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 (the C-terminal half of HKI) and competes directly with ATP, whereas in the alternative suggestion the inhibitor binds to an allosteric site (the 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.

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 isoforms, HKI and skeletal muscle hexokinase (HKII), are bound to the outer membrane of mitochondria and, in the case of HKI, are 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.

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-miniHKI was available for use from a previous study (32, 33). The transformer site-directed mutagenesis kit is from CLONTECH. T4 polynucleotide kinase and all the restriction enzymes are from Promega. Bio-gel hydroxyapatite resin is from Bio-Rad. Toyopearl DEAE-650M is from Tosohas. 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-β-D-sorbitol, deoxyribonuclease (DNase I), leupeptin, phenylmethylsulfonyl fluoride, and ampicillin are from Sigma. Glucose-6-phosphate dehydrogenase came from Roche Molecular Biochemicals. Isopropyl-1-thio-β-D-galactopyranoside 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 Nru I restriction site on the pET-11 vector to another unique Xho I site for the single point mutations. Double mutants were constructed by performing another single mutation in existing single-mutant plasmids. The primers for site-directed mutagenesis are 5’-GATCTTGGAGGAAGCAATTTCCGGTG-3’.

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TABLE I

| Enzyme                  | $k_{cat}$ | $K_m$ Glc $\mu M$ | $K_m$ ATP $\mu M$ | $K_i$ AnG6P $\mu M$ |
|-------------------------|-----------|-------------------|-------------------|---------------------|
| Wild-type HKI$^a$       | 63.6 ± 3.0| 68.2 ± 0.04       | 53 ± 1            | 37 ± 2              |
| Asp$^a$ → Ala$^a$       | 62.2 ± 1.2| 70.9 ± 0.07       | 49 ± 2            | 46 ± 2              |
| Gly$^a$ → Tyr$^a$       | 57.2 ± 3.5| 14.1 ± 0.01       | 38 ± 2            | 31 ± 1              |
| Ser$^a$ → Ala$^a$       | 72.3 ± 4.3| 0.59 ± 0.02       | 35 ± 2            | 48 ± 5              |
| Thr$^a$ → Ala$^a$       | 70.1 ± 1.8| 0.81 ± 0.06       | 42 ± 2            | 32 ± 2              |
| Asp$^a$ → Ala$^b$       | N.P.$^c$  | 0.43 ± 0.07       | 35 ± 2            | 35 ± 2              |
| Asp$^a$ → Glu$^b$       | N.P.$^c$  | 0.47 ± 0.07       | 37 ± 2            | 38 ± 3              |
| Asp$^a$ → Lys$^b$       | N.P.$^c$  | 0.44 ± 0.05       | 37 ± 3            | 23 ± 2              |

$^a$ From Ref. 31.
$^b$ From Ref. 37.
$^c$ Enzyme not purified.

for Thr$^356$ → Ala, 5'-CTGGATCTTGTTACTCTTCTTCTGGAATTCTC-3' for Gly$^67$ → Tyr, 5'-CTGTTGGGATGTGGAGGACACTTAC-3' for Asp$^361$ → Ala, 5'-CTGATCATCGGCTTGGAGCAACTGC-3' for Thr$^356$ → Ala, and 5'-CTCTTGCCCTGCTTGGAGGAAAC-3' for Asp$^362$ → Ala, where the modified codons are bold and underlined.

The oligonucleotide primers used for the selection of the mutant plasmid from the wild-type plasmid are: 5'-CAGCCTCCGGCTTCGAGAAGCCGACAG-3' for the conversion from the NruI site to the XhoI site and 5'-CTCCTCGGACTGGCAGGACAGG-3' for the conversion from the XhoI site back to the NruI site. Mutations were confirmed by sequencing the entire cDNA insert and coding for HKI.

Transformation of wild-type and mutant hexokinases—Transformed E. coli strain ZSC13, containing wild-type or mutant pET-11a-HKI, was grown in LB media at 37 °C to an $A_{600}$ of 0.6, whereupon the temperature was reduced to 22 °C, and isopropyl-$\beta$-D-thiogalactopyranoside was added to a final concentration of 0.4 mM. After induction, the cells were harvested and then resuspended in 25 mM KP, pH 7.5, 2 mM glucose, 1 mM EDTA, 0.4 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 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 fluid was passed through a DEAE column using a KP-buffer (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-buffer (pH 7.5). KCl gradient from 20 to 500 mM. Pooled fractions of HKI were further purified by preparative DEAE-high pressure liquid chromatography, as described elsewhere (31).

Preparation of AnG6P—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 Pi relief of G6P inhibition (34). The kinetic parameters 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%.

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 because of 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 HKI. The HKI Activity Assay and Kinetic Studies—HKI activity was determined by the glucose-6-phosphate dehydrogenase-coupled spectrophotometric 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 anticooperatively. The equilibrium constants for the dissociation of the first inhibitor molecule from HKI ($K_i$) and the second inhibitor molecule ($K_{ii}$) take on significantly different meanings in relation to site-specific affinity constants, as discussed below.

Circular Dichroism Spectra—Circular dichroism spectra were measured from 200 to 260 nm at room temperature by using a Jasco J710 circular dichroism spectrometer. The concentration of HKI used for circular dichroism measurements was 0.2 mM glucose in a buffer containing 2 mM Hepes (pH 7.8), 0.2 mM EDTA, and 0.05 mM $\beta$-mercaptoethanol.

RESULTS

In previous work (31) we mutated residues at the putative allosteric G6P pocket and found only a modest change (2-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 because of 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 HKI, which will be presented in detail elsewhere. Asp$^{322}$ of the C-terminal half interacts with the 2-hydroxyl group of G6P, whereas Asp$^{84}$, the residue in the N-terminal half corresponding to Asp$^{532}$, also interacts with the 2-hydroxy group of G6P. Asp$^{84}$ and Thr$^{680}$ residues of the C-terminal half, interact with the 1-hydroxyl group and 2-hydroxyl of G6P, respectively. Thr$^{322}$ 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$^{856}$ 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$^{57}$ of the N-terminal half can accommodate a mutation to a bulky side chain, which should block the binding of P$_1$ 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 because of a truncation of the gene that codes for HK1. 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 (2-fold or less) in the $K_i$ for
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G6P. On the other hand, the same mutations, when made in the putative binding locus of G6P at the active site of mini-HKI, eliminate G6P inhibition (Table III). These rather surprising and inexplicable 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 halves 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 binding sites. Evidently, both of the G6P binding sites (allosteric and active site) are functional in HKI, and G6P binding to either causes potent inhibition.

**DISCUSSION**

In 1951 Weil-Malherbe 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 with 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 and Fromm (42), that the kinetics of the reverse HKI reaction are normal Michaelin 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 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 because of 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 was used in the analysis of kinetic results obtained here and in a previous study (31). The rate equation for Scheme I is,

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

where $I$ and $S$ represent G6P and ATP, respectively (glucose is saturating and does not appear in Scheme I or in Equation 1), $V_m$ is the maximal velocity, $K_i$ is the Michaelis constant for ATP, and $K_i$ and $K_i$ 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 $K_i$ 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.

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

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

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 $K_i$ and $K_i$, respectively. These constants measure the dissociation of G6P from either the N-terminal half or the C-terminal half, whichever applies, when the alternative site in not occupied by G6P. $K_i$ represents the dissociation of G6P from the C-terminal site, when the N-terminal site is occupied by another molecule of G6P, and $K_i$ represents the dissociation of G6P from the N-terminal site, when the C-terminal site is occupied. Inhibitor binding to the two sites is random rapid-equilibrium. 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.
should effectively eliminate the (bles II and III). Furthermore, on the basis of Equations 1 and considerably in the full-length enzyme. On the other hand, a single HKI or mini-HKI (Tables I-III) are similar, Equation 2 predicts in the C-terminal half and the other in the N-terminal half of Kn

For wild-type HKI it is not possible to extract unique values for kinetic constant reported in Tables I-III) through Equation 3.

**Binding sites for G6P.** Fractional saturation (I) is related to kinetic constants reported in Tables I-III) through Equation 3.

\[
\frac{1}{K_c} = \frac{1}{K_1} + \frac{1}{K_2}
\]

(Eq. 3)

where \( K_c \) is the sum of constants \( K_1 \) and \( K_2 \). Equation 5 represents a binding isotherm with a stoichiometry of unity.

Independent binding of G6P to the active and 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 the 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 another mode remains, which assures virtually no diminution in G6P inhibition.

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