Purification and Characterization of a Bindable Form of Mitochondrial Bound Hexokinase from the Highly Glycolytic AS-30D Rat Hepatoma Cell Line

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ABSTRACT

Recent studies from this laboratory have demonstrated that a form of hexokinase characteristic of rapidly growing, highly glycolytic tumor cells is bound to an outer mitochondrial membrane receptor complex containing a M, 35,000 pore protein (D. M. Parry and P. L. Pedersen, J. Biol. Chem., 258: 10904-10912, 1983; R. A. Nakashima, et al., Cancer Research 48, 913-919, February 15, 1988). In new studies reported here the specificity of this receptor complex for binding hexokinase is defined, and a purification scheme is described which leads to a homogeneous and bindable form of the tumor hexokinase.

In the AS-30D hepatoma, hexokinase activity is elevated more than 100-fold relative to liver tissue. The relative increase in hexokinase activity is 8 times greater than that of any other glycolytic enzyme. Hexokinase is the only glycolytic enzyme of AS-30D cells to exhibit a mitochondrial/cytosplasmic specific activity ratio greater than 1, showing a 3.5-fold elevation in the mitochondrial fraction.

Purification of hexokinase is accomplished by preferential solubilization of the mitochondrial bound enzyme with glucose-6-phosphate, followed by high-performance liquid chromatography on gel permeation and anion exchange columns. The final fraction has a specific activity of 144 units per mg of protein, with a Km for glucose of 0.13 mM and for ATP of 1.4 mM. The purified tumor enzyme migrates as a single species upon sodium dodecyl sulfate: polyacrylamide gel electrophoresis with an apparent molecular weight of 98,000. Significantly, the purified tumor enzyme retains its activity for mitochondrial binding.

Additional results derived from chromatographic, polyclonal antibody, and amino acid analysis studies indicate that the predominant rat hepatoma hexokinase species is related most closely to isozymic form(s) of the enzyme commonly referred to as type II, and least related to the liver type IV isozyme (glucoinase).

INTRODUCTION

It has been widely observed that transformed cells exhibit increased rates of glucose utilization compared to normal cells from the same tissue of origin (1-8). Elevated glucose catabolism is important for the production of both energy (7) and required anabolic precursors in rapidly growing tumors. Previous studies from our laboratory (4-6) and others (9-11) have emphasized the role of the initial enzyme of glycolysis, hexokinase (EC 2.7.1.1), in maintaining high rates of glucose utilization in rapidly growing cancer cells. In comparison with normal cells from the same tissue of origin there is a large increase in hexokinase activity in highly glycolytic tumor cell lines (2-7, 9, 10). This is accompanied by a change in subcellular distribution of the enzyme from a predominantly cytosolic to a mitochondria-bound form (4-6, 9, 11-14). Mitochondria-bound hexokinase has been reported to be less sensitive to feedback inhibition by glucose-6-phosphate, a potent regulator of hexokinase activity in normal tissues (4, 9, 11). The bound enzyme may also obtain preferential access to mitochondrial generated ATP (13, 15). A direct correlation has been observed between mitochondrial bound hexokinase activity, tumor glycolysis, and tumor growth rate in cancer cell lines from a wide variety of tissues and species (3, 5, 6, 10).

A protein which promotes binding of rat brain hexokinase has been partially purified from the outer membrane of rat liver mitochondria (16). Several recent reports have suggested that the outer mitochondrial membrane pore protein, also known as VDAC or mitochondrial porin, might be identical to the hexokinase binding protein of rat liver (17, 18). Significantly, we have recently demonstrated directly that the pore protein forms at least one part of a hexokinase receptor complex on the outer membrane of tumor mitochondria (19).

Despite the apparent importance of mitochondrial bound hexokinase in tumor cell growth and metabolism very little is known about the molecular characteristics of the tumor enzyme. In the present study, we report the first purification to apparent homogeneity of mitochondrial bound hexokinase from a transformed cell line, the highly malignant AS-30D rat hepatoma. The purification technique, involving rapid separation of solubilized mitochondrial hexokinase by high-performance liquid chromatography, yields an apparently homogeneous enzyme of high specific activity which retains its ability to rebind to the outer mitochondrial membrane. We have also examined the specificity of the binding interaction between hexokinase and the outer mitochondrial membrane relative to that of other glycolytic enzymes.

MATERIALS AND METHODS

Chemicals. Acrylamide, bisacrylamide, sodium dodecyl sulfate, molecular weight standard proteins, Coomassie Brilliant Blue R-250, DEAE-cellulose (Cellex D), and 2-mercaptoethanol were obtained from Bio-Rad. Centricron 10 microconcentrators were purchased from Amicon. Dithiothreitol, NADP+, and glucose-6-phosphate dehydrogenase (Grade I) were from Boehringer Mannheim. Oligomycin, phenylmethylsulfonyl fluoride, bovine serum albumin (Fraction V, fatty acid free), NADH, and P1,P2-di(adenosine-5')pentaphosphate were from Sigma Chemical Co. Phenylisothiocyanate, constant boiling 6N HCl and amino acid standards were from Pierce. Enuls for the analysis of amino acid composition were obtained from the Waters Chromatography Division of the Millipore Corporation (Pico-Tag Eluents A and B). The detergents used were Lubrol WX from Grand Island Biological Co. and Triton X-100 from Research Products International Corp. All other chemicals and substrates used were of analytical grade or better and were obtained from standard chemical supply companies including Sigma Chemical Co., J. T. Baker Chemical Co., Calbiochem-Behring Corp., K and K Laboratories, Inc., Eastman Kodak Co., and Mallinckrodt Chemicals.

Animals and Tumor Cells. The AS-30D rat hepatoma cell line was obtained from Dr. Baltazar Reynafarje of the Department of Biological Chemistry, The Johns Hopkins University, School of Medicine, Baltimore, MD. Cells were grown in ascites form by i.p. injection into 100-150-g female Sprague-Dawley rats (from Holtzman Laboratories, Madison, WI). AS-30D cells were collected from ascites fluid and purified according to Parry and Pedersen (14). Animals were fed and watered ad libitum.

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Mitochondrial Isolation. Tumor mitochondria were isolated from AS-30D cells by the sonic cell disruption method of Parry and Pedersen (14). Rat liver mitochondria were prepared by the high-speed method of Bustamante et al. (20). In both cases after the cell homogenization step the mitochondria were resuspended in H medium* without bovine serum albumin or EGTA added. Mitochondria were either used fresh or else frozen in liquid nitrogen and thawed immediately prior to use.

Subcellular Localization of Glycolytic Enzyme Activities. Subcellular fractions of rat liver tissue and AS-30D cells were obtained by differential centrifugation of the homogenized cells according to Parry and Pedersen (14). Purified mitochondrial fractions were prepared as described above. Homogenates of rat liver or AS-30D cells were subjected to low speed (10 min at 20,000 × g) and high speed (45 min at 130,000 × g) centrifugations. The high speed supernatants were used as cytosolic fractions.

Enzyme assays were performed at 30 °C using a Gilford Model 252 spectrophotometer attached to a circulating water bath. All assay mixtures contained 13 μg/ml of oligomycin to inhibit F1-ATPase activity and 13 μM DAPP to inhibit adenylyl kinase. Samples containing membrane fractions were solubilized with 1 to 5 mg/mg of Lubrol WX prior to assay. Aldolase, enolase, and phosphoglucone isomerase were assayed according to Bergmeyer et al. (21). Lactate dehydrogenase activity was measured by the UV absorbance technique of Vassault (22). Pyruvate kinase was assayed according to Fujii and Miwa (23). Phosphofructokinase activity was measured as described by Duruibe and Tiejani (24). Hexokinase activity was determined according to Parry and Pedersen (14). All enzyme assays were coupled to either the oxidation of NADH via pyruvate kinase and lactate dehydrogenase, pH 7.9. Production of ADP by hexokinase was measured as described by Hidlip et al. (26) using a Pico-Tag work station (Waters Chromatography) to hydrolyze and derivatize the protein and a Waters high-performance liquid chromatography (HPLC) gel permeation column (Waters 1-60 and 300 SW columns) under the conditions described above. AS-30D hexokinase was purified by HPLC as described in a preceding section. A constant amount of catalytic activity of each of the hexokinases (0.5 units) was incubated at room temperature with rat liver mitochondria (1 mg protein) in 0.5 ml of H medium containing 10 mM MgCl2. After 15 min the suspensions were diluted with 0.9 ml of ice-cold H medium and centrifuged for 2 min at 12,800 × g in an Eppendorf microfuge. The supernatants were decanted, and the mitochondrial pellets containing bound hexokinase were assayed as described. Mitochondrial binding of hexokinase was assessed using rat liver mitochondria rather than AS-30D mitochondria in order to avoid problems arising from the presence of high levels of endogenous bound hexokinase activity in the tumor mitochondrial fraction.

Amino Acid Composition Analysis. The amino acid composition of purified AS-30D hexokinase was determined by the technique of Bidlingmeyer et al. (26) using a Pico-Tag work station (Waters Chromatography) to hydrolyze and derivatize the protein and a Waters high-performance liquid chromatography apparatus to analyze the derivatized amino acids. The AS-30D mitochondrial enzyme was purified as described above and lyophilized. The dried samples were hydrolyzed with 6 N constant boiling HCl (Pierce) in vapor phase for 24 h at 110°C. After hydrolysis the free amino acids were dried and derivatized with phenylisothiocyanate, forming the PTC derivatives. The PTC-amino acids were detected by absorbance at 254 nm using a fixed-wavelength detector (Waters Model 440). The amounts of PTC-amino acids eluted were calibrated to known injections of 250 pmol each of PTC-derivatized amino acids. The abbreviations used are: H medium, 210 mM D-glucitol:70 mM sucrose:5 mM EGTA:0.2 mM MgCl2:0.2 mM NaN3:0.6 mM phosphoenol pyruvate:2 mM ATP:55 units pyruvate kinase:0.5 mM glucose:6 units lactate dehydrogenase; pH 7.9. Production of ADP by hexokinase was measured as described by Hidlip et al. (26) using a Pico-Tag work station (Waters Chromatography) to hydrolyze and derivatize the protein and a Waters high-performance liquid chromatography (HPLC) gel permeation column (Waters 1-60 and 300 SW columns) under the conditions described above. AS-30D hexokinase was purified by HPLC as described in a preceding section. A constant amount of catalytic activity of each of the hexokinases (0.5 units) was incubated at room temperature with rat liver mitochondria (1 mg protein) in 0.5 ml of H medium containing 10 mM MgCl2. After 15 min the suspensions were diluted with 0.9 ml of ice-cold H medium and centrifuged for 2 min at 12,800 × g in an Eppendorf microfuge. The supernatants were decanted, and the mitochondrial pellets containing bound hexokinase were assayed as described. Mitochondrial binding of hexokinase was assessed using rat liver mitochondria rather than AS-30D mitochondria in order to avoid problems arising from the presence of high levels of endogenous bound hexokinase activity in the tumor mitochondrial fraction.

Preparation of Solubilized AS-30D Mitochondrial Hexokinase. Freshly prepared AS-30D mitochondria (5 to 10 mg/ml) were suspended in H medium with 1 mm phenylmethylsulfonyl fluoride, 1 mm EGTA, and 1 mM DTT added to inhibit protease activity. After addition of 1 mm glucose-6-phosphate the mitochondria were incubated for 15 min at 30°C. This resulted in the selective solubilization of mitochondrial hexokinase (12). Following centrifugation of the suspension at 20,000 × g for 15 min the resulting supernatant was centrifuged at 130,000 × g for 45 min to remove mitochondria and particulate contaminants. The high speed supernatant containing solubilized mitochondrial hexokinase was purified by HPLC as described below.

Chromatography of Hexokinase on DEAE-Cellulose. Hexokinase from either AS-30D mitochondria or normal rat liver was loaded onto a 0.9 × 6-cm column of DEAE-cellulose (Cellex D, from Bio-Rad) equilibrated with buffer containing 10 mm triethanolamine HCl, pH 7.6, 1.1 mM DTT, and 0.5 mM EGTA. The column was washed with 40 ml of equilibrating buffer to remove nondenatured proteins and then developed with a 40-ml linear gradient from 0 to 0.5 M NaCl in the same buffer at a flow rate of 18 ml/h. Fractions of 0.5 ml were collected and assayed for hexokinase activity as described above. The chromatography was performed at a temperature of 4°C.

Purification of AS-30D Mitochondrial Hexokinase by HPLC. AS-30D mitochondrial hexokinase was solubilized as described above. After filtration through a 0.45-μm HAWP filter (Millipore Corp.) the protein was injected onto two tandem gel permeation columns (Waters I-60 and 300 SW Protein Pak columns). The columns were prewashed with Buffer A [10 mM potassium phosphate (pH 7.0):10 mM glucose:0.5 mM EDTA:2.5 mM DTT] and the enzyme was eluted with the same buffer. Fractions containing peak hexokinase activity were collected and loaded directly onto a Waters DEAE-5PW Protein Pak anion exchange column equilibrated with Buffer A. After washing with Buffer A for 5 min the enzyme was eluted with a 50-ml linear gradient from 0 to 0.4 M NaCl in Buffer A. Hexokinase activity typically eluted as a sharp peak near the end of the gradient, of approximately 0.5- to 1.0-ml total volume. In experiments where low salt concentration was necessary, the enzyme was diluted with distilled water and reconcentrated by centrifugation for 45 min at 5000 × g in Centricon 10 microconcentrators (Amicon). The HPLC system (purchased from the Waters Chromatography Division, Millipore Corporation, Milford, MA) consisted of an M-45 pump, a Model 510 pump, a U6K manual injector with 2.0-ml sample loop, a Model 440 absorbance detector operated at 280 nm, a Model 680 automated gradient controller, and a Model 740 data module. The solvent flow rate was 0.5 ml per min. Buffer reservoirs were kept on ice during the chromatography, and fractions were transferred to an ice bucket immediately after elution in order to retain maximum hexokinase catalytic activity.

PAGE. Electrophoresis in the presence of SDS was performed as described by Laemmli (25) using a Bio-Rad Protean 1 slab cell apparatus. Proteins were precipitated with acetone prior to solubilization in 2% SDS, 5% 2-mercaptoethanol, 0.1 M glycine, pH 8.8. Samples were heated for 2 min at 100°C before loading onto the gel. Electrophoresis was performed for 2500 V-h overnight in a water-cooled apparatus.

Mitochondrial Binding of Hexokinase. Beef heart hexokinase and yeast hexokinase purchased from the Sigma Chemical Co. were purified by HPLC gel permeation chromatography on Waters I-60 and 300 SW columns under the conditions described above. AS-30D hexokinase was purified by HPLC as described in a preceding section. A constant amount of catalytic activity of each of the hexokinases (0.5 units) was incubated at room temperature with rat liver mitochondria (1 mg protein) in 0.5 ml of H medium containing 10 mM MgCl2. After 15 min the suspensions were diluted with 0.9 ml of ice-cold H medium and centrifuged for 2 min at 12,800 × g in an Eppendorf microfuge. The supernatants were decanted, and the mitochondrial pellets containing bound hexokinase were assayed as described above. Mitochondrial binding of hexokinase was assessed using rat liver mitochondria rather than AS-30D mitochondria in order to avoid problems arising from the presence of high levels of endogenous bound hexokinase activity in the tumor mitochondrial fraction.

* The abbreviations used are: H medium, 210 mM D-glucitol:70 mM sucrose:5 mM (4-tert-butylphenyl)-1-piperazineethanesulfonic acid, pH 7.4; EGTA, ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; DAPP, P30,P31-di(edenosine-5'-pentaphosphate); PTC, phenylthiocarbamyl.
tized amino acid standards (from Pierce). The chromatography was performed at 38°C at a flow rate of 1 ml/min. It should be noted that the conditions used in this analysis tend to result in a low recovery of cysteine residues.

Preparation of Polyclonal Antibodies to Tumor Hexokinase. Hexokinase solubilized from AS-30D mitochondria was purified as described above except that the HPLC sizing column was replaced with a 0.9- x 55-cm Sepharose CL-6B column. The resultant preparation showed only a single band upon SDS:PAGE exhibiting a molecular weight of 98,000. Purified AS-30D hexokinase (100 µg) was mixed with an equal volume of complete Freund's adjuvant and injected s.c. into the back of a male New Zealand white rabbit. The rabbit was boosted at 3-wk intervals with 100-µg injections of AS-30D hexokinase mixed with an equal volume of incomplete Freund's adjuvant. Serum containing polyclonal antibodies to hexokinase was prepared by allowing rabbit blood to clot overnight at 4°C, followed by centrifugation for 10 min at 700 x g. The resulting clear supernatant was decanted off and diluted 1:250 for use in Western blot experiments.

Assay of Immune Cross-Reactivity by Western Blot Analysis. The ability of polyclonal antibodies prepared against AS-30D hexokinase to cross-react with hexokinases from normal rat tissues was determined by Western blot analysis. Homogenates of rat brain, rat kidney, and rat liver, along with purified AS-30D hexokinase, were subjected to SDS:PAGE according to Laemmli (25). After electroblotting onto a nitrocellulose filter the proteins were incubated with a 1:250 dilution of rabbit serum containing polyclonal antibodies to AS-30D hexokinase. Antibody-reactive polypeptides were localized on the nitrocellulose filter by autoradiography following incubation with 125I-labeled Protein A.

RESULTS

Comparison of Glycolytic Enzyme Activities in AS-30D Hepatoma Cells and in Normal Rat Liver Tissue. We have compared the specific activities for a number of glycolytic enzymes in the AS-30D hepatoma and in normal rat liver (see Table 1). The most striking difference between normal and transformed cells is the great increase in low Km hexokinase in the tumor cell, representing a 136-fold increase in specific activity over normal rat liver. Elevated specific activities in the tumor were noted for several other key glycolytic enzymes, including phosphofructokinase (17-fold increase) and pyruvate kinase (10-fold increase).

Table 1: Comparison of glycolytic enzyme-specific activities in AS-30D hepatoma and normal rat liver tissue

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (units/mg)</th>
<th>Rat liver</th>
<th>Hepatoma</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase (low Km)</td>
<td>0.005 ± 0.001</td>
<td>0.68 ± 0.05</td>
<td>136</td>
<td></td>
</tr>
<tr>
<td>Phosphoglucone isomerase</td>
<td>0.35 ± 0.04</td>
<td>0.71 ± 0.14</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Phosphofructose kinase</td>
<td>0.01 ± 0.001</td>
<td>0.17 ± 0.04</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>Aldolase</td>
<td>0.04 ± 0.001</td>
<td>0.11 ± 0.01</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Enoiase</td>
<td>0.15 ± 0.02</td>
<td>0.66 ± 0.08</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>0.22 ± 0.02</td>
<td>2.28 ± 0.47</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>1.83 ± 0.32</td>
<td>5.24 ± 1.29</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

*At the glucose concentration (0.5 mM) used for this assay, liver glucokinase which has a very high Km for glucose (12 mM) does not contribute to the activity measured.

Specificity of Mitochondrial Binding of Hexokinase in AS-30D Hepatoma Cells. It has commonly been observed that most of the hexokinase in rapidly growing tumor cells is bound to the outer mitochondrial membrane (4–6, 9, 12–14, 29). The possibility that other glycolytic enzymes might also be associated with tumor mitochondria in a multienzyme complex has not been examined. Therefore, we investigated the subcellular localization of the glycolytic enzymes in the AS-30D hepatoma cell. A comparison of specific activities in the cytosolic and mitochondrial fractions is presented in Table 2. It is apparent from these data that the only enzyme which shows a markedly elevated specific activity in the mitochondrial fraction is hexokinase, with a specific activity over 3-fold higher in the mitochondrial fraction than in the cytosol. For all of the other glycolytic enzymes which we examined the ratio of specific activities in the mitochondrial fraction versus the cytosol was substantially less than one. These results emphasize that the binding of hexokinase to its outer mitochondrial receptor complex (14, 19) in the AS-30D tumor is a highly specific event.

Isozyme Composition of AS-30D Mitochondrial Hexokinase. We have characterized the AS-30D mitochondrial hexokinase by column chromatography on DEAE-cellulose. The pattern of elution of hexokinase from DEAE-cellulose columns has been used as a standard technique to define hexokinase isozyme types (30, 31). The elution profile of AS-30D mitochondrial hexokinase from DEAE-cellulose is shown in Fig. 1A. In these experiments the AS-30D hexokinase used was not HPLC purified, but rather was solubilized by glucose-6-phosphate treatment of AS-30D mitochondria. As shown in Fig. 1B, there are four isozymes of hexokinase present in normal rat liver tissue. Types I, II, and III represent forms with low Km for glucose and can be assayed at a glucose concentration of 0.5 mM. Type IV hexokinase (glucokinase) has a much higher Km for glucose and is assayed in 100 mM glucose. The bulk of the hexokinase present in normal rat liver is of the type IV isozyme. A comparison of the elution profiles of hepatoma mitochondrial hexokinase (Fig. 1A) with normal liver hexokinases (Fig. 1B) shows that there is a shift from predominantly high Km isoform (type IV) in normal liver to low Km isozymes in the transformed cell line. The bulk of the tumor enzyme coelutes from DEAE-cellulose columns with the type II isozyme of normal rat liver. A minor amount of the tumor mitochondrial hexokinase coelutes with the type I isozyme of normal liver. We have repeated this experiment 4 times with essentially identical results.

Purification of Mitochondrial Hexokinase from AS-30D Hepatoma Cells. Treatment of tumor mitochondria with millimolar concentrations of either ATP or glucose-6-phosphate results in the selective solubilization of bound hexokinase (9, 12, 14). We have used glucose-6-phosphate-solubilized mitochondrial hexokinase as the starting material for the purification of the bound
mitochondrial bound hexokinase in hepatoma cells

Freshly prepared AS-30D mitochondria were treated with 1 mM glucose-6-phosphate to solubilize bound hexokinase. Solubilized AS-30D hexokinase was further purified by chromatography on gel-permeation and DEAE-cellulose columns as described in "Materials and Methods." The Michaelis constants of purified AS-30D hexokinase were calculated from the intercepts of double reciprocal (Lineweaver-Burk) plots of velocity versus substrate concentrations. Average values were obtained from three different experiments for each substrate. The apparent K_m values obtained for glucose (0.13 mM) and ATP (1.41 mM) are comparable with values reported for crude solubilized mitochondrial hexokinase from the H-91 rat hepatoma (12). In comparing the kinetic properties of the purified AS-30D mitochondrial hexokinase with those reported for hexokinase isozymes from normal rat tissues, the tumor enzyme seems to be most similar to type II hexokinase purified from rat skeletal muscle with apparent K_m values of 0.2 mM for glucose and 1.11 mM for ATP (32).

Table 3 Purification scheme for mitochondrial hexokinase from AS-30D hepatoma cells

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Protein (mg)</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS-30D mitochondria</td>
<td>20.0</td>
<td>1.8 ± 0.4* (3)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Solubilized AS-30D hexokinase</td>
<td>1.2</td>
<td>23.9 ± 11.4 (15)</td>
<td>80</td>
<td>13</td>
</tr>
<tr>
<td>Gel permeation eluate</td>
<td>0.4</td>
<td>67.0 ± 21.9 (7)</td>
<td>74</td>
<td>37</td>
</tr>
<tr>
<td>DEAE-cellulose eluate</td>
<td>0.2</td>
<td>144.2 ± 26.7 (8)</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>

* Mean ± SD.
* Numbers in parentheses, number of samples for units of hexokinase activity per mg of protein in each fraction.

hexokinase isozyme from the highly glycolytic AS-30D rat hepatoma cell line. As shown in Table 3, freshly isolated AS-30D mitochondria contain 1.8 units/mg protein of bound hexokinase activity. Incubation for 15 min with 1 mM glucose-6-phosphate results in the solubilization of 80% of the bound hexokinase activity. The solubilized enzyme has an average specific activity of 23.9 units/mg protein, a 13-fold increase over whole AS-30D mitochondria.

The solubilized tumor enzyme was further purified by sequential chromatography on gel permeation sizing columns followed by a DEAE-cellulose anion exchange column. Chromatography on the HPLC gel permeation columns (Protein Pak 1-60 and 300 SW columns from Waters/Millipore) resulted in a 3-fold increase in specific activity of the tumor enzyme to 67 units/mg protein (see Table 3). Purification of this fraction by anion-exchange chromatography on a Protein Pak DEAE-5PW column (Waters/Millipore) resulted in a further 2-fold increase in specific activity to 144 units/mg (Table 3). Two peaks of hexokinase activity were observed in the DEAE-cellulose eluate. A low amount of activity, about 5% of total, eluted at a lower salt concentration (approximately 120 mM NaCl). This fraction was present at too low a concentration to work with and was routinely discarded. The second peak of activity, containing about 95% of total activity, was used in all subsequent experiments. Analysis of this second peak by SDS-PAGE showed it to consist of an apparently single Mr 98,000 polypeptide species (Fig. 2). Tumor hexokinase was also observed to migrate as a single peak of activity through gel permeation sizing columns during purification on HPLC (see "Materials and Methods"), consistent with a molecular weight of 98,000 (not shown). The specific activity of the final fraction, 144 units/mg protein, represents an 80-fold purification of hexokinase activity compared to the purified tumor mitochondrial fraction and a 212-fold purification compared to whole AS-30D cells (see Table 1).

The Michaelis constants of purified AS-30D hexokinase were calculated from the intercepts of double reciprocal (Lineweaver-Burk) plots of velocity versus substrate concentrations. Average values were obtained from three different experiments for each substrate. The apparent K_m values obtained for glucose (0.13 mM) and ATP (1.41 mM) are comparable with values reported for crude solubilized mitochondrial hexokinase from the H-91 rat hepatoma (12). In comparing the kinetic properties of the purified AS-30D mitochondrial hexokinase with those reported for hexokinase isozymes from normal rat tissues, the tumor enzyme seems to be most similar to type II hexokinase purified from rat skeletal muscle with apparent K_m values of 0.2 mM for glucose and 1.11 mM for ATP (32).

Fig. 1. Comparison of AS-30D hepatoma and rat liver hexokinase isozymes by DEAE-cellulose column chromatography. The isozyme composition of mitochondrial bound hexokinase (HK) from the highly glycolytic AS-30D rat hepatoma cell line (4) was compared with that expressed in control rat liver tissue (8). In A, hexokinase solubilized from AS-30D mitochondria was loaded onto a 0.9-x 6-cm column packed with DEAE-cellulose (Cellex D; Bio-Rad) equilibrated with 10 mM triethanolamine HCl (pH 7.6), 1 mM DTT, and 0.5 mM EGTA. The column was developed with a linear gradient from 0 to 0.5 M NaCl in the same buffer, and 0.5-ml fractions were collected and assayed for hexokinase activity. Data are plotted as the concentration of hexokinase activity versus fraction number. In B, liver tissue from female Sprague-Dawley rats was homogenized in H medium, centrifuged for 15 min at 20,000 x g, and loaded onto the same DEAE-cellulose column. The column was developed as described above, and fractions were assayed for hexokinase activity in 0.5 mM glucose (10) and glucose-6-phosphate to solubilize bound hexokinase. Solubilized enzyme has an average specific activity of 23.9 units/mg protein, a 13-fold increase over whole AS-30D mitochondria contain 1.8 units/mg protein of bound hexokinase activity. Incubation for 15 min with 1 mM glucose-6-phosphate results in the solubilization of 80% of the bound hexokinase activity. The solubilized enzyme has an average specific activity of 23.9 units/mg protein, a 13-fold increase over whole AS-30D mitochondria.

The solubilized tumor enzyme was further purified by sequential chromatography on gel permeation sizing columns followed by a DEAE-cellulose anion exchange column. Chromatography on the HPLC gel permeation columns (Protein Pak 1-60 and 300 SW columns from Waters/Millipore) resulted in a 3-fold increase in specific activity of the tumor enzyme to 67 units/mg protein (see Table 3). Purification of this fraction by anion-exchange chromatography on a Protein Pak DEAE-5PW column (Waters/Millipore) resulted in a further 2-fold increase in specific activity to 144 units/mg (Table 3). Two peaks of hexokinase activity were observed in the DEAE-cellulose eluate. A low amount of activity, about 5% of total, eluted at a lower salt concentration (approximately 120 mM NaCl). This fraction was present at too low a concentration to work with and was routinely discarded. The second peak of activity, containing about 95% of total activity, was used in all subsequent experiments. Analysis of this second peak by SDS-PAGE showed it to consist of an apparently single Mr 98,000 polypeptide species (Fig. 2). Tumor hexokinase was also observed to migrate as a single peak of activity through gel permeation sizing columns during purification on HPLC (see "Materials and Methods"), consistent with a molecular weight of 98,000 (not shown). The specific activity of the final fraction, 144 units/mg protein, represents an 80-fold purification of hexokinase activity compared to the purified tumor mitochondrial fraction and a 212-fold purification compared to whole AS-30D cells (see Table 1).

The Michaelis constants of purified AS-30D hexokinase were calculated from the intercepts of double reciprocal (Lineweaver-Burk) plots of velocity versus substrate concentrations. Average values were obtained from three different experiments for each substrate. The apparent K_m values obtained for glucose (0.13 mM) and ATP (1.41 mM) are comparable with values reported for crude solubilized mitochondrial hexokinase from the H-91 rat hepatoma (12). In comparing the kinetic properties of the purified AS-30D mitochondrial hexokinase with those reported for hexokinase isozymes from normal rat tissues, the tumor enzyme seems to be most similar to type II hexokinase purified from rat skeletal muscle with apparent K_m values of 0.2 mM for glucose and 1.11 mM for ATP (32).

Fig. 2. Analysis of purified AS-30D mitochondrial hexokinase by SDS-polyacrylamide gel electrophoresis. AS-30D mitochondrial hexokinase was purified by HPLC as described in "Materials and Methods." The purified enzyme was analyzed by SDS-PAGE on a 12 to 20% acrylamide gradient gel according to Laemmli (25). Peptide bands were visualized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad). Lane 1, purified AS-30D mitochondrial hexokinase (6 µg); Lane 2, whole AS-30D mitochondria (50 µg); Lane 3, molecular weight standards phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400).
Specific Binding of Purified AS-30D Hexokinase to Rat Liver Mitochondria. We have examined the effects of various treatments on the ability of purified AS-30D hexokinase to rebind to mitochondria and have compared the binding activities of purified hexokinases isolated from yeast, beef heart, and AS-30D rat hepatoma. As seen in Table 4, the purified AS-30D mitochondrial hexokinase retained its ability for mitochondrial binding. Under the conditions of this experiment, we observed binding of 74 milliunits of purified AS-30D hexokinase per mg of mitochondrial protein, or 63% of the binding activity observed with crude solubilized tumor hexokinase. The ability of mitochondria to bind hexokinase is dependent upon the type of hexokinase used (Table 4). Hexokinase purified from yeast exhibits very low binding activity, only 2% of that exhibited by solubilized tumor hexokinase. The binding capacity of the yeast enzyme was not significantly increased by increasing the amount of enzyme added to the mitochondria. Beef heart hexokinase exhibits moderate binding activity, 21% of the solubilized tumor hexokinase level.

It has been reported that binding of crude preparations of tumor hexokinase to its mitochondrial receptor is inhibited by the product glucose-6-phosphate and is highly sensitive to mild proteolytic treatment of the enzyme (9, 12, 14, 29). Significantly, mitochondrial binding of purified AS-30D hexokinase was almost completely inhibited by glucose-6-phosphate, while limited chymotryptic digestion resulted in the substantial loss of hexokinase binding activity (Table 4) without affecting catalytic activity (not shown). We conclude from these results that the mitochondrial hexokinase which we have purified from AS-30D cells retains its activity for specific binding to the outer mitochondrial membrane receptor.

Inhibition of Hexokinase Activity by Glucose-6-phosphate. The inhibitory effect of the product glucose-6-phosphate on hexokinase catalytic activity is shown in Fig. 3. In this experiment the ability of glucose-6-phosphate in the medium to inhibit either solubilized AS-30D hexokinase or mitochondrial bound AS-30D hexokinase was determined at different concentrations of inhibitor. Equal amounts of activity were added to each assay of either mitochondria-bound AS-30D hexokinase or else AS-30D hexokinase which had been solubilized from mitochondria by treatment with ATP. As shown in Fig. 3, glucose-6-phosphate is much more effective at inhibiting the activity of the soluble enzyme than the mitochondria-bound one. The 50% level of inhibition was reached at 0.1 mM glucose-6-phosphate for the soluble AS-30D hexokinase, while a 3.5-fold higher concentration of glucose-6-phosphate was required to inhibit the mitochondria-bound enzyme by an equivalent amount. These results confirm previous reports that mitochondrial hexokinase is less subject to product inhibition by glucose-6-phosphate than the soluble form of the enzyme (2, 4, 11).

Table 4 Specific reconstitution of purified tumor hexokinase with mitochondria

<table>
<thead>
<tr>
<th>Solubilized enzyme added</th>
<th>Bound hexokinase (milliunits/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solubilized AS-30D hexokinase</td>
<td>118 ± 9*</td>
</tr>
<tr>
<td>+ Glucose-6-phosphate</td>
<td>11 ± 3</td>
</tr>
<tr>
<td>Purified AS-30D hexokinase</td>
<td>74 ± 8</td>
</tr>
<tr>
<td>+ Glucose-6-phosphate</td>
<td>6 ± 2</td>
</tr>
<tr>
<td>Chymotrypsin treated AS-30D purifed hexokinase</td>
<td>4 ± 5</td>
</tr>
<tr>
<td>Purified beef heart hexokinase</td>
<td>25 ± 4</td>
</tr>
<tr>
<td>Purified yeast hexokinase</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

Rat liver, rather than tumor mitochondria, was routinely used to assess the capacity of hexokinases to bind to mitochondria. Although rat liver mitochondria have a lower binding capacity for hexokinase than do tumor mitochondria (9), the hexokinase receptor complex is present in liver mitochondria (16, 19) at an apparently reduced level. Moreover, liver mitochondria as isolated are essentially free of bound hexokinase, whereas it is difficult to prepare intact tumor mitochondria completely free of hexokinase. Differences in hexokinase binding between normal and tumor mitochondria most likely reflect quantitative differences in receptor levels rather than qualitative (i.e., chemical) differences in the respective receptors.

Discussion and Conclusion. We conclude from these results that the ability of isolated tumor mitochondria to bind hexokinase is dependent upon the type of hexokinase used (Table 4). Hexokinase purified from yeast exhibits very low binding activity, only 2% of that exhibited by solubilized tumor hexokinase. The binding capacity of the yeast enzyme was not significantly increased by increasing the amount of enzyme added to the mitochondria. Beef heart hexokinase exhibits moderate binding activity, 21% of the solubilized tumor hexokinase level.

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Amino Acid Composition of AS-30D Mitochondrial Hexokinase. The degree of homology exhibited between the different

Table 5 Comparison of amino acid compositions of hepatoma hexokinase and normal rat hexokinases

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>AS-30D hexokinase (this study)</th>
<th>Rat brain hexokinase (type I)*</th>
<th>Rat muscle hexokinase (type II)*</th>
<th>Rat liver hexokinase (type IV)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>10.1 ± 1.6*</td>
<td>11.3</td>
<td>10.4</td>
<td>10.2</td>
</tr>
<tr>
<td>Glu</td>
<td>11.9 ± 0.5</td>
<td>10.1</td>
<td>11.7</td>
<td>13.1</td>
</tr>
<tr>
<td>Ser</td>
<td>6.1 ± 1.5</td>
<td>5.5</td>
<td>5.9</td>
<td>6.9</td>
</tr>
<tr>
<td>Gly</td>
<td>13.3 ± 2.4</td>
<td>9.2</td>
<td>9.7</td>
<td>8.9</td>
</tr>
<tr>
<td>His</td>
<td>21.4 ± 0.4</td>
<td>2.0</td>
<td>2.6</td>
<td>2.4</td>
</tr>
<tr>
<td>Arg</td>
<td>6.7 ± 0.9</td>
<td>6.2</td>
<td>6.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Thr</td>
<td>5.6 ± 0.7</td>
<td>5.7</td>
<td>6.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Ala</td>
<td>7.1 ± 0.5</td>
<td>5.5</td>
<td>7.4</td>
<td>6.7</td>
</tr>
<tr>
<td>Pro</td>
<td>3.7 ± 0.5</td>
<td>4.1</td>
<td>2.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.7 ± 0.7</td>
<td>0.9</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Val</td>
<td>6.4 ± 1.2</td>
<td>7.4</td>
<td>7.4</td>
<td>7.1</td>
</tr>
<tr>
<td>Met</td>
<td>2.8 ± 1.0</td>
<td>3.4</td>
<td>3.4</td>
<td>4.0</td>
</tr>
<tr>
<td>Cys</td>
<td>1.6 ± 1.1</td>
<td>2.1</td>
<td>2.1</td>
<td>2.0</td>
</tr>
<tr>
<td>Ile</td>
<td>3.6 ± 0.8</td>
<td>5.7</td>
<td>4.1</td>
<td>3.8</td>
</tr>
<tr>
<td>Leu</td>
<td>9.2 ± 1.9</td>
<td>9.0</td>
<td>10.4</td>
<td>9.1</td>
</tr>
<tr>
<td>Phe</td>
<td>3.3 ± 0.8</td>
<td>4.1</td>
<td>4.1</td>
<td>4.0</td>
</tr>
<tr>
<td>Lys</td>
<td>4.8 ± 1.2</td>
<td>6.9</td>
<td>5.9</td>
<td>5.5</td>
</tr>
</tbody>
</table>

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hexokinase isozymes should be reflected in their amino acid compositions. A direct comparison of sequence homology requires the cloning and sequencing of the various hexokinase genes, a task which has not yet been performed for any organism above the level of yeast. In the absence of sequence data the relative homology between the tumor hexokinase isozyme and other isozymes from normal rat tissues can be estimated from amino acid composition data. The amino acid composition of purified AS-30D mitochondrial hexokinase was analyzed by reverse-phase HPLC of the PTC-derivatized amino acids as described in "Materials and Methods." Results for the tumor enzyme represent averaged values for five different preparations of purified AS-30D mitochondrial hexokinase. The data are presented in Table 5, along with the reported amino acid compositions of hexokinases type I (rat brain) (33), type II (rat skeletal muscle) (32), and type IV (rat liver) (34). It should be noted that these values were obtained from three different laboratories by different techniques.

Immune Cross-Reactivity of Hepatoma Hexokinase and Normal Rat Hexokinases. Polyclonal rabbit antibodies were prepared against the AS-30D mitochondrial hexokinase as described in "Materials and Methods." The ability of the anti-AS-30D hexokinase antibody to cross-react with hexokinases from normal rat tissues was determined by Western blot analysis using the anti-hexokinase antibody, followed by incubation with 125I-Protein A. Fig. 4 shows a densitometric trace of a representative autoradiograph. As shown in Fig. 4, Lane A, the purified AS-30D hexokinase reacts strongly with the rabbit antibody which was raised against it. Antibody cross-reactivity was seen with rat brain hexokinase (Fig. 4, Lane B), and with rat kidney hexokinase (Fig. 4, Lane C). No apparent cross-reactivity was observed with rat liver, the principal hexokinase isozyme of which is type IV (glucokinase) (Fig. 4, Lane D). In all three tissues a very strongly antibody-reactive M, ~66,000 peptide is observed (Fig. 4). The identity of this M, 66,000 peptide is unknown. However, it may represent a proteolytic product of hexokinase. From these results it would appear that tumor hexokinase exhibits some epitopic similarities with both rat brain and kidney hexokinases.

DISCUSSION

Results of experiments reported here provide new data relevant to the aberrant glucose catabolic properties of rapidly growing tumor cells. The data presented demonstrate that low \( K_m \) hexokinase activity is greatly elevated in the glycolytic AS-30D rat hepatoma cell line, over 100-fold higher than that detected in normal rat liver tissue. Previous studies have shown that the tumor hexokinase binds to a receptor complex located in the outer mitochondrial membrane (6, 9, 12, 14, 16–19). In the present report we demonstrate that hexokinase is the only glycolytic enzyme which apparently binds specifically to this mitochondrial receptor. The specific activity of AS-30D hexokinase in an isolated mitochondrial fraction was 3.5-fold higher than in a corresponding cytosolic fraction, while for all other glycolytic enzymes examined this ratio was substantially less than one. Mitochondrial binding of hexokinase has been reported to result in an increased affinity of the enzyme for substrate, a decrease in inhibition of enzyme activity by the product glucose-6-phosphate, and in a preferred access to mitochondrial synthesized ATP (2, 4, 5, 11–13, 15, 35). It has recently been demonstrated that the mitochondrial receptor for hexokinase consists at least in part of the outer membrane channel-forming protein (also known as VDAC or porin) (16–19). Since all hydrophilic, low-molecular-weight molecules, including ATP and ADP, must presumably cross the outer mitochondrial membrane through the channel-forming protein, it is easy to envision how hexokinase bound to this same protein could obtain preferential access to matrix-generated ATP. The specificity of this receptor for binding hexokinase would appear to be significant in supporting elevated glucose catabolism in tumor cells.

We report a rapid HPLC protocol for purification of AS-30D mitochondrial hexokinase which results in the purification to apparent homogeneity of a tumor enzyme of high specific activity (144 units/mg protein). Significantly, the purified tumor mitochondrial hexokinase retains its ability to rebind to the mitochondrial receptor. Moreover, binding is prevented by either glucose-6-phosphate or chymotrypsin, characteristic of hexokinase binding to the mitochondrial receptor (9, 12, 14, 29). It would thus appear that the purification technique used does not substantially affect the binding ability of AS-30D hexokinase.

Characterization of the AS-30D mitochondrial hexokinase by its kinetic and chromatographic properties indicates that the major form corresponds to the type II isozyme found in normal rat tissues. However, comparison of the amino acid compositions of normal and hepatoma hexokinases indicates that the tumor enzyme is not identical to any known hexokinase isozyme which has been purified from normal rat tissues. Polak and Wilson (36) observed that bindable forms of normal rat brain hexokinase contain an N-terminal binding domain which is required for interaction with the mitochondrial receptor. Presumably this binding domain is removed by limited proteolysis in those tissues in which hexokinase does not bind to the particulate fraction (9, 13, 36). It is possible that the difference in amino acid composition of tumor hexokinase may be related to the presence of a similar binding domain on the N-terminal
end of the protein. Since the tumor enzyme is N-terminally blocked, it has not yet been possible to sequence this domain of AS-30D hexokinase for comparison with the rat brain enzyme.

Studies with antibodies made against AS-30D hexokinase show immune cross-reactivity with normal hexokinases from rat brain and rat kidney, but not rat liver tissue. It should be noted that, under the conditions used, the results indicate either the presence or absence of epitopic similarities between tumor and normal isozymes, not how similar in structure the isozymes are. Still, we consider it significant that the anti-AS-30D antibody does not cross-react with type IV hexokinase (glucokinase), which is the predominant isozyme present in normal liver tissue. The type IV hexokinase of liver differs substantially from hepatoma hexokinase in other characteristics as well. The molecular mass of type IV hexokinase is 48,000 daltons (34), in contrast to the 98,000-dalton tumor enzyme. The Km for glucose of type IV hexokinase is two orders of magnitude higher than the tumor isozyme (12 mM versus 0.13 mM). Type IV hexokinase appears to be predominantly localized in the cytosol, while 60% of tumor hexokinase occurs bound to the outer mitochondrial membrane (14). Finally, the liver enzyme is not inhibited by the product glucose-6-phosphate, whereas the soluble tumor enzyme is significantly inhibited by physiological concentrations of glucose-6-phosphate. (It should be noted that the mitochondrial bound tumor enzyme is much less sensitive to glucose-6-phosphate inhibition). Thus, in the transformation from low glucose-utilizing normal liver to high glucose-utilizing hepatoma, the major isozyme of hexokinase shifts to a form with substantially different kinetic, physical, and regulatory properties.

Note Added in Proof.

Since this manuscript was submitted for publication Radajkovic and Ureta (37) have reported the purification of hexokinases Type B and C from the Novikoff hepatoma. Hexokinase B is derived from the mitochondria and is considered to be hexokinase II.

REFERENCES

Purification and Characterization of a Bindable Form of Mitochondrial Bound Hexokinase from the Highly Glycolytic AS-30D Rat Hepatoma Cell Line


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