ATP-Binding Site of Human Brain Hexokinase As Studied by Molecular Modeling and Site-Directed Mutagenesis†

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ABSTRACT: The interaction of ATP with the active site of hexokinase is unknown since the crystal structure of the hexokinase−ATP complex is unavailable. It was found that the ATP binding site of brain hexokinase is homologous to that of actin, heat shock protein hsc70, and glycerol kinase. On the basis of these similarities, the ATP molecule was positioned in the catalytic domain of human brain hexokinase, which was modeled from the X-ray structure of yeast hexokinase. Site-directed mutagenesis was performed to test the function of residues presumably involved in interaction with the tripolyphosphoryl moiety of ATP. Asp532, which is thought to be involved in binding the Mg^{2+} ion of the $MgATP^{2-}$ complex, was mutated to Lys and Glu. The k_{cat} values decreased 1000- and 200-fold, respectively, for the two mutants. Another residue, Thr680 was proposed to interact with the γ-phosphoryl group of ATP through hydrogen bonds and was mutated to Val and Ser. The k_{cat} value of the Thr680Val mutant decreased 2000-fold, whereas the k_{cat} value of the Thr680Ser decreased only 2.5-fold, implying the importance of the hydroxyl group. The K_m and dissociation constant values for either ATP or glucose of all the above mutants showed little or no change relative to the wild-type enzyme. The K_i values for the glucose 6-phosphate analogue 1,5-anhydroglucitol 6-phosphate, were the same as that of the wild-type enzyme, and the inhibition was reversed by inorganic phosphate (P_i) for all four mutants. The circular dichroism spectra of the mutants were the same as that of the wild-type enzyme. The results from the site-directed mutagenesis demonstrate that the presumed interactions of investigated residues with ATP are important for the stabilization of the transition state.

Hexokinase I, brain hexokinase, is one of four hexokinase isozymes found in mammalian tissue (Katzen & Schimke, 1965). It is believed to be the "pacemaker" of glycolysis in brain tissue and the red blood cell (Lowry & Passonneau, 1964; Rapoport, 1968). The kinetic mechanism of brain hexokinase is thought to be random bi-bi (Ning et al., 1969; Bachelard et al., 1971; Gerber et al., 1974), and a mechanism for its molecular regulation has been proposed (Ellison et al., 1975; Fromm, 1981; Wilson, 1984). Brain hexokinase exhibits regulatory properties that are unique among the isozymes of hexokinase, i.e., it is potently inhibited by its reaction product glucose 6-phosphate, and this inhibition can be reversed by physiological levels of inorganic orthophosphate (P_i) (Rudolph & Fromm, 1971; Fromm, 1981; Ureta, 1975). Brain hexokinase consists of two structural domains homologous to each other with approximately 50% identity and to yeast hexokinase with about 30% identity (Nishi et al., 1988). The catalytic properties of brain hexokinase are associated with the C-terminal domain, while the N-terminal domain is thought to be involved in molecular regulation (Nishi et al., 1988).

The structures of different crystal forms of the a and b isozymes of yeast hexokinase were investigated by Steitz and his collaborators (Anderson et al., 1978; Bennett et al., 1980; Harrison, 1985). They discovered that binding of glucose causes significant conformational changes in hexokinase which narrows ("closes") the active site cleft of the enzyme (Bennett et al., 1978). The published structures of hexokinase b complexed with orthotoluoylglucoseamine and hexokinase a complexed with glucose represent open and closed conformations of the enzyme (Anderson et al., 1978; Bennett et al., 1980; Harrison, 1985). A computer generated model for a mammalian hexokinase isozyme, glucokinase, complexed with glucose was also published (Charles et al. 1994). In spite of success in understanding the interaction of hexokinase with glucose, the interaction of the enzyme with ATP is still unclear. Steitz and collaborators have attempted to model ATP in the active site of the enzyme, based on the crystal structure of open hexokinase b soaked with 8-bromoadenosine monophosphate (Shoham & Steitz, 1980). The authors suggested that ATP may be bound nonspecifically to the open hexokinase form because the binding site for the phosphoryl group of the ligand seemed to form during the conformational transformation of the enzyme from the open to the closed state (Shoham & Steitz, 1980). In this study we used a different approach to model ATP within the active site of hexokinase.

Recently, it was reported that yeast hexokinase, glycerol kinase, actin, and heat shock protein hsc70 have similar ATP-binding domain structures although there are no overall primary sequence similarities among them (Bork et al., 1992;

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Kabsch et al., 1990; Flaherty et al., 1991; Hurley et al., 1993; Kabsch & Holmes, 1995). This proposal suggests an alternative approach for modeling ATP in the active site of hexokinase, namely, by superimposing the homologous ATP-containing structures on hexokinase. It permits building of ATP directly into the enzyme complexed with glucose. The reliability of this approach is based on a well-established principle of similar interactions of structural−functional homologues with their ligands (Rossmann et al., 1975). The obtained model of the ATP−enzyme complex pinpointed the putative ATP-binding residues of human brain hexokinase. This paper presents kinetic studies on the mutants of the presumed ATP-binding residues, Asp532 and Thr680. Our results suggest that Asp532 and Thr680 are essential for the catalytic reaction of human brain hexokinase.

EXPERIMENTAL PROCEDURES

Materials. Affi-Gel Blue Gel and Bio-gel hydroxyapatite were obtained from Bio-Rad Laboratories. Transformer Site-Directed Mutagenesis Kit (2nd version) was a product of Clontech. The Magic Minipreps DNA purification system was a product of Promega. Oligonucleotide synthesis and nucleotide sequence analysis were done by the Iowa State University Nucleic Acid Facility. *Nru*I was from New England Biolabs. *Xho*I was from Promega. The pET-11a plasmid was obtained from Novagen. *Escherichia coli* strains BL21(DE3) and TG-1 cells were obtained from Amersham Corp. Ampicillin, phenylmethanesulfonyl fluoride (PMSF),[1] ATP, NADP, NADPH, glucose 1,6-bisphosphate (Glu-1,6-BP), deoxyribonuclease I (DNase I), isopropyl 1-thio-β-D-galactopyranoside (IPTG), and 1,5-anhydro-D-sorbitol were products of Sigma.

Modeling the Brain Hexokinase Complexed with ATP and Mg^{2+}. The alignment of the sequences was made by analogy with the structure-based alignment of human glucokinase and yeast hexokinase b reported earlier (Charles et al., 1994). The program AMMP was used to build the models (Harrison, 1993). The force field was the sp3 variation of the UFF potential set (Harrison, 1993; Rappe et al., 1992). The amortized fast multipole algorithm was used for the nonbonded and electrostatic terms, and no cut off radius was applied. Crystallographically ordered solvent from yeast hexokinase was included. The open form was modeled by fixing the conserved atoms at their observed positions and building the remainder of the structure. Then the whole structure was minimized. A homotopy method (Harrison, 1995) was used to minimize the structures. In the homotopy method the targets for the minimization are gradually shifted from the current erroneous positions to the desired targets. The steps are controlled by a critical path parameter l which is slowly varied from 1 to 0. The homotopy was applied to the bonds and angle terms with the pyramid height torsion and nonbonded terms always being minimized. The path was tracked by minimization which approximates tracking a zero in the first derivative of the energy. Conjugate gradient minimization was used. The structure was minimized in two passes where the tracking parameter l was varied form 0.95 to 0 in 20 steps of 0.05. Procheck (Laskowshi et al., 1993) was used to check the stereochemistry at the end of the process, and no significant errors were found.

The closed form of hexokinase was initially modeled in the same manner, but starting from the yeast hexokinase a coordinate set. There is a region in one of the helices in the small domain of yeast hexokinase a which was poorly determined in the electron density (Harrison, 1985). When the model in that region was examined, it was found to be unsatisfactory, e.g., hydrophilic amino acids such as arginine were pointing into the domain and hydrophobic acids such as tyrosine or phenylalanine were pointing out into the solvent. The stereochemistry in that region was also poor. Therefore, it was decided to model the closed structure by using the yeast coordinates as guides for the backbone and deforming the open structure into the closed form. The same homotopy method was used with the homotopy applied to the α carbon restraints as well as the bonds and angles. The two modeled structures were similar in the small domain except where the poorly defined region was, and in that region the new model was much more consistent with the expected distribution of amino acid side chain positions.

The initial positions and conformations of $MgATP^{2-}$ complexes in the active site of brain hexokinase were obtained by superimposing the conserved residues of the ATP binding site of the enzyme (Bork et al., 1992) and the corresponding residues of either actin, heat shock protein, or glycerol kinase complexed with ligands (PDB entries are 1ATN, 1NGJ, and 1GLC, respectively). The model of the complexes of the closed form of brain hexokinase and ATP, which were built either from the ADP complexes of glycerol kinase or from the ATP complexes of actin, were further energy minimized with the same AMMP program. During the investigation of protein residues interacting with ATP, we manually adjusted the conformations of their side chains to check the possibility of hydrogen bond formation. If a distance between interacting groups was within 3.5−3.8 Å, the bond was considered as possible.

Expression of the Wild-Type and Mutant Human Brain Hexokinase I. The cDNA for human brain hexokinase I has been cloned into the expression vector pET-11a to generate pET-11a-HKI in our laboratory (Liu et al., 1991). pET-11a-HKI was transformed into *E. coli* strain BL21(DE3), a phage λ-lysogen containing the T7 RNA polymerase gene under control of the lacUV5 promoter. A 5-mL pregrowth culture of the transformed strain in M9 medium plus 40 mg/L ampicillin, 1 mM $MgSO_4$, and 4 mg/L glucose was grown overnight and then added to 500 mL of the same medium. The culture was shaken at 37 °C to early log phase (A_{600} = 0.4), and IPTG was then added to a final concentration of 0.4 mM to induce the T7 RNA polymerase gene. Cultures were shaken vigorously for an additional 24 h at 22 °C.

Purification of the Wild-Type and Mutant Forms of Human Brain Hexokinase I. The cells containing the overexpressed hexokinase were collected by centrifugation. About 30 g of wet cells (~30 mL) were suspended in 30 mL of suspension solution containing 50 mM Tris-HCl (pH 8.0), 10 mM glucose, 0.5 mM EDTA, 1 mM mercaptoethanol, and 2 mM PMSF. The cells were broken by French Press at a pressure of 1000 psi. This procedure was repeated twice. DNaseI solution (0.9 mL) (0.15 mL of 10 mg/mL DNase I stock plus 0.65 mL of 1 M $MgCl_2$) was mixed with the broken cells for 30 min. The broken cell suspension was

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl β-D-thiogalactopyranoside; Glu-1,6-BP, glucose 1,6-bisphosphate; Glu-6-P, glucose 6-phosphate; 1,5-an-G6P, 1,5-anhydroglucitol 6-phosphate; ANP, 5′-adenylyl β,γ-imidodiphosphate.

subjected to centrifugation for 50 min at 12 000 rpm. The supernatant fluid was dialyzed against 2 L of the dialysis buffer containing 50 mM Tris-HCl (pH 7.0), 10 mM glucose, 0.5 mM EDTA, and 1 mM mercaptoethanol. The buffer was changed twice. The dialyzed cell extract was loaded onto a 4 × 13 cm Affi-Gel Blue Gel column (Wilson, 1989) and washed with the same solution that was used for dialysis. The column was further washed with the buffer having the above composition but at pH 8.0 and then with the buffer containing 50 mM Tris-HCl (pH 9.0), 10 mM glucose, 0.5 mM EDTA, 1 mM mercaptoethanol, and 20% (v/v) glycerol. Human brain hexokinase I was eluted with 1.5 mM Glu-6-P in the same pH 9.0 buffer. The fractions containing human brain hexokinase were identified by SDS−polyacrylamide gel electrophoresis, pooled and subjected to ultrafiltration. Concentrated enzyme solution (5−10 mL) was loaded onto a 1 × 30 cm Bio-Gel hydroxyapatite column equilibrated with buffer containing 25 mM KP_i and 1 mM mercaptoethanol. The protein was eluted with 180 mL of a linear gradient solution of low ionic strength buffer containing 25 mM KP_i and 1 mM mercaptoethanol and 180 mL of high ionic strength buffer containing 0.3 M KP_i and 1 mM mercaptoethanol. The fractions containing pure human brain hexokinase were identified by SDS−polyacrylamide electrophoresis, collected, concentrated, and stored in 50% (v/v) glycerol at −20 °C.

Site-Directed Mutagenesis. Mutagenesis was performed by following the instructions provided with Transformer Site-Directed Mutagenesis Kit (2nd version) from Clontech. The oligonucleotides used for mutagenesis are as follows: 5′-TTCTTGGCCCTGAAGCTTGGAGGAAC-3′ for Asp532Lys, 5′-GCCCTGGAACTTGGAGG-3′ for Asp532Glu, 5′-TTGTTGGGGTCGGCAGCAATG-3′ for Thr680Val, and 5′-TTGTTGGGTCCGGCAGCAATG-3′ for Thr680Ser, where the underlines represent the mutated bases. The selective oligonucleotide sequence was chosen at the *Nru*I restriction site in the vector pET-11a and designed to change the *Nru*I site to a *Xho*I site. The sequence of the selective oligonucleotide is 5′-CAGCCTCGCCTCGAGAACGCCAG-3′, where the underline represents the *Xho*I site. The DNA sequence of the mutated gene was verified by DNA sequencing using the method of fluorescent dideoxychain termination sequencing.

Hexokinase Assay. Hexokinase activity was determined spectrophotometrically as described previously (Liu et al., 1991). For kinetic characterization, the amount of the wild-type and mutant human brain hexokinase was adjusted to give an activity of approximately 5×10^{-3} ΔOD_{340}/min. Human brain hexokinase was dialyzed against a solution containing 50 mM Tris-HCl (pH 7.0) and 1 mM mercaptoethanol to remove P_i. Glucose-6-phosphate dehydrogenase was dialyzed against the same solution to remove $(NH_4)_2SO_4$. Since glucose-6-phosphate dehydrogenase can use glucose as its substrate at high levels of the sugar, the amount of glucose-6-phosphate dehydrogenase used was as small as possible in order to preclude glucose oxidation. 0.1 unit of glucose-6-phosphate dehydrogenase was used in 1 mL of the assay system.

Kinetic Studies. The kinetic parameters of brain hexokinase were obtained by fitting initial-rate data to

$$\frac{1}{v} = \frac{1}{V_m}\left[1 + \frac{K_{m,ATP}}{[ATP]} + \frac{K_{m,Glu}}{[glucose]} + \frac{K_{iATP}K_{m,Glu}}{[ATP][Glucose]}\right] \quad (1)$$

where K_{iATP} is the dissociation constant for ATP. The data were analyzed using a MINITAB modification of a program originally written in OMNITAB (Siano et al., 1975) with an α value of 2.0.

Preparation of 1,5-an-G6P. 1,5-an-G6P was prepared as described previously (Ferrari et al., 1959).

Determination of Protein Concentration. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Circular Dichroism Measurement. Circular dichroism (CD) spectra were recorded using a Jasco J710 CD spectrometer as described previously (Zeng & Fromm, 1995).

RESULTS AND DISCUSSION

Purification of Wild-Type and Mutant Human Brain Hexokinase. The purification procedure outlined in this report is greatly simplified relative to previously reported methods (Zeng & Fromm, 1995), and the enzyme yield is increased (20−30%). Approximately, 20 mg of pure enzyme can be obtained from 10 L of *E. coli* culture. The procedure requires only two columns (Affi-Gel Blue Gel and Bio-Gel hydroxyapatite), dialysis, and ultrafiltration. Figure 1 shows the SDS−PAGE at each step of purification. The mutant enzymes, Asp532Lys, Asp532Glu, Thr680Val, and Thr680Ser, bind to the Affi-Gel Blue-Gel column, implying that their ATP binding ability is retained after mutagenesis.

Modeled Interaction of brain hexokinase with ATP and Mg^{2+}. The sequence alignment of the catalytic domain of human hexokinase with yeast hexokinase isomers a and b revealed 31% and 33% identity, respectively. The homology is especially high in the elements of secondary structure and in the regions of interactions with substrates (Figure 2). The model of the closed form of brain hexokinase, which was built from its open form by bending to yeast hexokinase a, is as similar to the prototype structure as the model built directly from hexokinase a; however, the geometry of its small lobe (Figure 3) is significantly improved.

Our modeling of the ternary complex of brain hexokinase was based on the assumption that the position of ATP and Mg^{2+} relative to the conserved residues of the active site must be consistent in all homologous phosphotransferases. The crystal structures of glycerol kinase ligated with ADP and Mg^{2+} (Hurley et al., 1993), the ATP-binding domain of actin ligated with ATP and Ca^{2+} (Kabsch et al., 1990), and the ATP-binding domain of heat shock protein ligated with ANP (5′-adenylyl-β,γ-imidodiphosphate) and Mg^{2+} (Flaherty et al., 1994) (Protein Data Base entries are 1GLC, 1ATN, and 1NGJ, respectively) were used for modeling. The superimposition of these enzymes on the model of the closed form of the catalytic domain of brain hexokinase demonstrated that all three ATP and Mg^{2+} analogues have similar conformations and positions in the active site of brain hexokinase (Figure 4). They fit the cavity of the active site without a serious overlapping with the protein. These observations together with high structural homology in the phosphoryl binding parts of the ATP binding sites of phosphotransferases provide for significant confidence in the model.

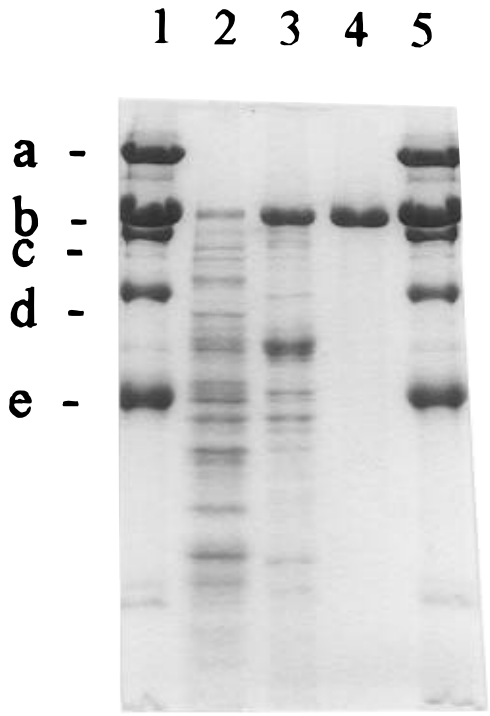

FIGURE 1: SDS−PAGE patterns for each step of the purification of wild-type human brain hexokinase. Lane 2, cell-free extract; lane 3, the enzyme fraction after the Affi-Gel Blue Gel column; and lane 4, the enzyme fraction after the Bio-Gel hydroxyapatite column. Lanes 1 and 5, protein standards; the molecular masses for the bands a, b, c, d, and e are 200, 116, 97.4, 66.2, and 45 kDa, respectively.

ADP from glycerol kinase is shifted approximately 2.5 Å away from the ATP molecules of actin and heat shock protein structures which have almost identical positions. The difference in the positions is greater for the adenine rings of the ligands (rms deviation is 3.0 Å) than for the β-phosphoryl group and Mg^{2+} (the deviations are less than 2 Å). This result correlates with the significant discrepancy between the adenine binding sites of glycerol kinase and actin. Adenine of ADP from glycerol kinase fits better into the active site of brain hexokinase. This agrees with the similarity of the adenine binding sites of these enzymes. Mg^{2+} and the phosphoryl groups of all three superimposed ATP analogues satisfactorily fit the model. However, all superimposed ATP analogues have the side chain of Thr863 overlapping with ribose. In all phosphortransferases except hexokinases, this position in the sequences is represented by a conserved glycine (Bork et al., 1992). The poor fit of ribose suggests either that brain hexokinase changes the orientation of Thr863 upon the binding of ATP or that ATP in hexokinases has a slightly different conformation than in the other enzymes.

The energy minimization of ATP in the active site of brain hexokinase removed the poor contact without a significant change in the position of the ligand. We used ATP and Ca^{2+} from superimposed actin as a starting model for the ligands. After the energy minimization, adenine of ATP shifted to a position close to that of adenine from the glycerol kinase structure. However, the γ-phosphoryl group resumed its initial position. The test minimization of the complex, with ADP and Mg^{2+} from glycerol kinase as a starting model, gave similar results.

The energy-minimized model of the active site of the enzyme with bound glucose, ATP and Mg^{2+} is presented in Figure 5. The ATP molecule is positioned in the cleft between two lobes of the enzyme. Adenine is bound to the big lobe and occupies a cavity between helix α9 (residues 784−788) and the loop, connecting strand β12 and helix α12 (residues 863−869).[2] Ribose is bound between side chains of Glu783 and Thr863. The loop residues 863−869 have a similar function in all homologous phosphotransferases, i.e., they form the basis of the adenosine-binding site. According to our modeling, one face of adenine is exposed to the solvent. This is quite different from other structurally known phosphotransferases. It is possible that, upon the binding of ATP, hexokinase undergoes further conformational change, leading it to a more closed conformation (Kabsch & Holmes, 1995). These hypothetical changes, however, should not significantly alter the part of the active site which binds the phosphoryl groups of ATP. The van der Waals model of the tertiary complex of hexokinase demonstrates that this part of the active site already fits closely to the ligands. The predictions of the model should be most accurate in this area.

The phosphoryl binding site consists of residues from two lobes or the catalytic domain of brain hexokinase. At one side, the tripolyphosphoryl moiety of ATP interacts with residues 680 and 681 of the β-turn between strands β-9 and β-10, and, at the other side, they interact with the loop connecting strands β-2 and β-3. Both elements are conserved within all homologous phosphotransferases (Bork et al., 1992; Kabsch et al., 1990; Flaherty et al., 1991). The ϵ-amino group of Lys621 is 4.6 Å from the γ-phosphoryl group. The modeling suggests that this residue can be involved in ATP binding.

In our model Mg^{2+} is coordinated by O1B of the β-phosphoryl group, and O3G of the γ-phosphoryl group of ATP and it is positioned relatively close to Asp532. The model suggests that Asp532 coordinates Mg^{2+} either by direct ionic interactions or through a mediating water molecule.

Our modeling of ATP bound to the closed form of hexokinase supports the suggestion of Steitz and collaborators that the correct binding of the tripolyphosphoryl moiety of ATP requires the closed form of hexokinase (Shoham & Steitz, 1980). The positions of all three superimposed ATP analogues, being in good agreement with each other, noticeably differ from that of 8-bromoadenosine monophosphate in the crystal structure of open yeast hexokinase (Figure 4). The γ-phosphoryl group of ATP in the crystallographically determined complex corresponds to α-phosphoryl group in our model and is about 6 Å away from the O6 hydroxyl of glucose. The γ-phosphoryl group of ATP in our model has a more realistic distance from O6 of glucose equal to 3.9 Å. Assuming that the two models represent the interaction of ATP with open and closed forms of hexokinase, respectively, we can conclude that interactions between the tripolyphosphoryl moiety of ATP and hexokinase are responsible for the formation of the productive ATP−hexokinase complex, and that the tripolyphosphoryl group binding site of hexokinase is not formed correctly in the open form of the enzyme.

The model proposes a number of residues in the active site which may be involved in hydrogen bond interactions with ATP (Figure 6). One of these residues (Arg539) was

[2] Here and after the nomenclature of the elements of secondary structure is adopted from the yeast hexokinase model of Steitz and co-workers (Anderson et al., 1978; Bennett et al., 1980).

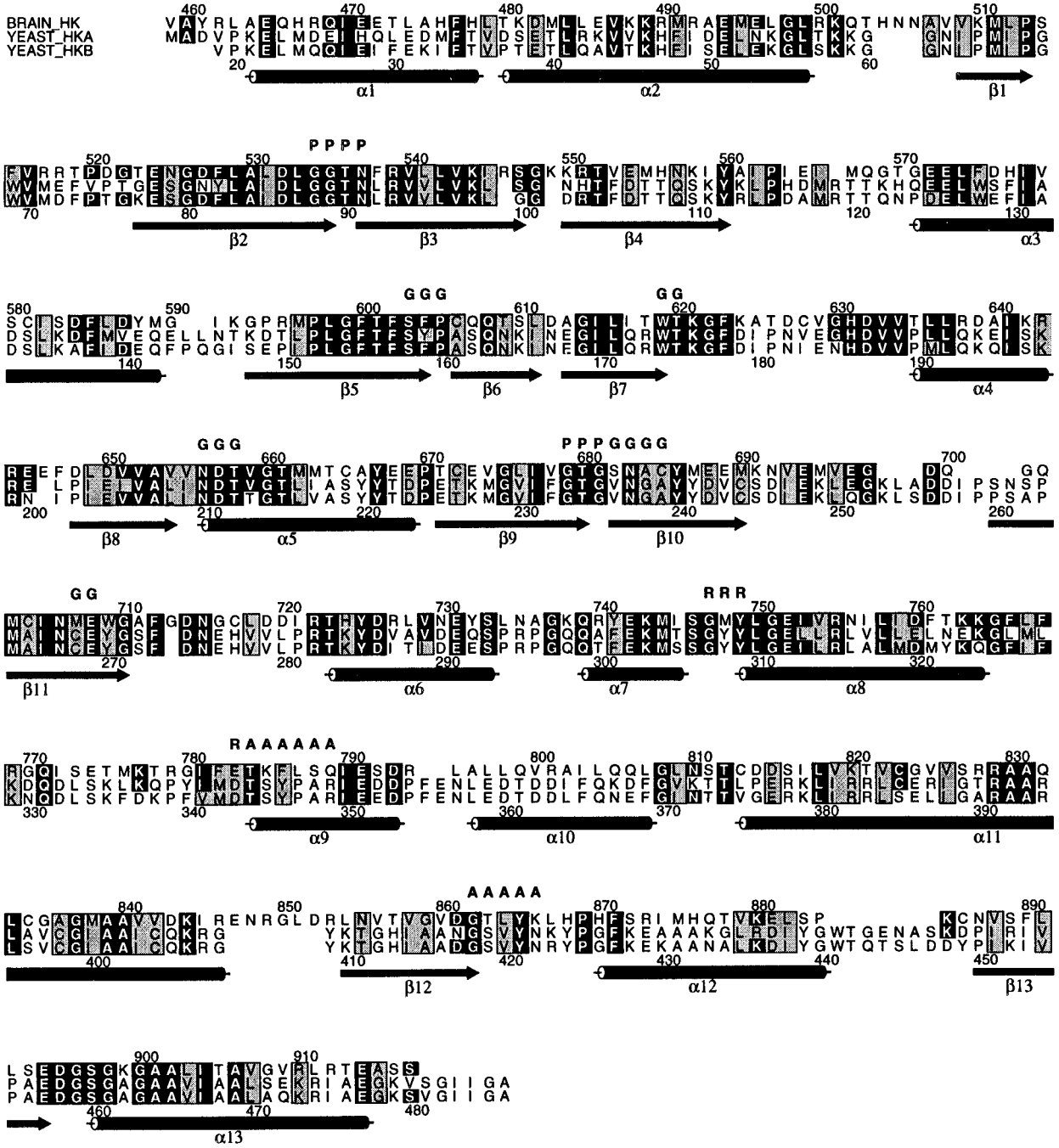

FIGURE 2: Structural alignment of amino acid sequences of the catalytic domain of human brain hexokinase and yeast hexokinases a and b. The residues mutually identical with those of brain hexokinase are outlined in black, and homologous residues are in shaded boxes. The elements of secondary structure are from the crystal structure of yeast hexokinase b. G, P, R, and A on the top of the brain hexokinase sequence indicate residues forming the binding sites for glucose, phosphoryl groups, ribose, and adenine, respectively. The picture was prepared using ALSCRIPT (Barton, 1993).

investigated by mutagenesis earlier (Zeng & Fromm, 1995). It was demonstrated that Arg539 is important for catalysis, but it is less important for ATP affinity. According to the model Arg539 interacts with α- and β-phosphoryl groups of the ligand. In glycerol kinase, the corresponding residue, Arg17, also interacts with the β-phosphoryl group of ATP. This conserved residue of hexokinases may be important for stabilization of productive conformations of the enzyme and ATP. Thus, the energy of presumable interaction is spent on promoting the transition state. In this study we investigated the function of two other residues predicted to interact with ATP.

Site-Directed Mutagenesis of Asp532 in the Putative ATP-Binding Site. Asp 532 is a conserved residue within phosphotransferases. Its presumed function in actin and heat shock protein is binding the Ca^{2+} ion and Mg^{2+} ion, respectively. Our model of the tertiary complex of human brain hexokinase supports the hypothesis that Asp532 might be involved in binding of the Mg^{2+} ion in the $MgATP^{2-}$ complex. To test its function, we mutated Asp532 to Lys and Glu. The kinetic parameters for the mutants, Asp532Lys and Asp532Glu, and the wild-type enzyme were determined and are shown in Table 1. The mutants, Asp532Lys and Asp532Glu, showed only small changes in K_m values for glucose and ATP, compared with the wild-type enzyme. However, the k_{cat} values of the mutants, Asp532Lys and Asp532Glu, exhibited 1000- and 200-fold decreases relative to the wild-type enzyme, respectively. The mutant Asp532Lys

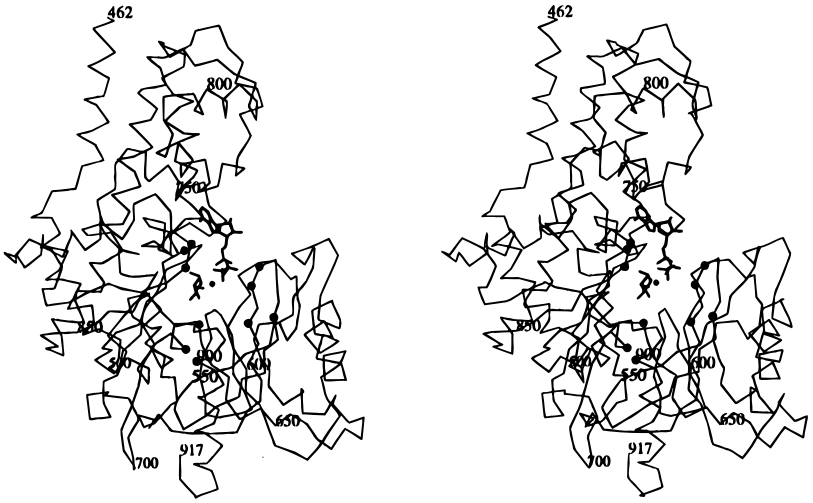

FIGURE 3: Stereoview of the Cα backbone of the modeled structure of the catalytic domain of brain hexokinase. The domain has a closed form. ATP, glucose, and Mg^{2+} ion bound in the cleft are shown with bold lines. The conserved residues within phosphotransferases are shown with filled circles.

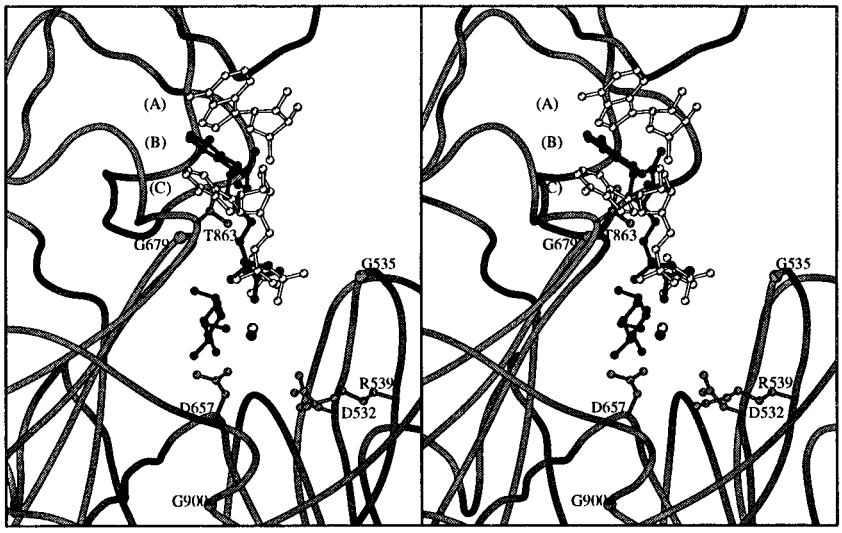

FIGURE 4: Stereoview of the ATP binding site of brain hexokinase (closed form) with ATP analogues superimposed from different crystal structures. The backbone of the enzyme is represented by smoothed coils. The side chains of conserved residues are drawn as ball-and-sticks. Glucose is shown in black. (A) The adenosine part of 8-bromoadenosine monophosphate from the crystal structure of yeast hexokinase b (Shoham & Steitz, 1980). (B) ADP and Mg^{2+} (filled circle) from glycerol kinase (Hurley et al., 1993). (C) ATP and Ca^{2+} (open circle) from actin (Kabsch et al., 1990). The Mg^{2+} and ATP analogues from heat shock protein (Flaherty et al., 1994) are not shown because their positions are identical with ligands from actin. The figure was prepared with MOLSCRIPT (Kraulis, 1991).

Table 1: Comparison of the Kinetic Data of the Wild-Type and the Mutant Forms of Hexokinase[a]

hexokinase	K_m(Glu) (μM)	K_m(ATP) (mM)	K_{iATP} (mM)	K_i (1,5-An-G6P) (μM)	k_{cat} (s^{-1})
wild-type	65 ± 6.6	0.49 ± 0.06	0.47 ± 0.09	26 ± 4.8	26.9
Asp532Lys	71 ± 6.4	0.57 ± 0.06	0.38 ± 0.05	25 ± 8.8	0.029
Asp532Glu	18 ± 6.0	1.42 ± 0.44	1.94 ± 0.44	29 ± 3.1	0.14
Thr680Val	33 ± 3.6	0.27 ± 0.04	0.40 ± 0.09	45 ± 11	0.013
Thr680Ser	43 ± 11	0.45 ± 0.10	0.45 ± 0.12	26 ± 2.4	11.6

[a] The values shown are the mean ± SD. The kinetic analysis was done with the computer program MINITAB.

was inhibited by the Glu-6-P analogue 1,5-an-G6P, with the same K_i values as that of the wild-type enzyme. P_i reversed the inhibition. The preserved affinities for the ligands and inhibitors as well as the mechanism of P_i activation indicate that the substituted side chains did not significantly alter the conformation either of the active or allosteric sites. The model supports this conclusion, demonstrating that Asp532 is positioned on the edge of the active site and there is enough room to accommodate the bigger side chains of the mutants. The conservative substitution of Asp532 to Asn reported earlier produced a similar effect on the activity (Baijal & Wilson, 1995). Thus, the dramatic decreases in activities of Asp532 mutants most probably come from the distortion of its interaction with the Mg^{2+} ion of the $MgATP^{2-}$ complex. The three mutations of Asp532 demonstrate that not only the charge but also the size of the residue are important for the catalysis.

The K_m values for ATP for the mutants Asp532Lys and Asp532Glu of hexokinase are the same as for the wild-type enzyme, indicating the interaction between Asp532 and the

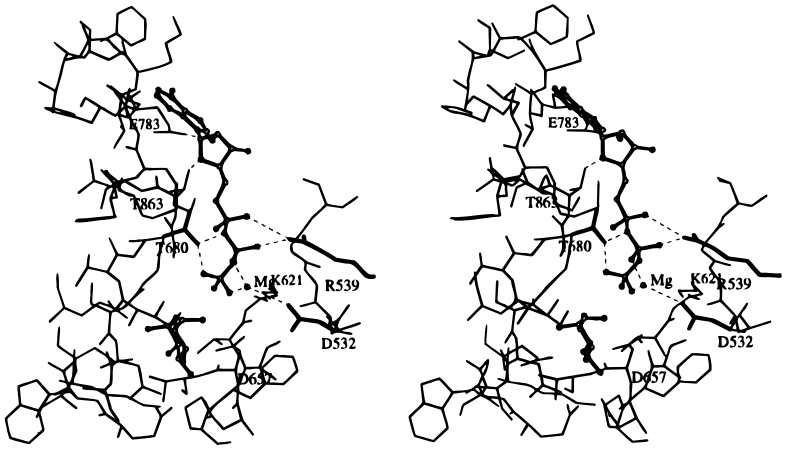

FIGURE 5: Stereodrawing of the ATP-binding site of the model of the ternary complex of brain hexokinase. Glucose and ATP are drawn in ball-and-stick, the filled balls are N, O, or Mg^{2+} atoms. The protein part of the complex is drawn with thin lines (except side chains of Asp532, Arg539, and Thr680). The hydrogen bonds and salt linkages between the protein and ligands are shown as dash lines. The figure was prepared with MOLSCRIPT (Kraulis, 1991).

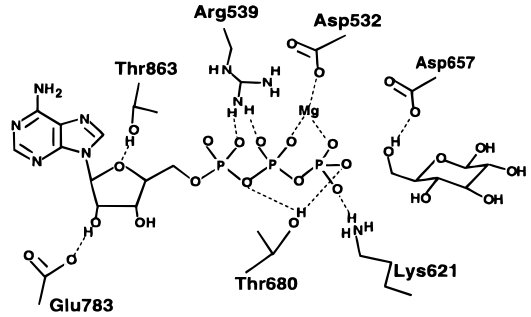

FIGURE 6: Schematic drawing of interactions of brain hexokinase with ATP, as predicted by modeling.

Mg^{2+} ion of the $MgATP^{2-}$ complex is not responsible for ATP binding affinity. Previous results (Rudolph & Fromm, 1971; Kosow & Ross, 1970) indicate the K_i value for the ATP^{4-} is about the same as the K_m value for $MgATP^{2-}$, a finding consistent with our present findings.

Site-Directed Mutagenesis of Thr680. Thr680 is conserved among sugar kinases. Our model of the ATP-binding site of human brain hexokinase predicted that the hydroxyl group of Thr680 may form hydrogen bonds to γ-phosphoryl group of ATP (Figure 6) and thus may be important for the catalytic reaction of hexokinase. Therefore, Thr680 was mutated to Ser and Val. Although the K_m values of both mutants for either glucose or ATP showed only slight or no change, the k_{cat} values for Thr680Val and Thr680Ser mutants exhibited 2000- and 2.5-fold decreases, respectively (Table 1). Since CD spectra for Thr680Val and Thr680Ser showed no difference relative to that of the wild-type enzyme, we suggest that no secondary conformation change occurs after mutagenesis. The dramatic decrease in the k_{cat} value for the Thr680Val mutant compared to the small change observed for Thr680Ser mutant suggests that the hydroxyl of Thr680 is important for catalysis. The location of Thr680 in the active site of hexokinase excludes any other involvement of its side chain in catalysis except for interaction with ATP. The small change in ATP affinity of Thr680 mutant indicates that similar to Arg539, the energy of interaction of this residue with ATP is used for stabilization of the transition state. If the proposed interaction between Thr680 and O1G of γ-phosphoryl group is correct, then this interaction may be involved in stabilization of the γ-phosphoryl group in the vicinity of the O6-hydroxyl group of glucose. The alternative function of the residue may be to stabilize the overall productive conformation of the ternary complex.

Pilkis et al. (1994) found that mutations in the human glucokinase gene are a common cause of an autosomal dominant form of non-insulin-dependent (type 2) diabetes mellitus. One mutant, Thr228Met, causes about a 2500-fold decrease in the k_{cat} value but no change in the K_m values either for glucose or for ATP relative to wild-type glucokinase. Thr228 corresponds to Thr680 in human brain hexokinase. The kinetic properties of the Thr228Met mutant is similar to those of the Thr680Val mutant in human brain hexokinase. Assuming human glucokinase has the same ATP-binding site as that of human brain hexokinase, Thr228 may interact with ATP in the same way as Thr680 does in hexokinase.

It is clear from Table 1 that the dissociation constants (K_{iATP}) for ATP are very similar to the K_m values for the nucleotide. This finding suggests that glucose, when present on the enzyme, does not alter the binding of ATP. This is based on the assumption that the kinetic mechanism of the mutants is not altered and is rapid-equilibrium random bi-bi as is the case for the wild-type phosphotransferase (Ning et al., 1969; Bachelard et al., 1971; Gerber et al., 1974)

All mutants of residues Asp532, Thr680, and Arg539 exhibit significant decreases in k_{cat} with only small changes in either K_{iATP} or K_m for ATP relative to the wild-type enzyme. All these residues are conserved within sugar kinases, and two of them (Arg539 and Asp532) are conserved in other phosphotransferases (Bork et al., 1992). The results of our modeling, as well as the analysis of functionally homologous enzymes (Kabsch et al., 1990; Flaherty et al., 1991; Hurley et al., 1993), suggest the involvement of these residues in interaction with the tripolyphosphoryl portion of ATP. Thus, we can conclude that the postulated interactions of these residues with ATP represent a classical example of utilization of binding energy for the stabilization of the transition state. According to Fersht (1985) the substrate-binding energy can promote catalysis either through the formation of "strain" in reacting compounds or by "induced-fit" conformational changes in the enzyme. It is difficult to say which factor is predominant in hexokinase. The crystallographic and kinetic data regarding the conformational

change of hexokinase induced by binding of ATP and glucose suggest the importance of the interactions with the tripolyphosphoryl moiety of ATP for that transformation. On the other hand, Asp532, through its interaction with Mg^{2+} ion, and Thr680 presumably by stabilizing the γ-phosphoryl group of ATP, represents the "strain concept" (Haldane, 1965). The importance of protein interactions with the tripolyphosphoryl moiety of ATP (or GTP) for the stabilization of the transition state seems to be a rather universal property of phosphortransferases. There are numerous examples in the literature where mutation of residues involved in such interactions produce significant changes in k_{cat} with little or no change in K_m: adenylate kinase (Arg132Met) (Dahnke & Tsai, 1994), adenylosuccinate synthetase (Gly15Val) (Kang & Fromm, 1994), the 70-kDa heat shock cognate protein (Asp199Ser) (Wilbands et al., 1994), and Rho protein (Asp265Asn) (Dombroski et al., 1988).

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