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Single Residue Determinants in the Binding of Recombinant Human Brain Hexokinase to the Mitochondrion

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Single residue determinants in the binding of recombinant human brain hexokinase to
the mitochondrion

by

Nidhi Shah

A dissertation submitted to graduate faculty
in partial fulfillment of the requirements for the degree of

MASTERS OF SCIENCE

Major: Biochemistry

Program of Study Committee:
Richard B. Honzatko, Major Professor
Scott Nelson
Donald Sakaguchi

Iowa State University
Ames, Iowa

2011

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The N-terminal segment (residues 1-15) of hexokinase I (HKI) is essential for the binding of HKI to the outer membrane of the mitochondrion. Whether the N-terminal segment is merely a hydrophobic anchor to the membrane or has specific residues that are key determinants in the mitochondrion-HKI interaction is unclear. Recombinant wild-type HKI binds to mitochondria, but the removal of residues 1-15 abolishes such binding. Mutations A4L, A8L and Q5P individually cause a 10-fold decreases (relative to wild-type enzyme) in HKI binding to mitochondria. In contrast, mutations Q5A, Y10L and T12I decrease binding by approximately twofold. The mutations did not affect the catalytic properties of the enzyme, and all HKI constructs remained monomeric to concentrations as high as 10 micromolar. Results here are consistent with a helical conformation for the N-terminus of HKI, with residues A4 and A8 defining a contiguous surface that does not tolerate large hydrophobic side chains.
CHAPTER 1: GENERAL INTRODUCTION

Hexokinase and its isozymes

Hexokinases catalyze the phosphorylation of glucose by transferring the $\gamma$-phosphoryl group from adenosine triphosphate (ATP) to the sixth-position hydroxyl group of glucose to give glucose-6-phosphate (Glc-6-P) (1). There are four isozymes of mammalian hexokinase (HK) namely types I, II, III and IV (1,2), which are located in different organs of the body. Liver contains all four types of hexokinases while kidney and intestine contain three (I-III) (2). Types I and II are found in epididymal fat pad, skeletal muscle, brain and heart (2). However, Type I is predominantly present in brain and kidney and Type II is predominant in skeletal muscle and epididymal fat pad (2). According to Katzen and Schimke, the properties of different types of hexokinase do not vary from tissue to tissue but are present in differing amounts depending on age, stability, and nutritional factors (2).

The formation of Glc-6-P by hexokinase commits glucose to alternative metabolic pathways: the formation of glycogen and short-term carbohydrate storage in liver; immediate use in energy production by glycolysis, and the formation of pentose phosphates for anabolic purposes (3) (Figure 1.1). Different metabolic pathways can be linked to the different organs in the body. These differences may be co-related to the differences between the structure; affinity for substrates and inhibitors and subcellular location of the isozymes. For example, HKI and HKII have a tail on the N-terminus that is important in binding of hexokinase to mitochondria. Whereas, HKIII and HKIV does not contain the N-terminus and hence is not bound to mitochondria. Thus, these isozymes may be associated with metabolic pathways other than glycolysis.
Types I-III have the molecular mass of 100 kDa while type IV, also known as glucokinase has a molecular mass of 50 kDa (3-5). HK types I-III are thought to have evolved by the duplication and fusion of an ancestral gene that encodes a 50 kDa hexokinase (3 and references therein, 5), the a result of which are N- and C- terminal halves of similar sequence (3-5). Moreover, these terminals have sequence similarity to that of yeast hexokinase and glucokinase, which are 50 kDa proteins (3 and references therein, 5). In spite of the sequence similarity the N- and C-terminal halves have different function for the isozyme Types I-III. For isozyme II, both halves have catalytic function, whereas for isozymes I and III, the C-terminal half has catalytic function and the N-terminal has a regulatory function (3, references within, 5). All three isozymes are inhibited by the product glucose-6-phosphate (Glc-6-P). It has been hypothesized that inhibition by Glc-6-P is the regulatory function of the N-terminal half of HKI and HKIII (3, 5).

Other differences between the three isozymes are substrate affinity, the role of the
inorganic phosphate (Pᵢ) in regulation and sub-cellular location. Of the four isozymes, HKIII has the highest affinity for glucose and lowest affinity for ATP. Glc-6-P inhibits isozyme I-III, but Pᵢ antagonizes inhibition by Glc-6-P in the Type-I isozyme only and being an inhibitor of isozymes II and III. Isozymes I and II have a conserved hydrophobic leader sequence at the N-terminus. Isozyme III lacks the hydrophobic leader sequence.

Immunolocalization studies demonstrate we association of isozymes Types-I and -II with mitochondria, whereas the Type-III isozyme is co-localized with the envelope of the nucleus (3 and references therein, 5). Hence, the hydrophobic leader sequence for the Types I and II isozymes confers mitochondrial binding properties. Table 1.1 summarizes the similarities and differences between the isozymes.

Table 1.1: Summary of the similarities and differences between mammalian isozymes.

<table>
<thead>
<tr>
<th>Properties</th>
<th>HKI</th>
<th>HKII</th>
<th>HKIII</th>
<th>HKIV</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW(kDa)</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>Tissues</td>
<td>Brain, kidney, RBCs</td>
<td>Skeletal muscle, fat</td>
<td>Liver, intestine, kidney</td>
<td>Liver, pancreas</td>
</tr>
<tr>
<td>$K_m^{\text{Glc}}$ (mM)</td>
<td>0.03</td>
<td>0.3</td>
<td>0.003</td>
<td>6</td>
</tr>
<tr>
<td>$K_m^{\text{ATP}}$ (mM)</td>
<td>0.5</td>
<td>0.7</td>
<td>1.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Function, N-terminal half</td>
<td>Regulatory</td>
<td>Catalytic</td>
<td>Regulatory</td>
<td>-</td>
</tr>
<tr>
<td>Function, C-terminal half</td>
<td>Catalytic</td>
<td>Catalytic</td>
<td>Catalytic</td>
<td>-</td>
</tr>
<tr>
<td>Inhibition by Glc-6-P</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Pi relief</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>-</td>
</tr>
<tr>
<td>Subcellular localization</td>
<td>Mitochondria</td>
<td>Mitochondria</td>
<td>Nuclear periphery</td>
<td>-</td>
</tr>
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</table>
Structure of Hexokinase and G6P Inhibition

Hexokinase I (HKI) is also called ‘brain hexokinase’ (6). According to Rose and Warms, brain hexokinase did not dissociate into subunits under their experimental conditions (7). Results obtained by Chou et al. (8) and Easterby (9) established HKI as a single polypeptide chain having the molecular weight of 100,000 kDa instead of 50,000 kDa subunits present in dimeric yeast hexokinase (8,9). In a significant extrapolation of these findings, Easterby (9) suggested that monomeric HKI evolved from a primordial yeast-like hexokinase through gene duplication and fusion (10). One half of the fused protein retained catalytic function, whereas the other half evolved a regulatory function. White and Wilson, however, suggest an alternative evolutionary process in which sensitivity to Glc-6-P had risen prior to gene duplication and fusion (12).

At high enzyme concentrations HKI crystallizes as a dimer (6,11,13-17). The N- and C-terminal halves of HKI have identical folds of the polypeptide chain and are connected by an α-helix (11, 14) (Figure1.1). Moreover, the N- and C-terminal half each has a binding site for glucose and Glc-6-P (13). In the presence of glucose, Glc-6-P occupies an allosteric site. In the C-terminal half the allosteric site partially overlaps the putative α-phosphoryl site of ATP (14) whereas the Glc-6-P pocket of the N-terminal half overlaps the high-affinity binding site for Pi (15).

The structures of crystallographic dimers are in conflict with result of solution studies. Firstly, HKI is a monomer at low concentrations (<0.5 mg/mL), both in the presence and absence of ligands (16,17). On the basis of equilibrium binding experiments, HKI has one high affinity site for G6P (18-20). In an attempt to nullify the effects subunit dimerization Liu et al (21) engineered HKI to be monomeric even at high enzyme
concentrations. Aleshin et al (11,14) crystallized the engineered monomer as a glucose/Glc-6-P complex and as a glucose/ADP complex and found two binding sites for Glc-6-P and two binding sites for ADP (Fig. 1.3). In parallel with this work Liu et al (21) demonstrated two mechanisms of Glc-6-P inhibition, one due to the binding of Glc-6-P to the N-terminal half, and the other due to its binding to the C-terminal half. To this day, however, the mechanism of Glc-6-P inhibition remains unclear. The existence of two mechanisms may be a consequence of two radically different environments for HKI in tissues. In the red blood cell, HKI is in the cytosol (not membrane associated), whereas in nerve tissue HKI is associated with the mitochondrion. Glc-6-P inhibition in the red blood cell may come through its interaction with the N-terminal domain, whereas Glc-6-P inhibition of HKI in nerve tissue may come through its interaction with the C-terminal domain.
Fig. 1.1. Hexokinase x-ray structure (PDB 1HKB). A. HKI dimer. B. HKI monomer. C-terminal half (red), N-terminal half (blue), and α-helical polylinker (yellow).
Fig. 1.2. Ligand binding to HKI (PDB 1HKC). A. HKI complexed with Glc (blue) and Glc-6-P (red) in a closed C-terminal conformation. B. HKI complexed with Pi (red spheres) and Glc (blue) in an open C-terminal conformation.
Mitochondrial binding of hexokinase

Hexokinase I binds to the outer mitochondrial membrane (7, 22, 23) through an 11-residue hydrophobic tail on its N-terminus (24). Xie and Wilson (24) proposed that the hydrophobic tail is helical and Mulichak et al. (13) proved it through their crystal structures. This helical tail binds to a porin in the outer mitochondrial membrane (25-27). Mitochondrial porin (also known as the voltage dependent anion channel or VDAC) has a β-barrel structure (28-31). VDAC is a 30 kDa protein that allows the voltage-dependent passage of selective anions through the outer membrane of the mitochondrion (34). There are three kinds of VDACs in humans, namely VDAC1, VDAC2 and VDAC3 (34). Human VDAC1 is most abundant and
is made of 19 β-strands with an α-helix located horizontally midway within the pore (Fig. 1.4). The α-helix is the presumed gate that controls the passage of anions (34).

Fig 1.4. Structure of mouse VDAC1 (PDB 2JK4). A. Side view B. Top view.

Hexokinase binds to mitochondria for perhaps two reasons: Hexokinase uses ATP as a substrate. The colocalization of hexokinase and VDAC gives hexokinase preferential access to intra-mitochondrial ATP (7). Experiments conducted in several laboratories support this hypothesis (5, 35-37). Growing tumor cells need large amount of energy produced through glycolysis. The amount of hexokinase was found to be greater in tumor cells as compared to normal cells (7). Hence, the discovery that hexokinase uses intramitochondrial ATP to initiate the glycolysis process was of great importance to understand the uncontrolled growth of cancer cells.

Nonetheless, when HKI binds to VDAC in patch-clamp experiments, anion conductance is terminated (38,39). By binding to VDAC, HKI blocks the pore. So how then does ATP reach the active site of HKI is unknown. Hence, we cannot say with absolute
certainty that hexokinase uses intramitochondrial ATP to initiate glycolysis.

Hexokinase also plays an important role in preventing apoptosis. Hexokinase is considered to be the “guardian of the mitochondria” (40) because it binds to VDAC on the outer mitochondrial membrane and prevents the release of apoptogenic factors (41). Mitochondria house an important co-factor for apoptosis, cytochrome c, which activates caspases for apoptosis (40). In order to release cytochrome c, the permeability transition pore (PTP) needs to be opened (41). PTP is formed by VDAC on the outer mitochondrial membrane, adenylate translocator (ANT) on the inner mitochondrial membrane and cyclophilin D and other proteins in the matrix (42). The opening of PTP results in loss of membrane potential, swelling of mitochondria, acidification of the cytosol and release of cytochrome c (42). Released cytochrome c thus activates Apaf 1 which initiates caspase activation and ultimately results in apoptosis (40).

Pro-apoptotic factors like Bax and Bak bind to VDAC and thus induce the release of cytochrome c from mitochondria (43-44). Binding of hexokinase to VDAC protects the mitochondrion against proteolytic degradation (45). It might also prevent the binding of Bax and Bak to VDAC. This in turn might prevent the opening of the PTP and release of cytochrome c. Hence, if hexokinase binding to VDAC is lessened or eliminated, mitochondrion-based apoptosis can be induced in cells.

**Hypothesis**

Tumor cells metabolize glucose at high rates due to presence of large amounts of hexokinase (45). Hexokinase acts as the guardian of the mitochondria (40) by binding to VDAC through its N-terminal residues (24). Chymostrypsin removes the hydrophobic tail on the N-terminus, and such proteolyzed hexokinase does not bind to mitochondria (7,32-33). Hence,
the first dozen residues are important in the binding of hexokinase to mitochondria. In our research, we hypothesized that, residues Alanine (4), Alanine (8) and Threonine (12), which fall on the same side of the N-terminal helix, interact with VDAC when hexokinase is bound to mitochondria. Hence, we conducted mitochondrial binding experiments using wild-type HKI and mutants A4L, A8L and T12I to an effect on the binding of HKI to mitochondria. Our findings indicate that positions 4 and 8 cannot have large hydrophobic side chains, most likely because this surface of the N-terminal helix binds to VDAC.

**Thesis Organization**

This thesis is organized into three chapters. Chapter 1 covers the general information and background about the enzymes hexokinase and voltage dependent anion channel (VDAC); their interaction with each other in the outer mitochondrial membrane and their structures. Problems and objectives of the thesis research are provided as well. Chapters 2 is a paper prepared for submission for publication in peer reviewed journals. Chapter 2 shows that the N-terminal hydrophobic tail has specific and essential amino-acid determinants that stabilize the binding of HKI to the mitochondrion. Conclusions and recommendations for future studies are in Chapter 3.

**References:**


19. Solheim LP, Fromm HJ. Kinetic evidence that the high-affinity glucose-6-phosphate site on hexokinase I is the active site. Biochem and Biophys Arch. 211, pp. 92-99. 1981.


CHAPTER 2: SINGLE-RESIDUE DETERMINANTS IN THE BINDING OF RECOMBINANT HUMAN BRAIN HEXOKINASE TO THE MITOCHONDRION

A paper to be submitted to the Journal of Biological Chemistry

Nimer Mehyar, Nidhi Shah, Lu Shen, Dong Yan, and Richard B. Honzatko

Abstract

The N-terminal segment (residues 1-15) of hexokinase I (HKI) is essential for the binding of HKI to the outer membrane of the mitochondrion. Whether the N-terminal segment is merely a hydrophobic anchor to the membrane or has specific residues that are key determinants in the mitochondrion-HKI interaction is unclear. Recombinant wild-type HKI binds to mitochondria, but the removal of residues 1-15 abolishes such binding. Mutations A4L, A8L and Q5P individually cause a 10-fold decreases (relative to wild-type enzyme) in HKI binding to mitochondria. In contrast, mutations Q5A, Y10L and T12I decrease binding by approximately twofold. The mutations did not affect the catalytic properties of the enzyme, and all HKI constructs remained monomeric to concentrations as high as 10 micromolar. Results here are consistent with a helical conformation for the N-terminus of HKI, with residues A4 and A8 defining a contiguous surface that does not tolerate large hydrophobic side chains.

1 This research was supported in part by National Institute of Health Research Grant NS 10546
2 To whom correspondence should be addressed.
Introduction

Hexokinases catalyze glucose phosphorylation by adenosine tri-phosphate (ATP) to produce glucose 6-phosphate (Glc-6-P). Mammals have four isozymes of hexokinase: I, II, III and IV (1). Hexokinase I (HKI) or brain hexokinase regulates glucose metabolism in brain tissue and the red blood cell (2). Hexokinases (I-III) have molecular masses of approximately 100 kDa (1), consisting of C- and N-terminal halves with significant levels of sequence identity to each other and to yeast hexokinase (3). Hexokinase IV (glucokinase) is a 50 kDa protein with a sequence similar to both C-terminal and N-terminal halves of hexokinase I-III and to yeast hexokinase (4). Similarities between mammalian isoenzymes evidently originate from the duplication and fusion of a primordial hexokinase gene similar to that of yeast hexokinase (5). A number of ligands inhibit HKI; however, Glc-6-P is probably the primary physiological inhibitor of the type-I enzyme (6). Glc-6-P inhibits HKI catalysis by binding with high affinity to either the C- or N-terminal halves (7,8). Under normal physiological conditions, inorganic orthophosphate (P$_i$) decreases Glc-6-P inhibition (9). P$_i$ binds tightly to the N-terminal half of HKI and relieves Glc-6-P inhibition by an allosteric mechanism that couples both halves of the enzyme (10) (11). At elevated levels, P$_i$ inhibits HKI by binding to the active site (12).

Despite structural similarities, hexokinases (I-III) are functionally different. Both halves of HKII support catalysis and are each sensitive to inhibition by Glc-6-P (13), whereas only the C-terminal half of HKI and HKIII supports activity (14,15). Nonetheless, P$_i$ can relieve Glc-6-P inhibition of HKI alone (4,7-8,12).
HKI co-localizes on the outer mitochondrial membrane (OMM) in brain tissue preferentially close to newly formed ATP inside the mitochondrion (16,17). In tumor cells, mitochondrion-associated HKII antagonizes Bax-induced cytochrome c release and in turn inhibits apoptosis (18-20). Bax interacts with permeability transition pore (PTP) resulting in loss of membrane potential, triggering of mitochondrial membrane permeabilization (MPP) and release of cytochrome c (21). Pro-apoptotic proteins such as Bax, Bak, and Bim interact with voltage-dependent anion channel (VDAC) forming an assembly of large channels likely used for cytochrome c passage from mitochondrion matrix to cytoplasm (22-24). VDAC is part of PTP, which in addition to VDAC, includes the adenylate translocator (ANT) of the inner mitochondrion membrane (IMM) (25-29), and cyclophilin D of the inner mitochondrion matrix (30-32). Released cytochrome c activates the caspase family of proteases in cytoplasm, which in turn triggers apoptosis (32-34). VDAC-1 overexpression causes apoptotic cell death (35,36). Hexokinase I and II, in association with VDAC, likely antagonize the formation of apoptotic channels. Some have gone so far as to label hexokinase as “guardian of the mitochondria” (37).

Over expression of N-terminally truncated VDAC-1 did not induce cells to release cytochrome c, and such cells were resistant to apoptosis (38). N-terminus peptide and other VDAC-1 based peptides bind to immobilized HKI, these peptides prevent HKI-protection of cells (39). Chemical modification of Glu 72 prevents HKI from binding to mitochondria (35). Mutating Glu 72 and other residues abolishes HKI-protection against apoptosis in cancer cells (40). Adding HKI to VDAC-1 reconstituted in lipid membrane reduced the channel conductance (25); however, the addition of HKI to mutant mouse VDAC-1 E72Q
reconstituted in lipid membrane did not block conductance in patch clamp experiments, indicating failure of HKI to bind to mutant VDAC-1 (40).

A number of small and physiological important ligands are capable of dissociating HKI from the mitochondrial outer membrane. These compounds include Glc-6-P and ATP (16). Skaff et al. (41) demonstrated wild-type recombinant HKI with properties comparable to native brain hexokinase including those of mitochondrial-binding and release. The recombinant construct has an intact membrane targeting element and a formyl group attached to the amino terminus of the polypeptide chain. The release properties of mutant constructs demonstrated the significance of Glc-6-P binding to the N-terminal half of HKI in promoting the release of the mitochondrion-associated enzyme. Nucleotide-induced release, on the other hand, follows a competitive binding model between HKI and nucleotides at a binding site on VDAC1 (unpublished data).

Early studies showed that digesting hexokinase I with proteases resulted in loss of mitochondrial binding properties (16). Rapid purification of rat brain hexokinase using HPLC decreased the portion of HKI devoid of mitochondria-binding properties (42,43). Rat-brain hexokinase with mitochondrion-binding properties intact is more hydrophobic than the non-bindable form (44). The two enzyme forms (with and without mitochondrial binding properties) have similar molecular weights and kinetic properties (45). N-terminal sequence of rat brain hexokinase was determined as a critical determinant of mitochondrial binding of HKI (46). The N-terminus of HKI from native tissue is putatively blocked by an acetyl group (47). Xie and Wilson (1988) proposed a helical conformation of the first 11 residues of HKI N-terminus. This conformation allows the insertion of the N-terminus in a lipid bilayer (46,48). The effect of monoclonal antibodies against rat brain hexokinase varied
from blocking mitochondrial binding to inhibiting Glc-6-P release (49). Mitochondrial-bound rat brain hexokinase was released upon exposure to monoclonal antibodies raised against the N-terminus (50).

Although some studies stress that hexokinase binding to mitochondria is determined by the hydrophobicity of the tail (51,52), HKI cellular co-localization with VDAC on OMM (17) implies more complex and specific interactions than general hydrophobic interactions with lipid. To date, no single residue has been proven essential to hexokinase association with the mitochondrion. Yet inspection of the putative N-terminal helix of HKI reveals amino acids with small side chains localize to only one face of the helix, whereas large side chains occupy all other faces. Does the helix surface defined by small side chains make critical contacts with a protein binding partner (presumably VDAC) in the outer membrane of the mitochondria? Indeed, the mutation of Ala 4 and Ala 8 to leucine abolished hexokinase association with the mitochondrion, whereas mutations at other residues have little or no effect. The discovery of a localized surface critical to hexokinase association with the mitochondrion provides a useful constraint in modeling possible HKI-VDAC complexes.

**Experimental Procedures**

*Materials—* ATP, Glc-6-P, ampicillin, deoxyribonuclease (DNase I), bovine serum albumin (BSA), leupeptin, protease cocktail inhibitor and phenylmethylsulfonyl fluoride (PMSF) came from Sigma. DEAE HPLC resin was a product of Tosohaas. *E.coli* strain BL21 (DE3) competent cells were from Novagen. Glucose-6-phosphate dehydrogenase was obtained from Roche Molecular Biochemicals. Isopropyl-1-thio-β-D-galactopyranoside (IPTG) came
from Anatrace. Sequencing PVDF membranes (ProSorb sample preparation cartridge) were purchased from Applied Biosystems.

*Construction of Wild-type Hexokinase and Mutant Plasmids*—Human brain hexokinase (HKI) had been cloned into pET-11a as reported in a previous study (41). Hexokinase mutants were created through PCR modification with oligonucleotide primers synthesized at the Iowa State University DNA Sequencing and Synthesis Facility. Mutants Ala4Leu, Ala8Leu, Thr12Ile, Gln5Ala and Gln5Pro were created with the following forward primers (and their reverse compliments) respectively:

\[
\begin{align*}
5'\text{CATATGATCGCCCTGCAGCTCCTGGCC}\text{-3'}; \\
5'\text{CGCAGCTCCTGCTCTATTACTTCACGGAGC}\text{-3'}; \\
5'\text{GCCTATTACTTCATTGAGCTGAAGGATG}\text{-3'}; \\
5'\text{GATCGCCGCGGCGCTCCTGGCC}\text{-3'}; \\
5'\text{GATCGCCGCGGCGCTCCTGGCC}\text{-3'}. \\
\end{align*}
\]

Iowa State University DNA Sequencing and Synthesis Facility confirmed all final constructs by sequencing the DNA of the entire gene. Mutant Y10L was used in previous studies (unpublished data).

*Expression and Purification of Wild-Type and Mutant Hexokinases*— Wild-type or mutant pET-211a-HKI plasmids were transformed into *E.coli* strain BL21 (DE3). Expression and purification of HKI enzymes were previously discussed (41). Protein concentrations employed the Bradford method with bovine serum albumin as a standard (53).

*Pig Liver Mitochondria Purification*— Pig livers were obtained from the Iowa State University Meats Laboratory shortly after slaughter. Mitochondrial purification is described
with modifications as discussed elsewhere (41). Both outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) integrities were measured as described previously (55, 56). Mitochondria were stored at –80º C in the presence of 5% dimethyl sulfoxide.

The HKI Activity Assay—Hexokinase activity was measured using the glucose 6-phosphate dehydrogenase (G6PDH)-coupled spectrophotometric assay. Assay solutions have 3mM MgCl₂, 3 mM DTT, 0.3 mM NADP and 10 µg/mL G6PDH in 50 mM Tris-HCl, pH 8.0, and concentrations of glucose and ATP:Mg²⁺ that varied from 1/5 x Kₘ to 5 x Kₘ. NADPH production was monitored at 340 nm wavelength. Reaction was initiated by the addition of enzyme and linear progress curves monitored for 3 minutes. Slopes were used to calculate initial rates in umole/min. The capacity of the coupling enzymes was verified by observing a twofold increase in velocity with a doubling of added hexokinase. Initial rate data were analyzed using GraFit (57).

Mitochondrial Binding and Release—Purified HKI was dialyzed twice against mitochondrial-binding buffer (250 mM sucrose, 5 mM Glc, 50 mM NaCl, 5 mM MgCl₂ and 50 mM HEPES, pH 7.4), and then diluted to 2 mg/mL using the same buffer. Thirty mg wet weight of mitochondrion, thawed on ice, was suspended in 1 mL of mitochondrial-binding buffer, and then collected by centrifugation at 11,000 × g for 5 minutes. Suspension and centrifugation steps were repeated twice. The pellet was suspended in 1 mL of mitochondrial-binding buffer with added HKI (2 mg/mL), protease cocktail inhibitor (0.25 mg/mL) and PMSF (1 mM). After 60 minutes on ice, the mixture of HKI and mitochondria was centrifuged at 11,000 × g for 5 minutes. Unbound HKI in the supernatant fluid was
decanted. Pelleted mitochondria were suspended in mitochondrial-binding buffer, less the MgCl₂, NaCl, and glucose, and centrifuged again. This wash step was twice-repeated.

HKI-bound mitochondria, prepared as above, is suspended in release buffer (250 mM sucrose and 50 mM HEPES, pH 7.4). 50 μL aliquots of this suspension were distributed to plastic micro-centrifuge tubes, to which were added a release agent (ATP, TNP-nucleotide, or Glc-6-P) at various concentrations. After 30 minutes at room temperature, the mitochondria were pelleted by centrifugation. HKI solubilized by nucleotide was removed by decanting the supernatant liquid. The mitochondrial pellet was suspended in wash buffer (250 mM sucrose and 5 mM HEPES, pH 7.4), and centrifuged. After discarding the supernatant fluid, pelleted mitochondria were suspended in 100 μL wash buffer and then assayed for hexokinase activity.

Results

Mitochondria integrity—The outer membrane integrity of the mitochondrion preparation was 90% as indicated by the cytochrome c oxidase activity assay (data not shown). Inner membrane integrity was confirmed by mitochondrial uptake of the cationic carbocyanine dye JC-1 (data not shown).

Rationale for the selection of HKI mutants—Xie and Wilson (46) predicted a helical structure for the first 11 residue sequence of rat brain HKI, which was confirmed subsequently the structure of the rat HKI (58) (Fig. 2.1). This sequence is identical to that of human brain HKI (Fig. 2.1). The helical conformation for these initial residues puts Ala 4, Ala 8 and Thr 12 on the same face of the helix (Fig. 2.1). Other faces of the helix have predominantly residues with large hydrophobic side chains. The alignment of small residues on one face of the N-
Fig. 2.1 **Structure of HKI.** A. Ribbon of HKI. The image shows the N-terminal $\alpha$-helix with positions 4, 8, and 12 in dark gray. Residues targeted for mutation (dark gray) lie on one side of the helix. Model adapted from crystal structure of HKI (PDB ID 1CZA). B. Stick model of the N-terminal helix (light gray). C. Human brain hexokinase (NCBI Reference Sequence: NP_000179.2) and rat brain hexokinase (GenBank: AAC20075.1) aligned by LALIGN (59).
terminal helix could facilitate packing of the helix against a relatively uniform surface of on the β-barrel of VDAC1. If so, then mutations of position 4, 8, and 12 to larger hydrophobic residues should decrease the binding of HKI to mitochondria. The mutation of position 5 to proline should destabilize the helix and thereby disrupt mitochondrial association.

*Size exclusion*— Size exclusion chromatography of wild-type HKI and mutants revealed a major band corresponding to a protein of mass 100 kDa (Table 2.1 and Fig. 2.2).

<table>
<thead>
<tr>
<th>HKI construct</th>
<th>WT</th>
<th>Y10L</th>
<th>A4L</th>
<th>A8L</th>
<th>T12I</th>
<th>Q5A</th>
<th>Q5P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW (kDa)</td>
<td>105 (7)</td>
<td>96 (4)</td>
<td>100 (6)</td>
<td>99 (9)</td>
<td>115 (10)</td>
<td>103 (6)</td>
<td>95 (5)</td>
</tr>
</tbody>
</table>

Linear regression equation of calibration curve (Figure 2.2) was used to calculate apparent molecular weights of wild type and mutant HKI.
Fig. 2.2. **Size exclusion chromatography of HKI constructs.** A. Elution profiles of standard proteins. B. Calibration curve of log molecular weights of standard proteins versus retention times. C. Elution profiles of wild-type and mutant HKI. The concentration of wild-type and mutant HKI was 1 mg/mL. A sample of volume 100 µL was injected onto a TSK-G3000SW type size-exclusion column and eluted with 5 mM glucose, 1 mM DTT, and 25 mM KPi, pH 7.4. Absorbance was monitored at 280 nm.
HKI and mutants properties—Purified proteins here were at least 95% pure on the basis of sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). Each hexokinase was prepared twice, and used immediately upon purification in mitochondrial binding, gel-filtration and kinetics experiments. Mutant hexokinases have $k_{\text{cat}}$, $K_m^{\text{Glc}}$ and $K_m^{\text{ATP}}$ comparable to those of wild-type HKI (Table 2.2). Edman degradation of wild-type and mutant hexokinases (400 picomole of purified enzyme) either provided no sequence information or weak signals for sequences beginning at residues 10 and 12. The vast majority of enzyme molecules in each sample had blocked N-terminal residues that resisted Edman degradation. Efforts to deformylate the N-terminus by incubation with 0.6 M HCl failed.

Table 2.2. Kinetic properties of HKI constructs.

<table>
<thead>
<tr>
<th>HKI construct</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m^{\text{ATP}}$ (mM)</th>
<th>$K_m^{\text{Glc}}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>92 (11)</td>
<td>0.58 (6)</td>
<td>40 (5)</td>
</tr>
<tr>
<td>Y10L</td>
<td>95 (15)</td>
<td>0.54 (4)</td>
<td>19 (3)</td>
</tr>
<tr>
<td>A4L</td>
<td>93 (7)</td>
<td>0.99 (8)</td>
<td>24 (3)</td>
</tr>
<tr>
<td>A8L</td>
<td>103 (15)</td>
<td>0.87 (7)</td>
<td>24 (5)</td>
</tr>
<tr>
<td>T12I</td>
<td>89 (9)</td>
<td>0.57 (5)</td>
<td>23 (4)</td>
</tr>
<tr>
<td>Q5A</td>
<td>98 (10)</td>
<td>0.60 (4)</td>
<td>20 (4)</td>
</tr>
<tr>
<td>Q5P</td>
<td>77 (4)</td>
<td>0.82 (2)</td>
<td>24 (6)</td>
</tr>
</tbody>
</table>

Determination of $k_{\text{cat}}$ employed concentrations of Glc and ATP of 1.6 mM and 9 mM respectively. Determination of $K_m^{\text{ATP}}$ employed at Glc concentration of 1.6 mM and concentrations of ATP of 0.3–7.5 mM. Determination of $K_m^{\text{Glc}}$ employed an ATP concentration of 9 mM and concentrations of Glc of 10–600 µM.
Wild-type and mutant hexokinase I binding to mitochondria—Scheme I represents the simplest equilibrium model that accounts for the binding of wild-type and mutant hexokinases to the mitochondria:

\[
E + Q \xrightleftharpoons{} EQ
\]

\[
K_1 = [EQ]/[E][Q]
\]

Scheme I

In Scheme I, \(K_1\) represents an association constant for the binding of the HKI construct (represented by \(E\)) to specific mitochondrial binding sites \(Q\). As demonstrated by previous work, nonspecific binding to the mitochondrion by HKI does not occur at significant levels. The ratio of enzyme bound to total \(E_o\) is as follows:

\[
R([E]) = \frac{K_1[E]}{1+K_1[E]} \quad \text{Eq. 1}
\]

The concentration of free specific mitochondrial sites \(Q\) is itself a function of the concentration of enzyme. Algebraic manipulation of the equilibrium expression in Scheme I and relationships for mass conservation of total enzyme \(E_o\) and total specific sites \(Q_o\) results in a quadratic relationship in \(E\):

\[
a[E]^2 + b[E] + c = 0
\]

where \(a = K_1\), \(b = K_1(Q_o-E_o)+1\), and \(c = -E_o\). The physical root of the quadratic equation gives \([E]\):

\[
[E] = -\{K_1(Q_o-E_o)+1\}/2K_1 + \{K_1^2(E_o-Q_o)^2+2K_1(E_o+Q_o)+1\}^{1/2}/2K_1 \quad \text{Eq. 2}
\]

Substitution of Eq. 2 into Eq. 1 provides a relationship for the fraction of enzyme bound to the mitochondria as a function of \(E_o\), \(Q_o\) and \(K_1\).

The fitting relationship is \(V = sR(E_o,Q_o,K_1)\) (Eq. 3), where \(s\) is a proportionality
constant that relates the fraction of bound enzyme $R$ to velocity $V$ in μmoles/min. Undefined quantities $s$, $E_0$, $Q_o$, and $K_1$ cannot be determined by a nonlinear least squares fit of the data from a single experiment. Hence, values for some of these quantities must come from other determinations. Firstly, one assumes the HKI-VDAC complex has an equal number of HKI and VDAC subunits. The value for $Q_o (7.1 \times 10^{-8} \text{ M})$ then is an estimate based on 42,000 VDAC molecules per mitochondrion (60), $7.2 \times 10^9$ mitochondria per 1 mg of total mitochondrial protein (61), and 0.014 mg of mitochondria in each 100 μL assay. Moreover, $s = Q_o \times \text{(specific activity)} \times \text{(assay volume)}$, where the specific activity for HKI is $6 \times 10^9$ μmole/min$^{-1}$ mole$^{-1}$ and the assay volume is liters. Hence, if the assumption of a 1:1 ratio of the HKI-VDAC complex is correct, then data can be fit to Eq. 3 using $K_1$ alone as an adjustable parameter. Data fit by this method appears in Fig. 2.3. Listed in Table 2.3 are values for $K_1$ for wild-type and mutant constructs of HKI.

*Glc-6-P induced release of Wild-type and mutant hexokinase I from mitochondria*— Glc-6-P releases wild-type HKI from mitochondria as well as T12I, Y10L and Q5A (Table 2.4). The model and fitting is as described previously (41). Release experiments could not be done for the A4L, A8L, and Q5P constructs due to poor binding to the mitochondrion.

*ATP-induced release of Wild-type and mutant hexokinase I from mitochondria*— ATP released wild-type, T12I, Y10L and Q5A hexokinases from the mitochondrion (Table 2.5 and Fig. 2.4). The model and fitting is as described by Nimer (PhD thesis). Release experiments could not be done for the A4L, A8L, and Q5P constructs due to poor binding to the mitochondrion.
Fig. 2.3. **HKI binding to pig liver mitochondria.** Plots represent velocities from varying concentrations of wild-type (○), T12I (▲), Y10L (■), Q5A (●), Q5P (□), A4L (△) or A8L (♦) bound to 1.8 mg, wet weight mitochondria (each fraction) in 250 mM sucrose and 50 mM HEPES, pH 7.5. The solid lines represent fitted curves using Eq. 3 with parameters given in the Results section and Table 2.3.
Table 2.3. **Fitted parameters for wild type and mutant HKI binding to pig liver mitochondria.**

<table>
<thead>
<tr>
<th>HKI construct</th>
<th>Apparent association constant $K_I$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$2.7 \times 10^4$ ($6.5 \times 10^2$)</td>
</tr>
<tr>
<td>Y10L</td>
<td>$1.4 \times 10^4$ ($7.3 \times 10^2$)</td>
</tr>
<tr>
<td>A4L</td>
<td>$3.6 \times 10^3$ ($1.6 \times 10^2$)</td>
</tr>
<tr>
<td>A8L</td>
<td>$3.5 \times 10^3$ ($1.5 \times 10^2$)</td>
</tr>
<tr>
<td>T12I</td>
<td>$2.0 \times 10^4$ ($7.0 \times 10^2$)</td>
</tr>
<tr>
<td>Q5A</td>
<td>$8.9 \times 10^3$ ($4.3 \times 10^2$)</td>
</tr>
<tr>
<td>Q5P</td>
<td>$4.5 \times 10^3$ ($1.5 \times 10^2$)</td>
</tr>
</tbody>
</table>

Parameter $K_I$ is defined in the Result section. HKI bound to 1.8 mg, wet weight mitochondria (each fraction) in 250 mM sucrose and 50 mM HEPES, pH 7.5 was measured by activity. Standard deviations in the last significant digits are given in parenthesis.
Table 2.4. **Fitted parameters for Glc-6-P-induced release of mitochondrial wild type and mutant HKI.**

<table>
<thead>
<tr>
<th>HKI construct</th>
<th>( a ) (( \mu M S^{-1} ))</th>
<th>( b ) (( \mu M ))</th>
<th>( c ) (( \mu M ))</th>
<th>( K_{ii} ) (mM)</th>
<th>( I_{0.5} ) (( \mu M ))</th>
<th>Relative velocity at ( I_{0.5} )</th>
<th>Limiting release</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.035 (7)</td>
<td>6 (2)</td>
<td>1.1 (4)</td>
<td>0.2 (1)</td>
<td>3.0 (1)</td>
<td>0.70 (5)</td>
<td>47 (3)</td>
</tr>
<tr>
<td>T12I</td>
<td>0.040 (6)</td>
<td>5 (2)</td>
<td>1.1 (3)</td>
<td>0.3 (1)</td>
<td>2.7 (3)</td>
<td>0.67 (6)</td>
<td>48 (3)</td>
</tr>
<tr>
<td>Y10L</td>
<td>0.028 (4)</td>
<td>4 (2)</td>
<td>0.9 (2)</td>
<td>0.4 (2)</td>
<td>2.8 (4)</td>
<td>0.63 (5)</td>
<td>54 (6)</td>
</tr>
<tr>
<td>Q5A</td>
<td>0.047 (1)</td>
<td>8 (1)</td>
<td>1.5 (1)</td>
<td>0.3 (1)</td>
<td>3.4 (4)</td>
<td>0.74 (2)</td>
<td>40 (2)</td>
</tr>
</tbody>
</table>

Parameters \( a, b, c, K_{ii}, \) and \( I_{0.5} \) previously described (41). Mitochondria with HKI bound in 250 mM sucrose and 50 mM HEPES, pH 7.5, were exposed to varying Glc-6-P concentrations for 30 minutes. Remaining mitochondria bound HKI was measured by activity. Standard deviations in the last significant digit are given in parentheses.
Table 2.5. **ATP-dependent release of HKI from pig liver mitochondria.**

<table>
<thead>
<tr>
<th>FHKI construct</th>
<th>$K_f$ (M$^{-1}$)</th>
<th>$K_s$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>$2.5 \times 10^6$ ($7.3 \times 10^4$)</td>
<td>$3.0 \times 10^3$ ($4.1 \times 10^2$)</td>
</tr>
<tr>
<td>T12I</td>
<td>$1.7 \times 10^6$ ($6.3 \times 10^4$)</td>
<td>$8.0 \times 10^3$ ($1.2 \times 10^5$)</td>
</tr>
<tr>
<td>Y10L</td>
<td>$4.9 \times 10^6$ ($1.9 \times 10^5$)</td>
<td>$3.0 \times 10^3$ ($5.0 \times 10^2$)</td>
</tr>
<tr>
<td>Q5A</td>
<td>$2.4 \times 10^6$ ($1.0 \times 10^5$)</td>
<td>$1.0 \times 10^4$ ($1.7 \times 10^3$)</td>
</tr>
</tbody>
</table>

Parameters are from an equilibrium model in which the binding of nucleotide and HKI to the mitochondrion is mutually exclusive. $K_f$ and $K_s$ are constants for the dissociation in M$^{-1}$ of HKI and ATP from the mitochondrion. Mitochondria with HKI bound in 250mM sucrose and 50 mM HEPES, pH7.5, were exposed to varying concentrations of ATP for 30 minutes. Remaining mitochondria bound HKI was measured by activity. Standard deviations in the last significant digits are given in parentheses.
Fig. 2.5. **ATP-induced release of wild-type and mutant HKI from pig liver mitochondria.** Mitochondria with bound wild type (Δ), T12I (○), Y10L (●) or Q5A (▲) in 250mM sucrose and 50 mM HEPES, pH7.5, were exposed to varying concentrations of ATP. Plots represent relative velocities from mitochondria-bound bound HKI that remains after exposure to varying concentrations of ATP. The solid lines represent fitted curves using fitting model described by Nimer et al. (unpublished data).
Discussion

$K_1$ of Scheme I represents binding affinity of HKI for the mitochondrion, but binding sites on the mitochondrion may be heterogeneous. VDAC molecules may exist as monomers, dimers, or in assemblies of large number. Nonetheless each class obeys the relationships of Scheme I, provided that HKI binds without cooperativity to large aggregates of VDAC. For a dispersive system, $K_1$ becomes a weighted average of association constants for each class of binding site:

$$K_1 = \frac{\sum K_1[Q_i]}{\sum [Q_i]}$$

where the summations extend over $i$ classes of binding sites with association constants $^iK_1$ and $[Q_i]$ is the concentration of mitochondrial binding sites in each class. The effect of site heterogeneity is a $K_1$ that depends on the concentrations of total enzyme $E_o$ and mitochondrial sites $Q_o$. For instance, high ratios of $E_o$ to $Q_o$ will result in low values for $K_1$, as subclasses of binding sites with low association constants contribute to the ensemble average. Wilson et al. (46) have published the only binding curve for which the number of mitochondrial binding sites $Q_o$ is in great excess over $E_o$. Applying the model used here to these data yields a $K_1$ of $1.4 \times 10^6$ M$^{-1}$. The number determined from (46) agrees well with the $K_1$ of nucleotide release assays (Table 2.5), but is 100-fold higher than the $K_1$ determined from binding assays (Table 2.3). The discrepancy lies in the relative abundance of HKI and mitochondria for the release and binding assays. Specifically for all data points in release assays $[HKI] \leq Q_o$, whereas for binding assays $[HKI] \geq Q_o$. The consequence of different experimental conditions is a $K_1$ that differs by 100-fold.

Pittler et. al. (47) proposed that an $N$-acetyl group blocks the N-terminus of native rat-brain hexokinase. In the present study, the N-termini of wild-type and mutant hexokinases resist
Edman degradation. Deformylation of C-terminal His-tagged human HKI removed the blocking group, resulting in a clear hexokinase sequence beginning with the N-terminal methionine (41). The same procedure, however, did not deblock recombinant HKI, used here, which is expressed from a vector which does not incorporate a C-terminal His-tag. There are now many reports of recombinant proteins expressed in E. coli with an N-acteyl group at the N-terminus (62). The status of the N-terminus of HKI constructs is critical, because the loss of either the blocking group or N-terminal residues could greatly alter the binding affinity toward the mitochondrion. Each of the constructs here has a blocked N-terminus, and hence, changes in binding properties relative to the wild-type enzyme must be a consequence of a specific mutation.

Several mechanisms could be responsible for the disruption of HKI binding affinity to the mitochondrion. Mutations could cause global conformational change in the protein; however, kinetic parameters clearly indicate active enzyme with wild-type properties. Moreover, the removal of residues from the N-terminus has no effect on the kinetic properties of HKI (or hexokinase Type-II) from human and other sources (63,64). Mutations could also change the state of aggregation of HKI, and thereby impede association with the mitochondrion, but constructs here are monomers up to peak concentrations used in mitochondrial binding assays. Hence, decreased binding affinities observed here are due the effect of the mutation on the specific interactions with the mitochondrion.

Although HKI binds to hydrophobic surfaces (48), specific binding of HKI to mitochondria cannot be explained merely by hydrophobic interactions with a lipid bilayer (46). Mutations at positions 4 and 8 identify the first single-residue determinants essential for the interaction of HKI with the mitochondrion. The loss of binding properties due to the Gln5→Pro mutation infers a helical conformation for the N-terminal residues of mitochondrion-bound HKI.
Hence, positions 4 and 8 define a contiguous surface that does not allow large residues, even if these residues are hydrophobic. Other positions seem less important, and allow conservative changes in the type of amino acid side chain, but no mutation improved mitochondrial binding.

Cyclic AMP-dependent protein kinase, D-AKP1, binding to mitochondria and endoplasmic reticulum is also controlled by a 15-residue N-terminal sequence homologous to that of HKI (52). Small hydrophobic side chains on residues corresponding to residues Ala\textsuperscript{4} and Ala\textsuperscript{8} in HKI tail are necessary for mitochondrial targeting (52). Moreover, the amount of HKI that binds to the mitochondrion diminishes as residues are removed from the N-terminal sequence (51). Thr\textsuperscript{12} is on the same helical face as Ala\textsuperscript{4} and Ala\textsuperscript{8}, but the mutation of position 12 to isoleucine has less impact on mitochondrial binding properties of HKI; however, the steric bulk at position 12 increases by only single methyl group, whereas positions 4 and 8 have additions of isopropyl groups. Ala\textsuperscript{4} and Ala\textsuperscript{8} are not likely critical for lipid interactions, but would define a relatively flat surface on the N-terminal helix. Such a surface would pack favorably against a flat exterior surface of the β-barrel of VDAC. Preliminary coarse grain modeling (Yang Gao and Richard Honzatko, unpublished results) indicates a preferred placement of the surface defined by positions 4 and 8 on the barrel of VDAC. This placement puts the N-terminal domain of HKI over the opening of the anion pore of VDAC, consistent with the channel-blocking properties of HKI.

Although N-terminal residues are essential for HKI association with the mitochondria, one cannot exclude the possibility of other residues elsewhere on HKI playing an essential role (65). Nucleotide (Nimer et al.) and Glc-6-P (41) release of mitochondrion-bound HKI implicate the pore of VDAC and the Glc-6-P binding pocket of the N-terminal half of HKI in the VDAC-HKI recognition. If VDAC favors an open conformation for the N-terminal half of HKI, then
Glc-6-P could disrupt essential contacts by driving the N-terminal domain to its closed conformation. The binding of nucleotides to VDAC could alter the conformation of the pore, disrupting the same VDAC-HKI interactions. Finally, the N-terminal hydrophobic helix of HKI continues beyond residue 15 and becomes highly charged with acid and basic side chains. Some of these charged residues are in position to interact with VDAC. Given the current work establishes a method of quantifying the effects of a mutation on mitochondrion binding affinity, one can systematically mutate residues suggested by a HKI-VDAC model to locate additional residues on HKI critical to mitochondrion-binding.
References

2. Lowry, O H; Passonneau, J V. (1964) J. Biol. Chem. 239, 31-42


29. Vyssokikh, M Y; Goncharova, N Y; Zhuravlyova, A V; Zorova, L D; Kirichenko, W; Krasnikov, B F; Kuzminova, A E; Melikov, K C; Melik-Nubarov, N S; Samsonov, A V; Belousov, W; Prisecpova, A E; Zorov, D B. (1999) *Biochemistry (Moscow)* **64**, 390-8


34. Lemasters, J J; Qian, T; Bradham, C A; Brenner, D A; Cascio, W E; Trost, L C; Nishimura, Y; Nieminen, A L; Herman, B. (1999) *J. Bioenerg. Biomembr.* **31**, 305-19


38. Abu-Hamad, S; Arbel, N; Calo, D; Arzoine, L; Israelson, A; Keinan, N; Ben-Romano, R; Friedman, O; Shoshan-Barmatz, V. (2009) *J. Cell Sci.* **122**, 1906-16


CHAPTER 3: GENERAL CONCLUSIONS

Loss of mitochondrial binding properties due to the loss of the 15 N-terminal residues of rat-brain HKI suggests that one or more of these residues are necessary for the binding of HKI to the membrane. No single-residue determinants of HKI mitochondrial-binding are known. This study reports two residues are essential for binding. Selective mutations amongst the first 15 residues have no effect on kinetic properties of HKI. And wild-type and mutant enzymes behave identically as monomers in solution. The N-terminus in all constructs is blocked by an unknown functional group that prevents sequencing by Edmund degradation. Mutant enzymes A4L and A8L do not bind to the mitochondrion. These residues define a contiguous surface of the N-terminal helix of HKI. That surface does not tolerate large hydrophobic side chains, indicating a close contact between it and a mitochondrial binding partner (VDAC-1).

It was hypothesized that the Q5P mutation would completely disrupt the helix and thus hexokinase would not bind to mitochondria. However, the results show that mutant hexokinase binds very weakly to mitochondria. These results suggest that other residues might be playing an important role in binding of hexokinase to VDAC and stabilizing it.

Further studies could include the mutation of charged residues 16, 17, 20 and 21, which are above the N-terminal tail, and to measure the effects on the binding properties of hexokinase. Similar binding studies can be done on a combination of mutations in the first 25 residues.
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