Communication

Functional Organization of Mammalian Hexokinase II

RETENTION OF CATALYTIC AND REGULATORY FUNCTIONS IN BOTH THE NH₂- AND COOH-TERMINAL HALVES*

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The mammalian hexokinase (HK) family includes three closely related 100-kDa isoforms (HKI–III) that are thought to have arisen from a common 50-kDa precursor by gene duplication and tandem ligation. Previous studies of HKI indicated that a glucose 6-phosphate (Glu-6-P)-regulated catalytic site resides in the COOH-terminal half of the molecule and that the NH₂-terminal half contains only a Glu-6-P binding site. In contrast, we now show that proteins representing both halves of human and rat HKII have catalytic activity and that each is inhibited by Glu-6-P. The intact enzyme and the NH₂- and COOH-terminal halves of the enzyme each increase glucose utilization when expressed in Xenopus oocytes. Mutations corresponding to either Asp-209 or Asp-657 in the intact enzyme completely inactivate the NH₂-terminal COOH-terminal half enzymes, respectively. Mutation of either of these sites results in a 50% reduction of activity in the 100-kDa enzyme. Mutation of both sites results in a complete loss of activity. This suggests that each half of the HKII molecule retains catalytic activity within the 100-kDa protein. These observations indicate that HKI and HKII are functionally distinct and have evolved differently.

The four mammalian hexokinases (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1) initiate glucose metabolism by catalyzing the conversion of glucose to glucose 6-phosphate (Glu-6-P). Hexokinases I–III (HKI–III) have a molecular mass of 100 kDa, a high affinity for glucose, and are feedback-inhibited by physiologic concentrations of Glu-6-P. Glucokinase (HKIV or GK), a 50-kDa protein with a lower affinity for glucose, is not inhibited by physiologic concentrations of Glu-6-P, nor is the 50-kDa yeast enzyme. The 100-kDa isoforms are thought to have arisen from a 50-kDa precursor through gene duplication and tandem ligation (1). The high degree of amino acid similarity between the NH₂- and COOH-terminal halves of a given HK with each other, with yeast HK, and with GK, supports this hypothesis (2–4). Additional support for a gene duplication event is provided by the demonstration of exon size conservation between GK and the NH₂- and COOH-terminal halves of HKII and by localization of the likely fusion site within the HKII gene (5, 6).

Analysis of the functional domains of the hexokinases has also been performed to learn how these enzymes evolved. Glucose and ATP bind to the COOH-terminal half of the 100-kDa HKI molecule, whereas Glu-6-P binds to the NH₂-terminal half (7–10). If a yeast-like HK is the precursor, it is necessary to hypothesize that the catalytic site is retained in the COOH half, but is lost in the NH₂ half of HKI and replaced by a regulatory site (1). The two halves of the HKI molecule have been obtained by proteolytic cleavage (11) and by construction of cDNA-based vectors that direct their expression (12, 13). As predicted from studies of the intact molecule, the COOH-terminal half of HKI is catalytically active, and the NH₂-terminal half is not (11–13). However, the observation that the COOH-terminal half is inhibited by Glu-6-P was not expected based on the behavior of the intact enzyme (11, 13). In view of these observations, a 50-kDa precursor with both catalytic and regulatory sites, such as exists in various marine organisms, silkworms, and schistosomes (14–17), could have been duplicated to form the 100-kDa hexokinases (11, 13). If so, the catalytic site in intact HKI must be lost or occluded in the NH₂-terminal half and retained in the COOH-terminal half, whereas the regulatory site could involve either or both halves. Although it is assumed that all of the 100-kDa hexokinases evolved similarly, and that they share structural and functional features, only the HKI isoform has been analyzed in detail. We show here that the NH₂- and COOH-terminal halves of rat and human HKII (N-HKII and C-HKII) both have catalytic activity in the intact enzyme and when expressed as discrete proteins. In addition, both halves of HKII are inhibited by Glu-6-P. These observations support the hypothesis that a 50-kDa precursor with both catalytic and regulatory sites was duplicated to form the 100-kDa hexokinases, and that HKII retains more of the characteristics of the ancestral gene than does HKI.

EXPERIMENTAL PROCEDURES

Expression and Purification of Fusion Proteins—The pGef plasmid, constructed by insertion of a bacterio phage T1 origin into the pGex-3x vector (Pharmacia Biotech Inc.), was linearized at a Small restriction site located adjacent to the glutathione S-transferase (GST) cDNA. The cDNAs encoding human HKII, N-HKII, and C-HKII (18, 19) were then ligated into pGef to obtain pGST-HKII, pGST-N-HKII, and pGST-C- HKII plasmids, respectively. pGST-N-HKII encodes amino acids 1–469, and pGST-C-HKII contains the coding sequence for amino acids 470–917 of human HKII. Escherichia coli, XL-1 Blue strain (Stratagene), were transformed with the recombinant plasmid and were grown to an A₆₀₀ of ~0.6 in LB medium containing 200 μg/ml ampicillin. Isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.4 mM, and the cells were incubated at ambient temperature for 4 h. Cells were collected by centrifugation (4,400 × g for 10 min at 4°C) and
resuspended in HB buffer (50 mM triethanolamine, pH 7.3, 50 mM KCl, 10 mM glucose, 1 mM dithiothreitol plus protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM benzamidine, 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride). The resuspended cells were then sonicated at 4°C for ~20 s, and the bacterial lysates were cleared of cellular debris by centrifugation (12,000 × g for 5 min at 4°C). The bacterial extract containing the fusion protein was applied directly to glutathione-Sepharose 4B (Pharmacia). The mixture was allowed to incubate at 4°C for 1 h. The suspension was centrifuged (12,000 × g for 3 min at 4°C), and the pellet was washed twice in 15 bed volumes of a HB buffer including the protease inhibitors and twice in 15 bed volumes of HB buffer. The fusion proteins were then eluted from the column with 0.75 ml of HB buffer containing 10 mM reduced glutathione. The eluted proteins were assayed for hexokinase activity as described below and were visualized on 7% SDS-polyacrylamide gels.

Polymerase Chain Reaction (PCR)-based Mutagenesis of Hexokinase II—Site-directed mutations were generated by two separate PCR reactions with pGST-HKII plasmid DNA as a template. To generate D209A, the primer A1943 (5'-GGTCCCAGTGGTCCGCACACGACC-3', the underlined letters here and below indicate base substitutions) and an upstream HKII specific primer C1474 (5'-GGTCCAGTGGTCCGCACACGACC-3') were used to amplify a 720-base pair (bp) segment of the human cDNA with the desired mutation. A second PCR was performed using a primer A2083 that is the reverse complement of A1943 and a downstream HKII specific primer A680 (5'-TGGTGATCGGCACCCGCTG-3') to amplify a 785-bp segment, whose first 28 bp are identical to the 3' end of the first PCR product. Equal amounts of the two purified PCR products were mixed, denatured and annealed, and used as a template for a third PCR reaction with primers C1474 and A680 to generate a 1477-bp product with the desired amino acid changes. The final PCR product was digested with BamHI and SalI and was used to replace the wild type BamHI-SalI restriction fragment in pGST-HKII plasmid.

To construct D657A, primers C477 (5'-CATTGTGCACGTGTGCGTAC-3') and C1191 (5'-AGTTGCAGTCTGCTGCTGACAGA-3') were used in the first PCR reaction, and primers A2083 (reverse complement of C1191) and B1046 (5'-TGTTGATCG-3') were used in the second PCR reaction. The third PCR product was digested with XhoI and ClaI and cloned into pGST-HKII. The PCR fragments inserted into pGST-HKII were validated by DNA sequencing.

Glucose Phosphorylation Assays—The K<sub>m</sub> values for glucose and ATP of HKII enzymes were determined by measuring the phosphorylation of [U-14C]glucose as a function of added glucose (0.1–3 mM) and ATP (0.2–10 mM). The apparent K<sub>m</sub> values for Glu-6-P were determined, with Glu-6-P ranging from 0 to 10 mM, at a glucose concentration of 0.1 mM and an ATP concentration of 10 mM for D209A and D657A and 5 mM for the other enzymes. An aliquot (5–10 μl) of purified protein was added to 90 μl of assay buffer containing 50 mM triethanolamine, pH 7.3, 20 mM MgCl<sub>2</sub>, 100 mM KCl, 1 mM dithiothreitol, and 0.05 μCi of [U-14C]glucose. After an incubation of 30 min at ambient temperature, the reaction was terminated with the addition of 200 μl of isopropanol alcohol. The [U-14C]Glu-6-P produced was isolated and quantified as described previously (20). The rate of glucose phosphorylation was linear during the assay, and the concentration of Glu-6-P at the termination of the assay was less than 0.1 mM (when not added to the assay; results not shown). The kinetic parameters were determined for each experiment by non-linear least-squares regression using an equation for substrate affinity or noncompetitive inhibition (21). The specific activity of the human enzymes, corrected for the contribution of the GST protein, was measured using a Glu-6-P dehydrogenase/NAD<sup>+</sup> -coupled assay (22). In this case, 1 unit of HK activity is defined as the amount required to produce 1 μmol of Glu-6-P in 1 min at 37°C.

Oocyte Studies—The expression of GLUT3 mRNA in Xenopus laevis oocytes was performed as described previously (23). Wild type and mutant rat mRNAs were obtained by using the Stratagene In Vitro Transcription Kit and rat HKII cDNA as the template (6). Oocytes were injected with 15 ng of GLUT3 mRNA with or without either 50 ng of HKII mRNA, 35 ng of C-HKII mRNA (encoding amino acids 469–917), or 25 ng of N-HKII mRNA (encoding amino acids 1–468). Co-expression of the rat HKIIs with GLUT3 had no effect on the rate of 3-O-methyl-glucose uptake (data not shown). Coupled transport and phosphorylation of glucose was measured by incubating oocytes in [2-<sup>3</sup>H]glucose with quantitation of [3H]2-O-glucose released (23). Protein extracts were obtained from 10 oocytes and were assayed for hexokinase activity (see above).

RESULTS AND DISCUSSION

The NH<sub>2</sub>- and COOH-terminal Halves of Human and Rat HKII Are Catalytically Active—The availability of the human HKII cDNA (18, 19) allowed us to test the N- and C-terminal halves of this isoform directly for activity. GST fusion proteins of intact HKII, N-HKII, and C-HKII were expressed in E. coli and purified to homogeneity (Fig. 1). The kinetic properties of these three proteins are shown in Table I. The specific activities of human N-HKII and C-HKII, although less than that measured for the intact enzyme, are comparable. All three enzymes had similar K<sub>m</sub> values for glucose, but C-HKII had a significantly lower affinity for ATP and Glu-6-P than did the other enzymes. Based on this experiment, we conclude that N-HKII and C-HKII are both active enzymes.

The purification of the human enzymes involved the binding of the GST fusion protein to a glutathione affinity column (Fig. 1). To evaluate whether the GST addition altered the kinetic properties of any of the enzymes, and to check for possible species differences, the mRNAs encoding the intact molecule, as well as the N- and C-terminal halves of rat HKII, were injected into X. laevis oocytes, and HK activity in the cell extracts was measured. In this cell-based system, as with the purified human HKII proteins, both the NH<sub>2</sub>- and COOH-terminal halves of rat HKII actively phosphorylate glucose (Table II). The kinetic parameters determined for the rat enzymes are similar to those observed for the human enzymes (Tables I and II); thus, the GST addition does not appear to affect the enzymes. The N-HKII and intact enzymes of both species have significantly higher affinities for ATP and Glu-6-P than does C-HKII. In addition, the intact HKII enzymes from both human and rat have kinetic parameters that resemble those previously observed in rat skeletal muscle, in which HKII is known to be the predominant isoform (Ref. 24 and Tables I and II).

No hexokinase activity was observed in extracts obtained after the injection of mRNAs encoding either N-HKII or C-HKII with a single Asp to Ala substitution (corresponding to Asp-209 and Asp-657 in intact HKII) into oocytes (data not shown). These results identify Asp-209 and Asp-657 as critical residues in the catalytic sites of N- and C-HKII, respectively, as is the corresponding amino acid in GK (Asp-205) and the single,
COOH-terminal catalytic site in HKI (Asp-657) (13, 25).

Having identified a single amino acid mutation that results in the loss of activity of rat N-HKII and C-HKII, we constructed COOH-terminal catalytic site in HKI (Asp-657) (13, 25). In this context, N-HKII is as active as C-HKII or the HKII, and C-HKII (see Tables I and III). D657A has a catalytic activity (D209A

| Table I |
|---|
| Kinetic measurements of human HKII and its NH2- and COOH-terminal halves |
| Specific activity | Km(glucose) | Km(ATP) | Ki(Glu-6-P) |
| units/mg | mM | mM | mM |
| HKII | 147 ± 4 | 0.34 ± 0.06 | 1.02 ± 0.14 | 0.21 ± 0.05 |
| N-HKII | 94 ± 50 | 0.46 ± 0.07 | 0.78 ± 0.26 | 0.11 ± 0.01 |
| C-HKII | 60 ± 3 | 0.51 ± 0.08 | 3.8 ± 0.2* | 2.7 ± 0.4* |

*Km or Ki value that is significantly different from that of intact human HKII (p < 0.05 as determined by one-way ANOVA).

COOH-terminal halves of HKII are active enzymes in the intact enzyme, and the Km values for glucose are comparable. It should be noted that these Km values are higher than those determined using the oocyte extracts (see Table II), which may reflect less than optimal access of glucose to the hexokinase due to limited transport or intracellular compartmentalization of the substrate. These results demonstrate that each HKII protein is active when expressed in an intact cell and each, in conjunction with the glucose transporter, results in increased glucose utilization by the cell.

Glu-6-P regulates the NH2- and COOH-terminal Halves of Human and Rat HKII—The demonstration that both halves of HKII are catalytically active distinguishes this enzyme from HKI in which only the COOH half is active (11–13). The ex-
pressed COOH half of HKI is inhibited by Glu-6-P and has a $K_i$ value similar to that of the intact enzyme (11). However, both expressed halves of HKI bind Glu-6-P (13), and Glu-6-P binds with higher affinity to the NH$_2$ half of HKI in the intact enzyme (9, 10). Thus, the mechanism of Glu-6-P inhibition in the intact HKI enzyme remains unclear. It was therefore of interest to determine whether each half of HKII is inhibited by Glu-6-P. As illustrated in Tables I and II, human and rat HKII and N-HKII and rat skeletal muscle HKII (24), all have comparable apparent $K_i$ values for Glu-6-P. The human and rat C-HKII enzymes have a significantly higher $K_i$ for Glu-6-P when compared to HKII and N-HKII. Also, as shown in Table III, D657A has a $K_i$ value similar to that observed for HKII and N-HKII, whereas D209A has a significantly higher $K_i$ value than either HKII or C-HKII. Thus, although both halves of HKII are sensitive to Glu-6-P inhibition, the NH$_2$ half most resembles the intact enzyme.

The present model for the evolution of hexokinases, based on studies with HKI, suggests that HKI–III evolved by duplication of a gene encoding a 50-kDa, Glu-6-P-sensitive hexokinase. As a consequence of the duplication process, the regulatory site in the COOH half and the catalytic site in the NH$_2$ half of the 100-kDa HKs are lost or masked. The observation that both the NH$_2$- and COOH-terminal halves of HKII have catalytic and Glu-6-P regulatory functions necessitates a re-evaluation of this hypothesis, at least as regards this isoform.

The results presented here suggest that HKII possesses properties that may allow it to play unique roles in glucose metabolism under different physiological conditions. According to this hypothesis, each half of HKII may respond to Glu-6-P levels differently since each has distinct $K_i$ values for Glu-6-P. In addition, the two halves of HKII are likely to show different activities over a range of intracellular ATP concentrations. Therefore, although the NH$_2$- and COOH-terminal halves of HKII carry out the same basic reaction in the intact enzyme, they may respond differently to substrate and inhibitor concentrations. These intriguing properties of HKII and their possible physiological significance warrant further investigation.

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