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EXPRESSION OF HUMAN BRAIN HEXOKINASE IN ESCHERICHIA COLI: PURIFICATION AND CHARACTERIZATION OF THE EXPRESSED ENZYME[1]

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Human brain hexokinase (hexokinase I) was produced in Escherichia coli from a synthetic gene under control of the bacteriophage T7 promoter. The expressed coding region derives from a human cDNA clone thought to specify hexokinase I based on amino acid sequence identity between the predicted translation product and hexokinase I from rat brain. The open reading frame from this cDNA was fused to the promoter and 5′ flanking region of T7 gene 10, and expressed in E. coli by induction of T7 RNA polymerase. Induced cells contained a hexokinase activity and an abundant protein of apparent molecular weight 100,000, neither of which was present in cells lacking T7 RNA polymerase. Enzyme purified to near homogeneity consisted of a 100,000 Da protein, the size predicted from the nucleotide sequence of the expressed cDNA. The purified enzyme had Michaelis constants of 32 μM and 0.3 mM for glucose and ATP, respectively, and bound to rat liver mitochondria in the presence of $MgCl_2$. Enzymatic activity was inhibited by glucose-6-P and this inhibition was relieved by inorganic phosphate. Deinhibition by phosphate is a property specific to brain hexokinase.

Hexokinase I, or brain hexokinase, is one of four hexokinase isozymes present in mammalian tissues. The other three isozymes, although essential components of tissue glycolysis, are not thought to be regulated at the molecular level (1-4). Hexokinase I, however, is a regulated enzyme and serves a critical role in controlling the rate of glycolysis in both brain and red blood cells. The hexokinase isozymes share some similar properties, e.g., both hexokinase I and hexokinase II are inhibited by glucose-6-P and bind to the mitochondrial outer membrane (5). Hexokinase I differs from hexokinase II, however, in that inorganic phosphate (Pi) prevents inhibition by glucose-6-P (1-3). This unique property of inhibition by the product glucose-6-P and deinhibition by Pi is the cardinal characteristic of hexokinase I.

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The kinetic and chemical mechanisms of hexokinases, as well as their physiological roles in metabolism, have been studied in detail (1-4). However, analysis of human brain hexokinase and its role as a critical regulator of glycolysis in brain tissue, has been hampered by the difficulty of obtaining sufficient amounts of pure enzyme from its native tissue. The availability of cDNA clones coding for hexokinase I (6) may overcome this problem by affording production of the enzyme in bacterial hosts.

This report describes the expression in E. coli of high specific activity human hexokinase I using a cDNA clone derived from kidney tissue and its purification and characterization.

EXPERIMENTAL PROCEDURES

Miscellaneous procedures Recombinant DNA manipulation was performed using standard procedures (7). Oligonucleotides were synthesized by the Iowa State University Nucleic Acid Facility using a Biosearch 8570EX automated DNA synthesizer. Single-stranded DNA (ssDNA) was prepared as described (8). Nucleotide sequence analysis was by the chain termination method (9). E. coli strain TG-1 (Amersham Corporation, Arlinton Hts., IL) was used for amplification of plasmids and/or production of ssDNA. Proteins were separated by polyacrylamide in the presence of SDS according to Laemmli (10). Protein concentration was determined by the method of Bradford (11). A cDNA clone encoding human hexokinase I was a generous gift of Dr. G.I. Bell.

Construction of hexokinase I expression vector pET-HKI The cDNA sequence used for expression of hexokinase I is included in plasmid pGEM4Z-HKI (6). The 1.1 kb NarI-BamHI fragment of pGEM4Z-HKI, containing the amino terminal 283 codons of the hexokinase I open reading frame, was ligated into pUC119 (8) digested with these two enzymes. The resultant plasmid, p119HK, was used in the oligonucleotide directed site-specific mutagenesis procedure of Kunkel (12) to create an NdeI recognition site that included the initiation codon of the hexokinase I open reading frame. The oligonucleotide used to introduce the mutation was 5'-ACCGCCAGC**ATAT**GATCGCC-3', which anneals to the coding strand of the cDNA. Presence of the mutation was verified by nucleotide sequence analysis. The NdeI-BamHI fragment containing codons 1-283 (nucleotides 1-850: ref. 6) of the cDNA was then purified from the mutant plasmid and ligated into plasmid pET-11a (Novagen, Inc., Madison, WI) to produce plasmid pINT. pET-11a contains the promoter and 5' flanking region of gene 10 from bacteriophage T7, followed immediately by a unique NdeI site. Codons 284-921 of the hexokinase cDNA, contained on the 1.9 kb fragment of pGEM4Z-HKI (nucleotides 851-2762: ref.6), was then ligated into the unique BamHI site of pINT to reconstruct the full length cDNA sequence in the final construction, expression plasmid pET-HKI. The BamHI site downstream of the open reading frame is located 10 nucleotides from the termination codon, immediately upstream of a T7 transcriptional terminator sequence provided by pET-11a.

Expression of hexokinase I pET-HKI was transformed into E. coli strain BL21(DE3) (Novagen Inc.), a phage lambda lysogen containing the T7 RNA polymerase gene under control of the lacUV5 promoter. A 5 ml pregrowth culture of the transformed strain in M9 medium (13), plus 40 mg/L ampicillin and 4 mg/L glucose, was grown overnight, then added to 500 ml of the same medium. The culture was shaken at 37°C to early log phase (A_{600} = 0.4). IPTG was then added to a final concentration of 0.4 mM, to

induce the T7 RNA polymerase gene. Cultures were shaken vigorously for an additional 24 to 40 hours at 22°C.

Purification of hexokinase I Cells were harvested by centrifugation at 4000 x g for 10 min, and washed once with 10 mM KPi, pH 7.0. The washed cell pellet was stored at -20°C overnight. Frozen cells were thawed in 120 ml 5 mM KPi, pH 7.0, containing 25 mg lysozyme and 1 mM PMSF. After 40 min at room temperature, the cell suspension was heated in a 55 °C waterbath for 3 min to complete lysis. To the mixture, 600 μl diluted DNAase (150 μl 10 mg/ml DNAase stock + 650 μl 1 M $MgCl_2$) was added, and the suspension was incubated at room temperature for 20 min. The lysate was centrifuged at 17,500 x g for 45 min. To each 100 ml of supernatant fluid, 10 ml 11% streptomycin sulfate was added with stirring. The suspension was centrifuged at 17,500 x g for 15 min. The supernatant fluid was pooled and solid $(NH_4)_2SO_4$ was added slowly with stirring to a final concentration of 25%. After centrifugation at 17,500 x g for 15 min, the supernatant fluid was pooled and additional solid $(NH_4)_2SO_4$ was added slowly to a final concentration of 35%. The suspension was centrifuged at 17,500 x g for 15 min and the supernatant fluid was discarded. The pellet was resuspended in a minimal volume of 50 mM KPi buffer, pH 7.0, containing 70 μl 2-mercaptoethanol and dialyzed against the same buffer overnight. The dialyzed fraction was injected onto a 21.5 x 150-mm Spherogen-TSK DEAE 5PW HPLC column. Elution was carried out at a flow rate of 1.0 ml/min. Solvents used were (A) 50 mM KPi, pH 7.0, containing 70 μl/L 2-mercaptoethanol and (B) 50 mM KPi, pH 7.0, containing 70 μl/L 2-mercaptoethanol, plus 1 M KCl. The enzyme was eluted by a series of linear gradients: 0-5 min, 100% A; 5-10 min, gradient to 35% B; 10-30 min, gradient to 60% B; 30-70 min, gradient to 80% B; 70-80 min, 20% A, 80% B, 80-90 min, gradient to 100% B; 90-95 min, 0% A, 100% B; 95-100, gradient back to 100% A. The fractions with high enzyme activity were pooled, concentrated, and dialyzed against 25 mM KPi, pH 7.0, containing 70 μl 2-mercaptoethanol overnight. The dialyzed sample was then injected onto a 100 x 7.8-mm Bio-Gel hydroxyapatite (HPHT) HPLC column. Solvents used were (A) 25 mM KPi, pH 7.0, (B) 0.5 M KPi pH 7.0. The elution gradients used were: 0-5 min, 100% A; 5-40 min, gradient to 70% A, 30% B; 40-50 min, gradient to 50% A, 50% B; 50-55 min, gradient to 0% A, 100% B, 55-57 min, washed with 100% B; 57-60 min, gradient back to 100% A. The fractions with high enzyme specific activity were pooled, concentrated, and dialyzed against 50 mM KPi buffer, pH 7.5, containing 0.1 M KCl, and 70 μl 2-mercaptoethanol.

Hexokinase activity assay Hexokinase activity was determined spectrophotometrically by following the increase in absorbance of NADPH at 340 nm (14). For the glucose-6-P inhibition studies, hexokinase activity was determined by measuring the formation of ADP, using a system coupled with excess pyruvate kinase and lactate dehydrogenase. The assay solution contained 0.1 M Tris-HCl, pH 8.0; 10 mM KCl, 5 mM ATP-Mg^{2+}, 0.5 mM NADH, 0.5 mM phosphoenolpyruvate, 2 mM $MgCl_2$, 5 μl lactate dehydrogenase/pyruvate kinase (Sigma, St. Louis) and appropriate glucose-6-P and Pi. One unit of hexokinase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of glucose-6-P per minute.

Binding of the expressed hexokinase to mitochondria Mitochondria were isolated from rat liver by using the procedure of Schraitman and Greenawalt (15). The freshly prepared mitochondria were used in binding experiments. The binding experiments were carried out by the procedure described by Arora and Pederson (16) in the presence of 10 mM $MgCl_2$.

RESULTS

pET-HKI directs expression of a hexokinase Induction of T7 RNA polymerase in cells containing pET-HKI resulted in accumulation of a 100

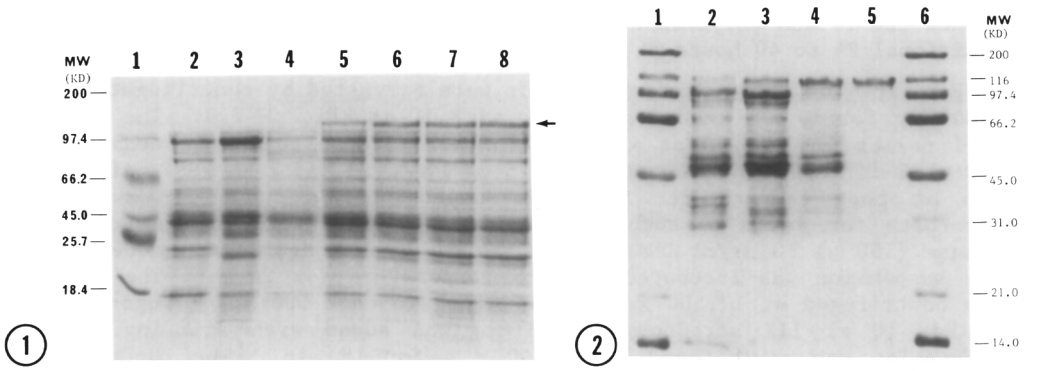

Fig. 1. SDS-PAGE of total cellular proteins of BL21(DE3) and BL21(DE3-HK). Cells were withdrawn from culture at different IPTG induction times. Proteins were analyzed on a 12% polyacrylamide gel and stained with Coomasie brilliant blue R-250. Lane 1, protein standards; Lane 2, proteins from control cell BL21(DE3) with IPTG induction; Lane 3, cytosol proteins from BL21(DE3-HK) without IPTG induction; Lane 4-8, cytosol proteins from BL21(DE3-HK) at 0, 10, 20, 30 and 40 hrs after IPTG induction, respectively. The solid arrow indicates the expressed protein.

Fig. 2. SDS-PAGE analysis of human hexokinase purification procedure. All samples were analyzed on a 12% polyacrylamide gel and stained with Coomasie brilliant blue R-250. Lane 1 and lane 6, protein standards; Lane 2, 6 μg of cell extract; Lane 3, 6 μg of ammonium sulfate precipitate; Lane 4, 4 μg of DEAE HPLC pool; Lane 5, 2 μg of HPHT HPLC pool.

KDa protein that was not present in host cells with no plasmid or when the polymerase was not induced (Fig. 1, lane 2 and 3, respectively). The maximal expression of this protein was observed after 40 hours at 22°C (Fig. 1, lane 7). Enzymatic assays of the total cell lysates showed the presence of a hexokinase activity that was not present in control cells (data not shown).

Purification of hexokinase Hexokinase was purified from induced E. coli containing pET-HKI as described in "Experimental Procedures". Samples from various steps in the purification protocol were analyzed by SDS-PAGE (12%) (Fig. 2) and the results are summarized in Table I.

Properties of the expressed human brain hexokinase The expressed hexokinase was purified to near homogeneity. The purified enzyme consists of a single polypeptide with a molecular weight of about 100,000 as judged from both SDS-PAGE (Fig. 1) and gel-filtration HPLC (data not shown). This hexokinase had a specific activity of 58 (Table I), which is close to the reported value for hexokinase I isolated from other sources as well as from human tissues (3,4,17). The Km values of the substrates were determined to be 32 μM and 0.3 mM for glucose and ATP, respectively.

Binding of the expressed human brain hexokinase to rat liver mitochondria It has been shown that soluble hexokinase can bind to liver mitochondria in the presence of magnesium (18) and this association has

Table I. Purification of expressed human hexokinase in E. coli

Purification Steps	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Cell-free extract	220	881	4.00	100	1.0
Streptomycin and $(NH_4)_2SO_4$	140	698	5.00	79.2	1.3
DEAE HPLC	29.8	506	17.0	57.4	4.3
HPHT HPLC	6.9	365	58.2	41.4	13

been suggested to be due to the presence of a specific peptide sequence at the N-terminus of the enzyme (19). Arora et al. (20) reported that ascites tumor hexokinase expressed in E. coli can bind to mitochondria in the presence of magnesium. Using their protocols, we obtained identical results with the expressed human hexokinase.

Inhibition of the expressed hexokinase by glucose-6-phosphate and deinhibition by Pi A unique characteristic of brain hexokinase is that the enzyme can be inhibited by its product, glucose-6-P, and the inhibition can be relieved by Pi (3,4). To confirm that the expressed protein is brain hexokinase, we carried out experiments to see whether the enzyme could be inhibited by glucose-6-P and whether the inhibition could be reversed by Pi. Fig. 3 shows a plot of (rate of hexokinase)$^{-1}$ vs glucose-6-P concentration. Pi concentrations up to 6 mM were without appreciable effect on the initial rate of the expressed hexokinase. However, in the presence of inhibitory concentrations of glucose-6-P, Pi was found to relieve the inhibition at a concentration of 6 mM.

DISCUSSION

We report here the construction of an expression vector for human hexokinase, expression of the enzyme in E. coli, and purification procedures for isolating high levels of pure expressed enzyme. Plasmid pET-HKI directs high-level expression in E. coli of a hexokinase coded for by a cDNA copy of a mRNA from human kidney cells. This enzyme was purified to near homogeneity. Kinetic analysis identifies the enzyme as hexokinase I, the major hexokinase isozyme of human brain tissue (1-4). The molecular weight of the expressed enzyme is approximately 100,000, in close agreement with that of hexokinase I purified from brain. The enzyme is inhibited by glucose-6-P, and binds to mitochondria in the presence of

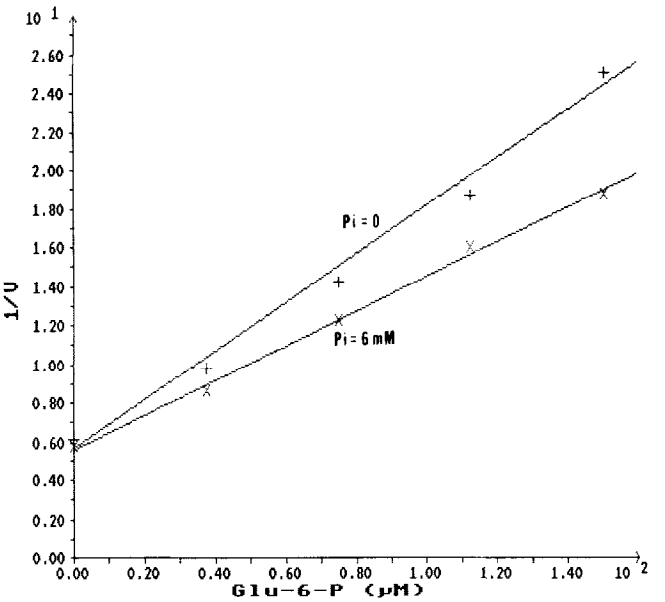

Fig. 3. Plot of (velocity of hexokinase)$^{-1}$ vs. glucose-6-P concentration at 0 and 6 mM Pi. Hexokinase activity was determined by measuring the formation of ADP, using a system coupled with pyruvate kinase and lactate dehydrogenase. The glucose-6-P and Pi concentrations are as indicated.

$MgCl_2$, properties shared by hexokinase I and hexokinase II. The expressed enzyme is distinguished from hexokinase II based on the fact that Pi relieves inhibition by glucose-6-P. Hexokinase I is the only hexokinase isozyme that displays this property (3,4).

While this research was in progress, Arora et al. (20,21) reported overexpression of hexokinase from mouse hepatoma cell lines. Their expressed enzyme has a specific activity of 0.2, a Km for ATP of 2.4 mM, and binds to mitochondria; however, its low specific activity and high Km for ATP indicate it is a unique hexokinase and may not be hexokinase I.

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