Structure/Function Relationships in Hexokinase

SITE-DIRECTED MUTATIONAL ANALYSES AND CHARACTERIZATION OF OVEREXPRESSED FRAGMENTS IMPLICATE DIFFERENT FUNCTIONS FOR THE N- AND C-TERMINAL HALVES OF THE ENZYME*

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Krishan K. Arora, Charles R. Filburn‡, and Peter L. Pedersen§
From the Laboratory for Molecular and Cellular Bioenergetics, Department of Biological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Hexokinases are comprised of two highly homologous ~50-kDa halves and are product-inhibited by glucose-6-P. Four amino acid residues, Ser205, Asp67, Glu708, and Glu742, located in the C-terminal half of the tumor mitochondrial enzyme have been shown to be essential for enzyme function (Arora, K. K., Filburn, C. R., and Pedersen, P. L. (1991) J. Biol. Chem. 266, 5359-5362). Here we have assessed also the role of the N-terminal half of the same enzyme.

Site-directed mutagenesis of residues predicted to interact with glucose in the N-terminal half, i.e., Ser205, Asp67, and Glu708, to Ala, have no effect on hexokinase activity. In addition, inhibition by hexose mono- and bisphosphates is unchanged for each of the mutant enzymes. Significantly, the overexpressed N-terminal polypeptide is devoid of catalytic activity but does have the capacity to bind ATP-agarose and be released with ATP and glucose-6-P. In contrast, the overexpressed C-terminal polypeptide is catalytically active and shows the same product inhibition pattern as the complete 100-kDa parent enzyme.

These results emphasize that the N-terminal half of tumor hexokinase is essential neither for catalysis nor product modulation. Rather, the N-terminal half may play another role, perhaps in modulation of the ATP/glucose-6-P-dependent binding of the enzyme to tumor mitochondria or by acting as a spacer between the outer mitochondrial membrane and the C-terminal catalytic unit.

Hexokinase (ATP:D-hexose 6-phosphotransferase, EC 2.7.1.1) commits glucose to catabolism by catalyzing its phosphorylation to Glc-6-P utilizing MgATP. In rapidly growing cancer cells, hexokinase is markedly elevated, and a large fraction (50-80%) of the total activity is bound to the outer mitochondrial membrane (1, 2), where it has preferred access to mitochondrially generated ATP (3). In mammals, there are four isozymes of hexokinase, i.e., Types I, II, III, and IV, which vary in their tissue distribution and kinetic properties (4). Of the four known isozymes of hexokinase, the major form found in tumors has properties in common with both the Types I and II isozymes (5). The Types I, II, and III isozymes, and the tumor enzyme, consist of a single polypeptide chain with a molecular mass of ~100 kDa, and all four are inhibited by the product Glc-6-P (6). The Type IV hexokinase (also known as glucokinase), similar to yeast hexokinase, is ~50 kDa and is insensitive to inhibition by Glc-6-P (6).

Recent studies have resulted in the cloning and sequencing of hexokinases from a variety of tissues including yeast (7, 8), human kidney (9), rat brain (10), rat skeletal muscle (11), rat liver (12, 13), and, from this laboratory, mouse hepatoma (14). The deduced amino acid sequence data indicate a significant identity between the mammalian forms and the tumor enzyme, as well as strong similarity to the yeast hexokinase and rat liver glucokinase (12, 14, 15). Thus, these studies provide evidence in support of the gene duplication-gene fusion hypothesis for the evolution of 100-kDa hexokinases from the 50-kDa yeast-like enzymes. It has been speculated (16, 17) that, as a result of such a duplication event, one of the halves may have evolved a regulatory function, whereas the other half retained the catalytic function of the enzyme. Significantly, the C-terminal half of the brain enzyme has been shown to comprise all its catalytic activity (18), whereas the N-terminal half is predicted to be involved in product inhibition by Glc-6-P (10, 19).

Based on amino acid sequence comparisons of tumor and other hexokinases with that of the yeast enzyme, the x-ray structure of which is known (20, 21), putative glucose and ATP binding domains have been predicted (12, 15). 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 (21) (see Fig. 1, A and B, and accompanying legends).

In our most recent studies (22), we have used the technique of site-directed mutagenesis for the first time to evaluate the role of amino acids predicted to interact with glucose in the C-terminal half of the tumor enzyme. We showed that all four conserved residues (Ser205, Asp67, Glu708, and Glu742) in the C-terminal half of tumor hexokinase, predicted from x-ray studies of the yeast enzyme to be within “contact” distance of glucose, are required for enzyme function. Interestingly, these 4 residues predicted to be within “contact” distance of glucose are also conserved in the N-terminal half of the enzyme. These correspond to Ser205, Asp67, Glu708, and Glu742 (see Fig. 1B).

In this report, we have evaluated the role of the N-terminal half of tumor hexokinase using two different approaches: (a) by assessing the requirement of conserved amino acid residues predicted to interact with glucose using the technique of site-
directed mutagenesis and (b) by characterizing overexpressed N- and C-terminal halves of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

Adenine and pyridine nucleotides, dithiothreitol, and glucose-6-phosphate dehydrogenase (Grade I) were obtained from Boehringer Mannheim. 1,5-Anhydro-β-glucitol, 5-thio-β-glucose-6-P, glucose-1,5-biphosphate were from Sigma. Sources of restriction and DNA-modifying enzymes and other molecular biological chemicals have been described previously (14). Oligonucleotide primers for site-directed mutagenesis were synthesized in the Protein/Peptide/DNA facility in the Department of Biological Chemistry, The Johns Hopkins University School of Medicine. The oligonucleotide-directed in vitro mutagenesis kit and the enhanced chemiluminescence detection kit for Western blotting were obtained from Amersham Corp. and were used according to the manufacturer's instructions.

**Methods**

**Recombinant DNA Procedures**—Unless specified, standard molecular biological protocols described by Sambrook et al. (23) were used.

**Glu185Ser**

Ser185 S → A  5'-CACATTGGCTTCCCTCGGTC-3'

Asp200 D → A  5'-GTTAAGCTCACAGTTGGGG-3'

Glu240 E → A  5'-GTATTACCCGAACGTTGGGCCCA-3'

Mutations were identified by sequencing of single strand DNA from M13 transformants. DNA sequencing was carried out by the dideoxy method (24) using a kit from Amersham.

**Plasmid Construction for Expression of the N-term. Half of Tumor Hexokinase**—Site-directed mutagenesis was performed according to the method of Taylor et al. (24) using a kit from Amersham. A 2.84-kb Xbal-Xbal fragment containing the entire coding region of tumor hexokinase and 50 base pairs of the 3' untranslated region was subcloned into the polylinker region of M13 mp19 at the XbaI site and was used as a template to make site-directed changes as described previously by Arora et al. (22). Site-directed mutations reported in this study and the sequence of the mutagenic primers, with nucleotide mismatches underlined, are as follows.

**SDS-PAGE and Protein Determination**—SDS-polyacrylamide gel electrophoresis was carried out in 1.5-mm-thick 10% acrylamide gels according to the method of Laemmli (28). Protein concentrations were determined by the bicinchoninic acid assay (Pierce Chemical Co.) using bovine serum albumin as the standard.

**Site-directed Mutations of Ser185, Asp200, and Glu240 in the Putative Glucose Binding Domain within the N-terminal Half of Hexokinase Have Little Effect on Catalytic Activity**—Previously, we showed that the 4 conserved amino acid residues Ser185, Asp200, Glu700, and Glu427 in the C-terminal half, predicted to interact with glucose based on x-ray crystallographic studies of the yeast enzyme (20, 21), are required for enzyme
function (22). Significantly, these amino acid residues are conserved also within the N-terminal half of tumor hexok
nase (Fig. 1B). In order to evaluate their role during the hexokinase-catalyzed reaction, 3 of the 4 conserved residues, Ser^155, Asp^209, and Glu^266, were mutated to Ala as described under "Methods" (see also Fig. 2A). Alanine was chosen for substitution, because it does not participate in hydrogen bonding to the hydroxyl groups of glucose. Hexokinase activity determined for both the overexpressed wild type and mutant enzymes is presented in Fig. 3A. It is clear that these point

![Structure of Hexokinase](image)

**Fig. 1.** A, computer graphics representation of the predicted structure of a mammalian hexokinase. The proposed structure is based on the fact that 100-kDa mammalian hexokinases, including tumor hexokinase, are twice the size of 50-kDa yeast-like enzyme. The two halves of tumor hexokinase designated as N- and C-terminal and shown in the figure in magenta and blue, respectively, share 68% amino acid sequence homology (14). The above structure was obtained by fusing the C terminus of one complete yeast hexokinase molecule with a second truncated molecule lacking the region corresponding to the first helix of the yeast enzyme (which is present in the N-terminal half but absent in the C-terminal half of the tumor and brain enzymes) and then rotating the newly joined molecule to eliminate any steric effects. The molecular model was generated using the RIBBONS program (42) with the Personalized system using Silicon Graphics IRIS 4D series workstation. The x-ray coordinates for yeast hexokinase were obtained from the Brookhaven Protein Data Bank. The figure illustrates the location of various "direct" contact residues believed to interact with glucose based on the x-ray studies of the yeast enzyme. The locations of these residues Ser^380, Asp^386, Glu^395, and Glu^397 in both halves are indicated. Numbers refer to the location in the sequence as determined by x-ray studies (21) and do not correspond directly to positions in the sequence as deduced from cDNA sequencing of the cloned gene (7, 8). Also, shown is the predicted direct "contact" lysine residue for ATP (Lys^95). B, schematic representation of the primary structure of tumor hexokinase illustrating putative glucose and ATP binding domains and residues proposed to be within contact distance of glucose and ATP. Four amino acid residues believed to interact with glucose in the two halves of tumor hexokinase correspond to Ser^155, Asp^209, Glu^266, and Glu^294 in the N-terminal half and Ser^261, Asp^373, Glu^58, and Glu^62 in the C-terminal half. In the tumor enzyme, the amino acid residue corresponding to Lys^301 of the yeast enzyme is Lys^558 in the C-terminal half. However, the corresponding lysine residue in the N-terminal half is replaced with a glutamic acid residue which is not shown.
Results described above on the intact enzyme suggest that the C-terminal half of tumor hexokinase may be responsible for catalysis and product inhibition and further indicate that Lys558 within the catalytic half is not involved in product inhibition.

The overexpressed C-terminal half of tumor hexokinase retains catalytic activity and the capacity to be product-inhibited, whereas the overexpressed N-terminal half has no detectable activity—In order to evaluate the role of the iso-

mutations have no major effect on the catalytic activity of hexokinase.

The S155A mutation consistently induced a slight activation (12–15%) of catalytic activity, whereas the D209A and E260A mutations induced a slight inhibitory effect (15–22%). These results remained the same in studies not presented here over a wide range of glucose and ATP concentrations.

In control experiments all recombinant proteins bearing mutations in the N-terminal half, i.e. S155A, D209A, and E260A, were shown to be stable in E. coli. This question was addressed by examining the overexpressed protein products of the wild type and mutant genes in SDS-PAGE (Fig. 4). All mutant proteins (lanes 2–4) show a band near 100 kDa with almost the same intensity as that for the wild type protein (lanes 1 and 6). These results were confirmed for each mutant protein by using Western blot analysis where predominantly one immunoreactive band comparable with that of the wild type was observed (results not presented).

Site-directed Mutations of Ser55, Asp209, and Glu260 within the Putative Glucose Binding Domain in the N-terminal Half of Hexokinase Also Have Little Effect on Its Capacity to be Inhibited by Glc-6-P Analog—Three proteins bearing mutations in the N-terminal half were tested for their capacity to be inhibited by Glc-6-P analogs. The Glc-6-P analog 1,5-AnG-6-P, which is a linear competitive inhibitor versus ATP, and a linear uncompetitive inhibitor versus glucose (Fig. 5, A and B), was used initially for the reasons given under “Methods.” Results presented in Table I show that no appreciable differences in $K_i$ (apparent) are seen between the mutant and the wild type enzymes in the presence of 1,5-AnG-6-P as inhibitor.

In our previous study (22) Lys558, the “invariant” lysine within the C-terminal half of tumor hexokinase predicted to interact with ATP, was mutated to Met, and this mutation decreased the relative $V_{max}$ by 70% of the wild type enzyme. We thought that this lysine may contribute also to the binding of the product Glc-6-P. For this reason, the K558M mutant protein was also tested for inhibition by 1,5-AnG-6-P in the present study. Again a $K_i$ (apparent) value similar to that of the wild type was obtained (Table I), indicating that the product inhibitory site within the C-terminal half does not involve Lys558.

In addition to Glc-6-P and 1,5-AnG-6-P, other analogs of Glc-6-P, viz. 5-thio-Glc-6-P and Glc-1,6-Pz, have been reported to be potent linear competitive inhibitors (versus ATP) of mammalian hexokinases (29). Therefore, all four mutant enzymes, S155A, D209A, E260A, and K558M, were tested for their inhibition by these hexose mono- and bisphosphate analogs of Glc-6-P. In every case, both at low and high ATP concentrations, essentially no difference in the inhibition pattern from that of the wild type enzyme was observed (results not presented).
FIG. 3. Effect of site-directed mutations within the N- and C-terminal halves of tumor hexokinase on its catalytic activity. Procedures for the wild type and mutant enzyme preparations, and hexokinase activity determinations, are described under “Methods.” The activity was measured at saturating concentrations of glucose (2.2 mM) and ATP (6.4 mM). Activities expressed as percentage of the wild type are shown as bar graphs. The data shown in A are means of at least four independent experiments. The error bars represent the standard error of mean. The average specific activity for the wild type enzyme was 270 ± 10 nmol of Glc-6-P formed per min/mg of protein (n = 6). For comparison, the relative activities are also shown for the corresponding site-directed mutants within the C-terminal half of tumor hexokinase.

FIG. 4. SDS-PAGE analysis of wild type (intact), mutant, and N- and C-terminal halves of hexokinase proteins. Wild type, N- and C-terminal halves, and mutant hexokinase proteins were overexpressed and subjected to SDS-PAGE exactly as described under “Methods.” Approximately 10–14 μg of protein from the particulate fraction was loaded onto each lane. Lanes 1 and 6, wild type; lane 2, S155A; lane 3, D209A; lane 4, E260A; lane 5, K558M; lane 7, C-terminal half; lane 8, N-terminal half. The position of a major single band corresponding to that of hexokinase near 100 kDa is indicated by an arrowhead to the left of lane 1. The position of a major single band corresponding to that of the N- and C-terminal halves near 50 kDa is indicated by an arrowhead to the right of lane 8. (The two additional bands near 46 kDa in lane 8 are immunoreactive with hexokinase antibody and are believed to be proteolytic products.) On the left are shown the positions for molecular size markers (in kDa): myosin (200), phosphorylase b (92.5), bovine serum albumin (69), ovalbumin (46), carbonic anhydrase (30), and trypsin inhibitor (21.5).

The overexpressed C-terminal half of tumor hexokinase exhibits significant catalytic activity, whereas the N-terminal half has no detectable activity. Although the specific activity of the C-terminal half is reduced relative to the wild type (i.e., 112 versus 220 nmol of Glc-6-P formed per min/mg of protein), it would seem on the basis of the above mutational analysis that this reduction may result because of differences in stability. In any case, it is important to note that the C-terminal half exhibits essentially the same kinetic patterns with and without product inhibitor (Fig. 6, A and B) as the intact enzyme (Fig. 5, A and B). Kinetic constants determined from these plots are summarized in Table II, where it is clear that the C-terminal half of tumor hexokinase interacts with glucose and ATP with very similar, and in most cases nearly identical, apparent affinities as the intact enzyme.

Taken together, these results not only indicate that the C-terminal half of the tumor enzyme contains the catalytic site but further demonstrate that the capacity of the intact enzyme to undergo product inhibition is due to its interaction with the C-terminal and not the N-terminal half of the enzyme.

The Isolated Overexpressed N-terminal Half of Tumor Hexokinase Interacts with ATP and Glc-6-P—Results presented above, both on mutant forms of the intact enzyme and on its overexpressed halves, indicate that the N-terminal half of hexokinase is neither catalytic nor involved in product inhibition. This raised the question about the role of the N-terminal half of hexokinase and prompted us to examine whether it can bind ligands like ATP and Glc-6-P. These experiments were done by mixing the N-terminal half with an ATP affinity agarose matrix as described under “Methods” and then using heat, ATP, or Glc-6-P to establish whether binding had occurred by monitoring any released protein with a hexokinase antibody. Fig. 7 demonstrates that the N-terminal half of hexokinase does bind to the ATP-agarose matrix as immunoreactive species are released by heating (lanes 1 and 2) and by adding ATP (lane 3) or Glc-6-P (lane 4). Although some proteolysis has occurred under the conditions used, these experiments indicate that the isolated N-terminal half of tumor hexokinase does exhibit the capacity to interact with both ATP and Glc-6-P. Moreover, this binding activity
terminal half of the molecule is less well conserved in the N-terminal half (10, 12, 14). This suggested different functions for the two halves of the molecule. Although evidence was obtained from biochemical studies of the brain enzyme that the C-terminal half is catalytic, and the N-terminal half binds Glc-6-P (18, 19), the finding that Glc-6-P also inhibits the catalytic activity of the C-terminal half raised doubts about the role of the N-terminal half in product inhibition.

Data presented in this report evaluate the role of the N-terminal half of hexokinase by using two different molecular biological approaches. Site-directed mutagenesis was the first approach used to assess the role of the N-terminal half of hexokinase. These experiments revealed that site-directed changes of the amino acid residues believed to interact with glucose within the N-terminal half affect neither the catalytic activity (Fig. 3A) nor the capacity of the enzyme to be inhibited by 1,5-AnGlc-6-P as the complete 100-kDa parent enzyme (Figs. 5 and 6). Moreover, the N-terminal half is devoid of any catalytic activity. These results provide direct evidence that the C-terminal half of the enzyme is specialized not only for catalysis but for product inhibition as well. Taken together, data obtained from site-directed mutagenesis and from overexpressed C- and N-terminal halves of hexokinase, indicate that the role of the N-terminal half of the enzyme is not to provide a locus for an "allosteric" product inhibitory site.

To further investigate whether the N-terminal region binds ATP and Glc-6-P, the isolated overexpressed fragment was subjected to further analysis and shown to interact with both ligands (Fig. 7). These findings lead us to believe that the N-terminal half, which is known to contain a 12-amino acid region at its N terminus, believed to be involved in membrane binding (14), may be essential for other reasons. One possibility is that the N-terminal half is required so that the relative levels of soluble and membrane-bound forms can be regulated in the cell by Glc-6-P and/or ATP. Significantly, it is well established that both Glc-6-P and ATP can release hexokinase from cellular membranes (2, 30). Moreover, the soluble enzyme is more sensitive to product inhibition by Glc-6-P (31). It is also possible that the N-terminal half serves as a spacer between the membrane and the catalytic C-terminal half of the enzyme, thus allowing the C-terminal half to

FIG. 5. Inhibition of the intact tumor hexokinase by 1,5-anhydroglucitol-6-P with ATP (A) or glucose (B) as the variable substrate. The procedure for preparing the particulate fraction of the intact enzyme and for assaying hexokinase activity are described under "Methods." The specific activity of the particulate fraction was 220 nmol of Glc-6-P formed per min/mg of protein. The concentration of fixed substrates, glucose (in Fig. 5A) and ATP (in Fig. 5B), were 2.2 and 6.4 mM, respectively. 1,5-Anhydroglucitol-6-P was 0 (C), 0.0174 (C), 0.0348 (A), or 0.987 (C) mM when ATP was variable substrate and 0 (C), 0.174 (C), 0.348 (A), or 0.696 (C) mM when glucose was variable substrate. Inset, a secondary plot of the slopes (for ATP) and of the intercepts (for glucose) derived from the double-reciprocal plot versus inhibitor concentrations.

appears to be specific as pretreatment of the N-terminal fragment with ATP abolishes the binding.

DISCUSSION

One of the most important properties of 100-kDa mammalian hexokinases is their capacity to be product inhibited by Glc-6-P, in contrast to the 50-kDa yeast and liver enzymes (29). The extensive amino acid sequence similarity between the two halves of the 100-kDa hexokinases (designated as N- and C-) have suggested that these proteins evolved from an ancestral 50-kDa yeast-like hexokinase by a process of gene duplication and gene fusion (16, 17). Significantly, both halves have predicted glucose and ATP binding domains. In particular, there is a very high conservation of amino acids ascribed to glucose binding in the two halves of the molecule (10, 12, 14). However, the putative ATP binding domain in the C-
particulate fraction of the C-terminal half, and for assaying hexoki-

nase. The concentration of fixed substrates, glucose (in Fig.

the particulate fraction was 112 nmol of Glc-6-P formed per min/mg

of protein. The values shown are averages of two independent experiments. The percent deviation from the mean did not exceed 10%, app.

Anhydroglucitol-6-P was 0

mutations in the

ence of improperly fold-

ed molecules within the population

Anhydroglucitol-6-P was 0

nucleotide half of hexokinase were studied on the complete

etrate with the next glycolytic enzyme,

interact more readily with the next glycolytic enzyme, i.e.

phosphoglucose isomerase.

In this study, the complete hexokinase molecule and its C-

and N-terminal halves were derived by overexpressing the respective proteins in *E. coli* and sedimenting the particulate fraction. It could be argued that in such preparations the presence of improperly folded molecules within the population may affect the interpretations described above. We believe this unlikely for two reasons. First, mutations in the N-terminal half of hexokinase were studied on the complete enzyme and an activity assay was used to monitor the results. Therefore, the effect of mutations only on active enzymes within the population were being monitored. Second, the isolated N-terminal fragment did bind ATP and Glc-6-P, a finding that would not be expected if this fragment were unfolded. However, in the latter experiment we cannot rule out the possibility that the N-terminal fragment was partially unfolded.

Consistent with some of the observations made in this study, White and Wilson (18) have shown using a biochemical approach that the C-terminal half of brain hexokinase has catalytic activity, whereas the N-terminal half is inactive. The mutational approach described here supports this conclusion as site-directed changes of conserved amino acid residues within the N-terminal half of the tumor enzyme, predicted to

**TABLE II**

Kinetic constants of the intact and the C-terminal half of
tumor hexokinase

Procedures for preparation of the intact and the C-terminal half

of hexokinase particulate fractions and hexokinase activity determi-

nations are described under "Methods." The specific activity of the

intact and C-terminal half preparations were respectively, 220 and

112 nmol of Glc-6-P formed per min/mg of protein. The values shown

are averages of two independent experiments. The percent deviation

from the mean did not exceed 10%, app.

<table>
<thead>
<tr>
<th>Kinetic constant</th>
<th>Intact hexokinase</th>
<th>C-terminal half of hexokinase</th>
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<tbody>
<tr>
<td>$K_{\text{app}}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>For Glucose (μM)</td>
<td>58</td>
<td>66</td>
</tr>
<tr>
<td>For ATP (mM)</td>
<td>1.02</td>
<td>0.95</td>
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<tr>
<td>$K_{\text{app}}^b$ (μM):</td>
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<tr>
<td>1,5-AnG-6-P vs. ATP</td>
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<td>118</td>
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<tr>
<td>1,5-AnG-6-P vs. Glc</td>
<td>180</td>
<td>165</td>
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$^a$ The apparent $K_a$ values were calculated from the double-reciprocal (1/v vs 1/s) plots shown in Figs. 5A and 6A (for ATP) and Figs. 5B and 6B (for glucose), respectively.

$^b$ The apparent $K_a$ values were calculated from the secondary plots of the slope (for ATP) and of the intercept (for glucose) versus inhibitor concentrations shown as insets in Figs. 5A and 6A, and 5B and 6B, respectively.

*Fig. 6. Inhibition of the C-terminal half of tumor hexokinase by 1,5-anhydroglucitol-6-P with ATP (A) or glucose (B) as the variable substrate. The procedure for preparing the particulate fraction of the C-terminal half, and for assaying hexokinase activity, are described under "Methods." The specific activity of the particulate fraction was 112 nmol of Glc-6-P formed per min/mg of protein. The concentration of fixed substrates, glucose (in Fig. 5A) and ATP (in Fig. 5B), were 2.2 and 6.4 mM, respectively. 1,5-Anhydroglucitol-6-P was 0 (○), 0.0174 (△), 0.0348 (△), or 0.087 (□) mM when ATP was variable substrate and 0 (○), 0.174 (△), 0.348 (△), or 0.686 (□) mM when glucose was variable substrate. Inset, a secondary plot of the slopes (for ATP) and of the intercepts (for glucose) derived from the double-reciprocal plot versus inhibitor concentrations.*

*Fig. 7. Binding of the N-terminal half of hexokinase to ATP affinity agarose. The experimental details for incubating the N-terminal half of hexokinase with ATP-agarose is described under "Methods." The proteins run on a 10% SDS-PAGE gel were immuno-blotted and probed also as detailed under "Methods." Lanes 1 and 2, proteins eluted from the matrix by heating at 95°C; lane 3, proteins eluted with ATP; lane 4, proteins eluted with Glc-6-P. The position of a major single band corresponding to that of the N-terminal half near 50 kDa is indicated by arrows. Protein recovery from the ATP affinity matrix was greater than 90%. On the left are shown the positions for molecular size markers (in kDa): myosin (200), phosphorylase b (92.5), bovine serum albumin (69), ovalbumin (46), car-bonic anhydrase (30), and trypsin inhibitor (21.5).*
interact with glucose, have very little effect on catalytic activity of the enzyme, and the N-terminal half alone is inactive. Significantly also, while this work was being processed for publication, Baijal and Wilson (32), using COS-1 cells to express the Type I brain enzyme, confirmed our earlier site-directed mutagenesis work on the C-terminal half of hexokinase (22). They showed also that conversion of the conserved serine (Ser155) in the N-terminal region of the brain enzyme has little or no effect on catalysis or product inhibition. These workers did not examine the effect of mutations on other amino acid residues within the N-terminal half of the enzyme. Also consistent with the work reported here are the recent studies of Magnani et al. (33), demonstrating that the C-terminal half of the Type I human placental hexokinase is product-inhibited by Glc-6-P.

Finally, another two points seem relevant to the work presented here. First, there has been controversy in the literature for many years as to whether mammalian hexokinases exhibit an allosteric site for product inhibition by Glc-6-P or whether inhibition by this product can be accounted for by binding to the catalytic site (29, 34-37). Certainly, the results presented here emphasize that the C-terminal half of the molecule separated from the N-terminal half contains both a catalytic site and a site for product inhibition and that mutations in the N-terminal half of the complete enzyme have little effect both on catalysis and product inhibition. Moreover, kinetic analyses reveal that the Glc-6-P analog 1,5-AnG-6-P is linearly competitive with the substrate ATP both in the intact enzyme (Fig. 5A) and in the C-terminal half (Fig. 6A). These data, together with recent NMR data on the brain enzyme indicating that glucose and Glc-6-P bind very nearly to the same region of the enzyme (38, 39), render unlikely the earlier suggestions (18, 19, 34) that mammalian 100-kDa hexokinases exhibit an allosteric site involved in product inhibition which is completely separate and distinct from the catalytic site. The second point related to the first concerns the evolutionary events that led to the mammalian 100-kDa hexokinases. Clearly, the results presented in this study support the view that these gene duplication products did not arise from 50-kDa unregulated yeast-like precursors which duplicated to provide a separate product inhibitory site. Rather, they seem to have arisen from 50-kDa hexokinase species like those found in marine organisms (40, 41) which had acquired already the capacity for product regulation, the purpose of gene duplication in this case then being to serve another role, perhaps in regulating membrane binding as indicated above.

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REFERENCES