The Roles of Glycine Residues in the ATP Binding Site of Human Brain Hexokinase*

Chenbo Zeng‡, Alexander E. Aleshin, Guanjun Chen§, Richard B. Honzatko, and Herbert J. Fromm¶

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

Mutants of hexokinase I (Arg539 → Lys, Thr661 → Ala, Thr680 → Val, Gly679 → Ala, Gly534 → Ala, and Gly679 → Ala), located putatively in the vicinity of the ATP binding pocket, were constructed, purified to homogeneity, and studied by circular dichroism (CD) spectroscopy, fluorescence spectroscopy, and initial velocity kinetics. The wild-type and mutant enzymes have similar secondary structures on the basis of CD spectroscopy. The mutation Gly679 → Ala had little effect on the kinetic properties of the enzyme. Compared with the wild-type enzyme, however, the Gly534 → Ala mutant exhibited a 4000-fold decrease in $k_{cat}$ and the Gly662 → Ala mutant showed an 11-fold increase in $K_m$ for ATP. Glucose 6-phosphate inhibition of the three glycine mutants is comparable to that of the wild-type enzyme. Inorganic phosphate is, however, less effective in relieving glucose 6-phosphate inhibition of the Gly662 → Ala mutant, relative to the wild-type enzyme and entirely ineffective in relieving inhibition of the Gly534 → Ala mutant. Although the fluorescence emission spectra showed some difference for the Gly662 → Ala mutant relative to that of the wild-type enzyme, indicating an environmental alteration around tryptophan residues, no change was observed for the Gly534 → Ala and Gly679 → Ala mutants. Gly662 → Ala and Gly534 → Ala are the first instances of single residue mutations in hexokinase I that affect the binding affinity of ATP and abolish phosphate-induced relief of glucose 6-phosphate inhibition, respectively.

Hexokinase catalyzes the phosphorylation of glucose, using ATP as a phosphoryl donor. Four isoforms of hexokinase exist in mammalian tissue (1). Hexokinase isoforms I, II, and III have molecular weights of approximately 100,000 and are monomers under most conditions. Amino acid sequences of isoforms I–III are 70% identical (2). Moreover the N- and C-terminal halves exhibit comparable catalytic activities for ATP. Glucose 6-phosphate inhibition of the three glycine mutants is comparable to that of the wild-type enzyme. Inorganic phosphate is, however, less effective in relieving glucose 6-phosphate inhibition of the Gly662 → Ala mutant, relative to the wild-type enzyme and entirely ineffective in relieving inhibition of the Gly534 → Ala mutant. Although the fluorescence emission spectra showed some difference for the Gly662 → Ala mutant relative to that of the wild-type enzyme, indicating an environmental alteration around tryptophan residues, no change was observed for the Gly534 → Ala and Gly679 → Ala mutants. Gly662 → Ala and Gly534 → Ala are the first instances of single residue mutations in hexokinase I that affect the binding affinity of ATP and abolish phosphate-induced relief of glucose 6-phosphate inhibition, respectively.

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‡ Present address: Div. of Molecular Oncology, Washington University School of Medicine, St. Louis, MO 63110.
§ Present address: Dept. of Microbiology, Shandong University, Jinan, Shandong 250100, People’s Republic of China.
¶ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, 1210 Molecular Biology Bldg., Iowa State University, Ames, IA 50011. Tel.: 515-294-4971; Fax: 515-294-0453; E-mail: hjfromm@iastate.edu.

EXPERIMENTAL PROCEDURES

Materials—Affi-Gel Blue and Bio-gel hydroxyapatite came from Bio-Rad. The Transfornet™ site-directed mutagenesis kit (2nd version) was a product of CLONTECH. The Magic Miniprep DNA purification system was a product of Promega. Oligonucleotide synthesis and nucleotide sequencing was done by the Iowa State University nucleic acid facility. NruI and XhoI were obtained from New England Biolabs and Promega, respectively. The pET-11α plasmid was purchased from No-

1 The abbreviation used is: Glu-6-P, glucose 6-phosphate.
The values shown are the mean ± S.D. The kinetic analysis was done with the computer program MINTAB (19). Figures in parentheses represent the p value % differences between the mutant and wild-type kinetic parameters. *p value % differences between the mutant and wild-type kinetic parameters is 0.05.

**Characterization of Mutants by Kinetics and Spectroscopy—**

The kinetic parameters of wild-type and mutant enzymes are in Table I. Compared with the wild-type enzyme, the Gly\(^{334}\) → Ala mutant showed a 4000-fold decrease in \(k_{cat}\), and 4-, 5-, and 3-fold increases in the \(K_m\) (or \(K_v\)) values for glucose, ATP, and 1,5-anhydroglucitol-6-phosphate (an analog that mimics Glu-6-P) (24) and which can be used in the hexokinase-glucose-6-phosphate dehydrogenase coupled spectrophotometric assay, respectively. The Gly\(^{626}\) → Ala mutant showed an 11-fold increase in the \(K_m\) for ATP relative to the wild-type enzyme (Table I), a 2-fold increase in the \(K_m\) for glucose, an 18-fold decrease in \(k_{cat}\) but little change in the \(K_m\) for 1,5-anhydroglucitol-6-phosphate. The mutation of Gly\(^{679}\) to alanine did not change kinetic properties, but the Gly\(^{679}\) → Ile mutation was relatively insoluble (20). \(P_i\) reverses Glu-6-P inhibition of the Gly\(^{679}\) → Ala mutant and the wild-type enzyme to the same extent, whereas \(P_i\) has a modest or no effect on the inhibition of the Gly\(^{626}\) → Ala and Gly\(^{534}\) → Ala mutants, respectively (Fig. 1).

The fluorescence emission spectra of the wild-type, Gly\(^{334}\) → Ala and Gly\(^{679}\) → Ala enzymes were identical, whereas the spectrum for the Gly\(^{626}\) → Ala mutant differed from that of the wild-type enzyme (Fig. 2). This difference is probably due to changes in the local environment of tryptophan residues. However, the CD spectra for the wild-type and all the mutant enzymes are identical (data not shown), suggesting no global conformational differences among these proteins.

The Thr\(^{661}\) → Ala mutant showed a 4-fold increase in the \(K_m\) for 1,5-anhydroglucitol-6-phosphate. The Thr\(^{661}\) → Val mutant exhibited a 9-fold decrease in \(k_{cat}\) relative to the wild-type enzyme. Other kinetic parameters for the two mutants were unaltered relative to those of the wild-type enzyme. The Arg\(^{339}\) → Lys mutant showed a 12-fold decrease in \(k_{cat}\) and little change in the \(K_m\) for either ATP or glucose or the \(K_v\) for 1,5-anhydroglucitol-6-phosphate.

**DISCUSSION**

Arg\(^{539}\) putatively interacts with the polyphosphoryl portion of ATP and stabilizes the transition state (19, 20). The Arg\(^{539}\) → Lys mutant is 10-fold more active than the Arg\(^{539}\) → Ile mutant (20), suggesting the importance of the positive charge at position 539. However, as the Arg\(^{539}\) → Lys mutant reported here is still 12-fold less active than wild-type hexokinase I, specific hydrogen bond interactions of the arginyl side chain are of equal importance in stabilizing the transition state. We have suggested, on the basis of previous work, that Arg\(^{539}\) may form salt bridges with oxygen atoms of the α- and β-phosphoryl groups of ATP (19). The observed properties of the Lys\(^{539}\) column, is consistent with the elevated \(K_m\) for ATP exhibited by this mutant (see below). The wild-type and mutant enzymes were more than 95% pure on the basis of SDS-polyacrylamide gel electrophoresis (data not shown).

### Table I

| Hexokinase | \(K_m\) (Glu) | \(K_m\) (ATP) | \(K_m\) (1,5-anhydroglucitol-6-phosphate) | \(k_{cat}\) |
|------------|--------------|--------------|-----------------------------------------|----------|
| Wild-type  | 6.5 ± 6.6    | 0.49 ± 0.06 (0.05) | 26 ± 4.8                               | 26.7 ± 0.92* |
| Gly\(^{334}\) → Ala | 287 ± 22.2 (0.05) | 2.2 ± 0.13 (0.05) | 82 ± 13 (0.05)                           | 0.006 ± 0.00 |
| Gly\(^{679}\) → Ala | 42 ± 6.4 (0.05) | 0.23 ± 0.04 (0.05) | 27 ± 6.0 (50)                           | 19.3 ± 1.35 |
| Gly\(^{626}\) → Ala | 118 ± 9.30 (0.05) | 5.5 ± 0.65 (0.05) | 28 ± 6.2 (50)                           | 1.50 ± 0.11 |
| Thr\(^{661}\) → Ala | 74 ± 17 (25.0) | 0.32 ± 0.10 (0.05) | 105 ± 19.1 (0.05)                        | 22.2 ± 2.11 |
| Thr\(^{661}\) → Val | 39 ± 13 (0.05) | 1.38 ± 0.29 (0.05) | 25 ± 5.9 (50)                           | 2.90 ± 0.32 |
| Arg\(^{339}\) → Lys | 52 ± 0.39 (0.05) | 0.43 ± 0.11 (20.0) | 19 ± 4.4 (0.05)                         | 2.20 ± 0.12 |

**Purification of Wild-type and Mutant Brain Hexokinase—**

The wild-type and mutant enzymes are identical (data not shown), suggesting no global conformational differences among these proteins.

**Hexokinase Assay—** Hexokinase activity was determined spectrophotometrically as described previously (21). The kinetic parameters depicted in Table I were obtained from initial rate data obtained from two or more experiments. The substrate concentrations in the kinetic experiments were varied from \(K_m/2\) to \(S_u\). At least three concentrations of the inhibitor 1,5-anhydroglucitol-6-phosphate, from below to above its \(K_m\), were used to evaluate its effect on the kinetics of the brain hexokinase enzyme.

**Methods—** 1,5-anhydroglucitol-6-phosphate was prepared as described elsewhere (22). Protein concentration was determined by the method of Bradford (23) using bovine serum albumin as a standard. CD spectra were recorded using a Jasco J710 CD spectrometer as described elsewhere (20).

**Fluorescence Emission Spectra—** The wild-type and mutant enzymes were dialyzed against 20 mM Hepes buffer (pH 7.0) containing 1 mM of β-mercaptoethanol. The enzyme concentration was 66.7 µg/ml. The fluorescence intensity was recorded over the wavelength range from 300–350 nm, using an excitation wavelength of 290 nm.

**RESULTS**

**Purification of the Wild-type and Mutant Human Brain Hexokinase—** The use of a 10-liter fermentor enhanced enzyme yield compared with 2-liter growth flasks. 20 liters of culture provided 70 mg of pure hexokinase I. The lack of retention of the Gly\(^{626}\) → Ala mutant on Affi-Gel Blue, a nucleotide affinity
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FIG. 1. Reversal of 1,5-anhydroglucitol-6-phosphate inhibited wild-type and mutant hexokinase enzymes by Pi. The concentrations of ATP are 0.25 mM (gray), 1 mM (black), and 4 mM (white) for the wild-type, Gly534 → Ala (G534A), Gly679 → Ala (G679A), Gly862 → Ala (G862A), Thr661 → Ala (T661A), Thr661 → Val (T661V), and Arg539 → Lys (R539K) enzymes, and 4 mM (gray), 8 mM (black), and 16 mM (white) for the Gly662 → Ala mutant. Pi concentration is 5 mM, and the concentration of 1,5-anhydroglucitol-6-phosphate is 100 μM. Reversal of 1,5-anhydroglucitol-6-phosphate inhibition (%) is defined as (A − B) × 100/B where A is activity in the presence of 1,5-anhydroglucitol-6-phosphate and P_i, and B is activity in the presence of 1,5-anhydroglucitol-6-phosphate.

We have probed the corresponding glycines by directed mutation, in the expectation that some of these glycines are linked to observed kinetic properties in hexokinase I. The mutation of Gly534 to alanine produced a dramatic effect on k_cat (4000-fold reduction) and modest effects on K_m for glucose and ATP. Gly534 is conserved in sequences of hexokinase, but according to our model (Fig. 3), its main chain torsion angles fall in the allowed region of the Ramachandran plot for alanine. Instead, Cβ of Ala534 is 3.6 Å from an oxygen of the β-phosphoryl group of ATP, but perhaps of greater significance is its 2.4 Å contact with backbone carbonyl 537 of an adjacent β-strand. Our model suggests then, the possibility of conformational change in the vicinity of residue 534 to relieve the close contact mentioned above. Such a local conformational change could influence Asp532, which on the basis of earlier work (19) plays a critical role in the stabilization of the transition state and may be involved in the binding of Mg_2^+. A larger perturbation on the active site due to the mutation of Gly534 to alanine is not likely, because K_m values for substrates are not influenced and CD spectroscopy indicates no change in secondary structure.

The Gly534 → Ala mutant represents the first instance whereby the mutation of a single residue has abolished P_i-induced relief of Glu-6-P inhibition in hexokinase I. Glu-6-P inhibition of the C-terminal half of hexokinase I (mini-hexokinase) cannot be reversed by P_i, implicating the N-terminal domain in the relief of inhibition (20). The mechanism by which P_i relieves Glu-6-P inhibition then evidently involves structural elements of both the N- and C-terminal halves of hexokinase I. Furthermore, the loss of P_i-induced relief of Glu-6-P inhibition in the Gly534 → Ala mutant is linked closely to position 534, as mutations of Asp532 to lysine and glutamate have little effect on this property (19).

Gly679 and Gly862 belong to reverse turns, which pack...
against each other in our model (Fig. 3). The main chain torsion angles put positions 679 and 862 in unallowed regions of the Ramachandran plot for alanine ($\phi = 111$, $\psi = 133$ for Gly679, $\phi = 70$, $\psi = 168$ for Gly862). Of the two mutants, Gly862 $\rightarrow$ Ala has the conformation of highest energy. Although the C$\beta$ atoms at positions 679 and 862 probably do not interact with ATP, they make unfavorable contacts in our model with backbone amide 863 (2.6 Å) and backbone amide 679 (2.7 Å), respectively. These unfavorable contacts may not be significant, however, as the mutation of Gly679 to alanine has no effect on the kinetic properties of the enzyme. Instead, the introduction of alanine at position 862 probably causes conformational changes in main chain torsion angles. Although the fluorescence spectra of the wild-type and Gly862 $\rightarrow$ Ala enzymes differ (indicating a perturbation in the local environment of tryptophan residues) their CD spectra are identical (indicating no change in secondary structure). Furthermore, the $K_i$ for 1,5-anhydroglucitol-6-phosphate and the $K_m$ for glucose are similar for the Gly862 $\rightarrow$ Ala mutant and the wild-type enzyme. Thus the mutation of Gly862 to alanine probably has an effect only on residues in the vicinity of position 862. Thr683, a residue conserved in hexokinase sequences, interacts with the ribose and base moieties of ATP in our model (Fig. 3). The Gly862 $\rightarrow$ Ala mutant, then, could influence interactions involving the base of ATP by perturbing the conformation or relative position of Thr683. The mutation of Gly862 to alanine increases the $K_m$ for ATP by 11-fold without large changes in other kinetic parameters and as such, represents the first mutation, which to our knowledge influences the binding affinity of ATP.

Mutations prepared here and from previous studies (19) show a trend that may be significant to the function of hexokinases in general. Mutations of hexokinase I, which putatively influence interactions involving the polyphosphoryl moiety of ATP, have no effect on $K_m$ but a large effect on $k_{cat}$. Conversely, the Gly862 $\rightarrow$ Ala mutant, which putatively influences interactions at the base moiety, has little effect on $k_{cat}$ but substantial effects on $K_m$ for ATP. Interactions involving the base of ATP are important for affinity, but polyphosphoryl-protein interactions are involved in the stabilization of the transition state. Conceivably, hexokinase I diverts energy from favorable interactions between the polyphosphoryl moiety of ATP and the enzyme to promote conformational changes that stabilize the

**Fig. 2.** Fluorescence emission spectra for the wild-type and Gly862 $\rightarrow$ Ala mutant form of human brain hexokinase.

**Fig. 3.** Stereoview of the model for ATP in its complex with hexokinase I. Positions 534, 679, and 862 are presented in their mutated states as alanines with *bold lines*. ATP and glucose (GLU) are also drawn in *bold lines*, and the *solid circle* represents Mg$^{2+}$.
transition state. This phenomenon is not without precedence. In adenylosuccinate synthetase from *E. coli* mutations involving protein interactions at the base of GTP affect $K_m$ (33), whereas interactions between the polyphosphoryl group of GTP and the protein (and Mg$^{2+}$) contribute to the stability of the transition state by driving conformational changes in the active site (34).

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