Glucose Phosphorylation

SITE-DIRECTED MUTATIONS WHICH IMPAIR THE CATALYTIC FUNCTION OF HEXOKINASE*

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Recent studies from this and other laboratories have resulted in the cloning and sequencing of hexokinases from a variety of tissues including yeast, human kidney, rat brain, rat liver, and mouse hepatoma. Significantly, studies on the hepatoma enzyme conducted in this laboratory (Arora, K. K., Fanciulli, M., and Pedersen, P. L. (1990) J. Biol. Chem. 265, 6481-6488) resulted also in its overexpression in Escherichia coli in active form. We have now used site-directed mutagenesis for the first time in studies of hexokinase to evaluate the role of amino acid residues predicted to interact with either glucose or ATP.

Four amino acid residues (Ser-603, Asp-657, Glu-708, and Glu-742) believed to interact with glucose were mutated to alanine or glycine, whereas a lysine residue (Lys-558) thought to be directly involved in binding ATP was mutated to either methionine or arginine. Of all the mutations in residues believed to interact with glucose, the Asp-657 → Ala mutation is the most profound, reducing the hexokinase activity to a level <1% of the wild type. The relative \( V_{max} \) values for Ser-603 → Ala, Glu-708 → Ala, and Glu-742 → Ala enzymes are 6, 10, and 6.5%, respectively, of the wild-type enzyme. Glu-708 and Glu-742 mutations increase the apparent \( K_m \) for glucose 50- and 14-fold, respectively, while the Ser-603 → Ala mutation decreases the apparent \( K_m \) for glucose 5-fold. At the putative ATP binding site, the relative \( V_{max} \) for Lys-558 → Arg and Lys-558 → Met enzymes are 70 and 29%, respectively, of the wild-type enzyme with no changes in apparent \( K_m \) for glucose. No changes were observed in the apparent \( K_m \) for ATP with any mutation.

These results support the view that all 4 residues predicted to interact with glucose from earlier x-ray studies may play a role in binding and/or catalysis. The Asp-657 and Ser-603 residues may be involved in both, while Glu-708 and Glu-742 clearly contribute to binding but are not essential for catalysis. In contrast, Lys-558 appears to be essential neither for binding nor catalysis.

Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) is the first enzyme of the glycolytic pathway that commits glucose to catabolism by catalyzing its phosphorylation to Glc-6-P with MgATP. In rapidly growing tumor cells, the enzyme hexokinase is markedly elevated and 50-80% of its activity is bound to the outer mitochondrial membrane (1-3), where it has preferred access to mitochondrially generated ATP (3). Of the four known hexokinases distinguished on the basis of charge (Types I, II, III, and IV), the major form found in tumors exhibits properties in common with both the Type I and II isozymes (4).

Recently, we have reported the primary structure of tumor mitochondrial hexokinase deduced from a full-length cDNA clone isolated from a highly glycolytic mouse hepatoma cell line (5). The tumor enzyme consists of 918 amino acids and has a mass of 102,272 daltons which is very close to that of the recently cloned and sequenced Type I hexokinase from rat brain (6) and human kidney (7). These three enzymes are twice the size of hexokinase Type IV (also called glucokinase) and yeast hexokinase. Similar to the Type I rat brain and human kidney hexokinases (6, 7), the tumor enzyme appears to have arisen from a gene duplication-gene fusion event resulting in a gene encoding a hexokinase with approximately two halves, each with a mass of 50 kDa which share close to 68% amino acid sequence homology (6). Significantly, the C-terminal half of the brain enzyme has been shown to account for all its catalytic activity (8), whereas the N-terminal half is predicted to be involved in product inhibition by Glc-6-P (6, 9).

Based on amino acid sequence comparisons of tumor, brain, kidney, and liver hexokinases with that of the yeast enzyme, the x-ray structure of which is known (10, 11), putative glucose and ATP-binding domains have been predicted (12). The glucose-binding domains, found in both the C- and N-terminal halves, include residues thought to interact directly with glucose in the yeast enzyme, i.e. Ser-158, Asp-211, Glu-269, and Glu-302 (11). These residues correspond, respectively, to Ser-603, Asp-657, Glu-708, and Glu-742 of the C-terminal half of the tumor enzyme (5).

Although x-ray studies using 8-bromo-AMP predict a nucleotide-binding site within the larger of the two lobes in the yeast enzyme (13), there appears to be little sequence homology in this region among various hexokinases. In contrast, a predicted ATP-binding region (12), based on sequence homology with the catalytic site of cAMP-dependent protein kinases (14), lies in the smaller of the two lobes (15, 16). This region includes Lys-111 in the yeast enzyme which has been reported to interact directly with an ATP affinity label (15). Also, the ATP analog, TNP-ATP, has been shown to bind a 50-amino acid peptide of yeast hexokinase which includes Lys-111 (16). This "invariant" lysine residue has been found in all hexokinases sequenced to date (5, 6, 7, 12) and corresponds to Lys-558 in the C-terminal half of the tumor enzyme.

In this report, we have used the technique of site-directed mutagenesis to evaluate the role of amino acid residues predicted to interact with either glucose or ATP in the C-terminal half of tumor hexokinase.

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1 The abbreviations used are: TNP-ATP, 2',3'-O-(2,4,6-trinitropheryl)-adenosine-5'-triphosphate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.
**EXPERIMENTAL PROCEDURES**

**Materials**

Sources of restriction and DNA-modifying enzymes and other molecular biological chemicals have been previously described (5). Oligonucleotide primers (19-mers) for site-directed mutagenesis were synthesized in the protein/peptide/DNA facility of the Department of Biological Chemistry, Johns Hopkins University School of Medicine. The oligonucleotide-directed in vitro mutagenesis kit was from Amersham Corp.

**Methods**

Oligonucleotide-directed Mutagenesis of Tumor Hexokinase—Site-directed mutagenesis was performed according to the method of Sambrook et al. (19) using a kit from Amersham Corp. A 2.84-kilobase XhoI-XbaI fragment containing the entire coding region of tumor hexokinase and 50 base pairs of the 3'-untranslated region was subcloned into the polyclin region of M13mp19 at the XbaI site and was used as a template to make site-directed changes. For Ser-603, the 19-mer long mutagenic primer was 5'-CACCTTCGCGTTCTCCCTCGT-3', and at the underlined base, codon TCG (Ser) was replaced with GGC (Ala). For Asp-657, the 19-mer primer was 5'-GGTTTGmGAAGATGATCAG-3', and at the underlined bases, codon GAC (Asp) was changed to GCC (Ala) or GGA (Gly). For Glu-742, the sequence of the 19-mer oligonucleotide was 5'-GGTTGmGTCCGAAGATGATCTACTCC-3', and at the underlined bases the co- don AAG for Lys was altered to either ATG for Met or AGG for Arg. A single oligonucleotide preparation was used to modify one residue to two different residues by adding 50% of both the bases at that particular position during synthesis of these oligonucleotides. Mutations were identified by sequencing single-strand DNA from M13 transformants. DNA sequencing was carried out by the dideoxy chain termination method of Sanger et al. (18) using Sequenase (version 2.0).

In order to transfer the mutants to the expression plasmid pKAXH (5) and to ensure that the site-directed mutations reported in this study were confined to the C-terminal half of the molecule, a repli- cating form DNA of M13 mutants was cut at two unique restriction sites, NcoI (at base 1365) and StuI (at base 2553). The 1188-base pair NcoI-StuI fragment was gel-purified and then recloned into the plasmid pKAXH from which the wild-type NcoI-StuI fragment had been removed. The desired mutations in the expression plasmid were reconfirmed by DNA sequence analysis.

**Recombinant DNA Procedures**—Unless specified, standard molecular biological protocols described by Sambrook et al. (19) were used.

Overexpression and Partial Purification of Wild-type and Mutant Hexokinase Proteins—Wild-type and mutant hexokinase proteins were overproduced in *Escherichia coli* under the control of the alkaline phosphatase (phoA) promoter system utilized recently by Arora et al. (5). Using this overexpression system, it was observed that, in response to the stress of overproduction of foreign proteins, bacterial gluco kinase was also induced. Therefore, a procedure was developed to separate the overproduced tumor hexokinase from induced *E. coli* glucokinase. Briefly, the procedure involved was as follows. 250 ml of a 12-14-h culture overexpressing hexokinase protein was sedimented at 12,000 × g for 10 min at 4 °C. This procedure was again centrifuged at 12,000 × g for 10 min at 4 °C. This procedure of washing the pellet with Tris-HCl/EDETA/NaCl buffer was repeated once again. After this step, >99% of the stress-induced glucokinase activity was eliminated and most of the overexpressed tumor hexokinase was recovered in the "particulate" fraction. The effect of site-directed mutations on hexokinase activity was tested using both the "Triton X-100 extract" and the detergent extracted particulate fractions. Both fractions yielded comparable results, but activity in the particulate fraction was higher in specific activity and much more stable, permitting storage for prolonged periods. Therefore, in this study results from the particulate fraction are reported.

**Hexokinase Activity Assay**—Hexokinase activity assays for both wild-type and mutant hexokinase protein preparations were performed spectrophotometrically essentially as described previously (2) using 2.3 mM glucose and 10 mM ATP in a coupled assay involving glucose 6-phosphate dehydrogenase and NADP. The particulate fractions were determined by the bicinchoninic acid assay (Pierce Chemical Co.) using bovine serum albumin as a standard.

**Western Blotting**—Immunoblots were performed on polyvinylidene difluoride membranes (Immobilon 18) using anti-hexokinase antibodies raised against purified AS-30D hepatoma hexokinase essentially as described previously (5).

**RESULTS**

**Site-directed Mutations of Ser-603, Asp-657, Glu-708, and Glu-742 in the Putative Glucose Binding Domain of Tumor Hexokinase**—In order to evaluate the role of amino acid residues predicted to interact with glucose during the hexokinase-catalyzed reaction, Ser-603, Asp-657, Glu-708, and Glu-742 were mutated to either Ala or Gly, as described under "Methods." These latter amino acids cannot participate in hydroxyl group binding, which is reflected in 50- and 14-fold increases in the apparent Kₘ for glucose compared with the wild type by at least 90%. A single mutation of Asp-657 to Ala has the greatest effect, reducing the activity to <1% that of the wild-type enzyme. Changing Glu-708, Glu-742, or Ser-603 to Ala reduces Vₘₕ by 10-15-fold or more relative to wild-type enzyme. Similar reductions in hexokinase activity are seen also when Glu-708, Glu-742, or Asp-657 are mutated to Gly (data not shown).

Apparent Kₘ values for both hexokinase substrates were determined for wild-type and all mutant enzymes except Asp-657 → Ala, the activity of which was too low to do meaningful kinetics. These values reported also in Table I, A show that all four site-directed mutations reduce hexokinase activity (Vₘₕ) by at least 90%. A single mutation of Asp-657 to Ala has the greatest effect, reducing the activity to <1% that of the wild-type enzyme. Changing Glu-708, Glu-742, or Ser-603 to Ala reduces Vₘₕ by 10-15-fold or more relative to wild-type enzyme. Similar reductions in hexokinase activity are seen also when Glu-708, Glu-742, or Asp-657 are mutated to Gly (data not shown).

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**Site-directed Mutations of Lys-558, Glu-708, and Glu-742 in the Putative Glucose Binding Domain of Tumor Hexokinase**—The ATP consensus sequence of protein kinases has a conserved lysine located...
Site-directed Mutations in Hexokinase

## Table I

| Mutation       | $K_{\text{m,glucose}}$ | $K_{\text{m,ATP}}$ | $V_{\text{max}}$ | Relative $V_{\text{max}}$ | Relative $V_{\text{m,ATP}}/K_{\text{m,glucose}}$ |
|----------------|------------------------|---------------------|-------------------|---------------------------|--------------------------------------------------|
| A. Glucose-binding domain and associated residues |                      |                     |                   |                           |                                                  |
| Wild type      | 0.045                  | 2.4                 | 100               |                           | 2.222                                            |
| Ser-603 → Ala  | 0.0084                 | 2.6                 | 5.9               |                           | 702                                              |
| Asp-657 → Ala  | a                      | a                   | b                 |                           | b                                                |
| Glu-708 → Ala  | 2.28                   | 2.7                 | 9.6               |                           | 4                                                |
| Glu-742 → Ala  | 0.62                   | 2.6                 | 6.4               |                           | 10                                               |

B. ATP-binding domain

| Mutation       | $K_{\text{m,glucose}}$ | $K_{\text{m,ATP}}$ | $V_{\text{max}}$ | Relative $V_{\text{max}}$ | Relative $V_{\text{m,ATP}}/K_{\text{m,glucose}}$ |
|----------------|------------------------|---------------------|-------------------|---------------------------|--------------------------------------------------|
| Wild type      | 0.046                  | 2.2                 | 100               |                           | 2.7                                              |
| Lys-558 → Arg  | 0.050                  | 2.2                 | 50                |                           | 70                                               |
| Lys-558 → Met  | 0.047                  | 2.1                 | 20                |                           | 69                                               |

### Notes

- The Asp-657 → Ala mutant hexokinase activity detected with the assay used was too low to permit an accurate kinetic analysis.
- The relative activity measured at 2.3 mM glucose plus 10 mM ATP was less than 1% that of wild type.

### Discussion

Data presented in this paper demonstrate for the first time that all four conserved amino acid residues predicted from x-ray crystallographic studies of the yeast enzyme (11) to be within “contact” distance of glucose may play significant roles in binding and/or catalysis, but apparently in different ways. Catalytic activity is virtually abolished when Asp-657 is changed to Ala, while Glu-708 → Ala and Glu-742 → Ala enzymes retain as much as 10% of the wild-type activity (Table I, A). Significantly, it has been suggested that the residue equivalent to Asp-657 in the yeast enzyme, i.e. Asp-211, is a catalytic base involved in the hexokinase reaction.
(10, 21). The conversion of Ser-603 to Ala reduces the activity markedly, consistent also with the recent finding (22) that a glucose analog, N-(bromoacetyl)-D-glucosamine, labels a region of rat brain hexokinase in the C-terminal half which includes the highly conserved residue Ser-603. The surprising decrease in apparent \( K_m \) for glucose, but the corresponding and greater decrease in \( V_{\text{max}} \), indicate that Ser-603 influences both glucose binding and catalysis, perhaps by affecting either transfer of the phosphoryl group of ATP to glucose or release of the product Glc-6-P.

Based on x-ray crystallographic studies of yeast hexokinase (10, 11), there is a deep central cleft that divides the molecule into two lobes. Upon binding glucose, the smaller lobe rotates 12° relative to the larger lobe, thus partially closing the cleft. This conformational change, from “open” to “closed” forms, induced by glucose is considered to be a necessary step for catalysis. The resultant enzyme-glucose binary complex, upon binding ATP, is predicted to then form a ternary complex which presumably undergoes another conformational change before catalysis occurs (23). It is possible that in both the Glu-708 → Ala and Glu-742 → Ala mutants the structure of the “open” form is altered such that the affinity for glucose is decreased, as suggested by the increased apparent \( K_m \) values for glucose (Table I, A). Thus, the magnitude of the resulting glucose-induced conformational change may be insufficient to promote a “productive” reaction pathway.

Finally, it seems important to compare results reported here using site-directed mutations with those recently reported on the yeast enzyme in which random mutations were made (24). Of the random mutations made, none correspond to residues mutated in this study, and of those residues that do affect activity, only Gly-235 and Asn-237 are predicted from studies of the crystal structure of a yeast enzyme-glucose analog complex to be within hydrogen bond distance of the sugar substrate (10). Certainly, additional studies will be necessary to better define the entire glucose-binding domain of hexokinase.

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