Identification of a Phosphate Regulatory Site and a Low Affinity Binding Site for Glucose 6-Phosphate in the N-terminal Half of Human Brain Hexokinase*

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Tsuei-Yun Fang‡, Olga Alechina, Alexander E. Aleshin, Herbert J. Fromm, and Richard B. Honzatko§

From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

Crystal structures of human hexokinase I reveal identical binding sites for phosphate and the 6-phosphoryl group of glucose 6-phosphate in proximity to Gly87, Ser88, Thr232, and Ser415, a binding site for the pyranose moiety of glucose 6-phosphate in proximity to Asp84, Asp413, and Ser415, and a single salt link involving Arg801 between the N- and C-terminal halves. Purified wild-type and mutant enzymes (Asp84Ala, Ser88Ala, Asp413Ala, Ser415Ala, Gly87Tyr, Ser88Ala, Thr232Ala, Ala, Asp413Ala, Ala, Ser415Ala, Ala, Ser449Ala, and Arg801Ala) were studied by kinetics and circular dichroism spectroscopy. All eight mutant hexokinases have $k_{cat}$ and $K_m$ values for substrates similar to those of wild-type hexokinase I. Inhibition of wild-type enzyme by 1,5-anhydroglucitol 6-phosphate is consistent with a high affinity binding site ($K_i = 50 \mu M$) and a second, low affinity binding site ($K_i = 0.7 \mu M$). The mutations of Asp84Ala, Gly87Tyr, and Thr232Ala listed above eliminate inhibition because of the low affinity site, but none of the eight mutations influence $K_i$ of the high affinity site. Relief of 1,5-anhydroglucitol 6-phosphate inhibition by phosphate for Asp84Ala, Ser88Ala, Asp413Ala, Ser415Ala, Gly87Tyr, and Thr232Ala binding at the N-terminal half of the enzyme (19). Two different mechanisms for the phosphate regulatory site are identified in crystal structures. (i) The phosphate regulatory site is at the N-terminal domain as identified in crystal structures. (ii) The glucose 6-phosphate binding site at the N-terminal domain is a low affinity site and not the high affinity site associated with potent product inhibition. (iii) Arg801 participates in the regulatory mechanism of hexokinase I.

Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) catalyzes the phosphorylation of the 6-hydroxyl of glucose, using ATP as a phosphoryl donor. Brain hexokinase (hereafter hexokinase I) is one of four isoforms found in mammalian tissue. In brain tissue and red blood cells hexokinase I participates in the regulation of glycolysis (1–5). Hexokinase IV (glucokinase) has properties similar to that of yeast hexokinase. It consists of a single polypeptide chain of molecular mass 50 kDa and is not inhibited by physiological levels of glucose 6-phosphate (Glu-6-P). Hexokinase isoforms I, II, and III have polypeptide chains of molecular mass 100 kDa. Glu-6-P is a potent inhibitor of isoforms I–III (5), but only for hexokinase I does phosphate (Pi) relieve Glu-6-P inhibition (6–8). Isoforms I-III have 70% identical amino acid sequences. Furthermore, the N- and C-terminal halves of isoforms I–III have similar amino acid sequences, putatively a consequence of the duplication and fusion of a primordial hexokinase gene (9–12).

The isolated C-terminal half of hexokinase I is catalytically active, whereas the N-terminal half by itself or in the context of the full-length protein has no catalytic activity (13–15). On the other hand, the N-terminal half of hexokinase I is involved in the Pi-induced relief of product inhibition on the basis of the following. (i) The isolated C-terminal half loses the property of Pi-induced relief of Glu-6-P inhibition (13, 16, 17). (ii) A chimeric hexokinase containing the N-terminal half of hexokinase I and C-terminal half of hexokinase II exhibits Pi-induced relief of Glu-6-P inhibition (18). (iii) The crystallographic structure of recombinant hexokinase I complexed with glucose and phosphate reveals a single site for phosphate, that being at the N-terminal half of the enzyme (19). Two different mechanisms of regulation, however, have been proposed by investigators. In one, Glu-6-P binds to the N-terminal half as an allosteric inhibitor and Pi competes directly with Glu-6-P. In the other, Pi binds to the N-terminal half and displaces Glu-6-P from the active site (C-terminal half) by way of an indirect mechanism. In both models the binding of phosphate to the N-terminal domain regulates catalysis at the C-terminal half by way of an allosteric mechanism.

Hexokinase I is a dimer in its crystalline complex with glucose and Glu-6-P (20). Glu-6-P and glucose bind at both the C- and N-terminal halves, and both halves adopt closed conformational states. The structure of a glucose-P complex of hexokinase I is also a dimer (19). Glucose and Pi bind at the N-terminal half only, and the C- and N-terminal halves are in opened and closed conformational states, respectively. Pi and the 6-phosphoryl group of Glu-6-P bind to the same residues at the N-terminal half, whereas the pyranose moiety of Glu-6-P binds at a separate locus. Gly87, Ser88, Thr232, and Ser415 are at the phosphate/6-phosphoryl binding site. Asp84Ala, Asp413Ala, and Ser449Ala interact with the pyranose moiety of Glu-6-P. Contact between the N- and C-terminal halves of hexokinase I occurs only by way of a transition helix and a salt link involving Arg801 (19, 20).

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‡ Present address: Dept. of Biological Sciences, Carnegie Mellon University, 4400 Fifth Ave., Pittsburgh, PA, 15213.

§ To whom correspondence should be addressed. Tel.: 515-294-6116; Fax: 515-294-0453; E-mail: honzatko@iastate.edu.

1 The abbreviations used are: Glu-6-P, glucose 6-phosphate; 1,5-AnG-6-P, 1,5-anhydroglucitol 6-phosphate; Glu-1,6-P 2, glucose 1,6-bisphosphate; IPTG, isopropyl-1-thio-D-galactopyranoside; kb, kilobase(s).
Presented here are the functional properties of wild-type and mutant hexokinases (Asp<sup>84</sup> → Ala, Gly<sup>87</sup> → Tyr, Ser<sup>88</sup> → Ala, Thr<sup>232</sup> → Ala, Asp<sup>413</sup> → Ala, Ser<sup>449</sup> → Ala, and Arg<sup>801</sup> → Ala), Gly<sup>87</sup> → Tyr and Thr<sup>232</sup> → Ala separately abolish PI relief of inhibition caused by the product analog 1,5-anhydroglucitol 6-phosphate (1,5-AnG-6-P). Hence the site for PI binding identified by crystallographic investigations is most likely the PI regulatory site of hexokinase I. Kinetic data from the wild-type enzyme infer the presence of high and low affinity inhibitory sites for 1,5-AnG-6-P. Asp<sup>84</sup> → Ala, Gly<sup>87</sup> → Tyr, and Thr<sup>232</sup> → Ala eliminate inhibition due to the low affinity site with no effect on the high affinity site. Glu-6-P interactions at the N-terminal half then are correlated with the low affinity binding site observed in kinetics. Finally, mutation of Arg<sup>801</sup> reduces PI relief of product inhibition and inhibition associated with the low affinity site. Arg<sup>801</sup> evidently plays a role in the transmission of the allosteric signal from the N-terminal half to the active site of hexokinase I.

**EXPERIMENTAL PROCEDURES**

**Materials**—A full-length cDNA of human brain hexokinase, cloned into the expression vector pET-11a (from Novagen) to produce pET-11a-HKI, was available from a previous study (21). The Altered Sites II in vitro Mutagenesis System, T4 polynucleotide kinase, T4 DNA ligase, plasmid pGEM-TZi(+) and restriction enzymes were from Promega. Bio-Gel hydroxyapatite came from Bio-Rad. Oligonucleotide synthesis and DNA sequencing were done at the Iowa State University Nucleic Acid Facility, Escherichia coli strain ZSC13, which does not contain endogenous hexokinase, was a gift from the Genetic Stock Center, Yale University. ATP, NADP, streptomycin sulfate, glucose 1,6-bisphosphate, endogenous hexokinase, was a gift from the Genetic Stock Center, Yale University. ATP, NADP, streptomycin sulfate, glucose 1,6-bisphosphate, and respectively protein molecular weight standards, wild-type hexokinase, and mutant hexokinase I fragment carrying the gene for the mutated hexokinase were determined by the method of Bradford (23), using bovine albumin as a standard.

**Construction of Mutant Hexokinase Genes**—The hexokinase gene was mutated according to the protocols of the Promega Altered Sites II in vitro Mutagenesis System with only slight modification. The pAL-TER-Ex1 vector from the mutagenesis system was modified first by inserting a small XbaI-BamHI fragment from the plasmid pGEM-TZi(+) into a CiaI site. An XbaI-ClaI fragment, containing the hexokinase cDNA of pET-11a-HKI, was inserted into the modified pAL-TER-Ex1 vector. The resulting construct served as the DNA template in mutagenesis work. The oligonucleotide primers used in site-directed mutations were 5′-TTC ATT GCC GCT GCT CTT GTT GG3′ (Asp<sup>84</sup> → Ala), 5′-C'TCG CAT GAT CTT TAC TTC CGA3′ (Gly<sup>87</sup> → Tyr), 5′-GAT CCT GTG GGG GCT TCC TTC CGA3′ (Ser<sup>88</sup> → Ala), 5′-CTG ATC ATC GAC GCC ACC AAC3′ (Thr<sup>232</sup> → Ala), 5′-GTT TCT TCC GCA GGA TCA1′ (Asp<sup>413</sup> → Ala), 5′-GTT GTC GAC GGA CCA TCC TAC ACA3′ (Ser<sup>449</sup> → Ala), 5′-GAG AGT GGC GCA AAG GGG GCT3′ (Ser<sup>449</sup> → Ala), and 5′-CTG CTC CAG GTC GCT ACG CAG3′ (Arg<sup>801</sup> → Ala), where the altered codons are in bold type. For mutations, Asp<sup>84</sup> → Ala, Gly<sup>87</sup> → Tyr, Ser<sup>88</sup> → Ala, Thr<sup>232</sup> → Ala, Asp<sup>413</sup> → Ala, Ser<sup>449</sup> → Ala, and Ser<sup>88</sup> → Ala, the XbaI-NcoI fragment carrying the gene for the mutated N-terminal half of hexokinase was ligated to the 7-kb XbaI-NcoI fragment of pET-11a-HKI to reconstruct the expression vector pET-11a-HKI. For the mutation Arg<sup>801</sup> → Ala, the XbaI-ClaI fragment carrying the whole gene of mutated hexokinase was ligated to the 5.5-kb XbaI-ClaI fragment of pET-11a-HKI to reconstruct the expression vector pET-11a-HKI. The whole XbaI-NcoI fragment carrying the mutated gene for the N-terminal domain or the XbaI-ClaI fragment carrying the mutated whole gene of hexokinase was sequenced to confirm the presence of only the desired mutation.

**Expression and Purification of Wild-type and Mutant Hexokinase I**—Wild-type and mutant forms of hexokinase I were produced by growing pET-11a-HKI transformed E. coli strain ZSC13. The pET-11a-HKI transformed E. coli was grown overnight in LB medium plus 40 μg/ml ampicillin and then added to a 100-fold volume of M9 medium with 100 μg/ml ampicillin. The culture was grown in a fermentor (with stirring of 200 rpm and a filtered air flow of 5.0 s.i.) or in flasks (with shaking at 220 rpm) at 37°C to early log phase (A<sub>600</sub> = 0.4), after which IPTG was added to a final concentration of 0.4 mm to induce the T7 RNA polymerase gene. The culture was grown for an additional 20–24 h at 22°C.

The wild-type and mutant forms of hexokinase were purified by using...
Bi Bi mechanism (7), which incorporates two inhibitory sites for Glu-6-P (Scheme I), is the simplest model that can account for the data of Fig. 2. In Scheme I, A, B, Q, and P are ATP, glucose, Glu-6-P, and ADP, respectively, bound productively to the C-terminal domain, I is 1,5-AnG-6-P bound to inhibitory sites, E is hexokinase I, and $K_{mA}$, $K_{mB}$, $K_A$, $K_Q$, and $K_P$ are dissociation constants. Scheme I presents equilibria between complexes which are pertinent to the conditions of kinetic investigations presented here. 1,5-AnG-6-P can interact with the free enzyme and with enzyme-ATP and enzyme-product complexes, but under conditions of saturating glucose and initial velocity protocols, these more complex schemes yield the same velocity relationship as does Scheme I. Equation 1, developed from Scheme I under conditions of saturating glucose and initial velocity protocols, is below:

$$1/v = (1/V_m)[1 + (K/A)(1 + I/K_i + f/K_{II})]$$  (Eq. 1)

In Equation 1, $v$ represents the velocity, $V_m$ the maximum velocity, A the concentration of ATP, I the concentration of 1,5-AnG-6-P, $K_A$ the dissociation constant for A from the EAB complex, $K_i$ the dissociation constant for B from the EAB complex, $K_{II}$ the dissociation constant for the EA complex, $K_{II}$ the dissociation constant for I from the EBI complex, and $K_{III}$ the dissociation constant for I from the EBI complex.

Equation 1 accounts for the wild-type data with a goodness-of-fit 2.2% and was used to fit inhibition data for all mutants (Table II). However, $K_{II}$ adopted negative values for Asp84 → Ala, Gly87 → Tyr, and Thr232 → Ala, and the reduction in the goodness-of-fit value for a two-site, relative to a single-site model did not validate the determination of $K_{III}$. Hence for Asp84 → Ala, Gly87 → Tyr, and Thr232 → Ala we report the complete loss of inhibitory function associated with the low affinity site. Indeed, the relevant plots for the Asp84 → Ala mutant, for instance, are consistent with a model of linear competitive inhibition up to a concentration of 1 mM 1,5-AnG-6-P (Fig. 2).

The P$_i$-induced relief of Glu-6-P inhibition was studied at two different concentrations of ATP and at several concentrations of 1,5-AnG-6-P and P$_i$. Mutations, Gly87 → Tyr and Thr232 → Ala, abolish the relief of Glu-6-P inhibition by P$_i$ (Figs. 3 and 4). Mutations, Asp84 → Ala, Ser88 → Ala, Ser449 → Ala, and Arg801 → Ala, reduce the effect of P$_i$ on Glu-6-P inhibition by approximately one-half relative to the wild-type enzyme (Fig. 4).

**DISCUSSION**

Crystal structures of recombinant human hexokinase I reveal a bound phosphate or phosphoryl group at the N-terminal half of the enzyme in the vicinity of Gly87, Ser88, Thr232, and...
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1,5-AnG-6-P inhibition constants for wild-type and mutant hexokinase I

The values shown are the mean ± S.D. 1,5-AnG-6-P is the competitive inhibitor of hexokinase I (versus ATP).

| Hexokinase | $K_i$ | $K_{ii}$ | $K_n$ |
|------------|-------|----------|-------|
| Wild-type  | 37.4 ± 1.7 | 54.9 ± 3.4 | 710 ± 40 |
| Asp$	extsuperscript{84}$ → Ala | 61.3 ± 1.9 | 74.6 ± 8.3 | —$	extsuperscript{c}$ |
| Gly$	extsuperscript{57}$ → Tyr | 30.7 ± 0.9 | 51.0 ± 5.2 | —$	extsuperscript{c}$ |
| Ser$	extsuperscript{52}$ → Ala | 46.2 ± 2.9 | 46.3 ± 5.9 | 1400 ± 190 |
| Thr$	extsuperscript{232}$ → Ala | 83.9 ± 4.9 | 81.1 ± 5.9 | —$	extsuperscript{c}$ |
| Asp$	extsuperscript{413}$ → Ala | 39.9 ± 1.6 | 29.4 ± 2.5 | 3400 ± 500 |
| Ser$	extsuperscript{415}$ → Ala | 42.9 ± 1.5 | 42.7 ± 5.0 | 1000 ± 90 |
| Ser$	extsuperscript{449}$ → Ala | 57.1 ± 2.3 | 54.4 ± 7.5 | 1100 ± 120 |
| Arg$	extsuperscript{501}$ → Ala | 59.7 ± 1.5 | 60.2 ± 5.4 | 4700 ± 1300 |

$^a$ $K_i$ was obtained by fitting data (plots of 1/velocity versus 1/[ATP]) at 2 mM glucose with inhibitor ranging from 0 to 100 µM to a model for linear competitive inhibition.

$^b$ $K_{ii}$ and $K_n$ were obtained by fitting data (plots of 1/velocity versus 1/[ATP]) at 2 mM glucose with inhibitor ranging from 0 to 1000 µM to a model for nonlinear (Equation 1) or linear competitive inhibition.

$^c$ Not supported by the data.

Ser$	extsuperscript{415}$ (19, 20). The hydroxyl groups of Ser$	extsuperscript{88}$, Thr$	extsuperscript{232}$, and Ser$	extsuperscript{415}$ hydrogen-bond to phosphate in the glucose-P, crystalline complex and to the 6-phosphoryl group of Glu-6-P in the glucose-Glu-6-P complex (Fig. 5). In addition, a bulky side chain at Gly$	extsuperscript{57}$ should block the binding of P, or the phosphoryl group of Glu-6-P. Residue 87 can accommodate bulky side chains without a significant perturbation to its backbone conformation. The mutations Gly$	extsuperscript{57}$ → Tyr, Ser$	extsuperscript{52}$ → Ala, Thr$	extsuperscript{232}$ → Ala and Ser$	extsuperscript{415}$ → Ala then should either weaken or completely block the interaction between the N-terminal half and phosphoryl groups. On the other hand, mutations of Asp$	extsuperscript{84}$, Asp$	extsuperscript{413}$, and Ser$	extsuperscript{449}$ to alanine should disrupt interactions between the pyranose moiety of product analogs and the N-terminal half of hexokinase I.

The findings here can be reconciled easily with one of two models for regulation of hexokinase I. The models differ primarily in the location of the functional site for Glu-6-P inhibition. Either Glu-6-P binds to the active site of the C-terminal half of the enzyme and competes directly with ATP or Glu-6-P binds to the N-terminal half and inhibits catalysis through an allosteric mechanism. Product analogs interact equally well with the high affinity site of mutant hexokinases and the wild-type enzyme (Table II), a result consistent with the direct inhibition of the active site by Glu-6-P and inconsistent with the allosteric model for Glu-6-P inhibition. The presence of bound Glu-6-P at the N-terminal half in the crystal structure of the complex of hexokinase I with glucose and Glu-6-P probably stems from the high concentration of inhibitor employed in the growth of that crystal form and the effects of dimerization at elevated protein concentrations. Indeed, kinetic data here show clearly the presence of a low affinity, inhibitory site which is sensitive to mutations at the Glu-6-P pocket of the N-terminal half of hexokinase I. As Glu-6-P stabilizes dimers of hexokinase I, the additional inhibition associated with the low affinity site may be a consequence of dimerization of the enzyme in the presence of high concentrations of product analog (25).

Previously reported mutations of the N-terminal half (Asp$	extsuperscript{84}$ → Asn (26), Ser$	extsuperscript{155}$ → Ala (15), and Asp$	extsuperscript{209}$ → Ala (15)) involve residues that bind to the pyranose group of Glu-6-P, as observed in the glucose/Glu-6-P crystal structure. Consistent with the properties of mutants reported here, mutations of the pyranose region of the Glu-6-P binding pocket of the N-terminal half of the enzyme have little effect on catalysis or the inhibition of catalysis. Previous experiments in kinetics, however, did not employ high concentrations of inhibitor so no data are available regarding the effects of these mutations on the low affinity inhibitory site.

Mutations of the Glu-6-P pocket of the C-terminal half of the enzyme (Asp$	extsuperscript{532}$ → Asn (26), Asp$	extsuperscript{532}$ → Glu (22), Asp$	extsuperscript{532}$ → Lys (22), Gly$	extsuperscript{534}$ → Ala (27), Ser$	extsuperscript{603}$ → Ala (14), Asp$	extsuperscript{657}$ → Ala (14), Thr$	extsuperscript{680}$ → Ser (22), Thr$	extsuperscript{680}$ → Val (22), Gly$	extsuperscript{862}$ → Ala (27) and Gly$	extsuperscript{866}$ → Val (26)), also have little effect on inhibition by product analogs. The mutations in the C-terminal half of hexokinase I listed above generally cause a significant loss of activity, and to compensate for low activity, enzyme concentra-
tions in assays were increased in some instances to levels at which hexokinase I could dimerize. Hence, reported kinetic parameters (22, 27) may reflect the properties of mutant dimers rather than mutant monomers. Further information is necessary regarding the state of aggregation of the C-terminal half mutants of hexokinase I before structure/function correlations can be made with certainty.

The results here directly confirm a functional role for the phosphate binding site observed in the glucose/Pi crystalline complex. Seven hydrogen bonds hold P$_i$ in place at the N-terminal domain, and on the basis of studies here, that site is indeed the functional regulatory site for P$_i$. Crystal structures also indicate the binding of Glu-6-P to

**Fig. 4.** Relief of 1,5-AnG-6-P inhibition induced by P$_i$ in wild-type and mutant hexokinases. Concentrations of ATP and P$_i$, respectively, are 0.625 and 3 mM (gray), 0.625 and 6 mM (hatched), 1.25 and 3 mM (black), and 1.25 and 6 mM (white). The concentrations of 1,5-AnG-6-P are those given in Fig. 3. Relief of 1,5-AnG-6-P inhibition (%) induced by P$_i$ is defined as $100 \times |A - B/A$ where A is the slope from plot of (relative velocity of hexokinase) $^{-1}$ versus 1,5-AnG-6-P concentration (as in Fig. 3) in the absence of P$_i$, and B is the slope from plot of (relative velocity of hexokinase) $^{-1}$ versus 1,5-AnG-6-P concentration in the presence of P$_i$. For mutant Asp$^{84}$ → Ala, Asp$^{113}$ → Ala, and Ser$^{449}$ → Ala only one concentration of ATP (1.25 mM) has been used.

**Fig. 5.** Stereoview of ligand binding sites at N-terminal half of recombinant human hexokinase I. A, interactions involving P$_i$ in the glucose-P$_i$ complex (19). B, interactions involving Glu-6-P in the glucose-Glu-6-P complex (20). Dashed lines represent donor-acceptor interactions. Drawing was done with MOLSCRIPT (28).

The single glucose molecule bound to the N-terminal domain near P$_i$ is omitted for clarity. Black circles represent sites for bound metal cations. Drawing was done with MOLSCRIPT (28).

**Fig. 6.** Overview of one of two polypeptide chains of the glucose-P$_i$ complex (19) showing the location of the Arg$^{901}$ salt link. One of the goals of the present study was to ascertain whether the crystalline hexokinase I dimers provide information regarding the structure-function properties of the isolated monomer in solution. Crystal structures indicate a single P$_i$ binding site at the N-terminal domain, and on the basis of studies here, that site is indeed the functional regulatory site for P$_i$. Crystal structures also indicate the binding of Glu-6-P to
the same site, and indeed the results here are consistent with a low affinity site for Glu-6-P at the N-terminal domain, which overlaps the functional regulatory site for Pi. The participation of Arg\textsuperscript{801} in a salt link with Asp\textsuperscript{251} and Glu\textsuperscript{252} stands as the only noncovalent interaction between the N- and C-terminal halves of hexokinase I (Fig. 6). Arg\textsuperscript{801} is the best candidate on the basis of crystal structures for the transmission of conformational changes in the N-terminal half to the active site. As a 50% reduction in the Pi relief of product inhibition attends the mutation of Arg\textsuperscript{801} to alanine, Arg\textsuperscript{801} is a likely participant in the allosteric mechanism of hexokinase I regulation. Other residues, however, also must participate in parallel with Arg\textsuperscript{801} in the allosteric regulatory mechanism of hexokinase I. Otherwise the mutation of Arg\textsuperscript{801} to alanine should abolish the Pi relief of product inhibition entirely. Interestingly, the residue corresponding to position 801 in hexokinase II is also an arginine, consistent with the appearance of phosphate-linked phenomena in the chimeric enzyme consisting of C-terminal half hexokinase II and N-terminal half hexokinase I (18). The results presented here regarding Arg\textsuperscript{801}, the regulatory site for Pi, and a low affinity site for Glu-6-P suggest that the crystal structures (19, 20) provide reasonable approximations of the free monomer in solution.

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