Functional Interaction between the N- and C-terminal Halves of Human Hexokinase II

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Mammalian hexokinases (HKs) I–III are composed of two highly homologous ~50-kDa halves. Studies of HKI indicate that the C-terminal half of the molecule is active and is sensitive to inhibition by glucose 6-phosphate (G6P), whereas the N-terminal half binds G6P but is devoid of catalytic activity. In contrast, both the N- and C-terminal halves of HKII (N-HKII and C-HKII, respectively) are catalytically active, and when expressed as discrete proteins both are inhibited by G6P. However, C-HKII has a significantly higher \( K_{\text{G6P}} \) for G6P (\( K_{\text{G6P}} \)) than N-HKII. We here address the question of whether the high \( K_{\text{G6P}} \) of the C-terminal half (C-half) of HKII is decreased by interaction with the N-terminal half (N-half) in the context of the intact enzyme. A chimeric protein consisting of the N-half of HKI and the C-half of HKII was prepared. Because the N-half of HKI is unable to phosphorylate glucose, the catalytic activity of this chimeric enzyme depends entirely on the C-HKII component. The \( K_{\text{G6P}} \) of this chimeric enzyme is similar to that of HKI and is significantly lower than that of C-HKII. When a conserved amino acid (Asp\(^{209}\)) required for glucose binding is mutated in the N-half of this chimeric protein, a significantly higher \( K_{\text{G6P}} \) (similar to that of C-HKII) is observed. However, mutation of a second conserved amino acid (Ser\(^{155}\)), also involved in catalysis but not required for glucose binding, does not increase the \( K_{\text{G6P}} \) of the chimeric enzyme. This resembles the behavior of HKII, in which a D209A mutation results in an increase in the \( K_{\text{G6P}} \) of the enzyme, whereas a S155A mutation does not. These results suggest an interaction in which glucose binding by the N-half causes the activity of the C-half to be regulated by significantly lower concentrations of G6P.

Hexokinases (ATP: d-hexose 6-phosphotransferase, EC 2.7.1.1; HK)\(^3\) catalyze the phosphorylation of glucose to glucose 6-phosphate (G6P). Type I, II, and III isozymes of mammalian HKs are ~100 kDa in size and are inhibited by the reaction product, G6P. Type IV HK (also known as glucokinase) is similar to yeast HK in that it has a relative mass of ~50 kDa and is insensitive to inhibition by physiologic concentrations of G6P. The deduced amino acid sequences of HKI–III reveal internal similarities between their N- and C-terminal halves and between each of these and yeast HK and glucokinase (1). This observation supports the hypothesis that the 100-kDa mammalian HKs evolved from the duplication and fusion of an ancestral 50-kDa HK (1, 2).

The N- and C-terminal halves of HKI show a marked functional difference despite the similarity of their amino acid sequences. The C-terminal half of HKI is catalytically active, whereas the N-terminal half is inactive (3–5). Whereas G6P binds to both halves of HKI (3, 5), the G6P regulatory site of HKI is thought to be in the N-half of the intact enzyme, and the C-half binding site is latent (1, 6). In contrast, we have shown that both the N- and C-terminal halves of human and rat HKII (N-HKII and C-HKII, respectively) have catalytic activity and that each is inhibited by G6P (7). However, the N- and C-terminal halves of HKII possess different kinetic characteristics: N-HKII has a slightly higher affinity for ATP than does C-HKII, and its \( K_{\text{G6P}} \) for G6P is about 20–30-fold lower than that of C-HKII (7). Furthermore, the \( K_{\text{G6P}} \) value of the intact 100-kDa HKII enzyme is similar to that of N-HKII (7), an observation confirmed by Tsai and Wilson (8).

Based on the crystal structure of yeast HK (9–12) and recently confirmed by a similar analysis of mammalian HKI (13–16), glucose is thought to bind to a cleft in the open conformation of the enzyme. This binding results in closure of the cleft and initiates the catalytic sequence. Asp\(^{211}\) is an important residue in yeast hexokinase. As the base catalyst, it binds the 6-hydroxy group of glucose, and this receives the phosphate moiety of ATP. Mutation of the corresponding residue to alanine in glucokinase (D205A) (17), the C-half of HKI (D209A) (18), and the N- and C-halves of HKII (D209A and D657A) (7) results in a complete loss of activity. In contrast, the carboxyl group of Ser\(^{155}\) in yeast HK interacts with the 3-hydroxyl group of glucose only in the closed conformation (1). Mutation of the corresponding amino acid to alanine in the C-half of HKI (S155A) (5) and the N- and C-halves of HKII in the intact enzyme (S155A and S603A) reduces the \( V_{\text{max}} \) of the enzyme to about 10% of that of the wild type (8). Thus, although the D209A mutation in mammalian HKs results in a loss of glucose binding and catalytic activity, the S155A mutation maintains glucose binding but reduces the \( V_{\text{max}} \).

Because the \( K_{\text{G6P}} \) of HKII is similar to that of N-HKII and significantly different from that of C-HKII, we focused the present analysis on how the activity of C-HKII is regulated by G6P in the intact enzyme. Mutations that have different effects on glucose binding in the N-half of HKII result in different values of \( K_{\text{G6P}} \). We here show that the regulation of the C-half by G6P in the intact HKII is influenced by the N-half of the molecule.

1 The abbreviations used are: HK, hexokinase; G6P, glucose 6-phosphate; GST, glutathione S-transferase; kb, kilobase pairs; PCR, polymerase chain reaction; G1c, glucose; NIDDM, non-insulin-dependent diabetes mellitus; C-half, C-terminal half; N-half, N-terminal half.

2 H. Ardehali, unpublished observations.
Experimental Procedure

Construction of NICII Chimeric Proteins—The human hexokinase I cDNA was provided by Dr. Graeme Bell (Howard Hughes Medical Institute, University of Chicago). A DNA fragment that encodes the N-half of HKI was prepared by PCR using B2392 (5'-GCCAGCAGTAGTCGCCCGGCA-3') as the 5' oligonucleotide and A2670 (5'-TTTACTCGCAACTCGCTGAGC-3') as the 3' oligonucleotide. A2670 contains a stop codon at position 1407, which is the putative fusion site between the N- and C-terminal halves of HKI. The pgf plasmid (7), constructed by insertion of a bacteriophage λ origin into the pGex-3x vector (Amersham Pharmacia Biotech), was linearized at a SmaI restriction site located adjacent to the glutathione S-transferase (GST) open reading frame. The PCR product that contains the N-half of HKI was then ligated into pGex-NHII to obtain pgGST-NHII. This plasmid encodes amino acids 1-469 of human HKI fused to the C-terminus of GST.

An NcoI site is conveniently located near the fusion site of the N- and C-terminal halves of both human HKI and HKII. NcoI cleaves at nucleotide 1366 in the open reading frame of both HKI and HKII. PGStN-HKI was digested with NcoI and BsrHII (a cleavage site about 1.2 kbp 5' of the GST start codon) to produce a fragment of ~3.2 kbp that contains the coding sequence of GST and the N-terminal half of HKI. This fragment was purified by size separation through agarose gel electrophoresis and was isolated using Spin-x tubes (Costar, Cambridge, MA). A GST-HKII expression plasmid, pgGST-HKII (7), was also digested with NcoI and BsrHII, and the larger fragment of about 5.2 kbp was isolated. These two fragments were ligated together to generate the plasmid pgGST-NHII-HKII, which encodes the chimeric protein GST-NICII.

PCR-based Mutagenesis of Hexokinase Constructs—Site-directed mutations were generated by two separate PCR reactions. The primers, templates, and PCR conditions used for the construction of the hexokinase mutants are illustrated in Table I. The PCR products were isolated by gel electrophoresis. Equal amounts (approximately 100 ng) of the two purified PCR products for each construct were mixed, denatured, annealed, and used as a template for a third PCR reaction with the outer primers, as described. By using these two primers, it was possible to selectively amplify a template that consisted of annealed PCR products 1 and 2 with the desired mutation. In the case of pgTSHKII-S155A, the final PCR product was digested with BamHI and SalI and was used to replace the wild-type fragment of pgGST-HKI restriction fragment in the pgGST-HKII plasmid. For pgGST-HKII-S603A, the third PCR product was digested with XhoI and ClaI and was used to replace the corresponding wild-type fragment of pgGST-HKI. In the case of pgST-NHKI-D209A and pgST-NHKI-S155A, the final PCR products were digested with SmaI and SalI and used to replace the wild-type fragment of pgST-N-HKI. PGST-NHKI-D209A and PGST-NHKI-S155A were ligated into NcoI and SmaI, and the smaller fragment was used to replace the corresponding fragment of pgST-HKII to generate the plasmids pgST-NICII-D209A and pgST-NICII-S155A. These plasmids encode the chimeric proteins GST-NICII-D209A and GST-NICII-S155A. The PCR fragments inserted into the wild-type plasmids were validated by DNA sequencing.

Expression, Purification, and Activity Measurements of Hexokinase Proteins—The expression and purification of GST-HK fusion proteins were carried out as described previously (7). Kinetic parameters were determined by a glucose phosphorylation assay that uses tracer [U-14C]glucose as substrate, as described previously (7). The expression and purification of GST and ATP were determined by measuring the phosphorylation of [14C]glucose. The modified equation is as follows.

\[
\frac{v}{[\text{Glc}]} = \frac{V_{\text{max}}[\text{ATP}]K_{\text{G6P}}}{K_{\text{ATP}} + K_{\text{G6P}} + [\text{ATP}] + [\text{G6P}] + [\text{Glc}]} + [\text{Glc}]
\]

(Eq. 1)

In this equation, [Glc], [ATP], and [G6P] refer to the concentrations of Glc, ATP, and G6P, respectively, and \( K_{\text{G6P}} \), \( K_{\text{ATP}} \), and \( K_{\text{G6P}} \) are the respective kinetic constants for glucose, ATP, and G6P. \( V_{\text{max}} \) is the maximum velocity, and \( v \) is the velocity of glucose phosphorylation.

Global Analysis—Kinetic data were analyzed using a nonlinear least squares curve-fitting program called Globals Unlimited (Department of Physics, University of Illinois, Urbana, IL). This global analysis program was originally developed for the analysis of multiple sets of fluorescence spectroscopic data (22, 23) and has been used to analyze other complex systems, such as coupled glucose transport and phosphorylation (24). The practical aspects of this approach have been reviewed (25). Global analysis was used to generate nonlinear least square fits to the kinetic parameters of the enzymes based on the hexokinase rate law (see above). The kinetic parameters (i.e. \( K_{\text{G6P}} \), \( K_{\text{ATP}} \), and \( K_{\text{G6P}} \)) were determined by analyzing the measured activities as a function of changes in each of the substrate and inhibitor concentrations.

Results and Discussion

The kinetic parameters of HKII, its two halves (N-HKII and C-HKII), and the D209A and D657A mutant enzymes are shown in Table II. In general, these are similar to the apparent values obtained previously by simple regression analysis (7). The exception is the \( K_{\text{G6P}} \), which is 10-fold lower on average, because the current method of analysis allowed for extrapolation to very low levels of the competitive ligand ATP.

The D209A mutation in HKII results in complete inactivation of the N-half of HKII (7), so the activity of the D209A enzyme is entirely contributed by its C-half. As noted with C-HKII, this mutant enzyme has a 20-30-fold higher \( K_{\text{G6P}} \) than HKII and N-HKII. Inactivation of the C-half of HKII (D657A) results in an enzyme with a \( K_{\text{G6P}} \) similar to that of N-HKII. However, neither the S155A mutation, which also inactivates the N-half of the enzyme, nor the S603A mutation affect the \( K_{\text{G6P}} \) of the intact enzyme (Table II). The \( K_{\text{G6P}} \) values for HKII, S155A, and S603A are between 39 and 45 \( \mu \text{M} \), which is in agreement with results reported by Tsai and Wilson (8). These data suggest that S155A and D209A have different roles in HKII, particularly with respect to the regulation of the catalytic activity of the C-half by G6P. Previous studies have shown that the D209A mutation in mammalian hexokinases results in a loss of glucose binding and catalytic activity, whereas the S155A mutation maintains glucose binding but reduces the \( V_{\text{max}} \) (5, 7, 17, 18). Therefore, the N-half of the D209A enzyme may remain in an open conformation, because the glucose-Asp\(^{155} \) contact is thought to trigger a conformational change from an open to a closed form. In contrast, the N-half of the S155A enzyme would undergo the conformational change associated with glucose binding, because the glucose-Ser\(^{155} \) contact occurs after the conformational change. We therefore hypothesize that a conformational change of the N-half is required for inhibition of the C-half at relatively low G6P concentrations.

Analysis of the kinetic parameters of the HKII enzyme, and its two halves, supports the hypothesis that the N-half influences the regulation of the C-half by G6P in the intact enzyme. The \( K_{\text{G6P}} \) of the intact enzyme is almost identical to that of N-HKII, despite the fact that the \( K_{\text{G6P}} \) of C-HKII is ~30-fold higher than that of the N-half. Detailed analysis of the G6P regulation of C-HKII in the intact enzyme is, however, complicated by the fact that both halves of HKII are active and both are sensitive to inhibition by G6P. We therefore constructed a chimeric enzyme in which C-HKII is the only catalytically active component and tested it for G6P inhibition.

The N-half of HKI is capable of binding glucose and G6P (1, 6), but it is catalytically inactive when expressed as a discrete protein and in the context of the intact HKI enzyme (Refs. 3–5 and data not shown). A chimeric protein consisting of the N-half of HKI and the C-half of HKII (NICII) allowed us to test...
Site-directed mutations were generated by two separate PCR reactions. The primers and template used in the PCR reactions, the annealing temperature, and the approximate size of each PCR product are shown in Table I.

**Methods**

**Construction of Hexokinase Mutants**

The primers and template used in the PCR reactions, the annealing temperature, and the approximate size of each PCR product are shown in Table I.

**Experimental Procedures**

The reaction mixtures contained 10 ng of the template plasmid DNA, 0.2 mM each of dNTPs, 0.1 nmol primers, and 1 unit of Vent polymerase (Perkin-Elmer) in 100 μl of a solution containing 50 mM KCl, 1.5 mM MgCl₂, 0.1% gelatin, and 10 mM Tris-HCl (pH 8.3). The reaction mixtures were denatured for 5 min at 94 °C, and then 30 cycles were performed, each consisting of denaturation at 94 °C for 1 min, annealing at the indicated temperature for 1 min, and extension at 72 °C for 1.5 min using a PTC-100 programmable thermocycler (MJ Research, Watertown, MA). The reactions were then incubated at 72 °C for an additional 10 min. The PCR products were gel isolated and used for a third PCR reaction as described under “Experimental Procedures.”

**Results**

The kinetic values of various enzymes with HKII are shown in Table II.

**Comparison of the kinetic values of various enzymes with HKII**

Each GST-HK fusion protein was partially purified to remove any endogenous hexokinase activity as described (7). Kinetic parameters were determined using the hexokinase rate law and the global analysis program as described under “Experimental Procedures.” The means ± S.E. were determined from three separate preparations of the enzymes and 17 different velocity measurements for each enzyme preparation.

| Km glucose (μM) | Km ATP (μM) | K₆₆P (μM) |
|-----------------|-------------|------------|
| HKII            | 0.36 ± 0.1  | 1.0 ± 0.24 | 45 ± 11  |
| N-HKII          | 0.45 ± 0.06 | 0.60 ± 0.14| 59 ± 16  |
| C-HKII          | 0.78 ± 0.15 | 4.1 ± 1.1* | 1733 ± 586* |
| D209A           | 0.40 ± 0.07 | 1.5 ± 0.11 | 1030 ± 82* |
| D657A           | 0.25 ± 0.06 | 0.61 ± 0.06| 17 ± 2    |
| S155A           | 0.39 ± 0.13 | 2.1 ± 0.5  | 40 ± 7    |
| S603A           | 0.53 ± 0.13 | 1.7 ± 0.5  | 39 ± 18   |

*K₆₆ or K₆₆ value that is significantly different from that of HKII (p < 0.05 as determined by one-way analysis of variance and appropriate post hoc testing).
Functional Organization of Mammalian Hexokinase II

TABLE III
Comparison of the kinetic values of various NICII chimeric enzymes with HKII

|            | \(K_{m}\) glucose | \(K_{m}\) ATP | \(K_{m}\) G6P |
|------------|-------------------|----------------|---------------|
| HKII       | 0.36 ± 0.1        | 1.0 ± 0.24     | 45 ± 11       |
| NICII      | 0.24 ± 0.04       | 1.8 ± 0.64     | 25 ± 8        |
| NICII-D290A| 0.49 ± 0.05       | 22 ± 0.05      | 208 ± 71      |
| NICII-S155A| 0.38 ± 0.03       | 4.6 ± 0.3e     | 61 ± 16       |

\(a\) \(K_m\) or \(K_{m}\) value that is significantly different from that of HKII (p < 0.05 as determined by one-way analysis of variance and appropriate post hoc testing).

This results in increased hepatic glucose utilization and storage. Glucokinase, when expressed in muscle cells, has a similar effect on glucose disposal (39). We hypothesize that HKII, because of the low affinity of the C-half for G6P, may serve the same purpose in insulin-sensitive, peripheral tissues. This could be accomplished by the binding of the enzyme to mitochondria and/or by some other effector that results in allosteric modulation of the enzyme that effectively uncouples the functional interaction of the N- and C-halves of the enzyme.

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