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Human hexokinase: multiple mechanisms of G6P inhibition

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Human hexokinase: Multiple mechanisms of G6P inhibition

by

Xiaofeng Liu

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

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Graduate College
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This is to certify that the Doctoral dissertation of

Xiaofeng Liu

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

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For the Major Program

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For the Graduate College
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<tr>
<th>Abbreviation</th>
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<tr>
<td>AnG6P</td>
<td>1,5-Anhydroglucitol 6-Phosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
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<td>Adenosine Triphosphate</td>
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ABSTRACT

Human hexokinase I catalyzes the first step in glycolysis, playing a key role in the energy metabolism of brain tissue. The chapters included in this thesis focus on structure-function relationships of hexokinase I. Glucose 6-phosphate (G6P), the reaction product, binds to hexokinase I with apparent negative cooperativity and inhibits catalysis (Chapter II). Both N- and C-terminal binding sites for G6P are functional, yet only one G6P molecule binds to hexokinase I.

A series of site-directed mutations were constructed in the base- and ribose-binding pocket of ATP in C-terminal half of hexokinase I G6P inhibition completely (Chapter III). The same mutations in the context of a truncated form of hexokinase I, which contains only the C-terminal half of the enzyme (mini-hexokinase), do not affect G6P inhibition. Obviously, the effects of the same mutations in full-length and mini-hexokinase I are different. On the basis of these results and structural information, we propose a model for allosteric G6P inhibition of hexokinase I. G6P binds to N-terminal half that reaches the active site of the C-terminal half. Furthermore, the results here suggest that the base- and ribose-binding pocket is the target of allosteric inhibition.

Chapter IV investigates the conformation of hexokinase I under different ligation states. The interface mutant engineered for the investigation is proved to be monomeric by small angle x-ray scattering and x-ray crystallography. The monomeric interface mutant and wild-type hexokinase I have essentially identical kinetic properties, thus evidently dimerization plays no role in hexokinase I function under conditions of in vitro. Small angle x-ray scattering data are consistent with the retention of a rod-like conformation under different ligation states, suggesting only subtle conformational changes in response to different ligands.

Chapter V reveals the ADP binding site in the C-terminal half of hexokinase I. Also, it suggests the location of allosteric interface between the N- and C-halves. Combining the structural information and kinetic results, the authors propose molecular mechanism of allosteric inhibition.
CHAPTER I. GENERAL INTRODUCTION

Literature Review

Hexokinase (ADP: D-hexose 6-phosphotransferase, EC 2.7.1.1) catalyses the conversion of glucose to glucose 6-phosphate:

\[ \text{Glucose} + \text{ATP} \cdot \text{Mg} \rightarrow \text{Glucose 6-phosphate} + \text{ADP} \cdot \text{Mg} \]

There are four different isoforms of mammalian hexokinase, named hexokinases I-IV (1-3). Hexokinase I—III have molecular weights of 100 kDa. Hexokinase IV, however, is a 50 kDa protein. Amino acid sequence alignments reveal significant sequence similarity among different isomers. The N- and C-domain of 100 kDa hexokinases share high sequence homology with each other (4-5), with that of hexokinase IV. In fact, hexokianse I—III may have evolved from a primodial gene through gene duplication and fusion (6).

The four isoforms also differ in their kinetic behaviors and tissue distributions. Hexokinases I—III are subject to potent inhibition by the reaction product G6P, while hexokinase IV, also called glucokinase, is not inhibited by G6P. G6P-inhibition can be reversed by inorganic phosphate in hexokianse I, but not in hexokinases II & III.

Hexokinase isomers also differ in their tissue distributions. Hexokinase I is found in almost all tissues, and is highly expressed in brain and red blood cells (1-3). Hexokinase II is mainly located in muscle and adipose tissue. Hexokinase III is expressed in very low levels, and is associated with the cell nucleus. Hexokinase IV is found mainly in liver, and uses excess blood sugar for glycogen synthesis and other important biological syntheses. Since the energy source for brain tissue is glucose, and hexokinase I is one of the key enzymes in glycolysis, studying the regulatory mechanism of hexokinase I in vitro can provide valuable
information in understanding its regulatory mechanism \textit{in vivo}. This thesis focuses on the structure-function relationships of human brain hexokinase I with emphasis on its mechanism of regulation.

\textbf{Physiological functions of hexokinase in brain tissue}-- Brain hexokinase in vivo is located on the surface of the mitochondrion (7). This location permits it to directly channel ADP and ATP in and out of the mitochondrial matrix, where most of the ATP is synthesized. As proposed, this organization increases the efficiency of energy production (8, 9).

Hexokinase I may be an oligomer when bound to mitochondria (10, 11). The state of oligomerization, however, is not clear (7, 11, 12). In solution hexokinase I is a monomer at physiological concentrations (1-10 \(\mu\)g/ml), but dimerizes when its concentration is higher than 1 mg/ml (12). Binding of human hexokinase I to rat liver mitochondria is cooperative with a Hill coefficient of 3.1 to 3.3 (7, 13, 14, 17).

Brain tissue contains hexokinase I exclusively (15, 16). The major subcellular location of hexokinase I is on the outer mitochondrial membrane, and the interaction seems to be specific and reversible, since G6P and high physiological concentrations of ATP can solubilize hexokinase from mitochondria (7, 14). Purified hexokinase I can re-associate with mitochondria. Electron microscopy studies show that hexokinase interacts with the mitochondrial outer membrane, at specific areas called \textit{contact sites} (18, 19, 20). The contact sites contain porin (VDAC) in the outer membrane, adenylate nucleotide translocator (ANT) in the inner membrane, and cyclophylin-D (Figure 1.1). This protein complex constitutes the mitochondria permeability transition pore (PTP), which putatively opens during apoptosis and necrosis. The possible role played by hexokinase I in this biological process (if any), remains to be determined.
Figure 1.1. Model for hexokinase I—mitochondria interaction. Hexokinase I interacts with porin (voltage dependent anion channel, or VDAC) in the outer membrane through its N-terminal hydrophobic tail. In the inner mitochondrial membrane, adenylate nucleotide translocator (ANT) and porin form a tight junction. Cyclophylin D (Cph. D) interacts with ANT. ATP transported from ANT-porin is channeled to the active site of hexokinase, and ADP is channeled back through the same complex. BPR, benzodiapine peripheral receptor.
The N-terminal 10 amino acid residues of human brain hexokinase are highly hydrophobic. A hydrophobicity analysis predicts that the sequence is membrane associable. Experimental results support this prediction: Mitochondrially bound hexokinase, after partial proteolysis, does not re-associate with mitochondria. The amino acid sequence analysis shows that the partially proteolyzed protein is missing its first 10 amino acids, suggesting the importance of this hydrophobic tail in membrane association (19, 21, 22). Aleshin examined the surface potential of human hexokinase I (unpublished results). The analysis showed that the C-terminus of hexokinase I is positively charged, while the N-terminus is negatively charged (Fig. 1.2). Human and rat hexokinase I show biphasic binding to rat liver mitochondria, with a Hill coefficient around 3 (14, 17). Furthermore, cross-linking of mitochondrial bound hexokinase suggests a tetramer (11). Combining all the experimental data together, Aleshin, (unpublished) proposed a model for hexokinase binding to mitochondria, as shown in Fig. 1.3.

Crystal structures of hexokinase I— The first crystal structure of hexokinase I (Fig. 1.4) with bound G6P and glucose was published in 1998 by Aleshin, et al. (10) and confirmed by Mulichak (23). Later Aleshin et al. (5) reported the structure of the hexokinase complex with Glc/Pi.

In their crystal structure, hexokinase I crystallized as a dimer, with two monomers in a head-to-tail association with each other. A monomer of hexokinase is composed of two structurally homologous N and C-domains, connected by a long interdomain α-helix. Each domain can be subdivided into two parts: the small subdomain and the large subdomain. Both N and C-domain has a G6P binding site (Fig. 1.4).
Figure 1.2. Surface potential of the hexokinase I monomer. The N-domain of hexokinase I is positively charged (purple). The C-domain of hexokinase I is negatively charged (red). The charge distribution is on the same face of hexokinase I,
Figure 1.3. A model of hexokinase I oligomer interacting with porin dimer. Hexokinase I subunits are shown in green and purple and the small subdomains are in darker green and purple. The N-terminal helices, which are inserted into the membrane, are blue. ADP molecules in the N-domain are yellow, ADP in C-terminal are red. Two possible locations of porin dimer are shown as concentric circles in pictures (a) and (b). Two porin molecules interact with each other and also with the targeting helices of hexokinase I. The model is consistent with either a tetramer or a dimer interacting with a porin dimer.
Figure 1.4. Hexokinase dimer complex with G6P/Glc. (a) Monomer of dimeric hexokinase I. The monomer adopts a rod-like conformation, with N- and C-domains connected by an interdomain α-helix. Each domain is divided into two parts: the small subdomain (yellow) and the large subdomain (white and purple). Glucose (red) and G6P (blue) are presented by space-filling models. (b) Interdomain contacts between hexokinase monomers. The three contact regions, labeled from 1 to 3, are marked in red.
Does the dimerization of hexokinase I in crystal structures play a role in regulating its activity? A triple mutation was introduced in subunit the interface of the hexokinase dimer to disrupt dimerization. This interface mutant was thoroughly studied by kinetics, small angle x-ray scattering and crystallography (24, 25). The results from crystallization and small angle scattering show that this interface mutant is a monomer. Kinetic properties of this interface mutant are essentially identical to those of wild-type hexokinase. Mitochondrial binding experiments were also done on this mutant (unpublished results), and its binding behavior is similar to that of wild-type hexokinase I. Hence, we have no direct evidence suggesting the dimerization is important in hexokinase regulation.

**Two models of regulation of hexokinase I in solution**— The first mechanism of G6P regulation of hexokinase activity was proposed by Fromm et al., based on kinetics (26, 27). G6P is a competitive inhibitor with respect to ATP (26, 27, 28, 29), a phenomenon that can be readily explained by ATP and G6P binding to a common site. White & Wilson (22) found that in solution, G6P protects the full-length enzyme and the truncated N-terminal domain from proteolysis, yet the inhibitor alone cannot protect the catalytic domain. Proteolysis studies suggest a high affinity G6P binding site at the N-terminal domain, the allosteric site, as proposed by Crane and Sols in 1954 (30). The isotope exchange experiment performed by Fromm and coworkers, as well as results obtained by other research groups, showed that G6P binds to hexokinase with a stoichiometry of 1 for each hexokinase molecule (9, 27). Based on this result, White and Wilson (22) assigned the inhibitory site to the N-terminal domain. White and Wilson (22) and Zeng and Fromm (33) demonstrated independently that the truncated catalytic C-terminal domain retains potent G6P inhibition. Wilson, et al. (12, 22, 31, and 32) suggested that this high affinity site for G6P at the catalytic domain is sterically
blocked in the full-length enzyme. One view, represented by Fromm and his coworkers, proposed that G6P directly inhibits hexokinase activity, on the other hand, Wilson et al. proposed that G6P regulates hexokinase activity by binding to an allosteric site at the N-domain. Crystal structures of human (5) and rat (23) hexokinase did not resolve the issue. Both crystal structures show one G6P binding site at each of half of hexokinase I.

In 1987 White and Wilson (34) proposed that the phosphate-binding site is located in the N-terminal domain. They demonstrated that phosphate protected the truncated N-terminal domain from proteolysis and that the G6P inhibition of the truncated C-terminal domain could not be reversed by P_i. According to Wilson (12) phosphate directly competes with G6P for an overlapping site in the regulatory N-terminal domain. Fromm and coworkers performed kinetic studies on phosphate effects. Their results also suggested a high affinity phosphate-binding site at N-domain of hexokinase I.

There are two models for G6P inhibition. G6P binds to the active site and prevents ATP from binding; or G6P binds to N-domain of hexokinase, and causes a long-range conformational change in active site that prevents ATP binding. Directed mutations of G6P sites (Chapter 2) was intended to address which G6P site, N-terminal site or C-terminal, is inhibitory.

The work published in Chapter 2 (35) of this thesis together with mutational investigation of the G6P binding site in the N-terminal domain by Fang et al. (36), allowed these authors to conclude that in hexokinase I both G6P binding sites are functional. The requirement of a single molecule of G6P binding to each hexokinase molecule suggests that the two sites may be anti-cooperative. However, it did not exclude the possibility of a single, high affinity binding site for G6P at either the N- or C-domain.
The crystal structure of the monomeric hexokinase mutant complexed with ADP/Glc at the active site provided new insights. In the crystal structure published by Aleshin, et al. (24), the interface between N- and C- domains has high thermal parameters, suggesting high flexibility. Furthermore, a comparison between the G6P and ADP complex shows that conformational changes in a small subdomain (flexible subdomain, Figure 1.4) may be important to G6P inhibition. The biggest conformational change is around the base and ribose binding area in the ADP binding site. Mutations around this area abolished G6P inhibition in full-length hexokinase, while potent G6P inhibition still remains in the truncated C-terminal half of hexokinase I. Evidently in hexokinase I, the N-terminal half has the high affinity G6P binding site that causes potent inhibition. G6P binding to N domain can displace ATP from the active site, through conformational changes. Furthermore, G6P regulates hexokinase activity only when ATP binds productively to the active site. If the ATP binding site is damaged, G6P binding to the N-domain cannot regulate hexokinase activity. These results will be discussed in Chapter III.

**Substrate Specificity of hexokinase**— In 1966 when Koshland proposed the “induced-fit” theory, he suggested that an enzyme's substrate specificity be evaluated in terms of binding and catalytic specificities (37). Binding specificity reflects how tightly the protein can bind substrates. The corresponding kinetic measurement is $K_m$ for the substrate. The lower the $K_m$ value, the tighter a protein binds a substrate. Catalytic specificity reflects how well an enzyme can catalyze a reaction. The corresponding parameter is $k_{cat}$, meaning how many turnovers an enzyme catalyzes within one second. The higher the $k_{cat}$ is, the better an enzyme's catalytic specificity. Overall catalytic efficiency, according to Koshland, is the
combination of the two terms: $k_{cat}/K_m$. Hence, an enzyme with extremely high $k_{cat}$ and low $K_m$ will have high efficiency.

Mammalian hexokinase has a high specificity toward sugar, and only glucose and fructose can bind to hexokinase with high affinity. As to the specificity toward nucleotides, little is known. It is generally perceived that ATP is the best substrate for hexokinase I in vivo, considering high cytosolic ATP concentrations (about 1 mM). Based on crystal structure of hexokinase I complexed with Glc/ADP, a series of mutations have been introduced to the base and ribose binding pockets of the ATP site, and evaluated by kinetics.

The crystal structure of hexokinase complexed with ADP obtained by Aleshin, et al., provides new insights regarding nucleotide specificity (25). A series of mutants have been constructed in the ATP binding pocket to abolish or severely weaken ATP binding. The results of the mutation in the ATP site were unexpected: In all mutant hexokinases, the apparent binding affinity for ATP is changed little, however, all the mutants show significantly lower $k_{cat}$ values (1-2% of wild-type value). Mutations at the phosphoryl binding sites of ATP show essentially the same effects (32, 33). ATP binding energy may be used to drive conformational changes in hexokinase. If ATP does not bind properly, it may not drive conformational changes in hexokinase that support effective catalysis. In this sense, hexokinase's specificity toward ATP is catalytic specificity. Kinetics studies performed with nucleotide triphosphates other than ATP support this idea. The binding affinities for ITP, GTP, CTP and UTP do not differ significantly from that of ATP; rather, their $k_{cat}$ values are significantly lower than that for ATP.
Dissertation Organization

This dissertation contains six chapters. Chapter I is a literature review, and provides general information about hexokinase I. Chapter II describes a series of directed mutations, which probe residues in the G6P site of the C-terminal domain. These investigations, combined with those of Fang et al., showed that both N- and C-terminal G6P binding sites can bind G6P with high affinity, and cause inhibition. G6P inhibition of hexokinase activity may exhibit negative cooperativity, that is, one G6P molecule can bind to either the N- or C-terminal site and cause potent inhibition, and the binding of one G6P to hexokinase prevents a second G6P molecule from binding. However, the work did not answer whether one G6P binding site, N- or C-domain, is dominant.

Chapter III is a manuscript. Mutations also involved in ATP binding provide experimental support for an allosteric mechanism of regulation of hexokinase activity by G6P. The conclusions drawn from mutagenesis are reinforced by studies of the effects of G6P inhibition on hexokinase activity supported by nucleoside triphosphates other than ATP.

Chapter IV is a paper published in *Biochemistry*. The paper combined results obtained from kinetic analysis and small angle x-ray scattering, and concluded that in solution, hexokinase does not undergo significant conformational changes in different ligation states. I made the mutation, purified the mutant hexokinase and performed kinetic studies to evaluate the functional properties of the mutant. The interface mutant and wild-type hexokinase I possess essentially identical properties.

Chapter V is paper published in the *Journal of Molecular Biology*. The work was originated by Dr. Aleshin and me, based on the kinetic results of the Thr{$^{536}$}→Ala enzyme. The mutation abolishes G6P binding to the C-domain. I made the Thr{$^{536}$}→Ala in the context
of triple interface mutant hexokinase, purified the quadruple mutant, and studied it kinetically. The quadruple mutant retained wild-type hexokinase properties except for a six-fold drop in specific activity.

The last chapter (Chapter VI) presents general conclusions about the work presented, as well as plans and suggestions for future research on hexokinase.

References


CHAPTER II: DUAL MECHANISMS FOR GLUCOSE 6-PHOSPHATE INHIBITION OF HUMAN BRAIN HEXOKINASE

A paper published in the Journal of Biological Chemistry

Xiaofeng Liu, Chang Sup Kim, Feruz T. Kurbanov, Richard B. Honzatko, and Herbert J. Fromm

Running Title: Glucose 6-P Inhibition of Brain Hexokinase

Abstract

Brain hexokinase (HKI) is inhibited potently by its product glucose 6-phosphate (G6P); however, the mechanism of inhibition is unsettled. Two hypotheses have been proposed to account for product inhibition of HKI. In one, G6P binds to the active site (C-terminal half of HKI) and competes directly with ATP, whereas in the alternative suggestion

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the inhibitor binds to an allosteric site (N-terminal half of HKI), which indirectly displaces ATP from the active site. Single mutations within G6P binding pockets, as defined by crystal structures, at either the N- or C-terminal half of HKI, have no significant effect on G6P inhibition. On the other hand, the corresponding mutations eliminate product inhibition in a truncated form of HKI, consisting only of the C-terminal half of the enzyme. Only through combined mutations at the active and allosteric sites, using residues for which single mutations had little effect, was product inhibition eliminated in HKI. Evidently, potent inhibition of HKI by G6P can occur from both active and allosteric binding sites. Furthermore, kinetic data reported here, in conjunction with published equilibrium binding data, are consistent with inhibitory sites of comparable affinity linked by a mechanism of negative cooperativity.

**Introduction**

Mammals harbor four hexokinase [ATP:d-hexose 6-phosphotransferase (2.7.1.1)] isozymes (1-3). One of these, brain hexokinase (HKI), is putatively the pacemaker of glycolysis in brain tissue and the red blood cell (4). Two isozymes, HKI and skeletal muscle hexokinase (HKII), are bound to the outer membrane of mitochondria, and in the case of HKI, juxtaposed to a porin-adenylate translocator complex (5-7). Only a small fraction of the potential HKI activity is used in brain tissue because of low concentrations of intracellular glucose and potent product inhibition by glucose 6-phosphate (G6P) (8, 9). Although HKII and HKI are both markedly inhibited by G6P, orthophosphate (P$_i$) reverses G6P inhibition of only HKI (10). In addition, P$_i$ reverses G6P-induced release of mitochondrially-bound HKI (5). Exactly how G6P functions as an inhibitor of HKI is unsettled (11, 12). Although most investigators now believe that G6P competes with ATP at
the active site of the enzyme (13-17), others suggest that G6P exerts its effect by binding to an allosteric site, topologically distinct from the active site (12, 18, 19). On the other hand, there seems to be general agreement regarding the kinetic mechanism of HKI as being rapid-equilibrium Random Bi Bi (20-22).

HKI arose putatively from the duplication and fusion of a primordial gene (23).

Human HKI has a molecular mass of 100 kDa, composed of two structurally similar halves. The two halves (C-terminal and N-terminal) share significant sequence homology (24). Catalytic activity of the enzyme is associated with the C-terminal half of HKI (14, 15, 25, 26), whereas the N-terminal half has a high affinity site for P_i, putatively responsible for the relief of G6P inhibition (14,15). Arora et al. (14) have suggested that the binding of G6P to this site releases HKI from mitochondria and is not involved in inhibition.

HKI arose putatively from the duplication and fusion of a primordial gene (23).

Recently published three-dimensional structures of human (27-29) and rat (30) HKI by x-ray crystallography reveals two globular halves held together by a connecting helix and a few hydrogen bonds. Each half is structurally similar to yeast hexokinase. In addition, the crystal structures revealed binding sites for G6P (28,30) and P_i (27). G6P binds to almost identical pockets at the C- and N-terminal halves of HKI, whereas the functional P_i site overlaps the 6-phosphoryl binding locus for G6P at the N-terminal half. Kinetic studies
indicate the presence of both high- and low-affinity binding sites for G6P on HKI (31). Presented here are the kinetic properties of several mutant forms of HKI, in which specific residues (individually and in combination) at G6P pockets are altered. The results support the following model: (i) G6P-binding to high affinity sites at either the N- or C-terminal pocket can independently cause potent inhibition of HKI. (ii) G6P-binding to HKI must be strongly anti-cooperative.

**Experimental Procedures**

**Materials**—A full-length cDNA of human brain hexokinase, cloned into an expression vector pET-11a (from Novagen) to produce pET-11a-HKI and pET-11d-miniHK, was available for use from a previous study (32,33). The Transformer site-directed mutagenesis kit is from Clonetech Laboratories, Inc. T4 polynucleotide kinase and all the restriction enzymes are from Promega. Bio-gel hydroxyapatite resin is from Bio-Rad. Toyopearl DEAE-650M is from Tosohaas. Oligonucleotide synthesis and DNA sequencing were done at the Iowa State University Nucleic Acid Facility. *Escherichia coli* strain ZSC13 (DE3), which does not contain endogenous hexokinase, was a gift from the Genetic Stock Center, Yale University. ATP, NADP, 1,5-Anhydro-D-sorbitol, deoxyribonuclease (DNAase I), leupeptin, PMSF, and ampicillin are from Sigma. Glucose 6-phosphate dehydrogenase came from Boehringer Manheim. IPTG is from BioWorld.

**Construction of Mutant Hexokinase genes**—The hexokinase gene was mutated according to the protocols of the Clontech Transformer site-directed mutagenesis kit. The mutant plasmid was selected from wild-type plasmids by switching a unique NruI restriction site on the pET-11 vector to another unique XhoI site for the single point mutations. Double
mutants were constructed by performing another single mutation existing in single-mutant plasmids. The primers for site-directed mutagenesis are

5’-GATCTTTGGAGGAGCAAAATTCTGGTG-3’ for Thr<sup>536</sup>→Ala,
5’-CTGGATCTTTGGTTACTCTTCCTTTCGAATTC-3’ for Gly<sup>87</sup>→Tyr,
5’-CTGTGGGAGTGGCAGGGACACTCTAC-3’ for Asp<sup>861</sup>→Ala,
5’-CTGATCATCGGCCTCTGGCAACCAATG-3’ for Thr<sup>232</sup>→Ala, and
5’-CTTGGCCCTGCTCTTTGGAGGAAACC-3’ for Asp<sup>532</sup>→Ala,
where the modified codons are in bold typeface and underlined.

The oligonucleotide primers used for the selection of the mutant plasmid from the wild-type plasmid are:

5’-CAGCCTCGCCTCGAGAACGCCAAG-3’ for the conversion from the NruI site to the XhoI site, and 5’-CCTCGCGTCGCAACGCCAAG-3’ for the conversion from the XhoI site back to the NruI site. Mutations were confirmed by sequencing the entire cDNA insert, coding for HKI.

**Expression and purification of wild-type and mutant hexokinase**— Transformed *E. coli* strain ZSC13, containing wild-type or mutant pET-11a-HKI, were grown in LB media at 37°C to an OD<sub>600</sub> of 0.6, whereupon the temperature was reduced to 22°C and IPTG added to a final concentration of 0.4 mM. Sixteen to 24 hours after induction, the cells were harvested and then re-suspended in 25 mM potassium phosphate (KP<sub>1</sub>) (pH7.5), 2 mM glucose, 1 mM EDTA, 0.4 mM 2-mercaptoethanol, 1 mM PMSF, and 3000 units of DNAase I at a temperature of 4°C. The cells were broken using a French press and centrifuged, after which the supernatant fluid was passed through a DEAE column, using a KP<sub>1</sub>-buffered (pH 7.5), KCl gradient from 0 to 0.5 M. The fractions containing HKI activity were concentrated and
then passed through a hydroxyapatite column using a KP$_1$ (pH 7.5) gradient from 20 to 500 mM. Pooled fractions of HKI were further purified by preparative DEAE-HPLC, as described elsewhere (31).

**Preparation of 1,5-anhydroglucitol 6-phosphate**—AnG6P was prepared as described elsewhere (34).

**Treatment of glucose 6-phosphate dehydrogenase**—Commercial glucose 6-phosphate dehydrogenase comes as an ammonium sulfate precipitate. Sulfate anion mimics the effect of P$_i$-relief of G6P inhibition (26). Thus, in order to avoid interference from sulfate, glucose 6-phosphate dehydrogenase was dialyzed against the activity assay buffer prior to use in kinetic experiments.

**The HKI activity assay and kinetic studies**—HKI activity was determined by the glucose 6-phosphate dehydrogenase coupled spectrometric assay (13). Hexokinase concentrations were determined by Bradford assays using bovine serum albumin as a standard (35). Initial rate data were analyzed by using a computer program written in MINITAB with an $\alpha$-value of 2.0 (36). In experiments with AnG6P the kinetic data were fit to a model for nonlinear competitive inhibition with respect to ATP, in which two molecules of inhibitor interact sequentially with HKI (29). This model, which hereafter we will call the stoichiometric model, can be used to evaluate either a system with two independent inhibitor sites or a system with two inhibitor sites, coupled by a mechanism of anti-cooperatively. The equilibrium constants for the dissociation of the first inhibitor molecule from HKI ($K_{i_1}$) and the second inhibitor molecule ($K_{i_2}$) take on significantly different meanings in relation to site-specific affinity constants, as discussed below.
Circular dichroism (CD) spectra—CD spectra were measured from 200 to 260 nm at room temperature by using a Jasco J710 CD spectrometer. The concentration of HKI used for CD measurements was 0.2 mg/ml in a buffer containing 2mM Hepes (pH 7.8), 0.2 mM glucose, and 0.2 mM β-mercaptoethanol.

Results

In previous work (31) we mutated residues at the putative allosteric G6P pocket and found only a modest change (two-fold or less) in the $K_i$ for G6P. Subsequently, Sebastian et al. (37) mutated the same residues at this site, and obtained similar results, but concluded that this site was the high-affinity binding site for G6P responsible for HKI inhibition. The results from both studies are summarized in Table I. In light of these divergent conclusions, we examined the functional consequences of mutations at the G6P-binding site at the C-terminal half of HKI. Our findings and conclusions are the subject of this report.

Purity of wild-type and mutant hexokinases—The purity of wild-type and mutant hexokinases was greater than 95% as judged by SDS-polyacrylamide gel electrophoresis (data not shown).

Secondary structure analysis—Circular dichroism spectra of mutant hexokinases and their cognate, wild-type forms, are essentially identical (data not shown), indicating no significant disruption of secondary structure or protein folding due to mutations.

Kinetic analysis of wild-type and mutant enzymes—The results in Tables I-III come from double reciprocal plots of reciprocal initial velocity versus reciprocal substrate concentration (data not shown). The data were subjected to goodness-of-fit analysis (36) using a variety of kinetic models. In all cases the G6P analog, AnG6P, which mimics the properties of G6P in assays of HKI (34), is a competitive inhibitor with respect to ATP and a
noncompetitive inhibitor relative to glucose. Kinetic parameters were obtained from the best-fit models, which registered goodness-of-fit values below 5%.

*Rationale for the selection of mutants*—Fig. 1 illustrates the structure of HKI with G6P bound at the active and allosteric sites. The illustration is based on a 1.9 Å resolution structure of a HKI monomer, which will be presented in detail elsewhere. Asp$^{532}$ of the C-terminal half interacts with the 2-hydroxyl group of G6P, whereas Asp$^{84}$, the residue in the N-half corresponding to Asp$^{532}$, also interacts with the 2-hydroxy group of G6P. Asp$^{861}$ and Thr$^{680}$, residues of the C-half, interact with the 1-hydroxyl group and 2-hydroxyl of G6P, respectively. Thr$^{232}$ interacts with the 6-phosphoryl group of G6P in the N-terminal half of HKI and corresponds structurally to Thr$^{680}$ of the C-terminal half. Ser$^{88}$ of the N-terminal half corresponds to Thr$^{536}$ of the C-terminal half, which hydrogen bonds to the 6-phosphoryl group of G6P. Gly$^{87}$ of the N-terminal half can accommodate a mutation to a bulky side chain, which should block the binding of P$_i$ or G6P, as noted previously (31).

Shown in Tables I-III are the results from single mutations of HKI and a form of HKI in which the N-terminal half is absent due to a truncation of the gene which codes for HKI. Hereafter we will refer to this truncated form of HKI as mini-HKI. Single mutations in HKI, either at the allosteric site (Table I) or the G6P binding locus at the active site (Table II) generally cause modest increases (two fold or less) in the $K_i$ for G6P. On the other hand, the same mutations, when made in the putative binding locus of G6P at the active site of mini-HKI, eliminates G6P inhibition (Table III). These results prompted a series of double mutations in HKI, which altered one residue in each of two G6P-binding sites (combined N- and C-terminal half mutations). The results of these experiments are in Table III. Evidently, elimination of G6P-inhibition comes about only as a consequence of mutations at both G6P-
Figure 1. Structure of a monomeric interface mutant of hexokinase I. G6P molecules are in dark gray. The illustration was drawn by MOLSCRIPT (47).
binding sites. Evidently, both of the G6P binding sites (allosteric and active) are functional in HKI, and G6P-binding to either causes potent inhibition.

**Discussion**

In 1951 Weil-Mahlerbe and Bone (38) reported that G6P is a noncompetitive inhibitor with respect to ATP in the HKI reaction. This finding, along with the high level of G6P specificity when compared to that of mannose 6-phosphate and fructose 6-phosphate, led Crane and Sols (18) to suggest that G6P binds to a site other than the active site, i.e., an allosteric locus. This view has been championed by other investigators (19), notably Wilson (12). On the other hand, kinetic studies from our laboratory (13), as well as many others (2, 21, 22, 39-41), showed that G6P is a competitive inhibitor with respect to ATP and a noncompetitive inhibitor with respect to glucose. Based upon these observations, we suggested that G6P competes with ATP at the active site, which is precisely what one would expect of a product inhibitor in a rapid-equilibrium Random Bi Bi kinetic mechanism. In support of the above were the findings of Sols (19), subsequently confirmed by Solheim and Fromm (42), that the kinetics of the reverse HKI reaction are normal Michaelian with a $K_m$ for G6P nearly equal to the $K_i$ for G6P in the forward reaction.

A major breakthrough in HKI research occurred when White and Wilson (25) cleaved the enzyme by proteolysis into polypeptides of nearly equal mass. Kinetic studies by these investigators (26), and subsequently by others (14,15), demonstrated that the C-terminal half of the enzyme contains the active site, whereas the N-terminal half of HKI is inactive. In addition, except for the reversal of G6P inhibition by P$_i$, the C-terminal half retained all of the kinetic properties of HKI (14,15,26). Hence, many investigators assigned the site for G6P inhibition to the C-terminal half (active site) and the site for P$_i$ relief of G6P inhibition
Table I. Kinetic parameters for mutations in the N-terminal half of hexokinase I

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>( k_{cat} ) (Sec(^{-1}))</th>
<th>( K_m ) ( ^{ATP} ) (mM)</th>
<th>( K_m ) ( ^{Glc} ) (µM)</th>
<th>( K_i ) ( ^{AnG6P} ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type HKI</td>
<td>61.2±0.9</td>
<td>0.44±0.07</td>
<td>41±2</td>
<td>18±2</td>
</tr>
<tr>
<td>Asp(^{84})→Ala(^{a})</td>
<td>63.6±3.0</td>
<td>0.68±0.04</td>
<td>53±1</td>
<td>37±2</td>
</tr>
<tr>
<td>Gly(^{87})→Tyr(^{a})</td>
<td>57.2±5.5</td>
<td>0.79±0.07</td>
<td>50±1</td>
<td>61±2</td>
</tr>
<tr>
<td>Ser(^{88})→Ala(^{a})</td>
<td>72.3±4.3</td>
<td>0.59±0.02</td>
<td>49±2</td>
<td>46±2</td>
</tr>
<tr>
<td>Thr(^{232})→Ala(^{a})</td>
<td>70.1±1.8</td>
<td>0.81±0.06</td>
<td>42±2</td>
<td>84±5</td>
</tr>
<tr>
<td>Asp(^{84})→Ala(^{b})</td>
<td>N.P.(^{c})</td>
<td>0.43±0.07</td>
<td>35±2</td>
<td>33±2</td>
</tr>
<tr>
<td>Asp(^{84})→Glu(^{b})</td>
<td>N.P.(^{c})</td>
<td>0.47±0.07</td>
<td>35±6</td>
<td>18±1</td>
</tr>
<tr>
<td>Asp(^{84})→Lys(^{b})</td>
<td>N.P.(^{c})</td>
<td>0.44±0.05</td>
<td>37±3</td>
<td>28±3</td>
</tr>
</tbody>
</table>

Table I footnotes.

\(^{a}\) Taken from (31). Data were fit to a model for simple competitive inhibition:

\[
1/v = 1/V_m[1 + K_S/S(1 + I/K_i)]
\]  

(Eq. 1)

\( v \) is the velocity, \( V_m \) is the maximal velocity, \( K_S \) is the Michaelis constant for ATP, and \( K_i \) is the inhibition constant for AnG6P. ATP is competitive versus AnG6P.

\(^{b}\) Taken from (37). Data were fit to a model for competitive inhibition with equation shown above (Eq. 1).

\(^{c}\) The wild-type and mutant hexokinases used in the kinetic experiments (37) were not purified.
Table II. Kinetic parameters for mutations in the C-terminal half of hexokinase I

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (units/mg protein/min)</th>
<th>$k_{cat}$ (Sec$^{-1}$)</th>
<th>$K_{m}^{ATP}$ (mM)</th>
<th>$K_{m}^{Glc}$ (µM)</th>
<th>$K_{i}^{AnG6P}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type HKI$^a$</td>
<td>61.2±0.9</td>
<td>102±2</td>
<td>0.44±0.07</td>
<td>41±2</td>
<td>18±2</td>
</tr>
<tr>
<td>Thr$^{536}→$Ala$^a$</td>
<td>5.8±0.05</td>
<td>9.7±0.08</td>
<td>1.80±0.06</td>
<td>43±1</td>
<td>25±2</td>
</tr>
<tr>
<td>Asp$^{861}→$Ala$^a$</td>
<td>1.9±0.1</td>
<td>3.2±0.2</td>
<td>0.93±0.13</td>
<td>32±2</td>
<td>29±3</td>
</tr>
<tr>
<td>Asp$^{532}→$Lys$^b$</td>
<td>Not Available$^c$</td>
<td>0.029</td>
<td>0.57±0.06</td>
<td>71±6</td>
<td>25±9</td>
</tr>
<tr>
<td>Thr$^{680}→$Val$^b$</td>
<td>Not Available$^c$</td>
<td>0.013</td>
<td>0.27±0.04</td>
<td>33±4</td>
<td>45±11</td>
</tr>
</tbody>
</table>

Table II footnote.

$^a$ Determined from experiments, which systematically vary the concentration of ATP and AnG6P. Concentrations of ATP vary from 1/3 to 3 times $K_{m}^{ATP}$. Concentrations of AnG6P are 0, 1, 2, 3, and 4 times $K_{i}^{AnG6P}$. All glucose concentrations are fixed at 2 mM. Mg$^{2+}$ concentrations are 3 mM above those for ATP. Data are fit to a model for simple competitive inhibition (Eq. 1, see Table I legend).

$^b$ Taken from (48).

$^c$ The specific activity is not reported.
Table III. G6P binding site mutations in mini-hexokinase and combined mutations in both N- and C- halves of hexokinase I

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activity (units/mg protein/min)</th>
<th>( k_{cat} ) (Sec(^{-1}))</th>
<th>( K_m^{ATP} ) (mM)</th>
<th>( K_m^{Glc} ) (µM)</th>
<th>( K_i^{AnG6P} ) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mini-HKI, Wild-type(^a)</td>
<td>55±1</td>
<td>45.6±0.9</td>
<td>0.50±0.12</td>
<td>52±4</td>
<td>25±2</td>
</tr>
<tr>
<td>Mini-HKI, Thr(^{536})→Ala(^a)</td>
<td>13.2±2</td>
<td>11±1</td>
<td>0.37±0.03</td>
<td>57±3</td>
<td>N.D. (^b)</td>
</tr>
<tr>
<td>Mini-HKI, Asp(^{861})→Ala(^a)</td>
<td>6.4±0.3</td>
<td>5.3±0.3</td>
<td>0.85±0.02</td>
<td>73±2</td>
<td>N.D. (^b)</td>
</tr>
<tr>
<td>Mini-HKI, Asp(^{532})→Ala(^a)</td>
<td>9.5±0.6</td>
<td>7.9±0.5</td>
<td>0.36±0.08</td>
<td>38±3</td>
<td>N.D. (^b)</td>
</tr>
<tr>
<td>Thr(^{232})→Ala/Thr(^{536})→Ala(^a)</td>
<td>10.4±0.4</td>
<td>17.3±0.7</td>
<td>0.90±0.03</td>
<td>61±5</td>
<td>N.D. (^b)</td>
</tr>
<tr>
<td>Gly(^{87})→Tyr/Thr(^{536})→Ala(^a,(^b)</td>
<td>3.2±0.1</td>
<td>5.4±0.2</td>
<td>1.08±0.06</td>
<td>108±8</td>
<td>N.D. (^b)</td>
</tr>
</tbody>
</table>

Table III footnotes.

\(^a\) mini-HKI is the truncated C-terminal half of hexokinase I, residues 455—917 of hexokinase I.

\(^b\) No inhibition by AnG6P for concentrations up to 500 µM.
to the N-terminal half (14,15). Our laboratory had suggested in 1975 that $P_i$ binds at an allosteric site on HKI (43).

The three dimensional structures of human HKI yielded an unexpected result in that G6P was bound to both the N- and the C-terminal halves of the enzyme (28,30). Earlier studies indicated the binding of only a single molecule of G6P to HKI (43-45). Subsequently, Fang et al. (31) showed that mutations in the G6P binding site of the N-terminal half of HKI caused only modest increases in the $K_i$ for G6P (Table I), and concluded that the N-terminal half could not be the site of potent G6P-inhibition. Shortly thereafter, Sebastian et al. (37), using recombinant rat HKI from COS cells, obtained similar results due to mutations of the G6P pocket of the N-terminal half of HKI (Table I); however, they concluded that the N-terminal site was indeed responsible for the potent inhibition of HKI by G6P.

The mechanism in Scheme I

$$
\begin{align*}
E & \rightleftharpoons EI \\
K_s & \\
E & \rightleftharpoons ES \\
K_e & \\
ES & \rightarrow E + P \\
K_m & \\
\text{Scheme I}
\end{align*}
$$

was used in the analysis of kinetic results obtained here and in a previous study (31). The rate equation for Scheme I is:

$$
\frac{1}{v} = \frac{1}{V_m} \left[ 1 + \frac{K_s}{S} \left( 1 + \frac{I}{K_I} + \frac{I^2}{K_i \cdot K_{II}} \right) \right] \\
(1)
$$

where $I$ and $S$ represent G6P and ATP, respectively (glucose is saturating and does not appear in Scheme I or in Eq. 1), $V_m$ is the maximal velocity, $K_s$ is the Michaelis constant for ATP, and $K_I$, $K_i$, and $K_{II}$ are inhibition constants for the binding of the first and second molecules of G6P, respectively. Scheme I is equally valid for the interpretation of kinetic data for inhibitor binding at independent sites with different affinities or for inhibitor binding to sites with identical affinities coupled by a mechanism of negative cooperativity. However, the
relationship of \( K_I \) and \( K_{II} \) to site affinity constants is model dependent. Most importantly, \( K_I \) does not have the same meaning for the wild-type and single-mutant enzymes.

\[
\frac{1}{V} = \frac{1}{V_m}\left[ 1 + \frac{K}{S} \left( 1 + \frac{I}{K_n} + \frac{I}{K_c} + \frac{I^2}{K_n \cdot K_{nc}} \right) \right]
\]

The results of Tables I—III are readily explained from the kinetic equation obtained from Scheme II:

Scheme II differs from Scheme I in that it explicitly defines binding sites for G6P at the N- and C-terminal halves of HKI. Site-specific constants for the dissociation of G6P from the N- and C-terminal halves are represented by \( K_n \) and \( K_c \), respectively. These constants measure the dissociation of G6P from the either the N-terminal half or the C-terminal half, whichever applies, when the alternative site in not occupied by G6P. \( K_{nc} \) represents the dissociation of G6P from the C-terminal site, when the N-terminal site is occupied by another molecule of G6P, and \( K_{cn} \) represents the dissociation of G6P from the N-terminal site, when the C-terminal site is occupied. Inhibitor-binding to the two sites is rapid-equilibrium, random. Hence, if one site is impaired by mutation, inhibition occurs by way of the other site.

Direct comparison of the kinetic equations based on Scheme I and Scheme II results in the following relationship:

\[
\frac{1}{K_I} = \left( \frac{1}{K_n} + \frac{1}{K_c} \right)
\]
For single mutants of HKI considered here, either $K_n$ or $K_c$ approaches infinity. Hence, Eq. 3 for single mutants reduces to $K_i = K_n$ or $K_i = K_c$, whichever applies. For the wild-type enzyme, however, $K_n$ and $K_c$ are related to $K_i$ (which is the kinetic constant reported in Tables I-III) through Eq. 3. For wild-type HKI it is not possible to extract unique values for $K_n$ and $K_c$ from kinetically determined $K_i$ values, unless an additional assumption is made. If we assume in the wild-type enzyme that $K_n$ and $K_c$ are equal, a reasonable assumption given the $K_i$ values in Tables I and II, then $K_n = K_c = 2K_i$. Hence, the approximate twofold increase in $K_i$ due to the mutation of one of the two G6P-binding sites is an expected result, and does not suggest the dominance of G6P-inhibition derived from the N-terminal site, as some have suggested (37).

Other than the above subtlety, Scheme II is relatively straightforward. The binding of I (G6P) occurs at two sites, one in the C-terminal and the other in the N-terminal half of HKI. As binding constants, obtained from kinetics for either HKI or mini-HKI (Tables I—III) are similar, Eq. (2) predicts no single mutation will alter the kinetics of inhibition appreciably in the full-length enzyme. On the other hand, a single mutation in mini-HKI or a double mutation in the full-length enzyme should eliminate inhibition, as has been observed (Tables II and III). Furthermore, on the basis of Eqs. (1) and (2), mutations made either in the C- or N-terminal half of HKI should effectively eliminate the $(I)^2$ term.

Although kinetic (31) and structural studies (27, 28, 30) strongly suggest that there are two binding sites on HKI for G6P and glucose, direct binding experiments implicate a stoichiometry of 1.0 for these ligands (43-45). These seemingly divergent findings can be rationalized by assuming a mechanism of negative cooperativity in ligand binding. The
kinetic studies of Fang et al. (31) revealed both weak and strong binding sites for G6P.

Fractional saturation ($\tilde{Y}$) is related to G6P concentration ($I$) as follows:

$$\tilde{Y} = \frac{K_1(I) + K_2(I) + 2K_3(I)^2}{1 + K_1(I) + K_2(I) + K_3(I)^2} \quad (4),$$

where $K_1$ and $K_2$ represent association constants for ligand-binding to free HKI and $K_3$ is the product of association constants which represents the binding of two molecules of G6P to HKI. If negative cooperativity pertains, then $K_3 \ll K_1, K_2$ and

$$\tilde{Y} \sim \frac{K_1(I) + K_2(I)}{1 + K_1(I) + K_2(I)} = \frac{K(I)}{1 + K(I)} \quad (5),$$

where $K$ is the sum of constants $K_1$ and $K_2$. Eq. (5) represents a binding isotherm with a stoichiometry of unity.

Independent binding of G6P to the active and the allosteric sites of HKI causes potent inhibition of HKI in vitro. HKI in vivo, however, is bound to the outer mitochondrial membrane. If as Arora et al. (14) suggest, the role of bound G6P at the allosteric site is to release HKI from the mitochondria, then G6P should not be bound to the allosteric site of mitochondrially-associated HKI. Under these physiological conditions, if G6P inhibition occurs, it will most likely occur at the active site. This observation is bolstered by recent work of Ardehali et al. (46), which reports G6P levels above 1 mM in perfused rat hearts, even though hexokinase II, present in that tissue is inhibited in vitro by G6P at micromolar levels. G6P binds with high affinity to the isolated N-terminal half of HKII and low affinity to the isolated C-terminal half, but in the full-length enzyme, high affinity inhibition dominates. These authors speculate, that HKII in its mitochondrially-associated state is inhibited weakly by G6P, and that interactions between its N- and C-terminal halves, which
are putatively responsible for potent G6P inhibition, are absent. In the case of HKI a fail-safe mechanism exists with respect to G6P inhibition, if one mode of G6P inhibition is lost, then the other mode remains, which assures virtually no diminution in G6P inhibition.

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CHAPTER III. MOLECULAR MECHANISMS OF G6P INHIBITION DIFFERENT FOR TRUNCATED AND FULL-LENGTH HEXOKINASE I

A manuscript prepared to be submitted to the *Journal of Biological Chemistry*

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Running title: Inhibition of Hexokinase I

**Abstract**

Crystal structures of hexokinase I implicate conformational changes near the ribose- and base-binding pockets of ATP in a proposed mechanism of allosteric inhibition of catalysis by glucose 6-phosphate. Mutations of Thr⁷⁸⁴ and Leu⁸⁶⁷ of the putative ATP pocket significantly reduce catalytic activity in both full-length hexokinase I and its truncated, C-terminal half. The same mutations of the full-length enzyme also abolish product inhibition (measured as inhibition by 1,5-anhydroglucitol 6-phosphate), but have little or no effect on product inhibition of the truncated, C-terminal half. 1,5-Anhydroglucitol 6-phosphate is less effective or completely ineffective as an inhibitor of full-length hexokinase I, using GTP.

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ITP, CTP or UTP as substrates in place of ATP, but inhibits catalysis by the truncated, C-terminal half, using the same substrates. For nucleoside triphosphates other than ATP, $K_m$ values are approximately 2-8 mM, implicating a common mode of substrate recognition that may involve only the polyphosphoryl moiety of the nucleotide. The above strongly infers different molecular mechanisms of product inhibition for full-length hexokinase I and its truncated, C-terminal half. Product inhibition of the latter is probably due to the direct binding of glucose 6-phosphate to the active site, whereas inhibition of the full-length enzyme is most likely by way of an allosteric mechanism.

**Introduction**

Hexokinase (ATP: d-hexose 6-phosphate transferase, EC 2.7.1.1) catalyzes the first step in glycolysis: the ATP-dependent phosphorylation of glucose to glucose 6-phosphate (G6P). Mammals possess four isozymes of hexokinase, the expression of which varies with tissue type (1-3). Hexokinase I is the predominant isozyme in brain tissue, binding to the outer membrane of mitochondria (4), where it putatively interacts with permeability transition pores composed of porin and adenylate nucleotide translocator (5-7). ATP, Mg$^{2+}$, Pi and G6P influence the association of hexokinase I with mitochondria (8,3). In fact, hexokinase I may use permeability transition pores to directly exchange ADP/ATP with the inner matrix of the mitochondrion (9-10).

Mammalian hexokinases I-III have $M_r$ of 100 kDa, approximately twice the mass of yeast hexokinase and mammalian hexokinase IV (glucokinase). The 100 kDa hexokinases evolved putatively by the duplication and fusion of a primordial gene (11-14). The N- and C-terminal halves of the 100 kDa hexokinases are homologous to each other and to 50 kDa hexokinases. Both domains of hexokinase II phosphorylate glucose, whereas the N-terminal
halves of hexokinases I and III have no catalytic function (15-16).

Ligand-binding sites in crystallographic structures of hexokinases are primarily at a cleft between small and large folding domains of the polypeptide chain (17-24). The C-terminal half of full-length hexokinase I binds glucose to the cleft. ADP, G6P and Pᵢ bind to a set of overlapping sites in proximity to the bound glucose molecule. The N-terminal half also binds glucose at a site essentially identical to that of the C-terminal half. G6P and Pᵢ bind to overlapping sites of the N-terminal half, near the glucose molecule, but adenine nucleotides do not bind to the G6P/Pᵢ-pocket of the N-terminal half. Instead adenine nucleotides bind at a distinct locus near the N-terminus of the enzyme (21,22). The functional role of this alternative adenine nucleotide site is unclear.

Inhibition of hexokinase I by G6P is competitive with respect to ATP (25). Product inhibition (both the kinetic mechanism and binding affinity) is unaltered by the truncation of the N-terminal half from hexokinase I (15,26,27). At low concentrations, Pᵢ relieves product inhibition of hexokinase I, but only if the Pᵢ/G6P binding site of the N-terminal half is available or functional (28,29). G6P and Pᵢ are mutually exclusive in binding to hexokinase I (30). At elevated concentrations (above 10 mM), Pᵢ is an inhibitor of catalysis (30), presumably binding directly to the active site of the C-terminal half.

In spite of the wealth of data regarding G6P-ligation of hexokinase I, the molecular mechanism of product inhibition remains unsettled. In fact, investigators have proposed at least three different mechanisms: (i) G6P binds only to the active site of the C-terminal half and is displaced by the binding of Pᵢ to the N-terminal half (30). (ii) G6P only binds to the N-terminal half, inhibiting catalysis by way of an allostERIC mechanism (31). Pᵢ relieves inhibition by directly displacing G6P from the N-terminal half. (iii) G6P binds to both the C-
and N-terminal halves, but the binding is anti-cooperative (32). P; blocks G6P from the N-terminal half and stabilizes a conformational state of hexokinase I that also blocks G6P from the C-terminal half. Each of the proposed schemes faces a similar challenge in allowing only one tightly bound molecule of G6P for each molecule of hexokinase I (33-35).

The strongest evidence in favor of model i above is the absence of any change in the affinity constant for G6P or the kinetic mechanism of inhibition in response to the truncation of the N-terminal half from the full-length enzyme (15,26,27). Presented below are the first single mutations of full-length hexokinase I that completely eliminate product inhibition. The mutations target residues of the base/ribose pocket of ATP, and are distant from the G6P binding pocket of the C-terminal half. Indeed, the same mutations have little effect on product inhibition of the truncated, C-terminal half of hexokinase I. Along with other results reported here, the above infers a different molecular mechanism for product inhibition of full-length and truncated forms of hexokinase I. The most likely mode of inhibition for the truncated C-terminal half is the direct competition of G6P with ATP for the active site, whereas for the full-length enzyme competition between G6P and ATP is most likely by way of an allosteric mechanism.

**Experimental**

**Materials** — cDNA for full-length human hexokinase, cloned into expression vector pET-11a (from Novagen) to produce pET-11a-HKI, was available from previous studies (29,32). Digestion of the cDNA for the full-length enzyme with restriction endonucleases NcoI and BamHI produced a cDNA fragment for the C-terminal half of hexokinase I, which was cloned into pET-11d (Novagen) to create pET-11d-miniHKI (28,32). The Transformer site-directed mutagenesis kit is from Clontech Laboratories, Inc. T4 polynucleotide kinase
and all restriction enzymes are from Promega. Bio-gel hydroxyapatite resin is from Bio-Rad. Toyopearl DEAE-650M is from Tosohaas. Oligonucleotide synthesis and DNA sequencing were done at the Iowa State University Nucleic Acid Facility. *Escherichia coli* strain ZSC13 (DE3), which does not contain endogenous hexokinase, was a gift from the Genetic Stock Center, Yale University. ATP, GTP, UTP, CTP, NADP, 1,5-Anhydro-D-sorbitol, deoxyribonuclease (DNAase I), leupeptin, phenylmethylsulfonyl fluoride (PMSF), and ampicillin were from Sigma. Glucose 6-phosphate dehydrogenase came from Boehringer Manheim. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) came from BioWorld.

*Construction of Mutant Hexokinase Genes*—The hexokinase gene was mutated according to protocols from the Clontech Transformer site-directed mutagenesis kit. The primers for site-directed mutagenesis were as follows:

5'-GGCATCTTTGAGGGGAAAGTTTCTCTTCT-3' for Thr$^{784}$→Ala;  
5'-GGCATCTTTGAGGCTGAAGTTTCTCTTCT-3' for Thr$^{784}$→Leu;  
5'-GGGACACTCTACAAGGCTCATCCACACTTCTCC-3' for Leu$^{867}$→Ala;  
5'-GGGACACTCTACAAGTACCATCCACACTTCTCC-3' for Leu$^{867}$→Tyr.

Modified codons in the list above are underlined and in bold typeface. Mutant plasmid were selected from wild-type plasmids by changing a unique *NruI* restriction site on the pET-11 vector to a unique *XhoI* site for the single point mutations. The oligonucleotide selection primer was 5'-CAGCCTCGCC**CTCGAG**AACCAGCCAGCAAG-3' (modified bases underlined in bold typeface). All mutations were confirmed by sequencing the entire cDNA insert, coding for hexokinase I.
Expression and purification of wild-type and mutant hexokinase—Transformed *E. coli* strain ZSC13, containing wild-type or mutant pET-11a-HKI, were grown in LB media at 37° C to an OD<sub>600</sub> of 0.6, whereupon the temperature was reduced to 22° C and IPTG added to a final concentration of 0.4 mM. Sixteen to 24 hours after induction, the cells were harvested and then re-suspended in 25 mM KP<sub>i</sub> (pH 7.5), 2 mM glucose, 1 mM EDTA, 0.4 mM 2-mercaptoethanol, 1 mM PMSF, and 3000 units of DNAase I at a temperature of 4° C. Cells were disrupted by use of a French press. After centrifugation the supernatant fluid was passed through a DEAE column, using a KP<sub>i</sub>-buffered (pH 7.5), KCl gradient from 0 to 0.5 M. The fractions containing hexokinase I were concentrated and then passed through a hydroxyapatite column using KP<sub>i</sub> (pH 7.5) gradient from 20-500 mM. Pooled fractions of hexokinase I were further purified by preparative DEAE-HPLC, as described elsewhere (17). Expression and purification of the truncated C-terminal half followed the same procedure.

Preparation of 1,5-anhydroglucitol 6-phosphate (AnG6P)—AnG6P was prepared as described elsewhere (36). AnG6P has properties of inhibition and binding to hexokinase I that are indistinguishable from those of G6P. The coupling enzyme, glucose 6-phosphate dehydrogenase, used in kinetics assays of hexokinase I (see below) does not recognize AnG6P as a substrate.

Treatment of glucose 6-phosphate dehydrogenase—Commercial glucose 6-phosphate dehydrogenase comes as an ammonium sulfate precipitate. Sulfate anion mimics P<sub>i</sub>-relief of G6P inhibition (15). Thus, in order to avoid interference from sulfate, glucose 6-phosphate dehydrogenase was dialyzed against the activity assay buffer (see below) prior to use in kinetics experiments.
**Hexokinase I Activity Assay and Kinetics**—Hexokinase I activity was determined by the glucose 6-phosphate dehydrogenase coupled spectrometric assay (25). Concentrations of hexokinase I were determined by Bradford assays using bovine serum albumin as a standard (37). Assays were performed in 100 mM Tris-Cl buffer, pH 7.4. Substrate, inhibitor and Mg$^{2+}$ concentrations are provided as footnotes to data tables. Initial rate data were analyzed by using a computer program written in MINITAB with an $\alpha$-value of 2 (38). In experiments with AnG6P the kinetic data were fit to a model for linear competitive inhibition with respect to ATP, in which one molecule of inhibitor interacts with hexokinase I. AnG6P is, in fact, a nonlinear competitive inhibitor with respect to ATP for wild-type hexokinase I (29). The nonlinear dependence of inhibition on the G6P concentration, however, becomes significant only as concentrations of AnG6P exceed 0.5 mM. As this report focuses on the mechanism of inhibition of AnG6P due to its interaction at a single, high-affinity binding site, maximum inhibitor concentrations are maintained well below 0.5 mM.

**Circular dichroism (CD) spectra**—CD spectra were measured from 200—260 nm at room temperature by using a Jasco J710 CD spectrometer. The concentration of hexokinase I used for CD measurements was 0.2 mg/ml in a buffer containing 2 mM Hepes (pH 7.8), 0.2 mM glucose, and 0.2 mM β-mercaptoethanol.

**Results**

**Rationale for Direct Mutations**—Aleshin et al. (22) have proposed an allosteric mechanism in which G6P-ligation of the N-terminal half displaces ATP from the active site of hexokinase I. The binding of G6P putatively induces a rotation of the N-terminal half
about the helix, which connects the two halves of the enzyme (Figure 1). Loop 241—253 of
the N-terminal half is in contact with the flexible subdomain of the C-terminal half. Hence,
as the N-terminal half rotates to a new orientation, it induces a conformational change in the
flexible subdomain of the C-terminal half. Two different orientations of the N-terminal half
(G6P-bound and P_i-bound), putatively stabilize two conformational states of the flexible
subdomain of the C-terminal half.

The conformation of Thr^{784} may differ significantly in the two states of ligation of the
N-terminal half. In crystal structures of G6P-ligated hexokinase I (17, 22), Thr^{784} protrudes
Backbone carbonyl 747 also hydrogen bonds with the 2'-hydroxy of the adenine nucleotide.
Mutation of Thr^{784} to alanine or to leucine will disrupt the hydrogen bond network of the
ADP-ligated active site, but neither side chain has unacceptable contacts in the G6P-bound
conformation.

Leu^{867} and Thr^{683} together make numerous nonbonded contacts with the base and
ribosyl groups of ADP. Depending on which ligand is present, the side chain of Thr^{863} also
hydrogen bonds with the 6-phosphoryl group of G6P or the α-phosphoryl group of ADP.
Leu^{867} and Thr^{683} have similar conformations and interactions with each other in both G6P-
into the ribose/base pocket of ATP. In contrast, the side chain of Thr^{784} hydrogen
bonds with backbone amide 751 and backbone carbonyl 747 in the ADP-ligated active site
(Figure 2). and ADP-ligated active sites (22). Leu^{867}, however, may be important only to
adenine nucleotide interactions with the active site of hexokinase I. Mutations of Leu^{867} to
alanine and tyrosine then should test the importance position 867 in the recognition of ATP.
Product inhibition of the truncated, C-terminal half clearly cannot work through the allosteric
mechanism, outlined above. Instead, G6P probably binds directly to the active site with its 6-
Figure 1. Cartoon representation of the proposed allosteric mechanism of G6P inhibition of catalysis in hexokinase I. The conformation of monomeric hexokinase varies in response to ligation of its active site (C-terminal half) and its vestigial active site (N-terminal half). In the presence of low concentrations of G6P, the allosteric interface between the N- and C-terminal halves, maintains the flexible subdomain in an ATP-antagonistic state, in which Thr^{784} blocks the base-binding pocket for the nucleotide (A). In the presence of elevated concentrations of G6P, the N- and C-terminal halves are decoupled, with G6P bound directly to the active site, overlapping the ATP pocket (B). Sufficient levels of P_i displace G6P from the N-terminal half and allow a rigid-body rotation of that half relative to the C-terminal half. The allosteric interface now stabilizes the ATP-compatible conformation of the flexible subdomain, removing Thr^{784} as a steric obstacle to ATP association (C). ATP binds to the P_i-stabilized conformer of hexokinase (D).
phosphoryl group overlapping the α- and β-phosphoryl binding sites of ADP (Figure 2). The G6P-pocket is well separated from Thr^{784} and Leu^{867}. Hence, the two proposed mechanisms of product inhibition, allosteric for the full-length enzyme and direct for its truncated, C-terminal half, should respond differently to mutations at positions 784 and 867. From past work Thr^{536}, a residue of the C-terminal half, binds directly to the 6-phosphoryl group of G6P in crystal structures. Its mutation to alanine abolishes product inhibition of the C-terminal half, but has little effect on inhibition of the full-length enzyme (32). Conversely, if the proposed model for allosteric regulation is valid, mutations of Thr^{784} and/or Leu^{867} should influence product inhibition of the full-length enzyme, but have little or no effect on inhibition of the truncated C-terminal half.

Expression, Purification, and Secondary Structure Analysis of Wild-type and Mutant Enzymes—Wild-type and mutant hexokinases behaved similarly through purification protocols. All proteins studied here were at least 95% pure on the basis of SDS-PAGE (data not shown). The CD spectra of mutant and wild-type hexokinases were identical within experimental error (data not shown).

Functional Properties of Hexokinase I Mutants—Mutations at positions 784 and 867 reduce $k_{\text{cat}}$ by approximately 100-fold relative to the wild-type constructs (Table I). The sites of mutation are remote from the glucose pocket, and indeed we observe at most a twofold effect on the $K_m$ for glucose.

The data of Fig. 3 are representative of responses by wild-type hexokinases (both full-length and truncated) and by alanine mutants of the truncated C-terminal half to a systematic variation in the concentrations of ATP and AnG6P at saturating glucose. These data fit a model of simple competitive inhibition by AnG6P with respect to ATP:
Figure 2. Active site of hexokinase I. *(top)* ATP- and *(bottom)* G6P-ligated states as defined by crystallographic structures of an engineered monomer of hexokinase I (22). Selected side-chains, ATP, G6P, and glucose are represented by ball-and-stick models. The ATP molecule from the top panel is transformed as a wire frame model to the bottom panel to illustrate steric contacts between it, Thr^{784} and bound G6P. This drawing was done by MOSSCRIPT (40).
In Eq. 1, \( v \) is the velocity, \( V_m \) is the maximal velocity, \( K_s \) is the Michaelis constant for ATP, and \( K_i \) is the inhibition constant for AnG6P. Goodness-of-fit values for all data sets of Table I are 5% or lower. The \( K_m \) for ATP is generally insensitive to mutations at positions 784 and 876, increasing at most by threefold. On the other hand, position 784 and 867 mutations abolish inhibition by AnG6P for the full-length enzyme, but have little effect on product inhibition of the truncated, C-terminal half.

The absence of a major change in the \( K_m \) of ATP due to mutations at the ATP pocket suggests a fundamental error in the identification of the ribose/base binding pocket or alternatively the recognition of ATP by a mechanism independent of its base/ribose moiety.

Assays of wild-type hexokinase I, and its truncated C-terminal half, with nucleoside triphosphates other than ATP, namely ITP, GTP, CTP and UTP, reveal catalytic rates approximately 30-fold less than that for ATP, and \( K_m \) values varying from 2 to 8 mM (Table II). Perhaps more significantly, AnG6P did not inhibit catalysis of the full-length enzyme supported by GTP, UTP or CTP, and exhibited a five-fold increase in IC\textsubscript{50} for AnG6P in catalysis supported by ITP. In contrast, AnG6P potently inhibited the truncated, C-terminal half of hexokinase I, regardless of the nucleoside triphosphate used in support catalysis.

**Discussion**

Several lines of evidence support the binding of G6P directly to the active site of the truncated, C-terminal half of hexokinase I. Crystal structures of the full-length enzyme reveal G6P bound to a site that overlaps the ATP pocket at the C-terminal half (22). The kinetic mechanism of G6P inhibition of the truncated, C-terminal half is competitive with respect to ATP (25). Directed mutations of residues that interact with G6P abolish product
Table I. Kinetic parameters for wild type and mutant forms of hexokinase I

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity (units/mg protein/ min)</th>
<th>$k_{\text{cat}}$ (sec$^{-1}$)</th>
<th>$K_m$ ATP (mM)</th>
<th>$K_m$ Glucose (µM)</th>
<th>$K_i$ AnG6P (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type, fHK$^e$</td>
<td>61.2±0.9</td>
<td>102±2</td>
<td>0.44±0.01</td>
<td>41±2</td>
<td>18±2</td>
</tr>
<tr>
<td>Wild-type, cHK$^e$</td>
<td>55±1</td>
<td>45.6±0.9</td>
<td>0.5±0.1</td>
<td>52±4</td>
<td>25±2</td>
</tr>
<tr>
<td>Thr$^{784}$→Ala, fHK</td>
<td>0.81±0.05</td>
<td>1.35±0.09</td>
<td>0.90±0.08</td>
<td>110±20</td>
<td>—$^d$</td>
</tr>
<tr>
<td>Thr$^{784}$→Leu, fHK</td>
<td>0.64±0.07</td>
<td>1.1±0.1</td>
<td>1.14±0.07</td>
<td>28±2</td>
<td>—$^d$</td>
</tr>
<tr>
<td>Thr$^{784}$→Ala, cHK</td>
<td>1.5±0.1</td>
<td>0.90±0.08</td>
<td>1.2±0.1</td>
<td>33±1</td>
<td>40±2</td>
</tr>
<tr>
<td>Leu$^{867}$→Ala, fHK</td>
<td>0.55±0.01</td>
<td>0.92±0.08</td>
<td>0.91±0.05</td>
<td>55±4</td>
<td>—$^d$</td>
</tr>
<tr>
<td>Leu$^{867}$→Tyr, fHK</td>
<td>0.93±0.02</td>
<td>1.60±0.03</td>
<td>1.8±0.1</td>
<td>28±3</td>
<td>—$^d$</td>
</tr>
<tr>
<td>Leu$^{867}$→Ala, cHK</td>
<td>1.2±0.04</td>
<td>0.72±0.02</td>
<td>0.64±0.05</td>
<td>40±3</td>
<td>74±5</td>
</tr>
</tbody>
</table>

Table I footnotes.

$^a$ fHK is full-length hexokinase I, and cHK is the truncated C-terminal half of hexokinase I, residues 455—917 of hexokinase I.

$^b$ Determined from experiments, which systematically vary the concentration of ATP and AnG6P. Concentrations of ATP vary from 1/3 to 3 times $K_m$ ATP. Concentrations of AnG6P are 0, 1, 2, 3, and 4 times $K_i$ AnG6P. All glucose concentrations are fixed at 2 mM. Mg$^{2+}$ concentrations are 3 mM above those for ATP. Data are fit to a model for simple competitive inhibition (Eq. 1).

$^c$ Determined from experiments, which systematically vary the concentration of glucose. Glucose concentrations are 10, 20, 40, 60, 80, 100, 150, 200, and 300 µM. All ATP concentrations are fixed at 5 times $K_m$ ATP. Mg$^{2+}$ is 3 mM in excess of the concentration of ATP.

$^d$ No detectable inhibition by AnG6P up to 1 mM.

$^e$ Taken from (32).
Figure 3. Plots of initial velocity versus ATP at selected concentrations of AnG6P for mutants of the truncated, C-terminal half of hexokinase I. The mechanism of inhibition by AnG6P with respect to ATP for the (top, A) Thr$^{784}$→Ala and (bottom, B) Leu$^{767}$→Ala mutants enzymes is competitive. Details of the experiment are in the text and footnotes to Table I. Concentrations of AnG6P used in experiments: •, 0; ▲, 100 μM; ▲, 200 μM; ◆, 300 μM; □, 400 μM.
inhibition of the truncated, C-terminal half (32).

Evidence equally compelling also supports a high-affinity association of G6P with the N-terminal half of hexokinase I. G6P at low concentrations protects the truncated, N-terminal half from proteolysis (31). Mutations at the G6P pocket that abolish product inhibition of the truncated, C-terminal half of hexokinase I, do not influence product inhibition of the full-length enzyme (32). Crystal structures reveal a high-affinity binding site for G6P at the N-terminal half of the enzyme (22). Results here clearly reveal fundamental differences in the molecular mechanisms of product inhibition for full-length and truncated hexokinase I. Mutations of Thr$^{784}$ or Leu$^{867}$ to alanine have little effect on product inhibition of the truncated, C-terminal half of hexokinase I, but abolish AnG6P inhibition of the full-length enzyme. AnG6P inhibits the wild-type C-terminal half in catalysis supported by GTP, ITP, CTP, and UTP, but is ineffective as an inhibitor of the full-length, wild-type enzyme in the presence of the same nucleoside triphosphates. Product inhibition of the truncated, C-terminal half is due unambiguously to the binding of G6P directly to the active site. In this binding mode, the 6- phosphoryl group of AnG6P will compete directly with the polyphosphoryl moiety of any nucleoside triphosphate. If AnG6P were to bind to the full-length enzyme in the same fashion, then it should inhibit catalysis regardless of the mutations considered here or the choice of nucleoside triphosphate. In fact, AnG6P is a potent inhibitor of the full-length, wild-type enzyme only with respect to ATP.

The observations above are consistent with the proposed mechanism of allosteric inhibition due to product-ligation of the N-terminal half. In that mechanism, G6P-ligation of the N-terminal half of hexokinase I leverages a conformational change involving Thr$^{784}$,
Table II. Kinetic parameters of full-length and truncated hexokinase I with common nucleoside triphosphates

<table>
<thead>
<tr>
<th>Nucleoside Triphosphate</th>
<th>Specific activity (units/mg protein/min)</th>
<th>$k_{cat}$ (sec$^{-1}$)$^a$</th>
<th>$K_m^{NTP}$ (mM)$^a$</th>
<th>IC$_{50}$-AnG6P (μM)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full-length enzyme:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>54±1</td>
<td>91±2</td>
<td>0.53±0.04</td>
<td>20±1</td>
</tr>
<tr>
<td>ITP</td>
<td>1.8±0.2</td>
<td>3.0±0.2</td>
<td>2.9±0.7</td>
<td>94±4</td>
</tr>
<tr>
<td>GTP</td>
<td>2.1±0.9</td>
<td>4±2</td>
<td>2.9±0.5</td>
<td>—$^c$</td>
</tr>
<tr>
<td>UTP</td>
<td>2.25±0.06</td>
<td>3.8±0.1</td>
<td>5.9±0.4</td>
<td>—$^c$</td>
</tr>
<tr>
<td>CTP</td>
<td>1.62±0.03</td>
<td>2.68±0.06</td>
<td>3.3±0.2</td>
<td>—$^c$</td>
</tr>
<tr>
<td>Truncated, C-terminal half:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>44±1</td>
<td>36.7±0.5</td>
<td>0.47±0.02</td>
<td>28±1</td>
</tr>
<tr>
<td>ITP</td>
<td>2.20±0.05</td>
<td>1.83±0.04</td>
<td>1.95±0.09</td>
<td>38±1</td>
</tr>
<tr>
<td>GTP</td>
<td>1.99±0.04</td>
<td>1.66±0.03</td>
<td>4.5±2</td>
<td>12.8±0.7</td>
</tr>
<tr>
<td>UTP</td>
<td>1.0±0.1</td>
<td>0.8±0.1</td>
<td>7.8±2</td>
<td>28.4±0.3</td>
</tr>
<tr>
<td>CTP</td>
<td>1.37±0.05</td>
<td>1.14±0.04</td>
<td>4.6±0.2</td>
<td>6.2±0.9</td>
</tr>
</tbody>
</table>

Table II footnotes.

$^a$ Determined from a series of assays in which the concentration of glucose was 2 mM and the concentrations of the nucleoside triphosphates varied from 1/3 to 3 times $K_m^{NTP}$.

$^b$ Concentration of AnG6P that causes 50% inhibition. NTP concentrations were equal to their respective $K_m$ values, and the glucose concentration was 2 mM.

$^c$ No detectable inhibition by 1 mM AnG6P.
which impairs ATP recognition (see Results). The absence of product inhibition in the mutant enzymes studied here may arise from the failure of the G6P-ligated, N-terminal half to stabilize the G6P-inhibited active site. On the basis of modeling, however, none of the mutations should destabilize the G6P-inhibited conformation of the active site. Instead, the mutations should destabilize the ATP-ligated active site, and indeed, $k_{cat}$ falls 100-fold. Rather than the loss of G6P-inhibition, the mutant enzymes behave as if they are inhibited even in the absence of product. Hence, the insensitivity to AnG6P may be the consequence of a stable, G6P-inhibited conformation of the active site.

ATP is not well recognized as a substrate by the six mutant enzymes ($K_m^{ATP}$ of approximately 1 mM, $k_{cat}$ 1% of wild-type levels). Arguably mutant enzymes here recognize ATP about as well as the wild-type enzyme recognizes other nucleoside triphosphates ($K_m^{NTP}$ of 3—8 mM, $k_{cat}$ 3% of levels sustained by ATP). Changes at positions 784 and 867 seem not to perturb the polyphosphoryl pocket of ATP, for as noted above, AnG6P still potently inhibits mutants of the truncated C-terminal half. On the other hand, residues 784 and 867 are in contact with the base/ribose of ATP, and hence mutations there should perturb base recognition of ATP by the full-length and truncated hexokinases. The enzymes resulting from mutations at positions 784 and 867 probably share a common mode of recognition of nucleoside triphosphates, are primarily by way of the polyphosphoryl moiety.

Why, then, is $k_{cat}$ reduced 100-fold, but $K_m$ for ATP only doubled in response to the mutations? Most likely the free energy of interaction between ATP and the active site drives the C-terminal half of the enzyme into a conformation that facilitates catalysis. Crystal structures of hexokinase I have revealed the C-terminal half either in an opened conformation, to which glucose binds weakly (18), or in a closed conformation, which binds
glucose and G6P with high affinity (22). As the $K_m$ for glucose is low for the mutant and wild-type enzymes (Table I), the C-terminal half is probably in a closed conformation during the rate-limiting step of catalysis. The closed ATP-conformer for the 784 and 867 mutant hexokinases must differ, however, from the closed ATP-conformer of the wild-type enzyme. Recently, a glucose/ADP complex of wild-type hexokinase (Aleshin, A. E. and Honzatko, R. B., unpublished), in which the polyphosphoryl moiety is productively bound to the active site, has revealed a C-terminal half in a slightly more opened conformation than that of the glucose/G6P complex. Conceivably, glucose/G6P-ligation of the active site may result in an "over-closed" conformation, which is unfavorable for the productive association of ATP. Catalysis observed from the position 784 and 867 mutant enzymes may arise from this "over-closed" conformer, rather than from the slightly more opened conformer, which is energetically accessible to the glucose/ATP-complex of the wild-type enzyme.

If product inhibition of the full-length enzyme is by way of an allosteric mechanism, as the results here infer, then G6P cannot bind with high affinity to the C-terminal half of the full-length enzyme. The mechanism, which prevents the association of G6P with the active site of the full-length enzyme, is unclear. Occlusion of G6P pocket by the N-terminal half of the enzyme is unlikely mechanism, as discussed elsewhere (39).

Mutations that abolish G6P-ligation of the N-terminal half (29,32), must then "turn-on" the G6P-binding pocket of the C-terminal half. The restoration of G6P-binding to the C-terminal half requires a long-range conformational change in the full-length enzyme. An N-terminal half, unable to ligate $P_i$ or G6P, probably favors an opened conformation. Structural elements from the N-terminal half, in contact with the C-terminal half, would move significantly in a transition from a closed to opened conformer, and result in a significant perturbation to the coupling interface between the N- and C-terminal halves of the full-length
Figure 4. Image of a model for hexokinase I with an opened N-terminal half. Presumably, an opened N-terminal half would force significant conformational changes on the C-terminal half in order to avoid unacceptable steric interactions.
enzyme (Figure 4). In crystal structures of an engineered monomer of hexokinase I, the
contact interface is decoupled, and G6P at micromolar concentrations binds to both halves of
the enzyme (22). If a decoupled C-terminal half binds G6P with high affinity, then possibly
the perturbation caused by the opening of the N-terminal half will restore high-affinity
binding of G6P to the C-terminal half. Hence, in past work, the elimination of potent product
inhibition required mutations at G6P binding sites of both the N- and C-terminal halves of
hexokinase I (32).

References

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CHAPTER IV. NONAGGREGATING MUTANT OF RECOMBINANT HUMAN HEXOKINASE I EXHIBITS WILD-TYPE KINETICS AND ROD-LIKE CONFORMATIONS IN SOLUTION

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Abstract

Hexokinase I governs the rate-limiting step of glycolysis in brain tissue, being inhibited by its product, glucose 6-phosphate, and allosterically relieved of product inhibition by phosphate. On the basis of small-angle X-ray scattering, the wild-type enzyme is a monomer in the presence of glucose and phosphate at protein concentrations up to 10 mg/mL, but in the presence of glucose 6-phosphate, is a dimer down to protein concentrations as low as 1 mg/mL. A mutant form of hexokinase I, specifically engineered by directed mutation to block dimerization, remains monomeric at high protein concentration under all conditions of ligation. This nondimerizing mutant exhibits wild-type activity, potent inhibition by glucose 6-phosphate, and phosphate reversal of product inhibition. Small-angle X-ray scattering data from the mutant hexokinase I in the presence of glucose/phosphate, glucose/glucose 6-phosphate, and glucose/ADP/Mg^{2+}/AlF_{3} are consistent with a rodlike conformation for the monomer similar to that observed in crystal structures of the hexokinase I dimer. Hence, any mechanism for allosteric regulation of hexokinase I should maintain a global conformation of the polypeptide similar to that observed in crystallographic structures.

Introduction

Hexokinase I (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) governs the flow of glucose into the energy metabolism of brain tissue and red blood cells (1). The type I isozyme [one of four isozymes found in mammals (2)] consists of a single polypeptide of molecular mass 100 kDa, which shows 50% sequence identity between its N- and C-terminal halves (3, 4). The sequence identity putatively is a consequence of duplication and fusion of a primordial hexokinase gene (5-8). Hexokinase I uses ATP to phosphorylate the 6-hydroxyl
group of glucose and is inhibited potently by the product, glucose 6-phosphate (G6P).

Hexokinase I is distinct from isozymes II-IV in that inorganic phosphate (P_i) at physiological levels can relieve inhibition by G6P (9-11).

At low concentrations (below 10 μg/mL of enzyme) typical for kinetics investigations, hexokinase I is putatively a monomer (12). At elevated concentrations (above 10 mg/mL), hexokinase I aggregates and appears as a dimer in all crystallographic structures (13-16). The extent to which dimerization of hexokinase I perturbs the conformation of the monomer is unclear. Crystallographic dimers have hinge elements which permit rigid-body movements of structural domains within the N- and C-terminal halves. More significantly, the long transition helix between the two halves of a monomer has the propensity to bend and/or reorient (13, 16). A bent or reoriented transition helix could allow a monomer to adopt a radically different conformation in solution, in which the N-terminal half of hexokinase I establishes an extensive new interface with the C-terminal half. The new interface would involve elements of the active site of the C-terminal half. Hence, in order to develop a correct model for allosteric regulation in monomeric hexokinase I, the limits of conformational change in the monomer under various states of ligation must be defined with greater certainty.

Unfortunately, experimental methods currently available to probe macromolecular conformation have in common a requirement for high protein concentration in solution. In the case of hexokinase I, the enzyme concentrations necessary for the growth of crystals, and for experiments in NMR and light scattering, creates in solution a mixture of dimers and monomers of the wild-type enzyme. Reported below are the properties of a mutant hexokinase I, in which residues critical to the stability of the dimer have been changed by
directed mutation. On the basis of small-angle X-ray scattering data, the resulting mutant exists in solution as a monomer at elevated concentrations of enzyme, either in the presence or absence of ligands. Furthermore, this nonaggregating form of hexokinase I has kinetic properties nearly identical to those of the wild-type enzyme. Under all conditions of ligation explored here, the nonaggregating mutant has a rodlike conformation in solution, which is similar to that observed for a single polypeptide chain of the crystalline dimer. Hence, allosteric regulation of catalysis in monomeric hexokinase I probably involves an interface between the N- and C-terminal halves, similar to ones currently defined by crystal structures.

**Materials and Methods**

_Materials—_A full-length cDNA of human brain hexokinase, cloned into an expression vector pET-11a (from Novagen) to produce pET-11a-HKI, was available from a previous study (17). The Transformer Site-Directed Mutagenesis Kit is from Clontech Laboratories. Inc. T4 polynucleotide kinase, T4 DNA ligase, plasmid pGEM-7Z(+), the Magic Miniprep DNA purification system, and restriction enzymes were from Promega. 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, 1,5-anhydro-D-sorbitol, deoxyribonuclease I (DNAase I), leupeptin, phenylmethanesulfonyl fluoride (PMSF), and ampicillin were from Sigma. Glucose 6-phosphate dehydrogenase came from Boehringer Mannheim. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) was from BioWorld. Bio-gel hydroxapatite came from Bio-Rad and DEAE-Toyopearl 650M resin was from TosoHass.
Rationale for Mutations—The critical interactions which putatively stabilize crystallographic dimers of hexokinase I are at the 1-6 interface (Figure 1). The 1-6 interface is preserved in two conformational states of the dimer, which differ significantly from each other (13, 14). Glu280, Arg283, and Gly284 are at the 1-6 interface in crystallographic dimers and yet are remote from known ligand binding sites of the N-terminal half and from the interface between N- and C-terminal halves within a monomer. The mutation of Glu280 and Arg283 to alanine eliminates hydrogen bonds between monomers of crystallographic dimers and the mutation of Gly284 to tyrosine introduces steric hindrance. The main chain angles ($\varphi = -113^\circ$, $\psi = 2^\circ$) and the structural context of Gly284 permit the introduction of a tyrosyl side chain, which would project out from the surface of the protein.

Construction of Mutant Hexokinase Genes—Mutagenesis was performed according to protocols provided with the Transformer Site-Directed Mutagenesis Kit, except that alkaline denaturation/ethanol precipitation was employed to produce single-strand plasmids instead of heat denaturation. The triple mutation, (Glu$^{280}$→Ala/Arg$^{283}$→Ala/Gly$^{284}$→Tyr), was introduced by means of an oligonucleotide of the following sequence:

5'-CAGAGTGTGACAGGGCAATTGACGCATATTCCCTCAACCCTGG-3'

where altered codons are in boldface type. The primer used for selection, 5'-GAGCCTCGCCTCGAGAAGCCAGCAAG-3', converts the unique NruI site to another unique XhoI site. The mutation was confirmed by sequencing the entire insert by the fluorescent, dideoxy chain termination method (Iowa State University DNA Facility).

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 overnight in LB medium plus 40 $\mu$g/mL ampicillin. The resulting inoculant
Figure 1. Stereoview of the 1-6 interface of the hexokinase I dimer. Two polypeptide chains are represented in different line widths. The main-chain elements of residues, which contribute nonbonded contacts to the 1-6 interface, are in bold lines with black dots representing the positions of mutated residues. Residues 242-250 of the N-terminal half and 796-813 of the C-terminal half may be important to the allosteric mechanism of hexokinase I. Glucose molecules (space filling representation) at the N- and C-terminal halves mark the approximate locations of allosteric and active sites, respectively. The image is drawn with MOLSCRIPT (34).
was added to a 100-fold volume of M9 medium with 100 \( \mu g/mL \) ampicillin. The culture was grown in a fermentor (with stirring of 200 rpm and a filtered air flow of 6 L/min) at 37 °C to early log phase \( (A_{600} = 0.4) \), after which isopropyl-\( \beta \)-D-thiogalactopyranoside (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 I were purified by streptomycin sulfate precipitation, DEAE-anion exchange column chromatography, hydroxyapatite column chromatography, and DEAE 5PW Spherogel TSK-G HPLC chromatography as described elsewhere (13, 14, 18). The final step of purification separated the enzyme into two electrophoretically indistinguishable components. The leading fraction, which contained 75% of the total activity, was used in studies reported here.

**Measurement of Protein Concentration**—Concentrations of wild-type and mutant hexokinases used in kinetics assays were determined by the method of Bradford (19), using bovine serum albumin as a standard. Protein concentrations for small-angle scattering experiments were determined spectrophotometrically at a wavelength of 280 nm, using an extinction coefficient of 0.52 mL mg\(^{-1}\) cm\(^{-1}\).

**Preparation of 1,5-Anhydroglucitol 6-phosphate**—The barium salt of 1,5-anhydroglucitol 6-phosphate (AnG6P) was prepared as described elsewhere (20). A precisely weighed amount of the barium salt of AnG6P was dissolved in distilled water by adding HCl. Barium ions were removed by titration with a stoichiometric amount of 1 M Na\(_2\)SO\(_4\) followed by centrifugation.

**Hexokinase Assay and Kinetics**—Hexokinase activity was determined by the Glucose-6-phosphate dehydrogenase coupled spectrometric assay as described elsewhere.
Mutant and wild-type enzymes were dialyzed against 50 mM Hepes, pH 7.5, just prior to their use in activity assays. Glucose 6-phosphate dehydrogenase was dialyzed exhaustively in order to remove ammonium sulfate. Assays were performed in Hepes/sodium acetate (50 mM/100 mM, pH 7.8). Care was taken to minimize the introduction of phosphate and/or sulfate as contaminants of assay components. Mg\(^{2+}\) was added to each assay to a concentration of 2 mM in excess of the ATP concentration. The concentration of hexokinase used for activity assays was 1.2-2.0 \(\mu\)g/mL. The concentrations of AnG6P used in the determination of \(K_i\) and \(K_{ii}\) were 0, 100, 200, 300, and 400 \(\mu\)M, at ATP concentrations of 4.0, 2.3, 0.90, 0.60, 0.45, and 0.30 mM. Phosphate relief of product inhibition employed an ATP concentration of 0.60 mM, \(P_i\) concentrations of 3 and 6 mM, and concentrations for AnG6P as above for the determination of \(K_i\) and \(K_{ii}\). Initial rate data were analyzed by using a computer program written in MINITAB language with an \(\alpha\) value of 2.0 (17). Data representing the relation of reciprocal velocity to concentrations of ATP and AnG6P were fitted to a kinetic model for nonlinear competitive inhibition:

\[
\frac{1}{v} = \left(\frac{1}{V_m}\right) \times \left[1 + \left(\frac{A}{V_m}\right) \times \left(\frac{1}{K_i + \frac{I^2}{K_{ii}K_i}}\right)\right]
\]

In the above, \(v\), \(V_m\), and \(A\) represent initial velocity, maximum velocity, and ATP concentration, respectively, and \(K_i\), \(K_{ii}\) and \(K_{iii}\) represent dissociation constants for ATP, a high affinity binding site for AnG6P, and a low affinity site for AnG6P, respectively. Equation 1 and its associated kinetic scheme were presented in previous work (18).

Circular Dichroism Measurements—CD spectra were measured from 200 to 260 nm at room temperature from samples of mutant and wild-type hexokinases (protein concentrations of 200 \(\mu\)g/mL in 2.0 mM Hepes, pH 7.5, 0.1 mM glucose, and 0.25 mM \(\beta\)-mercaptoethanol, with and without 1 mM G6P, using a Jasco J710 CD spectropolarimeter.
**Modeling of Monomers**—Crystal structures of hexokinase I dimers [PDB accession codes 1HKB (14) and 1HKC(13)] are points of departure in modeling plausible conformational states of monomeric hexokinase I. The models are used in the calculation of scattering intensities (see below). A single polypeptide chain and the entire dimer of the Glc/G6P complex [PDB accession code 1HKB (14)] were adopted as models for monomers and dimers in solution, respectively. In these models, both the N- and C-terminal halves are in a closed conformation (14). Models with open N- and/or C-terminal halves were built by superposition of an open N-terminal half (using its small domain) and/or an open C-terminal half (using its large domain) onto corresponding domains of a single polypeptide chain from the Glc/G6P complex. Models for open N- and C-terminal halves come from a previous study (13).

Other models were developed in order to ascertain the global conformational state of the monomer under different conditions of ligation. Small-angle scattering intensities are sensitive to conformational changes, which alter the gross structure or anisometry. Hence, distinguishable models of the monomer (at the resolution of small-angle scattering data) require repositioning of the N-terminal half relative to the C-terminal half so as to significantly change the global conformation of the polypeptide chain. The calculated scattering intensities are insensitive, however, to the exact details of the conformational change. Hence, we arbitrarily let the axis of the transition helix define the principal direction of the monomer and the Cα atom of residue 475 (which lies at the C-terminal end of the transition helix) be a point of articulation. Fixing the C-terminal half in place, the transition helix and its attached N-terminal half can be reoriented by rotations about an axis perpendicular to the transition helix. Superposition of the N-terminal half of hexokinase I
onto the C-terminal half reveals a different orientation of the first helix of the N-terminal half relative to the transition helix. The axes of the transition helix and the first helix of the superimposed N-terminal half define a plane. The rotation axis used to generate models here is perpendicular to this plane.

**Small-Angle X-ray Scattering**—X-ray scattering data were collected on the EMBL x33 camera (22-24) on the storage ring DORIS III of the Deutsches Elektronen Synchrotron (DESY) using multiwire proportional chambers with delay line readout (25). At the sample-detector distance of 3 m and a wavelength $\lambda$ of 0.15 nm, the sampled range of momentum transfer was $0.1 < s < 2 \text{ nm}^{-1}$ [$s = (4\pi \sin \theta)/\lambda$, where $2\theta$ is the scattering angle]. Scattering intensities were measured from either wild-type or the interface mutant hexokinases (protein concentrations varying from 0.4 to 16 mg/mL) in a buffer containing Hepes (50 mM, pH 7.8), dithiothreitol (2 mM), glucose (1 mM) and $\beta$-mercaptoethanol (1 mM) with either (i) G6P (1 mM)/sodium acetate (100 mM), (ii) $P_i$ (1 mM)/sodium acetate (100 mM), or (iii) ADP (10 mM)/NaCl (100 mM)/Mg(NO$_3$)$_2$ (20 mM)/NaF (5 mM)/ Al(NO$_3$)$_3$ (0.5 mM). The data were normalized to the intensity of the incident beam, and corrected for detector response. Scattering due to the buffer was subtracted, and the resulting difference curves were scaled to the enzyme concentration. All procedures involve statistical error propagation using the program SAPOKO (D.I.S. and M.H.J.K., unpublished material).

The forward scattering $I(0)$ and radius of gyration $R_g$ of individual curves were evaluated using the Guinier approximation, $I_{\exp}(s) = I(0)\exp(-s^2R_g^2/3)$, which is valid for $(sR_g) < 1.3$ (26) and also from the entire scattering curve using the indirect transform package GNOM (27). The maximum dimensions of the hexokinases were estimated by use of the orthogonal expansion program ORTOGNOM (28). The extrapolated forward scattering value
was used to evaluate the molecular mass of the solute after normalization against the reference scattering from solutions of bovine serum albumin.

Scattering curves from models were calculated using the program CRYSOLO (29), which takes into account the scattering from the solvation shell at the surface of the protein model. The model is covered by a hydration layer of thickness 0.3 nm with an adjustable density $\rho_b$, which may differ from that of the bulk solvent $\rho_s$. The scattering from a particle in solution is

$$I(s) = \left( |A_a(s) - \rho_s A_s(s) + \delta \rho_b A_b(s)|^2 \right) \Omega$$

(Eq. 2)

where $A_a(s)$ is the scattering amplitude from the particle in vacuo, $A_s(s)$ and $A_b(s)$ are, respectively, the scattering amplitudes from the excluded volume and the hydration layer, both with unitary density $\delta \rho_b = \rho_b - \rho_s$, and the brackets ($\langle \rangle$) stand for the average over all particle orientations [$\Omega$ is the solid angle in reciprocal space, $s = (s, \Omega)$]. The program uses the multipole expansion of the scattering amplitudes to facilitate the spherical average in eq 2.

For solutions containing only monomers, calculated scattering curves (derived from models built as described above) were fit to experimental scattering curves using two free parameters, the excluded volume of the particle, and the contrast of the hydration layer $\delta \rho_b$.

For solutions containing mixtures of monomers and dimers, the volume fractions of each component was determined by fitting the experimental data with linear combinations of calculated scattering curves from monomers and dimers, using the program OLIGOMER (D.I.S, unpublished material).
Results

Protein Purity and Circular Dichroism (CD) Spectra—On the basis of SDS-PAGE, wild-type and interface mutant enzymes were at least 95% pure (data not shown). The CD spectra of the wild-type enzyme and the interface mutant enzyme coincide. The addition of G6P to 1 mM did not influence the CD spectrum of either enzyme (data not shown).

Kinetic Studies—Kinetic parameters for wild-type and mutant hexokinases are identical to within experimental uncertainty (Table 1). The $K_m$ for ATP and the $K_i$ for AnG6P differ for the wild-type and mutant enzymes by approximately 3 and 4 standard deviations, respectively. The reported standard deviations, however, derive from the random distribution of data about the model for a specific experiment and do not reflect systematic errors, which may arise from variations in stock solutions and different preparations of enzyme. The $K_m$ for ATP of the wild-type enzyme, for instance, has varied from 0.4 to 1.2 mM with an average value of 0.6 mM. Hence, the observed $K_m$ for the mutant is indistinguishable from that of the average for the wild-type enzyme. The $K_i$ for AnG6P is sensitive to the level of phosphate contamination in the ATP stock solution, as well as the precise determination of ATP concentration. The specific activity of wild-type and mutant enzymes under the conditions of assay here are 61 ± 7 and 60 ± 4 µmol mg$^{-1}$ min$^{-1}$, respectively. These values are equal to those reported for hexokinase I purified from natural sources. The wild-type and interface mutant enzymes follow the nonlinear competitive mechanism (Eq 1, goodness of fit 5%). The two enzymes exhibiting comparable values for $K_i$ and $K_{ii}$. The high uncertainty in the $K_{ii}$ value is a consequence of the threshold concentration (400 µM) of AnG6P, which is too low for an accurate determination of a low affinity interaction. Enzyme velocities are very low and subject to significant error at millimolar concentrations of AnG6P, complicating kinetic...
Table 1. Kinetic parameters for wild-type and interface-mutant hexokinases.

<table>
<thead>
<tr>
<th>Kinetic Parameter</th>
<th>Wild-type hexokinase</th>
<th>Interface mutant hexokinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{\text{cat}} ) (s(^{-1}))</td>
<td>100(10)</td>
<td>100(6)</td>
</tr>
<tr>
<td>( K_m ) (Glucose) (μM)</td>
<td>61(6)</td>
<td>57(5)</td>
</tr>
<tr>
<td>( K_m ) (ATP) (mM)</td>
<td>1.2(0.2)</td>
<td>0.66(0.06)</td>
</tr>
<tr>
<td>( K_i ) (AnG6P) (μM)</td>
<td>15(2)</td>
<td>23(2)</td>
</tr>
<tr>
<td>( K_{II} ) (AnG6P) (mM)</td>
<td>1.4(0.4)</td>
<td>3(2)</td>
</tr>
<tr>
<td>( P_i ) relief (^3) (%)</td>
<td>72(6)</td>
<td>80(6)</td>
</tr>
</tbody>
</table>

Table I footnote.

\(^1\) Values in parentheses are standard deviation.

\(^2\) \( K_i \) and \( K_{II} \) were obtained by fitting data (plots of \(1/\text{velocity}\) versus \(1/[\text{ATP}]\) at 2 mM glucose with AnG6P ranging from 0 to 400 μM) to a model for nonlinear competitive inhibition (Eq. 1).

\(^3\) Percent relief of AnG6P inhibition by \( P_i \) was determined as follows: \(100 \times (A - B)/A\), where \(A\) is the slope from the plot of (relative velocity of hexokinase)\(^1\) \( v \) vs. AnG6P concentration (as in Fig. 2) in the absence of Pi and \(B\) is the slope from the plot of (relative velocity of hexokinase I)\(^1\) \( v \) vs. AnG6P concentration in the presence of 6 mM Pi.
Figure 2 Models of hexokinase I used in the analysis of small-angle scattering data. In all images, the C-terminal half is on the bottom, small domains (residues 75-209 and 448-465 of the N-terminal half and residues 523-657 and 896-913 of the C-terminal half) are yellow, large domains (residues 210-447 of the N-terminal half and 658-895 of the C-terminal half) are green, the interdomain helix (residues 448-475) is purple, and ball-and-stick representations of bound glucose, Glc-6-P and ATP are red, blue, and purple, respectively. The ligand binding sites are those observed in crystal structures (13, 14) except for that of ATP which comes from a model (14). P<sub>i</sub> binds to the N-terminal half at the phosphoryl site of G6P. (a) A single polypeptide chain from the G6P/glucose complex (PDB accession label 1HKB), in which both halves are in a closed conformation (14). (b) The model corresponding to a rotation angle of -20° (see text for definition of the rotation angle). Note the loss of contacts between the N- and C-terminal halves. (c) The model corresponding to a rotation angle of 35°. The relative orientations of the transition helix (purple) and the corresponding helix (residues 16-27) of the N-terminal half are the same. (d) The model corresponding to a rotation angle of 80°. The N-terminal half is in direct contact with G6P-binding site of the C-terminal half. (e) A different orientation of the model in (a) to facilitate comparison to the model of (f) where both halves are in an open conformation. Images of hexokinase I drawn with MOLSCRIPT (34) and RASTER3D (35).
assays under conditions, which in principle should allow a more precise determination of $K_{ii}$. Phosphate relieves inhibition by AnG6P for wild-type and interface-mutant hexokinases to approximately the same extent. The kinetic parameters for the wild-type enzyme essentially are unchanged from those of the previous study (18), except for $k_{cat}$, which differs slightly due to differences in the buffer system used here (Hepes/sodium acetate, pH 7.8) and in the previous investigation (Tris, pH 7.4).

_Models Employed in Calculating X-ray Scattering_—Crystal structures of hexokinase I reveal different relative orientations for the first helix of the N-terminal half and the structurally corresponding transition helix, which lies between N- and C-terminal halves. A rotation of 35° about the axis defined in the Materials and Methods and Figure 2 brings the transition helix into alignment with the first helix of the superimposed N-half and results in a monomer with a more compact structure, similar to a bent rod. A rotation of 80° allows the N-terminal half to come in contact with elements of the active site at the C-terminal half (Figure 2). A rotation of 80° creates a new interface, which could transmit the effects of G6P binding at the N-terminal half to the active site of the C-terminal half. Further positive rotation results in unacceptable steric interactions. Rotations in the opposite direction (hereafter defined as rotations through negative angles) initially produce a more extended monomer (-20° rotation) with no contacts between the N- and C-terminal halves. Further negative rotation leads to bent-rod structures, which are indistinguishable by small-angle scattering from those structures generated by positive rotations. Hence, rotations over the range -20 to 80° sample a full range of global conformations for hexokinase I at the resolution of small-angle scattering data.
Table 2: Experimentally Determined Radii of Gyration, Molecular Masses, and Maximum Intramolecular Distances Calculated from GNOM for Wild-Type and Interface-Mutant Hexokinases

<table>
<thead>
<tr>
<th>Sample</th>
<th>concn (mg/mL)</th>
<th>radius of gyration (nm)</th>
<th>$M_r$ (kDa)</th>
<th>$D_{max}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, Glc/Pi</td>
<td>14.4</td>
<td>4.70(0.02)</td>
<td>120</td>
<td>15</td>
</tr>
<tr>
<td>7.2</td>
<td>4.53(0.06)</td>
<td>108</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>4.22(0.09)</td>
<td>95</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>WT, Glc/G6P</td>
<td>14.6</td>
<td>4.57(0.04)</td>
<td>250</td>
<td>15</td>
</tr>
<tr>
<td>7.3</td>
<td>4.38(0.06)</td>
<td>240</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td>4.61(0.09)</td>
<td>255</td>
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<tr>
<td>0.9</td>
<td>4.53(0.15)</td>
<td>265</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>mutant, Glc/Pi</td>
<td>16.9</td>
<td>4.18(0.04)</td>
<td>92</td>
<td>15</td>
</tr>
<tr>
<td>8.4</td>
<td>4.65(0.07)</td>
<td>95</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>4.34(0.09)</td>
<td>87</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>4.43(0.16)</td>
<td>83</td>
<td>15</td>
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<tr>
<td>mutant, Glc/G6P</td>
<td>14.1</td>
<td>4.32(0.06)</td>
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<tr>
<td>7.0</td>
<td>4.37(0.09)</td>
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<tr>
<td>3.5</td>
<td>4.35(0.17)</td>
<td>114</td>
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<tr>
<td>1.8</td>
<td>4.37(0.15)</td>
<td>121</td>
<td>15</td>
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<tr>
<td>0.9</td>
<td>4.34(0.24)</td>
<td>119</td>
<td>15</td>
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</tr>
<tr>
<td>0.4</td>
<td>4.23(0.37)</td>
<td>108</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>mutant, Glc/ADP</td>
<td>12.9</td>
<td>4.04(0.06)</td>
<td>102</td>
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<td>6.5</td>
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<td>3.2</td>
<td>4.41(0.12)</td>
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<td>15</td>
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<tr>
<td>0.8</td>
<td>4.12(0.23)</td>
<td>121</td>
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</table>

Table 2. footnote.
1 Values in parentheses are standard deviations.
2 Samples contain wild-type (WT) enzyme or interface-mutant enzyme in the presence of Glc/Pi, Glc/G6P, or Glc/ADP/Mg$^{2+}$/AlF$_3$ under conditions provided in the Materials and Methods.
3 Standard deviations of $M_r$ and $D_{max}$ are about 15 and 7%, respectively.
Small-Angle X-ray Scattering--Molecular weight determinations by small-angle scattering rely on an accurate determination of hexokinase I concentration. The direct determination of hexokinase I concentration, however, has a 10% uncertainty. This coupled with systematic errors in the acquisition of scattering data for each of the concentration series of Table 2 translates into a 15% uncertainty in the determination of molecular weight. The variations in molecular weight, reported in Table 2, reflect the anticipated level of uncertainty.

Invariants of the scattering curves [the radius of gyration ($R_g$) and the apparent molecular weight of the macromolecule] are consistent with a mixture of monomers and dimers for the wild-type enzyme (Table 2, Figure 3). In contrast, solutions of the interface-mutant enzyme yielded molecular weights and $R_g$ values comparable to those of a monomer for all concentrations and buffers. Quantitative estimates of the composition of the hexokinase mixtures were obtained using OLIGOMER (see Materials and Methods). In the presence of fixed concentrations of G6P or P_i, the volume fraction of dimers increases with the concentration of the wild-type enzyme (Table 3). P_i stabilizes the wild-type monomer relative to the dimer, whereas G6P stabilizes the dimer relative to the monomer. A significant portion of the wild-type enzyme is dimeric in the presence of G6P, even at the lowest enzyme concentration (0.9 mg/mL). The scattering curves from the interface-mutant enzyme in all solutions were superimposable (Figure 4), the invariants of each curve being indicative of monomeric hexokinase I. Hence, data for the interface-mutant enzyme can be compared directly to calculated scattering curves based on various models for the hexokinase I monomer. The models and scattering curves were generated as described in the Materials and Methods. To test whether the extended conformation of the monomer, observed in the
Figure 3. Small-angle X-ray scattering from the wild-type enzyme in Glc/G6P. Curves 1-4 correspond to 14.6, 7.3, 3.6, and 0.9 mg/mL enzyme, respectively. Observed data are represented by circles. Calculated curves (solid lines) are based on a subunit of the Glc/G6P crystalline complex (rotation, 0°; N- and C-terminal halves closed).
Table 3: Volume Fractions Expressed as Percent Monomers and Dimers of Hexokinase I Present in the Solution on the Basis of Small Angle Scattering

<table>
<thead>
<tr>
<th>Sample</th>
<th>concn (ng/mL)</th>
<th>$\chi^2$</th>
<th>$&lt;M_r&gt;$ (kDa)</th>
<th>$&lt;R_g&gt;$ (nm)</th>
<th>Monomer (%)</th>
<th>Dimer (%)</th>
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</thead>
<tbody>
<tr>
<td>WT, Glc/G6P</td>
<td>14.6</td>
<td>0.670</td>
<td>196</td>
<td>4.41</td>
<td>3.7(1.0)</td>
<td>96.3(0.8)</td>
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<tr>
<td></td>
<td>7.3</td>
<td>0.464</td>
<td>192</td>
<td>4.41</td>
<td>8.0(1.6)</td>
<td>92.0(1.2)</td>
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<tr>
<td></td>
<td>3.6</td>
<td>0.451</td>
<td>182</td>
<td>4.39</td>
<td>17.9(2.6)</td>
<td>82.1(1.9)</td>
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<tr>
<td></td>
<td>0.9</td>
<td>0.283</td>
<td>179</td>
<td>4.39</td>
<td>20.5(7.8)</td>
<td>79.5(5.5)</td>
</tr>
<tr>
<td>WT, Glc/Pi</td>
<td>14.4</td>
<td>1.011</td>
<td>124</td>
<td>4.26</td>
<td>76.2(0.8)</td>
<td>23.8(0.5)</td>
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<tr>
<td></td>
<td>7.2</td>
<td>0.666</td>
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<td>4.21</td>
<td>89.4(1.1)</td>
<td>10.6(0.8)</td>
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<td>3.6</td>
<td>0.618</td>
<td>99.9</td>
<td>4.16</td>
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<td>0.1(1.3)</td>
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<tr>
<td></td>
<td>1.8</td>
<td>0.537</td>
<td>98.7</td>
<td>4.16</td>
<td>101.3(2.5)</td>
<td>-1.3(1.6)</td>
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<tr>
<td></td>
<td>1.0</td>
<td>0.665</td>
<td>105</td>
<td>4.19</td>
<td>95.4(4.0)</td>
<td>4.6(2.6)</td>
</tr>
<tr>
<td>mutant, Glc/G6P</td>
<td>14.1</td>
<td>0.522</td>
<td>105</td>
<td>4.19</td>
<td>95.1(1.1)</td>
<td>4.9(0.8)</td>
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<td></td>
<td>7.0</td>
<td>0.369</td>
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<td>4.19</td>
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<td>3.5</td>
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<td>4.14</td>
<td>103.6(2.5)</td>
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<td>6.5</td>
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<td>4.17</td>
<td>97.6(1.9)</td>
<td>2.4(1.4)</td>
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<tr>
<td></td>
<td>3.2</td>
<td>0.370</td>
<td>106</td>
<td>4.19</td>
<td>93.9(3.3)</td>
<td>6.1(2.3)</td>
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<td>0.8</td>
<td>0.398</td>
<td>79.9</td>
<td>4.03</td>
<td>120.1(6.4)</td>
<td>-20.1(4.5)</td>
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</table>

Table 3 footnotes.

1 Values in parentheses are standard deviations. 2 Samples contain wild-type (WT) enzyme or interface-mutant enzyme in the presence of Glc/Pi, Glc/G6P, or Glc/ADP/Mg$^{2+}$/AlF$_3$ under conditions provided in the Materials and Methods. 3 Goodness of fit expressed as $\chi^2 = (N - 1)^{-1}\Sigma[(I(s_j) - I_{exp}(s_j))/\sigma(s_j)]$, where $N$ is the number of data points, $I(s_j)$ the calculated curve, and $I_{exp}(s_j)$ and $\sigma(s_j)$ are the experimental data and their standard deviations, respectively. 4 $<M_r>$ is the volume-fraction, weighted average of the monomer $M_r$ (100 kDa) and the dimer $M_r$ (200 kDa). 5 $<R_g>$ is the volume-fraction, z-average of the radii of gyration of the hydrated monomer (4.16 nm) and dimer (4.42). 6 Volume fractions evaluated by OLIGOMER (see Materials and Methods). The program does not restrict the fractions to be within the range 0.0-1.0, hence negative values are possible.
Figure 4. Agreement in calculated scattering curves and observed scattering data. Scattering data are from the interface mutant enzyme in Glc/Pi (1), in Glc/ADP/Mg^{2+}/AlF₃ (2), and in Glc/G6P (3), and the wild-type enzyme in Glc/Pi (4). Each curve is offset along the vertical axis in order to facilitate comparisons between observed data (open circles) and the calculated scattering (solid lines) from a subunit of the Glc/G6P crystalline complex (rotation, 0°; N- and C-terminal halves closed).
Table 4: Goodness-of-Fit between Experimental and Calculated Scattering Curves for the Interface Mutant of Hexokinase I

<table>
<thead>
<tr>
<th>model for calculated data</th>
<th>goodness-of-fit for interface mutant in</th>
<th>rotation (deg)</th>
<th>C-terminal</th>
<th>N-terminal</th>
<th>Glc/P$_1$</th>
<th>Glc/G6P</th>
<th>Glc/ADP/Mg$^{2+}$/AlF$_3$</th>
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<tr>
<td></td>
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<td>0</td>
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<td>closed</td>
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<td>closed</td>
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<td>closed</td>
<td>closed</td>
<td>0.641</td>
<td>0.462</td>
<td>0.557</td>
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</table>

Table 4 footnote.

1 Calculated curves are determined from the models of Figure 2, using the program CRYSOL (29). The last four entries of Table 4 correspond to models of Figure 2, panels f, a, b, c. and d, respectively. Models for the first two entries of Table 4 are not illustrated here.
Figure 5. Calculated and observed scattering curves from the interface mutant enzyme in Glc/P$_i$. Observed data are represented by circles. Calculated scattering curves are for the N- and C-halves closed with a rotation of 0° (solid line), 35° (short dash), and 80° (long dash).
crystallographic dimers, is a consequence of dimerization, models of Figure 2b and Figure 2c were generated using rotation angles of 35 and 80°, respectively. These more compact models, however, gave cattering curves which fit the observed data poorly (Figure 5. Table 4). Calculated scattering curves from extended models, such as those of Figure 2a and 2b, where the rotation angle is either 0 or -20°, provided excellent fits to the observed data (Figure 5, Table 4). The quality of the fit was insensitive to models based on an extended monomer with open and/or closed domains (Figure 2, panels e and f). Models, which fit the observed data well, have in common a rodlike conformation. This rodlike conformation exists in the presence of ligand combinations (Glc/Pi, Glc/G6P, or Glc/ADP/Mg^{2+}/AlF_3), which should sample all of the anticipated conformational states of hexokinase I.

The radius of gyration computed from the atomic model of the monomer is 4.1 nm. whereas the experimental values are systematically higher (about 4.2 nm). The increase is due to a higher solvent density in the hydration shell (using CRY SOL, contrasts in the hydration layer 8b between 14 e/nm^3 and 38 e/nm^3 for all the fits). These contrasts correspond to bound water of about 1.1 g/cm^3 and to a protein hydration of about 0.23 g of H_2O/g of dry protein, in agreement with results obtained previously for other proteins in solution (30).

**Discussion**

Small-angle scattering data are consistent with appreciable dimer formation for wild-type hexokinase I in the presence of 1 mM G6P and 2 mM glucose, whereas in the presence of 1 mM Pi and 2 mM glucose, the enzyme is primarily monomeric. The results are in agreement with analytical ultracentrifugation studies of hexokinase I from bovine brain, which indicate enzyme monomers at total protein concentrations of up to 7 mg/mL and a
dimer in the presence of G6P at total enzyme concentrations of 1-1.8 mg/mL (31). Either P\textsubscript{i} at 10 mM or ATP at 5 mM antagonize dimerization induced by G6P. Easterby (32) reported approximately 9% dimer in the absence of G6P at total enzyme concentrations of 2.5 mg/mL of hexokinase I from heart. Dimerization is favored by the reduction of ionic strength and pH. Sedimentation coefficients increase for hexokinase I from rat brain in the presence of G6P at low protein concentration (12). P\textsubscript{i} reverses the observed increase in sedimentation coefficient. The antagonistic effects of G6P and P\textsubscript{i} putatively demonstrate two conformational states for the monomer, but the same study reported monotonic increases in sedimentation coefficients with increases in total enzyme concentration, a phenomenon not easily reconciled with the existence of a single state of aggregation for the enzyme. Indeed, the sucrose gradient used in the latter study could profoundly stabilize the hexokinase dimer. Polyethylene glycol, for instance, induces crystallization of hexokinase dimers; the effect of polyethylene glycol on the bulk properties of an aqueous solution is not unlike that of a high concentration of sucrose.

In contrast to the wild-type enzyme, the interface mutant remains a monomer at all protein concentrations in the presence of all ligand combinations (Table 3). Furthermore, the mutant enzyme retains a rodlike conformation in solution consistent with Figure 2, panels a, b, e, and f. Small-angle scattering data were insensitive to conformational differences between G6P- and P\textsubscript{i}-bound states of the mutant, with either state explained equally well by models with opened or closed domains (Table 4). No significant change occurs in the radius of gyration of the mutant enzyme in response to antagonistic ligands. Observed changes in sedimentation coefficient for the wild-type enzyme in sucrose density gradients then are not a
likely consequence of conformational differences in monomers, but rather may be due to ligand-induced change in the state of aggregation of the wild-type enzyme.

Small-angle scattering data reported here place distinct limits on the conformational changes in monomeric hexokinase I in response to ligand association. Whether it be inhibited by the product, relieved of that inhibition by phosphate, or complexed with putative transition-state analogues (Glc/ADP/Mg\(^{2+}\)/AlF\(_3\)), the monomer retains a rodlike conformation similar to that observed for a single polypeptide chain of crystallographic dimers. The interface between the N- and C-terminal halves observed in crystal structures involves loop 242-250 of the N-terminal half and residues 796-813 of the C-terminal half (Figure 1). The mutation of Arg801, involved in a salt link between the N- and C-terminal halves of the crystalline dimer, reduces phosphate relief of product inhibition (18), a finding which also implicates the interface above in the allosteric mechanism. Hence, existing crystal structures of hexokinase dimers provide a reasonable approximation of the conformation of hexokinase monomers in solution.

Conformational changes associated with the allosteric mechanism of hexokinase I must maintain an extended, rodlike structure. Four kinds of conformational change are possible: (i) rotating the transition helix, as done in the modeling here; (ii) bending the transition helix at its approximate midpoint; (iii) translating the N- and C-terminal halves in opposite directions along the axis of the transition helix; (iv) twisting the N- and C-terminal halves in opposite directions about the axis of the transition helix. Types i-iii above require rotations, bending, and/or displacements of a magnitude that retains an extended molecule with an overall length of 15 nm, whereas any twist (type iv conformational change) would not alter overall length. Conformational changes within either the N- or C-terminal half (such
as domain movements between open and closed conformers) are probably of great importance to function, but do not cause a detectable change in the length and/or shape of hexokinase I. So far, crystallographic structures of dimers have revealed only a tendency for the transition helix to bend modestly at its mid-section (13).

Small-angle scattering data are inconsistent with any model, whereby the N-terminal half occludes the G6P pocket of the C-terminal half of the monomer (4). For the N-terminal half to block the G6P pocket of the C-terminal half, the monomer must assume a bent conformation (rotation angle of 80°), which is not observed. Yet, in equilibrium binding experiments, only one G6P molecule binds to hexokinase I, whereas in crystallographic dimers two molecules of G6P bind to each polypeptide chain (one each at the N- and C-terminal halves). Hence, some mechanism in the monomer other than occlusion must make the binding of G6P to the N- and C-terminal halves mutually exclusive. The data here are consistent with a model in which interactions between the N- and C-terminal halves statically perturb one of the G6P pockets or a model in which the G6P pockets in the N- and C-terminal halves are coupled by a mechanism of negative cooperativity. Crystal structures of the interface mutant under different states of ligation, could provide more insight regarding interactions between ligand binding pockets of the N- and C-terminal halves. To this end crystals are now available for the interface mutant enzyme in its complex with G6P, which diffract to 1.9 Å resolution.

References


CHAPTER V. CRYSTAL STRUCTURES OF MUTANT HEXOKINASE I REVEAL MULTIPLE ADP BINDING SITES AND CONFORMATIONAL CHANGES RELAVANT TO ALLOSTERIC REGULATION

Alexander E. Aleshin¹, Christina Kirby¹, Xiaofeng Liu¹, Gleb P. Bourenkov², Hans D. Bartunik², Herbert J. Fromm¹, Richard B. Honzatko¹*

Abstract

Hexokinase I, the pacemaker of glycolysis in brain tissue, is composed of two structurally similar halves connected by an α-helix. The enzyme dimerizes at elevated protein concentrations in solution and in crystal structures; however, almost all published data reflect the properties of a hexokinase I monomer in solution. Crystal structures of mutant forms of recombinant human hexokinase I, presented here, reveal the enzyme monomer for the first time.

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Key Words: hexokinase I; brain hexokinase; X-ray structure; glycolysis; allosteric enzyme.

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time. The mutant hexokinases bind both glucose 6-phosphate and glucose with high affinity to their N and C-terminal halves, and ADP, also with high affinity, to a site near the N terminus of the polypeptide chain. Exposure of the monomer crystals to ADP in the complete absence of glucose 6-phosphate reveals a second binding site for adenine nucleotides at the putative active site (C-half), with conformational changes extending 15 Å to the contact interface between the N and C-halves. The structures reveal distinct conformational states for the C-half and a rigid-body rotation of the N-half, as possible elements of a structure-based mechanism for allosteric regulation of catalysis.

**Introduction**

Hexokinase catalyzes the first step of glycolysis, the phosphorylation of glucose by ATP to produce glucose 6-phosphate (G6P). In brain tissue and the red blood cell, hexokinase regulates glucose metabolism (Lowry & Passonneau, 1964). The enzyme from brain (called the type-I isozyme) binds to the surface of mitochondria (Polakis & Wilson, 1985; Xie & Wilson, 1988; Wicker et al., 1993; Beutner et al., 1996, 1997, 1998). Here, it interacts putatively with permeation pores, composed of porin (a channel protein that spans the outer membrane of the mitochondrion) and adenylate translocase (a channel protein that spans the inner membrane). The stacked assembly of translocase, porin and hexokinase permits the direct exchange of adenine nucleotides between the mitochondrial matrix and the hexokinase active site (de Cerqueira Cesar & Wilson, 1998). The kinetic properties of hexokinase I, bound to mitochondria and free in solution, are qualitatively similar (Rose & Warms, 1967; Purich & Fromm, 1971; de Cerqueira Cesar & Wilson, 1998).

Hexokinase isozymes I-III have a molecular mass of approximately 100 kDa (Katzen & Schimke, 1965), and consist of two halves, each of which resembles yeast hexokinase in
sequence and structure (Schwab & Wilson, 1989; Wilson, 1995). Isozyme IV (glucokinase) has a molecular mass of 50 kDa, and is similar in sequence and structure to both halves of isozymes I-III. The sequence similarity between the N and C-terminal halves putatively stems from the duplication and fusion of a primordial hexokinase gene (Easterby & O'Brien, 1973; Holroyde & Trayer, 1976; Ureta, 1982; Manning & Wilson, 1984).

Although isozymes I-III share the same structural organization, they differ significantly in functional properties. Both halves of hexokinase II support catalysis, whereas the N-terminal halves of hexokinases I and III are inactive (White & Wilson, 1989; Arora et al., 1993). G6P potently inhibits isozymes I-III, but only for hexokinase I does orthophosphate (P_i) relieve product inhibition (Ureta, 1975; Ellison et al., 1975; Fromm, 1981). In contrast to isozymes I-III, glucokinase (like hexokinase from yeast) is insensitive to G6P.

In crystal structures, G6P binds to both halves of hexokinase I (Aleshin et al., 1998a, b; Mulichak et al., 1998). Indeed, G6P binds with high affinity to the truncated N-terminal half of hexokinase I (White & Wilson, 1989) and potently inhibits the truncated C-terminal half (White & Wilson, 1989; Magnani et al., 1992; Zeng & Fromm, 1995). Single mutations, which eliminate G6P-binding to either the N or C-terminal half, have little or no influence on G6P inhibition (Zeng et al., 1996; Fang et al., 1998; Sebastian et al., 1999; Liu et al., 1999). If mutations at both G6P binding sites are combined, then product inhibition is abolished (Liu et al., 1999). Evidently, the binding of G6P to either the N-half (allosteric inhibition) or the C-half (direct inhibition) of the enzyme inhibits catalysis. Data from equilibrium binding and kinetics experiments, however, support only one high-affinity binding site for G6P to wild-type hexokinase I (Chou & Wilson, 1974; Ellison et al., 1975; Mehta et al., 1988), suggesting
the dominance of one inhibitory mechanism or the possibility of anti-cooperative binding of G6P to the N and C-terminal halves (Liu et al., 1999).

The mechanism of P-relief of product inhibition presents yet another paradox. On the basis of crystal structures (Aleshin et al., 1998b) and directed mutations (Fang et al., 1998), P associates with high affinity to the N-terminal half at the binding site for the 6-phosphoryl group of G6P. Hence, P can displace G6P from the N-terminal half. P and G6P-ligated N-terminal halves, however, have nearly identical conformations (Aleshin et al., 1998b). How, then can the C-terminal half respond differently to a regulatory domain that has the same global conformation in its inhibited and inhibition-relieved states?

At enzyme concentrations typical for kinetics investigations, hexokinase I is a monomer (Wilson, 1995; Aleshin et al., 1999), but at enzyme concentrations used for crystallizations hexokinase I is a dimer (Aleshin et al., 1998a, b, c; Mulichak et al., 1998). As the contact surface of the putative allosteric interface is small relative to that between subunits in the dimer, the dimer may be of limited value in understanding the allosteric mechanism. In an effort to address this potential shortcoming, we present crystal structures of an engineered monomer of hexokinase I, which for the first time reveal an ADP-ligated active site of hexokinase and an unexpected binding site for ADP at the N-terminal half. The ADP-ligated active site suggests a Mg\textsuperscript{2+}-ATP complex of hexokinase, which is in agreement with proposed metal-ATP complexes of related enzymes and for the hydrated Mg\textsuperscript{2+}-ATP complex of yeast hexokinase derived from solution studies. The ADP-binding site at the N-half is proximal to structural elements required for membrane association of hexokinase, reflecting a possible role for the N-half in the exchange of adenine nucleotides with the mitochondrial matrix. ADP and G6P-ligated monomers of hexokinase I reveal different conformational
states for the C-terminal half, one of which permits the association of ATP and the other antagonizes ATP binding. Rigid-body movements of the N-terminal half in response to the binding of P$_i$ and G6P, may differentially stabilize the two conformational states of the C-half.

**Results and Discussion**

*General properties of engineered hexokinase monomers*—Kinetic parameters for the engineered monomer of hexokinase I (the triple mutant, Glu$^{280}$→Ala, Arg$^{283}$→Ala, Gly$^{284}$→Tyr), are comparable to those of the recombinant, wild-type enzyme (Table 1). The triple mutant retains sensitivity to G6P and P$_i$, which clearly demonstrates a functional allosteric system. Small-angle X-ray scattering experiments reveal no dimerization of the triple mutant up to concentrations of 16 mg/ml in the presence or absence of ligands (Aleshin *et al.*, 1999). Consistent with data from X-ray scattering is the growth of a new crystal form (space group $P2_1_2_1_2$, $a$=145.66 Å, $b$=145.80 Å, and $c$=58.13 Å), which has but one polypeptide chain in the asymmetric unit and no intermolecular contact characteristic of those in the hexokinase dimer. Indeed, intermolecular contacts in crystals of the triple mutant differ completely from those of previous crystal forms of hexokinase I (Aleshin *et al.*, 1998a, b, c; Mulichak *et al.*, 1998). In spite of the differences in lattice contacts, the triple mutant retains the same rod-like conformation of the hexokinase I subunit observed in crystals of the dimer (Figure 1), consistent with the findings of small-angle X-ray scattering (Aleshin *et al.*, 1999). The N and C-terminal halves of the monomer have one molecule each of bound G6P and Glc. Each half is in a closed conformation, similar to the closed conformation of yeast hexokinase (Bennett & Steitz, 1980). The two structural domains (small and large), which exist in each half of hexokinase I, form a compact structure due to a 17° rotation of the small domain
### Table 1. Kinetic Parameters for Wild-Type and Mutant Hexokinases

<table>
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<th>$k_{cat}$</th>
<th>$K_m^{ATP}$</th>
<th>$K_m^{Glc}$</th>
<th>$K_i^6$</th>
<th>Pi-relief$^6$ (%)</th>
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<td>WT$^1$</td>
<td>601±5</td>
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<td>Triple$^2$</td>
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<td>Quadruple$^3$</td>
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<td>44.9±3.5</td>
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<td>85±6</td>
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<tr>
<td>Thr$^{536}$→Ala-mini$^4$</td>
<td>44±5</td>
<td>0.37±0.03</td>
<td>56.8±2.9</td>
<td>N.D.$^7$</td>
<td>N.D.$^7$</td>
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**Table I footnotes.**

$^1$Wild-type hexokinase I.

$^2$Triple (Glu$^{280}$→Ala, Arg$^{283}$→Ala, Gly$^{284}$→Tyr) mutant of hehokinase I.

$^3$Quadruple (Glu$^{280}$→Ala, Arg$^{283}$→Ala, Gly$^{284}$→Tyr, Thr$^{536}$→Ala) mutant of hehokinase I.

$^4$Thr$^{536}$→Ala mutant of truncated C-half (residues 455-917) of hexokinase I. $^5K_i$ was obtained from plots of 1/velocity versus 1/[ATP] at 2mM glucose with AnG6P ranging from 0 to 400 μM.

$^6$Percent relief of AnG6P inhibition by Pi was determined as follows: 100 × (A – B)/A, where A is the slope from the plot of (relative velocity of hexokinase)$^1$ versus AnG6P concentration in the absence of Pi and B is the slope from the plot of (relative velocity of hexokinase)$^1$ versus AnG6P concentration in the presence of 6 mM $P_i$.

$^7$Not detectable.
relative to the large domain (Aleshin et al., 1998b). The open conformation has been observed in hexokinase I for only the C-terminal half, in a crystalline dimer grown from a solution of Glc and P$_1$ (Aleshin et al., 1998b). ADP is required for the growth of crystals of the triple mutant, and indeed one molecule of ADP binds to the N-terminal half at a site remote from that of G6P and Glc binding (Figure 1).

The quadruple mutant (Glu$^{280}$→Ala, Arg$^{283}$→Ala, Gly$^{284}$→Tyr, and Thr$^{356}$→Ala), employed in ligand soaks of ADP, combined the non-dimerizing properties of the triple mutant along with an active site still competent with respect to catalysis, but insensitive to direct inhibition by G6P (Table 1). Indeed, crystals of the quadruple mutant are isomorphous to those of the triple mutant, but require a 500-fold increase in G6P concentration in order to grow. (Crystals of either the triple or quadruple mutants have appeared only in the presence of Glc, ADP and G6P). Hence, in the preparation of ADP-quadruple mutant complexes, G6P is removed from crystals by repeated exchange with ADP solutions (see Experimental). Ligand exchange reduces the resolution of X-ray diffraction from 2.0 to 2.8 Å (Table 2), suggesting the onset of conformational changes in the ADP-quadruple mutant, which disrupt crystalline molecule of ADP bound to the N-terminal half at the site noted above in the triple mutant (Figure 1).

Statistics from data collection and refinement for each of the structures are presented in Table 2. On the basis of PROCHECK (Laskowski et al., 1993), all structural parameters are equivalent to or better than those from structures of comparable resolution. Only two out of 898 residues fall into generously allowed regions of the Ramachandran plot. Estimates of the overall root-mean-square error in atom positions (Murshudov & Dodson, 1997) are 0.16 Å for the G6P/Glc complex and 0.45 Å for the ADP/Glc complex.

**ADP-ligated active site**—In the ADP/Glc complex, the base moiety of ADP is located
Figure 1. Overview of (a) the ADP/Glc-monomer complex and (b) the G6P/Glc-monomer complex of hexokinase I. The large and small domains of the N and C-halves are purple and yellow, respectively. The flexible subdomain (residues 766-812) is dark purple. The side-chains of the residues 281, 283, and 284, which were altered by mutation to block dimerization, are red. ADP molecules are cyan, glucose molecules are green, the phosphate and G6P molecules are dark blue. This drawing was made with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merritt & Murphy, 1994).
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<td>14.1 (0.35)</td>
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<td>— *</td>
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<td>39.7 (1.00)</td>
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Table 2 footnotes.

*Hexokinase I with mutations Glu²⁸⁰→Ala, Arg²⁸³→Ala, Gly²⁸⁴→Tyr. #Hexokinase I with mutations Glu²⁸⁰→Ala, Arg²⁸³→Ala, Gly²⁸⁴→Tyr, Thr⁵³⁶→Ala. $R_{merge}$ = $\Sigma_i |I_i - <I_i>| / \Sigma_i I_i$. ²Otwinowski et al. (1993), Steller et al., (1997), Collaborative Computational Project, Number 4, (1994). & R-factor = $\Sigma ||F_{obs}|-|F_{calc}|| / \Sigma |F_{obs}|$, for all reflections $|F_{obs}| > 0$. ³R-factor based on 5% of the data excluded from refinement. †Obtained by trial-and-error adjustment during refinement. *Not bound. ³Estimated value.
order (see below). The ADP-quadruple mutant has one molecule of ADP and Glc bound to a closed C-terminal half, one molecule of P, and Glc bound to a closed N-terminal half, and a between helices α9 (residues 783-791) and α11' (residues 862-867, Figure 2). The 6-amino group and the N-1 atom of ADP hydrogen bond with backbone carbonyl group 785 and the side-chain of Ser788, respectively (Table 3). The 2'-hydroxyl group hydrogen bonds with backbone carbonyl group 747. The side-chain of Thr784 is poorly ordered, but one of its staggered conformers could hydrogen bond with the 2'-hydroxyl group as well. The β-phosphoryl group of ADP occupies the same site as the 6-phosphoryl group of G6P, hydrogen bonding with Thr863 (side-chain and amide backbone), Thr680 (side-chain and amide backbone), and Ala536 (amide backbone). The α-phosphoryl group of ADP hydrogen bonds to backbone amides 536 and 537, as well as the side-chains of Asn537 and Thr863. The binding sites for G6P and ADP overlap, providing a structural basis for a mechanism of direct inhibition of catalysis, which must be the dominant mechanism in mutants of hexokinase I, which cannot bind G6P to their N-terminal halves (Liu et al., 1999) (Figure 2). ADP binding to the active sites of the quadruple mutant and glycerol kinase (Hurley et al., 1993) are similar, except for their β-phosphoryl groups (Figure 3). The γ-phosphoryl group in the quadruple mutant points away from the 6-hydroxyl group of Glc, the productive binding of that group being blocked by loop 532-539. The conformation of loop 532-539 is identical with that observed in the G6P/Glc complexes of the wild-type enzyme. Hence, the mutation of Thr536 to alanine does not perturb the loop. The conformation of loop 532-539 in the open active site of hexokinase I (Aleshin et al., 1998b), however, differs significantly from that in the closed active site, and would allow productive association of the adenine nucleotide
Figure 2. Ligation of the C-terminal half of monomeric hexokinase I. Top, The ADP/Glc-bound active site with ADP and Glc drawn in green, selected side-chains in orange, elements of the small domain in yellow, and segments of the large domain in purple. Dotted red lines designate donor-acceptor interactions. The electron density (2F_{obs}-F_{calc}, blue color) covering the ADP molecule is from an omit map, contoured at 1 σ. Bottom, The G6P/Glc-bound active site. ADP in black, taken from the top illustration, represents the overlap with G6P. This drawing was made with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merrit & Murphy, 1994).
Figure 3. Comparison of the ADP/Glc monomer complex of hexokinase (left) to the ADP-complex of glycerol kinase (right). The large domain of hexokinase is purple and the small domain yellow. Dotted red lines indicate donor-acceptor interactions. The β-phosphoryl group of ADP is not productively bound in the ADP/Glc monomer complex due to the steric influence of loop 532-539. In the conformation observed for the open C-half (green), however, loop 532-539 allows productive binding of the β and γ-phosphoryl groups of ATP (black lines) with Mg$^{2+}$ (black sphere). Comparable interactions involving the ADP molecule are observed in a ligated complex of glycerol kinase (Hurley et al., 1993). This drawing was made with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merrit & Murphy, 1994).
Using loop 532-539 from the open active site in the context of the closed C-terminal half, one can model a productive conformation for the \( \beta \) and \( \gamma \)-phosphoryl groups of Mg\( ^{2+} \)-ATP (Figure 4). The model for the Mg\( ^{2+} \)-ATP/Glc complex of hexokinase I shares features of metal-ATP complexes of related enzymes (glycerol kinase, actin and hsp70). Thr863 of the ADENOSINE motif (Bork et al., 1992) interacts with the ribosyl and \( \alpha \)-phosphoryl groups of ATP, Asp532 (PHOSPHATE I motif) interacts with Mg\( ^{2+} \) through coordinated water molecules, and loop 679-681 (PHOSPHATE II motif) has conserved interactions with the \( \beta \) and \( \gamma \)-phosphoryl groups of ATP. The 6-hydroxyl group of Glc is 3 Å from the \( \gamma \)-phosphorus atom and oriented for an in-line displacement reaction. The terminal oxygen atoms of the \( \gamma \)-phosphoryl group interact with Lys621 and/or Ser603, backbone amide group 681 and the metal cation. Asp657 hydrogen bonds with the 6-hydroxyl group of glucose as a proton acceptor, and is a putative catalytic base. The mutation of Asp657 to alanine reduces \( k_{cat} \) by at least 100-fold (Arora et al., 1991). No functional group of the protein is evident as a catalytic acid (protonation of ADP as a leaving group), but two of the water molecules coordinated to the Mg\( ^{2+} \) could transfer a proton to the \( \gamma \)-phosphoryl group of ATP. Loop 532-539 in the open conformation does not interact with Mg\( ^{2+} \)-ATP, but a modest (1-2 Å) conformational movement toward the \( \beta \) and \( \gamma \)-phosphoryl groups of ATP would allow hydrogen bond formation with backbone amide groups 534-536 and/or the side-chain of Thr536. Mutation of Gly534 to alanine results in a 4000-fold reduction in \( k_{cat} \) (Zeng et al., 1998). The model is consistent as well with independent determinations of metal hydration and the stereochemistry of metal-coordinated ATP in hexokinase complexes. Electron paramagnetic resonance (EPR) spectra indicate the coordination of four water molecules to
Figure 4. Stereoview of the Mg$^{2+}$-ATP/Glc complex of the active site of hexokinase I. The illustration is based on a model derived from the ADP/Glc monomer complex of hexokinase I. Structural elements in purple and yellow are from the large and small domains, respectively, of the C-terminal half of hexokinase I. Elements in green represent loop 532-539 in its open conformation. Dotted red lines designate donor-acceptor interactions. Mg$^{2+}$ and its coordinate bonds are black, with four red spheres representing coordinated water molecules. The 6-hydroxyl group of Glc is in-line with respect to the $\gamma$-phosphoryl group of ATP, with Asp657 acting as a catalytic base. This drawing was made with MOLSCRIPT (Kraulis, 1991) and RASTER3D (Merrit & Murphy, 1994).
the cation in complexes of yeast hexokinase, and the absence of direct metal-protein coordination (Olsen & Reed, 1993). As Mg$^{2+}$ in the model occupies the pyranose-binding pocket for G6P (see below), ample room is available for a completely hydrated cation. Indeed, Asp532 and Arg539 of the small domain may hydrogen bond with the metal-coordinated water molecules. Such interactions indirectly link the small to the large domain and should stabilize a closed conformation for the C-terminal half (Figure 4). The left-handed isomer of chromium-ATP binds to hexokinase 15-fold more tightly than the right-handed isomer (Rawlings et al., 1993). Mg$^{2+}$-ATP (Figure 4) adopts the stereochemistry of the left-handed chromium-ATP complex; the right-handed complex will fit into the active site, but the distance between the 6-hydroxyl group of Glc and the $\gamma$-phosphorus atom of ATP is 2-2.5 Å (as opposed to 3 Å for the left-handed complex). On the basis of the present study, we cannot determine which of the two chromium-ATP complexes best represents the productive Mg$^{2+}$-ATP complex.

**G6P-ligated active site**— G6P (2.0 Å resolution, average B of 26 Å$^2$) reveals a different orientation of the pyranosyl group of G6P (Figure 2), relative to that reported originally in the dimer complex (Aleshin et al., 1998a). In the monomer complex, the pyranose moiety of G6P is rotated by 180° about its C5-C6 bond, leaving interactions between its critical 2-hydroxyl group a Electron density in the vicinity of bound nd the enzyme unchanged, but interchanging protein-ligand interactions involving its 1 and 3-hydroxyl groups (Table 3). This new conformation for G6P provides an adequate fit of the electron density from the 2.8 Å resolution dimer complex. Given the superior order and resolution of the G6P/Glc monomer complex in the vicinity of the G6P-binding pocket, the conformation for G6P in the dimer structure is probably incorrect. An updated deposition for
the G6P/Glc dimer complex reflects the conformation observed here for the G6P/Glc monomer complex.

The C-terminal halves of the G6P/Glc and ADP/Glc complexes are in closed conformations. Yet significant conformational differences exist between the C-terminal halves of the two structures. Thr784, which is in contact with the ribose and base moieties of ADP, projects into the ADP pocket in the G6P/Glc monomer complex (Figure 5). Electron density difference maps between the G6P/Glc and ADP/Glc complexes reveal conformational changes, which extend over residues 776-812 (Figure 5). Aleshin et al. (1998a) called this region the flexible subdomain, because it diverged conformationally from corresponding segments in the N-terminal half of hexokinase I and in yeast hexokinase. Indeed, this region is sensitive to ligation of the adenine pocket, and has high thermal parameters for main-chain atoms. Conformational changes in Thr784 directly perturb residues 774-786, which connect helix α8 to helix α9 (Figure 5). The side-chain of Ile781 is in contact with the side-chain of Leu807. Hence, the conformational change in Thr784 is relayed through non-bonded contacts between Ile781 and Leu807 to segment 792-814. That segment is putatively the C-terminal side of the allosteric interface of hexokinase I (Fang et al., 1998). The most significant conformational change in segment 792-812 involves loop 792-798. Here, the electron density in omit maps is weak (Figure 6), but reproducible in multiple data sets for the ADP/Glc and G6P/Glc monomers. Hence, a conformational difference in the adenine pocket (Thr784) results in perturbations throughout the flexible subdomain of the C-terminal half, and reach the putative allosteric interface of hexokinase I.

Yeast hexokinase (G6P-insensitive), hexokinase from Schistosoma mansoni (G6P-sensitive, and directly inhibited) and hexokinase I (G6P-sensitive, with direct and allosteric
Table 3. Selected Interactions from the G6P/Glc- and ADP/Glc-Monomer Complexes.

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<th>Distance (Å)</th>
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Table 3. footnotes.

\(^a\)Distances are from the G6P/Glc-monomer complex.

\(^b\)Distances are from the ADP/Glc-monomer complex.

\(^c\)Lattice contact.

\(^d\)Thr\(^{536}\) in wild type hexokinase I.
Figure 5. Conformational differences in the flexible subdomain of the C-terminal half of the ADP/Glc- and G6P/Glc-complexes. The Cα trace of the ADP/Glc- (bold lines) and the G6P/Glc-complexes diverge in the flexible subdomain (residues 792—814). Two conformations of Thr784 are shown explicitly. The contact interface between halves of the hexokinase I subunit involve interactions between the flexible subdomain and loop 241—253 of the N-terminal half.
inhibition) may exhibit specific steps in the evolution of hexokinase structure and G6P inhibition. In the closed Glc complex of yeast hexokinase (Bennett & Steitz, 1980), a sulfate anion binds to the 6-phosphoryl site, but does not induce a conformational change in the segment equivalent to loop 532-539 of hexokinase I. In hexokinase from S. mansoni, the sulfate anion at the 6-phosphoryl site induces a conformational change in the corresponding loop (Mulichak et al., 1998), similar to that observed for hexokinase I (Aleshin et al., 1998a, b). Hence, a pattern is emerging that associates G6P sensitivity in hexokinases with the conformational mobility of segments corresponding to loop 532-539 in hexokinase I. The residues in yeast hexokinase and S. manosi, corresponding to Thr784, adopt a conformation similar to that of the ADP/Glc-complex of hexokinase I. They do not have the conformation observed for Thr784 in the G6P/Glc-complex of hexokinase I, even though the hexokinase from S. mansoni is arguably in an inhibited state. Evidently, regions corresponding to the flexible subdomain in hexokinase I are inflexible in enzymes from yeast and S. mansoni, both of which are 50 kDa proteins with no allosteric regulation. Hence, the flexible subdomain observed in hexokinase I may be linked to the evolution of its allosteric mechanism.

*Ligand interactions at the vestigial active site of the N-terminal half*— The N-terminal half of hexokinase I binds ligands at a locus corresponding to the active site of hexokinases. We refer to this site as the vestigial active site, in order to distinguish it from yet another binding site at the N-terminal half for adenine nucleotides (Figure 1). The vestigial active site binds either G6P/Glc or P_i/Glc in dimer (Aleshin et al., 1998a, b, c) and monomer complexes (reported here). Regardless of the ligand combination (G6P/Glc or P_i/Glc) or the state of oligomerization of hexokinase I, the N-terminal halves are always in a closed conformation. The 6-phosphoryl group of G6P and P_i occupy the same site. Glc and the G6P/P_i interactions
Figure 6. Electron density and refined models for the flexible subdomain in the ADP/Glc- and G6P/Glc-complexes of hexokinase I. Electron density from an omit map \( (2F_{\text{obs}} - F_{\text{calc}}) \), contoured at 1\( \sigma \) from the G6P/Glc-complex, with models for the G6P/Glc-complex (bold black lines) and the ADP/Glc-complex (red lines) superimposed (top). Electron density from an omit map \( (2F_{\text{obs}} - F_{\text{calc}}) \), contoured at 1\( \sigma \) from the ADP/Glc-complex, with models for the G6P/Glc-complex (black lines) and the ADP/Glc-complex (bold red lines) superimposed (bottom).
with the vestigial active site correspond closely to those of Glc and G6P at the C-terminal half (Table 3).

Specific mutants of hexokinase I, which cannot bind product at the C-terminal half, are still potently inhibited by G6P (Liu et al., 1999). The mode of inhibition must be allosteric, and for such mutants P_i is an antagonist of G6P. The structural basis for P_i-G6P antagonistism is clearly the common binding site for the phosphate moiety. No report in the literature, however, has yet to explain how nearly identical, closed N-terminal halves can evoke different functional responses from the C-terminal half. The obvious difference in P_i and G6P is the pyranose moiety. The 2-hydroxyl group of the pyranose moiety is absolutely required for potent G6P inhibition (Crane & Sols, 1954). The 2-hydroxyl group of G6P interacts with the side-chain of Asp84 and the backbone amide and side-chain of Ser449. Evidently, the G6P-Ser449 interaction changes the conformation of the polypeptide backbone relative to that of the P_i/Glc-bound N-terminal half (Figure 7). The localized conformational change due to the ligation of Ser449 by G6P could well influence the global conformation of hexokinase I. Ser449 lies in the first turn of the helix (transition helix), which connects the N-terminal half to the C-terminal half. The N-terminal halves of monomer and dimer complexes, grown in the presence of G6P, have nearly identical orientations relative to their transition helices. The dimer complex co-crystallized with P_i, however, differs significantly in the relative orientation of its N-terminal half and transition helix. Superposition of the N-terminal halves from the G6P/Glc monomer and the P_i/Glc dimer, using the first half of the transition helix, reveals a 6° rotation of the entire N-terminal half about the Cα atom of Ala451 (Figure 7). The rigid-body rotation of the N-terminal half, will necessarily influence the position of loop 241-253, relative to the C-terminal half. Loop 241-253 is the N-terminal side of the
putative allosteric interface, interacting through hydrogen bonds and steric contacts with the flexible subdomain of the C-terminal half (Aleshin et al., 1998a, b, Fang et al., 1998).

**ADP-association with the N-terminal half**—ADP binds to the N-half at a site well removed from its vestigial active site (Figure 1). The ADP molecule bound at the N-half is well ordered (average thermal parameter of 34 Å²), and even after washing crystals of the G6P/Glc monomer extensively in buffers free of ADP, electron density remains at this site. The base moiety of ADP is stacked between the side-chains of Phe380 and Arg30 (Figure 8). The 6-amino group and the endocyclic N-1 atom hydrogen bond with the protein directly or through a bridging water molecule (Figure 8, Table 3). Arg30 hydrogen bonds with the 2'-hydroxyl group, and His27 either hydrogen bonds with the 3'-hydroxyl group or Asp23, depending on the orientation of its side-chain. The latter interaction may allow ADP to exert a direct influence on the orientation of the N-terminal helix, which is essential for the association of hexokinase I with mitochondria (Polakis & Wilson, 1985; Xie & Wilson, 1988; Wicker et al., 1993; Beutner et al., 1996, 1997, 1998). Arg425 and Arg426 bind to the pyrophosphoryl group of ADP. Magnesium cations, although present in the crystallization buffer, are not associated with the ADP molecule. An additional hydrogen bond to the β-phosphoryl group of ADP comes from a lattice contact (Table 3), which may explain the absolute requirement for ADP in the growth of the monomer crystal form.

The ADP site at the N-terminal half of hexokinase I is probably functional and not an artifact of lattice contacts. 8-Azido-ATP labels rat hexokinase I somewhere within the first 100 residues (Nemat-Gorgani & Wilson, 1986), suggesting the presence of this ADP binding site on hexokinase I in solution. (Note in Figure 8 the proximity of ADP to several residues with sequence numbers below 100). An ADP (or related) molecule bound to this site could mediate
Figure 7. Conformational differences in P$_i$- and G6P-bound N-halves of hexokinase I.

Stereoview of the N-half from the Pi/Glc-dimer complex (bold lines) superimposed on the N-half of the G6P/Glc-monomer complex, using the entire N-half as the basis for superposition (top). The superposition reveals a significant difference in the conformation of Ser449, which is a recognition element for the 2-hydroxyl of G6P. Stereoview of the N-half of the Pi/Glc-dimer complex (bold lines) superimposed on the N-half of the G6P/Glc-monomer complex, using the N-terminal half of the transition helix as the basis for superposition. The entire N-half pivots about the N-terminus of the transition helix by a rotation of 6°.
Figure 8. ADP binding site at the N-terminal half of hexokinase I. The ball-and-stick model represents the ADP molecule. Electron density is from an omit map \((2F_{\text{obs}}-F_{\text{calc}})\), contoured at 1.4 \(\sigma\) from the G6P/Glc-complex. Donor-acceptor interactions are indicated by dotted lines.
association of hexokinase I with the mitochondrial membrane. Alternatively, the ADP site at
the N-half could be a way-station in the transport of adenine nucleotides to and from the
mitochondrial matrix. The direct transport of adenine nucleotides to and from membrane-bound hexokinase I has been suggested by several investigations (BeltrandelRio & Wilson, 1992; Laterveer et al., 1995; de Cerqueira Cesar & Wilson, 1998). The ADP-binding site is
not blocked by dimer formation and, thus, can bind to hexokinase dimers. Hexokinase I
putatively binds to mitochondria as an oligomer of uncertain composition (Xie & Wilson,

Allosteric mechanism of hexokinase I—The monomer complexes here reveal distinct
conformational states for the C-terminal half, one of which is ATP-compatible, whereas the
other is ATP-antagonistic (Thr784 blocks the base moiety of ATP from its binding site).
Evidently, the N-terminal half also has two conformational states, differing by a 6° rotation
about the N-terminal end of the transition helix. These observations suggest an allosteric
mechanism in which the N-terminal half stabilizes the ATP-compatible C-half in one of its
two orientations and the ATP-antagonistic C-half in the other orientation (Figure 9).

Interactions between the flexible subdomain of the C-terminal half and loop 241-253
of the N-terminal half putatively define the allosteric interface. These structural elements are
coupled in dimer crystal structures, but in the monomer complexes here, they do not interact.
A decoupled monomer is probably the major conformer in solution, whenever G6P
concentrations are sufficiently high to saturate both binding sites of the enzyme (Figure 9). At
low concentrations of G6P, however, product binds to the N-half and stabilizes the ATP-
antagonistic state of the flexible subdomain. P_i displaces G6P, allowing the N-half to stabilize
the ATP-compatible state.
The model above is consistent with that proposed by White & Wilson (1987, 1990). It has the same shortcoming as well. If the G6P-ligated, N-terminal half stabilizes the ATP-antagonistic state of hexokinase I, it should also facilitate the direct binding of G6P to the C-terminal half. Hence, G6P inhibition of hexokinase I should exhibit a second-order dependence on the concentration of product-inhibitor. Although G6P is a non-linear competitive inhibitor with respect to ATP, the association of the second molecule of G6P is much weaker (by two orders) than the first (Fang et al., 1998). In fact, equilibrium binding studies reveal only one high-affinity site on hexokinase I for G6P in the presence of Glc (Chou & Wilson, 1974; Ellison et al., 1975; Mehta et al., 1988). Thus, either the two G6P pockets are coupled anticooperatively (as suggested by Liu et al., 2000) or the direct binding of G6P to the C-terminal half is impaired. The structure-based mechanism proposed here cannot be reconciled easily with the anticooperative binding of G6P.

White & Wilson (1990) suggest that the G6P-binding pocket of the C-terminal half is blocked by the N-terminal half. In all crystal structures of hexokinase I grown in the presence of G6P, however, product is bound to both the N and C-terminal halves. The N-terminal half does not directly block the G6P pocket of the C-terminal half. The pyranose pocket for G6P at the C-terminal half, however, differs in one respect from the corresponding pocket of the N-terminal half. In the open, C-terminal half of the Pi/Glc-dimer of hexokinase I, Asp895 forms a salt-link with Arg539 (Aleshin et al., 1998b). The Asp895-Arg539 salt link corresponds to residue pair Ser447-Arg91 of the N-terminal half, which clearly cannot form the corresponding interaction. The Asp895-Arg539 salt link may perturb the conformation of Ser897, which corresponds to Ser449 of the N-terminal half. Hence, a structural element
Figure 9. Allosteric states of the hexokinase I monomer. The conformation of monomeric hexokinase varies in response to ligation of its active site (C-terminal half) and its vestigial active site (N-terminal half). In the presence of low concentrations of G6P, the allosteric interface between the N- and C-terminal halves, maintains the flexible subdomain in an ATP-antagonistic state, in which Thr784 blocks the base-binding pocket for the nucleotide (A). In the presence of elevated concentrations of G6P, the N- and C-terminal halves are decoupled, with G6P bound directly to the active site, overlapping the ATP pocket (B). Sufficient levels of Pi displace G6P from the N-terminal half and allow a rigid-body rotation of that half relative to the C-terminal half. The allosteric interface now stabilizes the ATP-compatible conformation of the flexible subdomain, removing Thr784 as a steric obstacle to ATP association (C). ATP binds to the Pi-stabilized conformer of hexokinase (D).
critical for the recognition of the 2-hydroxyl group of G6P may not be available in the open C-terminal half.

The significance of the above salt-link is certainly testable by directed mutation, as is the role of Thr784 in mediating product inhibition of catalysis. The mutation of Thr784 to alanine abolishes G6P inhibition of hexokinase and the mutation of Asp895 to alanine, alone causes a threefold decrease in the inhibition constant (denoted $K_i$ by Fang et al., 1998) for the weak G6P interaction (unpublished results). These results, as yet preliminary, are consistent with the structural mechanism presented here.

**Experimental**

**Experimental Materials**— A full-length cDNA of human brain hexokinase, cloned into an expression vector pET-11a (from Novagen) to produce pET-11a-HKI, was available from previous studies (Fang et al., 1998; Aleshin et al., 1999). The Transformer site-directed mutagenesis kit is from Clonetech Laboratories, Inc. Phage T4 polynucleotide kinase and all restriction enzymes are from Promega. Bio-gel hydroxyapatite resin is from Bio-Rad. Toyopearl DEAE-650 M is from Tosohaas. The Iowa State University Nucleic Acid Facility performed oligonucleotide synthesis and DNA sequencing. *Escherichia coli* strain ZSC13 (DE3), which does not contain endogenous hexokinase, was a gift from the Genetic Stock Center, Yale University. ATP, ADP, NADP, 1,5-anhydro-D-sorbitol, deoxyribonuclease I (DNase I), leupeptin, phenylmethylsulfonyl fluoride (PMSF), ampicillin and polyethylene glycol 8000 (PEG 8000) are from Sigma. Glucose-6-phosphate dehydrogenase is from Boehringer Manheim. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) is from BioWorld. Directed mutation, expression, purification and kinetics The triple mutant of hexokinase I (Glu$^{280}$ → Ala, Arg$^{283}$ → Ala, Gly$^{284}$ → Tyr) was constructed and kinetically characterized in a
previous study (Aleshin et al., 1999). The quadruple mutant was constructed by directed mutation of the triple mutant, according to the protocols of the Clontech Transformer site-directed mutagenesis kit. The mutant plasmid was selected from wild-type plasmids by switching a unique NruI restriction site on the pET-11 vector to another unique XhoI site for the single point mutation. The primer for mutation Thr$^{536}\rightarrow$ Ala is 5'-GATCTTGGAGGAGGCAAATTTCCGTG-3', where the modified codon is in bold typeface and underlined. The oligonucleotide primers used for the selection of the mutant plasmid from the wild-type plasmid are 5'-CAGCCTCGCCCGCAAGCACGCCAGCAAG-3', for the conversion from the Nru I site to the XhoI site, and 5'-CCTCGCCGCTCGCGACGCCAGCAAG-3', for the conversion from the XhoI site back to the NruI site. Mutations were confirmed by sequencing the entire cDNA insert, coding for hexokinase I. *E. coli* strain ZSC13, transformed with mutant pET-1la-HKI, was grown in LB medium at 37°C to an A600 of 0.8, whereupon the temperature was reduced to 22°C and IPTG added to a final concentration of 0.4 mM. At 16 to 24 hours after induction, the cells were harvested and then re-suspended in 25 mM KP$_i$ (pH 7.5), 2 mM glucose, 1 mM EDTA, 0.4 mM 2-mercaptoethanol, 1 mM PMSF, and 3000 units of DNase I at a temperature of 4°C. The cells were broken using a French press and centrifuged, after which the supernatant was passed through a DEAE column, using a KP$_i$-buffered (pH 7.5), KCl gradient from 0 to 0.5 M. The fractions containing HKI were concentrated and then passed through a hydroxyapatite column using a KP$_i$-buffered (pH 7.5), KCl gradient from 20 mM-500 mM. Pooled fractions of mutant hexokinase I were further purified by DEAE-HPLC as described (Aleshin et al., 1998a). Hexokinase activity was determined by a coupled spectrometric assay, using G6P dehydrogenase (Fromm & Zewe, 1962). 1,5-Anhydroglucitol 6-phosphate
(AnG6P) is a surrogate for G6P in kinetic studies of G6P inhibition (the coupling enzyme degrades G6P but not AnG6P) and was prepared as described (Ferrari et al., 1959).

Commercial glucose-6-phosphate dehydrogenase comes as an ammonium sulfate precipitate. Sulfate anion, which mimics the effect of Pi in the relief of G6P inhibition (White & Wilson, 1989), was removed by dialysis. Hexokinase concentrations were determined by Bradford (1976) assays using bovine serum albumin as a standard. Initial rate data were analyzed by using a computer program written in MINITAB with an $\alpha$-value of 2.0 (Siano et al., 1975). In experiments with AnG6P, the kinetic data were fit to a model for non-linear competitive inhibition with respect to ATP, in which two molecules of inhibitor interact sequentially with hexokinase I (Fang et al., 1998).

**Crystal preparation**—Crystals of the triple and quadruple mutants were grown by the hanging drop method. Two protocols produced the same crystal form. In the first, hexokinase I mutants were transferred from a storage buffer (30 mM KP$_i$ (pH 7.5), 1 mM Glc, 1 mM β-mercaptoethanol) to a buffer consisting of 25 mM ADP (pH 7.5), 1 mM Glc. Equal volumes (2.5 μl) of the protein solution (20 mg/ml of mutant enzyme in the buffer above) and a precipitant solution (14-18% (w/v) PEG 8000, 100 mM 2-N-morpholinoethanesulfonic acid (Mes), pH 5.8-6.5, 200 mM sodium acetate, 20 mM ADP, 2 mM Al(NO$_3$)$_3$, 10 mM NaF, 30 mM magnesium acetate) were mixed. The drops were equilibrated against 0.7 ml of the precipitant solution. One crystal (dimensions, 0.15 mm×0.15 mm ×0.05 mm) appeared per droplet. Although G6P was not added, the crystalline enzyme had two G6P molecules bound with high occupancies. G6P was produced by hexokinase from glucose and an ATP contaminant in the ADP. The amount of ATP impurity (0.16%) was determined by a yeast hexokinase, G6P dehydrogenase coupled spectrometric assay (Fromm & Zewe, 1962), using
excess glucose and NADP. The importance of G6P for the growth of this crystal form follows from the high ligand occupancy, and from the failure to grow crystals with purified ADP.

In the second crystallization protocol, the protein was dialyzed against 1 mM Glc, 20 mM G6P (pH 7.5), and then concentrated to 20 mg/ml. Equal volumes (2.5 µl) of the protein solution and a precipitant solution (14-18% PEG 8000, 100 mM Mes (pH 5.8-6.5), 150-250 mM sodium acetate and 20 mM ADP) were mixed. The high concentration of G6P, used in the second protocol, provided larger crystals (0.4 mm×0.3 mm×0.2 mm). Data reported in column 1 of Table 2 comes from a crystal grown by the second protocol, but the structure of the crystal grown by the first procedure is identical. Prior to crystal soaks, contaminating ATP was removed from ADP by the coupled assay system above. A solution containing 250 mM impure ADP, three units each of yeast hexokinase and G6P-dehydrogenase, 10 mM magnesium acetate, 1 mM NADP, and 1 mM Glc was incubated for two hours at 24°C, and then filtered through a pressure concentrator with a molecular mass cut-off of 14 kDa. The purified ADP was stored frozen.

Crystals of the quadruple mutant grew only by the second protocol (elevated G6P concentration). G6P was removed from crystals by soaking in G6P-free solutions. Crystals were transferred to 20% PEG 8000, 50 mM Mes (pH 6.5), 50 mM sodium acetate, 1 mM DTT, 40 mM magnesium acetate, 100 mM purified ADP, one unit of G6P-dehydrogenase, and NADP for five hours. The G6P-dehydrogenase/NADP guarantees the removal of any G6P that may be formed by the action of hexokinase on the ligand mixture. Data from these crystals appear in column 2 of Table 2. Glycerol was added to soaking solutions to a final concentration of 25% (v/v) prior to flash-freezing crystals in liquid nitrogen.
Data collection—X-ray diffraction data were collected from the crystal of Table 2, column 1, on the beamline of Dr Bartunik at Deutsches Elektronen Synchrotron (DESY), using a MAR CCD detector. The data from crystals of Table 2, column 2, were collected at BioCARS, Sector 14, at the Advanced Photon Source (APS), Argonne National Laboratory. Data were processed with programs Denzo/Scalepack (Otwinowski et al., 1993), DPS/Mosfilm (Steller et al., 1997) and Scala (Collaborative Computational Project, Number 4, 1994).

Structure determination and refinement—The crystal structure of the G6P/Glc monomer complex (Table 2, column 1) was determined by molecular replacement, using the subunit from G6P/Glc dimer complex (PDB entry 1HKB). The N and C-halves of hexokinase I were positioned independently in the unit cell. The program AmoRe (Navaza, 1994) was used in calculation of rotation and translation functions, based on data to 4 Å, and then used again in the rigid-body refinement of the N and C-halves, against data to 3 Å resolution (final rigid-body R-factor of 0.37).

Model building and refinement—Refinement of the G6P/Glc monomer complex (Table 2, column 1) used ARP (Lamzin & Wilson, 1993), Refmac (Murshudov et al., 1997), programs from the CCP4 package, and parameters provided by Engh & Huber (1991). The refinement was monitored by the decline in R_free, based on 5% of the data randomly culled from the refinement process. The protein molecule was split into its four domains, which were refined as rigid bodies. The program ARP, combined with Protin, Refmac, and Sigmaa, was used for restrained refinement and the calculation of electron density maps. Adjustments to the model were accomplished with XtalView (McRee, 1992) and O (Jones et al., 1991). The first 80% of water molecules were added automatically (3σ cut off applied to an F_obs−F_calc
Fourier map) and refined with the solvent-building routine of ARP. Restrained refinement by Refmac with anisotropic scaling and minimization by the maximum likelihood method were used. The final 20% of water molecules were added manually and ambiguous areas were rebuilt using omit electron density maps produced with Refmac and Sigmaa. Data for the ADP/Glc-monomer complex (Table 2, column 2) were of modest resolution, making the use of ARP impractical. The model for the ADP/Glc complex reflects restrained refinement of individual parameters, but such refinement did not improve $R_{\text{free}}$. The conformations of ligands and protein residues were verified by the comparison of electron density maps from two crystals, prepared independently by the same protocol. Generally, water molecules were fit to peaks of difference density maps higher than 3σ, only if the corresponding positions were occupied in the high-resolution structures of the G6P/Glc monomer complex.

Exceptions to this rule were made in areas that had undergone a conformational change. The analysis of root-mean-square deviations in the positions of C atoms of different models employed the program LSQKAB of the CCP4 package. This program was also used to superimpose the glycerol kinase structure (Hurley et al., 1993) onto the hexokinase I structures, and to determine the differences in the orientation of individual domains in hexokinase I structures.

**Data Bank accession numbers**—Coordinates for the G6P/Glc and ADP/Glc monomer complex have been deposited in the RCSB Protein Data Bank under the accession labels 1CZA and 1DGK, respectively.

**References**


CHAPTER VI. GENERAL CONCLUSIONS

The research included in this thesis contributes to our understanding of the regulation of human hexokinase I. Brain tissue depends heavily on glucose as an energy source. The reaction catalyzed by hexokinase I commits glucose to metabolism in brain cells.

Chapter II proposes a mechanism of negative cooperativity in G6P binding and inhibition. Mutations in G6P binding sites in either the N- and C-terminal half of hexokinase I do not abolish G6P inhibition. However, the same mutations in the truncated hexokinase I (mini-hexokinase I) and mutations on both G6P binding sites abolish G6P inhibition completely. Hence, G6P binding sites, in the N- or C-domain, are functional in regulating hexokinase I. Combining results from isotope exchange by Fromm et al., which shows a G6P: hexokinase I stoichiometry of 1, we propose negative cooperativity in G6P inhibition. Yet, the work in Chapter II does not answer whether one of the two G6P sites is dominant.

Chapter III presents an allosteric mechanism of G6P inhibition. A comparison of hexokinase structures complexed with ADP/Glc and G6P/Glc reveal conformational changes in C-domain, implying the importance of the flexible subdomain in the C-half. The mutations in the base- and ribose-binding pocket of ATP in hexokinase I abolish G6P inhibition completely, while the same mutations do not affect G6P inhibition of mini-hexokinase. Obviously, the inhibition mechanisms for full-length and mini-hexokinase I are different. We assign the mechanism for mini-hexokinase I to be direct inhibition, and the mechanism for full-length hexokinase I to be allosteric. Presumably, G6P binding to N-domain of hexokinase I causes long-range conformational changes, which suppresses G6P binding to C-domain. The molecular basis of the suppression needs to be investigated.
Chapter IV investigates the possible influence of dimerization observed in hexokinase I crystal structure. An interface mutant has been constructed and studied by kinetics and small angle x-ray scattering. The interface mutant and wild-type hexokinase I possess essentially identical kinetic properties. The interface mutant has been proved to be monomeric by small angle x-ray scattering and crystallization. Monomeric hexokinase I was then used in small angle scattering experiments to investigate conformational changes under different ligation states. Structural models have been built to calculate the radius of gyrations of hexokinase I, and the calculated values were used to fit experimental data collected by small angle x-ray scattering. The results suggest a rod-like conformation of hexokinase I in different ligation states, implying only subtle conformational changes in hexokinase I are likely. In addition, the kinetic results show no evidence of dimerization in hexokinase regulation.

Chapter V describes the crystal structures of monomeric hexokinase. For the first time the ATP binding site in C-domain of hexokinase I is revealed with high resolution. Comparisons of this structure with the hexokinase/G6P structure reveals conformational changes in hexokinase I under G6P and ATP states, and thus provides a molecular basis for G6P inhibition. Also, the allosteric interface between N- and C-domains of hexokinase I is implied by the current proposed mechanism of allosteric inhibition. The discovery of a nucleotide binding site in the N-domain introduces additional considerations regarding its function.
Perspectives for Future Research on Human Hexokinase

There remain many questions regarding the regulation studies of hexokinase I. If the allosteric mechanism is presumably dominant, then how is G6P binding to the C-domain suppressed? What structural elements are responsible for this suppression? The question may be answered by new crystal structures, electron paramagnetic resonance (EPR) and fluorescence studies.

Studying the regulation mechanism of hexokinase I in vivo needs to be done as well. In human brain, hexokinase I binds to the mitochondrial outer membrane. The molecular interactions between hexokinase I and the mitochondrial permeability transition pore (PTP) provides another fertile field of research. The possible role (if any) that hexokinase I plays in regulating energy production and cell apoptosis are only two examples. Besides, the study of hexokinase I in association with mitochondria, another question waits to be answered: Why does nature keeps two functional G6P sites in hexokinase I? The retention of two functional sites may be related to cellular locations of hexokinase I in different tissue types. In red blood cells, hexokinase I is in the cytosol, thus it may be inhibited allosterically by G6P binding to N-domain. In brain tissue, hexokinase I is crowded at the contact sites, and presumably forms a tetramer. Under this condition, the conformational changes required in allosteric inhibition may not be available due to restraints of oligomerization. Hence, G6P may regulate hexokinase activity of mitochondrially associated enzyme by binding to active site.
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