was obtained by plotting the peak areas against amounts of NADPH (Fig. 2B).

Selective thermoinactivation of hexokinase isozymes. The aliquots of samples (40 μl), prepared by gel filtration on a Sephadex G-25 were combined with 360 μl thermoinactivation mixture (100 mmol/l potassium gluconate, 50 mmol/l mannitol, 0.2 mmol/l EGTA, 0.1 mmol/l deferoxamine, 1 mmol/l glucose, 5% glycerol, 5 mmol/l CHAPS, 1 mmol/l DTT, 2.5 mmol/l KF, and 10 mmol/l TEA-MOPS, pH 7.4, at 21°C) and kept on ice before assay. For analysis of chromatographic fractions, aliquots of chromatographic fractions (40 μl) were combined with 160 μl of the thermoinactivation mixture. Three aliquots of samples (50 μl each) were kept on ice for 30 min; another three were incubated in a 45°C water bath for the same time. To assay hexokinase activity, as described above, the reaction was started by mixing 50 μl of the incubated sample with 50 μl of concentrated reaction mixture.

Selective thermoinactivation of CrK was measured at 30°C by spectrophotometric monitoring of the generation of NADPH at 340 nm in a coupled enzymatic reaction (hexokinase/Glc-6-P DH) (28). The reaction was initiated with the addition of 25 mmol/l phosphocreatine to 2.5 ml of reaction mixture composed of 100 mmol/l potassium gluconate, 10 mmol/l Mg-aceate, 1 mmol/l ADP, 0.05 mmol/l P₁, P-Di(O-adenosine-5')-pentaphosphate, 0.1 mmol/l deferoxamine, 0.2 mmol/l EGTA, 2 mmol/l DTT, 0.2 mg/ml BSA, 2.5 mmol/l glucose, 1 mmol/l NADPH, 3 mmol/l Tris-HCl (pH 7.4), 0.1 mmol/l deferoxamine, and 50 mmol/l imidazole buffer (pH 6.8) after a 2-min preincubation with the sample (2 μl of the soluble fraction or 10 μl of the extract from the particulate fraction).

Phosphoglucone isomerase. Activity of phosphoglucone isomerase (PGI) was measured at 30°C with fructose-6-phosphate as the substrate (reverse
reaction) (29). The generation of glucose-6-phosphate was measured by spectrophotometric monitoring of the reduction of NADP at 340 nm in the presence of Glc-6-P DH. The reaction was initiated with the addition of 5 mmol/l fructose-6-phosphate to 2.5 ml reaction mixture, the latter composed of 100 mmol/l potassium gluconate, 50 mmol/l mannitol, 0.2 mmol/l EGTA, 0.1 mmol/l deferoxamine, 0.5 mg/ml BSA, 1 mmol/l NADP, 1.0 U/ml Glc-6-P DH, and 10 mmol/l TEA-MOPSO (pH 8.0 at 21°C) after a 1-min preincubation with the sample (50 µl of the soluble fraction or the extract from the particulate fraction).

Statistics. All enzyme activities were run in triplicate. The mean and SD are presented in the tables and figures. For all 11 biopsies, specific hexokinase activity was measured twice, before and after gel filtration, in the thermal inactivation assay. These data are shown in Table 1, Table 2, and Fig. 3. The estimated hexokinase activities from both measurements were very close (±5%). The chromatograms were used to calculate total hexokinase activity and the activities of hexokinase isozymes (Table 2) and were based on data from three biopsies.

RESULTS

Assay development

HPLC measurement of NADPH: improving detection sensitivity for hexokinase assay. The chromatogram of a typical HPLC separation of the hexokinase reaction mixture on a reverse-phase column is presented in Fig. 2A, trace 1. The observable generation of NADPH in the reaction mixture takes place even in the absence any external source of hexokinase (Fig. 2A, trace 2). This generation could be explained by minute hexokinase contamination in the auxiliary enzyme Glc-6-P DH or by glucose oxidizing capacity of Glc-6-P DH itself because background levels of NADPH increase with an increasing concentration of Glc-6-P DH and glucose in the assay. The main problem in HPLC analysis of reduced pyridine nucleotides is instability of NADPH in small volumes of diluted solutions (30). To neutralize the catalytic activity of adventitious iron, and probably other transient metals, in the oxidation of reduced pyridine nucleotides, 100 µmol/l

![Graph](image-url)

**TABLE 1**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>U/mU CrK</th>
<th>U/g wet wt*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particulate</td>
<td>0.64 ± 0.11</td>
<td>3.88 ± 0.65</td>
</tr>
<tr>
<td>Soluble</td>
<td>0.07 ± 0.03</td>
<td>0.45 ± 0.22</td>
</tr>
<tr>
<td>Soluble + particulate</td>
<td>0.72 ± 0.12</td>
<td>4.33 ± 0.66</td>
</tr>
</tbody>
</table>

Data are means ± SD. *Normalized on the base of total CrK activity.

![Graph](image-url)

**FIG. 1.** Latency of hexokinase activity in homogenate and particulate fraction of human skeletal muscle. The samples were frozen-thawed three times with liquid nitrogen/30°C water bath in the presence or absence of 0.6 mol/l KCl. The hexokinase activity was measured using a Waters HPLC system equipped with an ultraviolet detector as described in RESEARCH DESIGN AND METHODS. The hexokinase activity of samples after KCl treatment is expressed as 100%.

![Graph](image-url)

**FIG. 2.** HPLC analysis of NADPH in hexokinase reaction mixture. A: A 10-µl aliquot of incubated and then quenched reaction mixture was injected into the column. The reaction mixture was incubated for 40 min as a blank (trace 2) or in the presence of an aliquot of the particulate fraction (trace 1). B: The calibration curve was obtained by plotting the peak areas against the amount of injected NADPH.
Deferoxamine mesylate was introduced to the reaction mixture (31). We use a high concentration of methanol (60%, vol/vol) to denature enzymes and stop the reaction. Then, 10 mmol/l hydrazine was added to the methanol to destroy NADP after termination of the reaction (30), and 30 mmol/l 2-amino-2-methyl-1-propanol was added to protect generated NADPH from destruction by hydrazine and to provide alkaline conditions that stabilize NADPH (30). During an HPLC analysis of 70 samples taking 8 h, the concentration of NADPH in samples was stable despite the ambient temperature (21–25°C) of the sample tray. Also, we did not find a significant difference in peak areas for NADPH standards separated by HPLC at pH 6.0 or pH 7.5 in the mobile phase. We used a mobile phase adjusted to pH 6.0 in most experiments because separation at pH 6.0 provides better peak symmetry. Introduction to the mobile phase of 2 mmol/l ascorbate plus 0.1 mmol/l deferoxamine mesylate to prevent possible oxidation of reduced pyridine nucleotides during separation did not affect peak area of NADPH.

Proportionality of hexokinase reaction with time and amount of sample. It was found that except for an initial short lag period (~90 s), the hexokinase reaction is absolutely linear for at least 50 min of incubation. To reduce the possible error due to an initial lag period, a time count for a 40-min reaction was started after a 30-s warm-up of the reaction tube in a water bath.

To prevent possible interference by sample components in the hexokinase assay, samples used in the reaction mixture were diluted to the limits imposed by the sensitivity of the assay. In experiments on hexokinase activity in homogenate fractions, it was found that velocity of the reaction depends, in a linear manner, on the final tissue dilution in the reaction mixture across a range from 1:2,000 to 1:5,000. In experiments on hexokinase activity in chromatographic fractions, it was found that velocity of the reaction depends linearly on the volume of the fraction aliquot that is added to the reaction mixture, in a range from 5 to 25 μl.

**Total, soluble, and particulate-bound hexokinase activity of human skeletal muscle homogenate**

The latency of hexokinase activity. We compared the effect of 0.6 mol/l KCl on hexokinase activity of whole muscle homogenate and on hexokinase activity of soluble and particulate fractions. Data on the effect of 0.6 mol/l KCl on hexokinase activity are presented in Fig. 1. As shown in Fig. 1, addition of 0.6 mol/l KCl to samples of human skeletal muscle homogenate and a subsequent triple cycle of freezing and thawing with liquid nitrogen and a 30°C water bath increases hexokinase activity of muscle homogenate by 2–4 fold. The freezing-thawing...
cycle in the absence of KCl, but in the presence of the detergent CHAPS, did not significantly affect hexokinase activity. Treatment of the particulate fraction with 0.6 mol/l KCl increased hexokinase activity by severalfold (Fig. 1). The increase of homogenate hexokinase activity by KCl that was observed was due to this effect on the particulate fraction because KCl did not affect hexokinase activity in the soluble fraction.

**Distribution of hexokinase activity between soluble and particulate fractions.** We compared the distribution of total hexokinase activity between soluble and particulate fractions in skeletal muscle from 11 healthy lean research volunteers. The results are shown in Fig. 3. Sonication was used in the presence of KCl/Triton X-100 to convert hexokinase activity in the particulate fraction to a soluble form. Hexokinase activity was 2.78 ± 0.6 U/g wet wt tissue in the particulate fraction and 0.31 ± 0.1 U/g wet wt tissue in the soluble fraction.

To estimate the amount of hexokinase in the particulate fraction that is bound to subcellular structures versus the amount that is mechanically trapped within the myofibrillar matrix, we compared the distribution of hexokinase activity between soluble and particulate fractions with the distribution of the activity of CrK and PGI. CrK has some affinity to myofibrils and mitochondria (32,33), whereas PGI is considered as a truly cytosolic enzyme (34). For correct estimation of hexokinase, CrK and PGI enzyme activities were measured not only in the soluble and particulate fractions, but also in the solution that was left after brief washing of tissue samples to remove any blood. The results of these experiments are presented in Fig. 3. As can be seen in Fig. 3, the incubation of a 10-mg piece of tissue for 5 min on ice resulted in the release of ~30% of CrK, ~30% of PGI, and only traces of hexokinase activities. The different compartmentalization of hexokinase and the other two enzymes can be seen from the distribution of activities between the soluble and particulate fractions. The soluble fraction contains >90% of CrK and PGI and only 10% of total hexokinase activity (Fig. 3). From these results, we estimate that not more than 10% of skeletal muscle hexokinase is mechanically trapped within the myofibrillar matrix. Therefore, the great majority of the activity of hexokinase in the particulate fraction can be explained by binding to subcellular structures, most probably to mitochondria, rather than trapping of hexokinase, as has also been established for hexokinase distribution in transformed cell cultures and neuronal tissue (16,18).

In Fig. 3, hexokinase activity is expressed as activity per gram of actual wet weight of tissue. Because of the small weight of tissue specimens used in these experiments, the inclusions of collagen, fat, or blood could significantly affect this estimation of specific hexokinase activity. It has been shown that the activity of CrK in human muscle does not depend on muscle fiber type, sex, or physical training and therefore can be used as a reliable criterion for an estimation of the amount of functionally active muscle fibers in biopsy samples (35). In Table 1, hexokinase activities are expressed per unit of total CrK activity in tissue samples and per gram of tissue wet weight. The wet weight of tissue samples has been normalized on the basis of CrK activity, assuming that the tissue sample with the highest CrK activity indicates the true wet weight of viable muscle fibers (35). As can be seen from Table 1, the total hexokinase activity in human skeletal muscle is ~4 U/g wet wt or 0.7 U/mU CrK.

**Hexokinase isoforms in the soluble and particulate fractions of human skeletal muscle homogenate**

**Ion-exchange chromatography.** To estimate the activities of hexokinase isoforms I and II in preparations from human skeletal muscle, we used analytical ion-exchange chromatography on DEAE-Agarose, DEAE-Tricacyl, and Mono Q sorbents. Aliquots of samples obtained from the Sephadex column were applied to ion-exchange columns. These results are presented in Fig. 4A and B. The amount of soluble fraction that was applied to the column was twice the amount of the particulate fraction on the basis of tissue wet weight. Three well-defined peaks (peak S, I, and II) were found in chromatograms of the soluble extract prepared from particulate fractions (Fig. 4A and B). More than 90% of the hexokinase activity applied to the column was recovered as the sum of these three peaks. For chromatograms of the particulate fraction, the activity that was recovered in peak I represented ~70–80% of total activity. For the soluble fraction, peak I represents ~30% of total activity. The comparison of the chromatograms of the particulate and soluble fractions show that most hexokinase activity (80–90%) in human muscle resides in the particulate fraction, and this is in agreement with data of direct analysis (Fig. 3).

For identification of peaks from ion-exchange chromatograms of human skeletal muscle, we used three criteria: 1) position of a peak in the KCl gradient in comparison with known standards, 2) thermal stability (1), and 3) the effect of glucose to inhibit the hexokinase III isozyme (36). As sources of hexokinase I and hexokinase II isozymes, we used extracts from rat brain and rat skeletal muscle, respectively, prepared by the same methods as preparations from human muscle. It is well established that rat brain contains mostly the hexokinase I isozyme, whereas rat skeletal muscle is highly enriched by the hexokinase II isozyme (1). The chromatographic profile of hexokinase activity in rat brain and rat skeletal muscle is presented in Fig. 5A. Brain contained only one peak of hexokinase activity (peak I) that eluted at ~0.2 mol/l KCI. According to studies of Chou and Wilson (37), peak I represents the hexokinase I isozyme. The extract from rat skeletal muscle contains relatively minor amounts of hexokinase in peak I, with the majority of hexokinase activity being eluted (at ~0.3 mol/l KCl) as peak II. According to the early studies of Grossbard and Schimke (1), peak II can be identified as the hexokinase II isozyme. The extract from the homogenate of rat skeletal muscle also contains peak S. Comparison of the chromatographic profiles of hexokinase activity in extracts from rat brain homogenate, rat skeletal muscle, and human skeletal muscle, on the basis of the respective positions of the peaks developed by the KCl gradient and by the order of the peak elutions, suggests that peak I and peak II in chromatograms of samples from human skeletal muscle represent the hexokinase I and hexokinase II isozyme, respectively (Fig. 4).

Additional support for the identification of hexokinase isoforms in human skeletal muscle was obtained from experiments on the thermal inactivation of hexokinase activity in chromatographic fractions. Purified hexokinase
Isozymes have different heat stabilities. In the absence of glucose, hexokinase I is more thermally stable than hexokinase II (1). However, our experiments on thermal inactivation of hexokinase in chromatographic fractions were complicated by the presence in those fractions of the thermal stabilizers glucose and glycerol. To overcome the stabilizing effects of glycerol and glucose, we diluted the chromatographic fraction by fivefold and introduced 5 mmol/l of the detergent CHAPS to the inactivation medium. The applicability of these conditions, for selective thermal inactivation of hexokinase II in chromatographic fractions, was examined on preparations from rat brain and rat skeletal muscle. A mixture of homogenates from rat skeletal muscle and rat brain in a ratio of 20:1 was separated on DEAE-Sepharose. Most of the hexokinase activity was eluted from the column in two well-resolved and approximately even peaks (peak I and peak II), which correspond to hexokinase I and hexokinase II isozymes, respectively (Fig. 5B). Diluted aliquots of these chromatographic fractions were incubated at 45°C for 45 min and then assayed for hexokinase activity. Control aliquots were kept on ice before analysis. As can be seen in Fig. 5B,
heating did not affect the hexokinase activity of peak I and completely eliminated the hexokinase activity in peak II. Heating destroyed hexokinase activity in peak S.

These empirically derived conditions for thermal inactivation were then applied to samples from human muscle. Presented in Fig. 6A is a chromatographic profile of hexokinase activity in the particulate fraction from human skeletal muscle before and after heating the chromatographic fractions at 45°C for 45 min. As can be seen in Fig. 6A, heating destroyed peaks S and II but did not affect hexokinase activity in peak I. These data clearly indicate that the particulate fraction from human skeletal muscle contains substantial activity of the hexokinase I isozyme.

To estimate the effect of glucose concentration on hexokinase activity in chromatographic fractions, we separated extracts from the particulate fraction of human skeletal muscle by FPLC on a Mono Q column for better peak resolution and then assayed hexokinase activity at low (0.5 mmol/l) and high (10 mmol/l) glucose concentrations. The data from this experiment is presented in Fig. 6B. FPLC separation on a Mono Q column revealed three peaks (S, I and II) and an additional small peak [I(n)] (Fig. 6B). This smaller peak could represent hexokinase I isozyme truncated by proteolysis, as previously suggested (38). None of the hexokinase peaks showed a decrease in activity at high glucose concentrations (Fig. 6B). The inability of glucose at high concentrations to inhibit hexokinase activity of peaks S and II suggests that peaks S and II do not represent the hexokinase III isozyme. Peak S manifests a high thermal lability (Fig. 6A) and probably represents the hexokinase II isozyme.

Kinetic analysis of thermal inactivation of hexokinase activity in particulate and soluble fractions from human skeletal muscle. In preliminary experiments, we found that incubation of particulate fractions from human muscle at 45°C for 60 min destroyed >80% of total hexokinase activity. These results clearly contradicted chromatographic data showing that 80% of total hexokinase activity in the particulate fraction corresponds to thermally stable hexokinase I isozyme. However, after ion-exchange chromatography of the particulate fraction, heating of chromatographic fractions did allow easy differentiation of hexokinase I and hexokinase II isozymes (Fig. 6A). The reason for rapid thermal inactivation of hexokinase I isozyme bound within the particulate fraction probably originates from the destabilizing effects of surrounding lipids or proteins on the structure of bound hexokinase I. The conversion of particulate bound hexokinase to a soluble form by KCl/Triton X-100 extraction, as described in RESEARCH DESIGN AND METHODS, followed by subsequent gel filtration of the extract and then introduction to a thermal inactivation medium including 5 mmol/l CHAPS to reduce protein and lipid interactions, prevents rapid thermal inactivation of hexokinase I at 45°C. Using the described procedure for sample preparation for thermal inactivation, we found only a 25% decline in hexokinase activity in the particulate fraction in the first 5 min (Fig. 7), and the rest of the activity, belonging to hexokinase I isozyme, showed little loss during the next 55 min of heating. The soluble fraction treated by the same methods (excepting sonication) showed an identical two-phase kinetic pattern of thermal inactivation. During the rapid phase of decline of activity, likely corresponding to inactivation of type II isozyme, there was an ~60% loss of hexokinase activity in the soluble fraction (Fig. 7).

The comparative analysis of hexokinase isozymes contents in soluble and particulate fractions. The data from chromatography and thermal inactivation experiments concerning the content of hexokinase I and II isozymes in the particulate and soluble fractions from
human skeletal muscle is summarized in Table 2. The areas for the thermally labile peaks S and II were used to calculate the content of hexokinase II. A high correlation exists between chromatography and thermal inactivation data concerning the content of hexokinase I and II isoenzymes in soluble and particulate fractions and is presented in Table 2. The data presented in Table 2 shows that, on the basis of thermal inactivation methods, hexokinase I and II accounted for 76 and 24%, respectively, of total hexokinase activity. Nearly all (95%) of hexokinase I activity was found in the particulate fraction (0.52 ± 0.09 vs. 0.03 ± 0.01 U/mU CrK, particulate vs. cytosolic), which is in accord with the high affinity of hexokinase I isoform for mitochondria (3,39). Hexokinase II activity was largely bound to the particulate fraction (72%), but 28% was found within the cytosolic fraction. Thus, within the particulate fraction, the relative contributions of hexokinase I and hexokinase II were 81 and 19%, respectively, whereas within the cytosolic fraction, the relative contributions for hexokinase I and hexokinase II were 37 and 63%, respectively (Table 2).

**Assay development.** The well-known method for assay of hexokinase, in small samples of muscle tissue, is based on fluorometric monitoring of the generation of NADPH in a coupled enzymatic reaction with Glc-6-PDH. This method has received wide acceptance from laboratories engaged in studies of muscle metabolism (30). The amplification of the assay by “enzyme cycling” significantly increases sensitivity (30). However, the application of this high-sensitivity method of enzyme cycling to analyze hexokinase activity in hundreds of chromatographic fractions is too time-consuming and thus impractical. We found that HPLC analysis of the reaction mixture offers a reasonable alternative in sensitivity and time for chromatographic experiments performed with small tissue samples. The HPLC separation with fluorescence detection allows a direct estimation of the concentration of NADPH in the reaction mixture with a very high sensitivity and without interference by other components. The signal from the HPLC fluorescent detector is linear from 2 to 200 pmol of injected NADPH (Fig. 2B). In the presence of hydrazine and 2-amino-2-methyl-1-propanol in the stopping reagent, these samples can be stored indefinitely at −80°C before HPLC analysis and without change in NADPH concentration.

**Hexokinase isoforms in human skeletal muscle.** Hexokinase isoforms I and II are expressed in skeletal muscle (3). Rat skeletal muscle contains mostly type II enzyme (1,5), whereas human muscle contains approximately equal amounts of type II and type I isoforms (19,21,40). Our data (based on two approaches—ion-exchange chromatography and a modified method of thermal inactivation) reveal that in human skeletal muscle, 70–75% of hexokinase activity corresponds to the type I isoform, and 25–30% of activity belongs to the thermally labile type II isozyme (Table 2). Thermally labile hexokinase II exists in human skeletal muscle as two chromatographically distinct forms: peak S and peak II (Figs. 4 and 6). The same forms were also found in rat skeletal muscle (Fig. 5) but were not found in extracts from rat brain that contain solely the hexokinase I isoform (Fig. 5A). The reason for partial elution of type II hexokinase without retention on the column will require future studies.

**Subcellular distribution of hexokinase isoforms in human skeletal muscle.** Hexokinase activity in a particulate cellular fraction, denoting, but not specifically proving mitochondrial binding, has been reported in many mammalian cells cultures and tissues. A relatively small amount of particulate-bound hexokinase was found in rat skeletal muscle (5,34) in contrast to brain tissue, where >80% of hexokinase is found within the particulate fraction (41). As can be seen in Fig. 3 and Table 1, in human skeletal muscle, ~90% of total hexokinase activity is bound within the particulate fraction.

Using chromatography and thermal inactivation methods, we found distinct differences in the distribution of hexokinase I and II among the soluble and particulate fractions of human muscle homogenate. As shown in Table 2, 95% of type I hexokinase activity was found in the particulate fraction. Type II hexokinase activity was largely bound to mitochondria (72%), but 28% was found within the cytosolic fraction. Thus, within the particulate fraction, the relative contributions of hexokinase I and hexokinase II were 81 and 19%, respectively, whereas within the cytosolic fraction, the relative contributions for hexokinase I and hexokinase II were 37 and 63%, respectively (Table 2). In the soluble fraction, approximately the same ratio between these two hexokinase isoforms was found, as was previously reported by Vogt et al. (21), in which the thermal inactivation method was used to identify hexokinase I and II. However, our results on hexokinase isoforms in the particulate fraction differ substantially from the data of Vogt et al. (21). They found that 90% of hexokinase in the particulate fraction was due to a thermally labile enzyme and interpreted this thermal lability to represent solely hexokinase

**Discussion**

**Assay development.** The well-known method for assay of hexokinase, in small samples of muscle tissue, is based on fluorometric monitoring of the generation of NADPH in a coupled enzymatic reaction with Glc-6-P DH. This method has received wide acceptance from laboratories engaged in studies of muscle metabolism (30). The amplification of the assay by “enzyme cycling” significantly increases sensitivity (30). However, the application of this high-sensitivity method of enzyme cycling to analyze hexokinase activity in hundreds of chromatographic fractions is too time-consuming and thus impractical. We found that HPLC analysis of the reaction mixture offers a reasonable alternative in sensitivity and time for chromatographic experiments performed with small tissue samples. The HPLC separation with fluorescence detection allows a direct estimation of the concentration of NADPH in the reaction mixture with a very high sensitivity and without interference by other components. The signal from the HPLC fluorescent detector is linear from 2 to 200 pmol of injected NADPH (Fig. 2B). In the presence of hydrazine and 2-amino-2-methyl-1-propanol in the stopping reagent, these samples can be stored indefinitely at −80°C before HPLC analysis and without change in NADPH concentration.

**Hexokinase isoforms in human skeletal muscle.** Hexokinase isoforms I and II are expressed in skeletal muscle (3). Rat skeletal muscle contains mostly type II enzyme (1,5), whereas human muscle contains approximately equal amounts of type II and type I isoforms (19,21,40). Our data (based on two approaches—ion-exchange chromatography and a modified method of thermal inactivation) reveals that in human skeletal muscle, 70–75% of hexokinase activity corresponds to the type I isoform, and 25–30% of activity belongs to the thermally labile type II isozyme (Table 2). Thermally labile hexokinase II exists in human skeletal muscle as two chromatographically distinct forms: peak S and peak II (Figs. 4 and 6). The same forms were also found in rat skeletal muscle (Fig. 5) but were not found in extracts from rat brain that contain solely the hexokinase I isoform (Fig. 5A). The reason for partial elution of type II hexokinase without retention on the column will require future studies.

**Subcellular distribution of hexokinase isoforms in human skeletal muscle.** Hexokinase activity in a particulate cellular fraction, denoting, but not specifically proving mitochondrial binding, has been reported in many mammalian cells cultures and tissues. A relatively small amount of particulate-bound hexokinase was found in rat skeletal muscle (5,34) in contrast to brain tissue, where >80% of hexokinase is found within the particulate fraction (41). As can be seen in Fig. 3 and Table 1, in human skeletal muscle, ~90% of total hexokinase activity is bound within the particulate fraction.

Using chromatography and thermal inactivation methods, we found distinct differences in the distribution of hexokinase I and II among the soluble and particulate fractions of human muscle homogenate. As shown in Table 2, 95% of type I hexokinase activity was found in the particulate fraction. Type II hexokinase activity was largely bound to mitochondria (72%), but 28% was found within the cytosolic fraction. Thus, within the particulate fraction, the relative contributions of hexokinase I and hexokinase II were 81 and 19%, respectively, whereas within the cytosolic fraction, the relative contributions for hexokinase I and hexokinase II were 37 and 63%, respectively (Table 2). In the soluble fraction, approximately the same ratio between these two hexokinase isoforms was found, as was previously reported by Vogt et al. (21), in which the thermal inactivation method was used to identify hexokinase I and II. However, our results on hexokinase isoforms in the particulate fraction differ substantially from the data of Vogt et al. (21). They found that 90% of hexokinase in the particulate fraction was due to a thermally labile enzyme and interpreted this thermal lability to represent solely hexokinase
II. This difference, from our data, can be explained by differences in the conditions of thermal inactivation used in the two studies. In our preliminary experiments, it was found that most of the hexokinase activity bound in the particulate fraction is highly temperature sensitive. This finding appeared to be in sharp contrast with data from particulate fraction is highly temperature sensitive. This difference from our data, can be explained by differences in the conditions of thermal inactivation used in the two studies. In our preliminary experiments, it was found that most of the hexokinase activity bound in the particulate fraction, belonged to the thermally stable isoinzyme type I (Table 2). However, after conversion of bound hexokinase to a soluble form and inclusion of the detergent CHAPS in the thermal inactivation mixture to reduce nonspecific lipid-protein and protein-protein interactions, we found excellent agreement between chromatography and thermal inactivation data (Table 2). Our data confirm that heating of untreated particulate fractions cannot be used for the estimation of hexokinase isozyme composition in these fractions, as has been recently pointed out by Wilson (23). However, our data showed that with certain preparation of the sample and proper conditions, thermal inactivation is a reliable and valuable tool for the study of hexokinase isoforms.

There are no definitive data on the localization of bound hexokinase within human muscle. The association of hexokinase with mitochondria was shown in brain tissue and transformed cells (15–18). In mitochondria, hexokinase is bound on the outer mitochondria membrane at contact sites, probably as part of the permeability transition pore, which includes the adenine nucleotide translocator, porin, and other proteins (42–44). Porin is considered to be a “hexokinase binding protein” (42). The proximity of bound hexokinase to the adenine nucleotide translocator establishes a “privileged access” of the enzyme to ATP generated by oxidative phosphorylation. This is one reason for the high level of mitochondria-bound hexokinase in some lines of rapidly growing transformed cells (16). The decreased sensitivity of bound hexokinase to inhibition by glucose-6-phosphate might additionally contribute to a high glycolytic rate in transformed cells (45). The “privileged access” of hexokinase to ATP obviously makes cellular bioenergetics more protected and reliable, and a detailed consideration of the functional significance of this binding of hexokinase is presented in a review by Wilson (3). The high level of hexokinase binding in human skeletal muscle (Fig. 3) in comparison with rat muscle (34) probably reflects better protection of human muscle bioenergetics in the situation of intense physical activity.

The existence and role of nonmitochondrial binding sites for hexokinase have been previously considered in connection with a potential role of hexokinase in mediating the transport of glucose (3,46). It was shown that stimulation of macrophages by phorbol ester activates hexose transport and induces the translocation of hexokinase to the cell surface. Cytochalasin D, an inhibitor of actin microfilament polymerization, prevents phorbol-induced translocation of hexokinase and activation of hexose transport (47). These data indirectly indicate binding of hexokinase with microfilaments. A model for participation of hexokinase in glucose transport (“loose-coupling” model) suggests binding of enzyme in the vicinity of the transporter and that this may decrease exit of glucose from the cell (48). A similar model was used to explain insulin-induced activation of glucose transport in adipocytes (49). A very tight coordination between appearance of insulin sensitivity and expression of hexokinase II and GLUT4 type transporter during ontogenesis of rat skeletal muscle has been reported (50). This coordination can reflect a specific function of the hexokinase II isozyme in muscle insulin-regulated glucose transport.

Hexokinase II has a lesser affinity to mitochondria than hexokinase I (39). Our data show enrichment of the soluble fraction by hexokinase II in comparison with the particulate fraction (Table 2). For that reason, potentially, hexokinase II might be more easily translocated to the vicinity of the glucose transporter in plasma membrane under stimulation by insulin. In addition, the sensitivity of hexokinase II expression to exercise and hormones make hexokinase II an ideal tool for fine-tuning glucose transport in relation to the demands of muscle bioenergetics.

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