Role of the N-terminus of recombinant human brain hexokinase in mitochondrial binding

by

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To my parents, Mohan Maskey and Shanti Maskey

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ABSTRACT

The binding of hexokinase Types I and II to mitochondria is a putative checkpoint in apoptosis. Evidence supports the specific binding of residues 1 through 15 of hexokinase Type I (HKI) to the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane. Ala4 and Ala8 of HKI play critical roles in mitochondrial binding. Substitution of these residues individually with leucine causes the loss of binding properties. Identified here is the significant role of the methionine at position 1 in the binding of HKI to mitochondria. The following conclusions can be drawn from the properties of mutant HKI constructs: Recombinant wild-type HKI is blocked at the N-terminus. The replacement of Ile2 with threonine results in a recombinant HKI with an unblocked amino terminus that does not bind to mitochondria. The extension of HKI at the N-terminus by duplicating its first four residues (putting amino acids at positions 0, –1, –2, and –3) retains mitochondrial binding properties. The mutation of Ile at position –2 to threonine results in an unblocked amino terminus that still binds to mitochondria. The mutation of Met1 to leucine in the extended construct greatly reduces mitochondrial binding. The conclusion: blocking of the N-terminal methionine is not a significant factor in mitochondrial binding of HKI. Instead, it is the specific structure of the methionine side chain that enables a strong binding interaction. Side chains at positions 1, 4, and 8 define a contiguous face on the N-terminal α-helix of HKI critical mitochondrial binding.

CHAPTER 1: GENERAL INTRODUCTION

Hexokinase in Metabolism

The first step in the metabolism of glucose (Glc) is usually phosphorylation catalyzed by hexokinase (ATP: D-hexose 6-phosphate transferase, EC 2.7.1.1) to produce glucose-6-phosphate (Glc-6-P). Selective expression of isozymic forms of hexokinase, differing in catalytic and regulatory properties as well as subcellular localization, is likely to be an important factor in determining the pattern of Glc metabolism in mammalian tissues/cells (1). Mammals have four types of isozymes for hexokinase: I, II, III, IV. Hexokinase I (HKI), which is also known as brain hexokinase regulates the glucose metabolism in brain tissues, kidney and red blood cells (2). Hexokinase I, II and III have the molecular weights of approximately 100 kDa (3), which has both C- and N- terminal halves with significant levels of sequence identity to each other and also to the yeast hexokinase (4). Similarities between mammalian isozymes evidently originate from the duplication and fusion of a primordial hexokinase gene similar to that of yeast hexokinase (5). According to Katzen and Schimke, the properties of the different types of hexokinase do not vary much from tissue to tissue, but are present in different amounts depending on age, stability and nutritional factors (3). There are number of ligands that inhibit brain hexokinase, but Glc-6-P, which is also the product of glucose phosphorylation, is probably the main physiological inhibitor (6). Glc-6-P inhibits brain hexokinase by binding to either the C- or N-terminal halves with high affinity (7, 8). Under normal physiological conditions, however, inorganic orthophosphate (Pi) seems to alleviate the inhibition caused by the Glc-6-P in brain hexokinase (9). Pi binds to the N-terminal half of the HKI and lessens Glc-6-P inhibition by an allosteric mechanism that

couples both halves of the enzyme (10, 11). At the higher concentration, Pi inhibits HKI by binding to the active site (12).

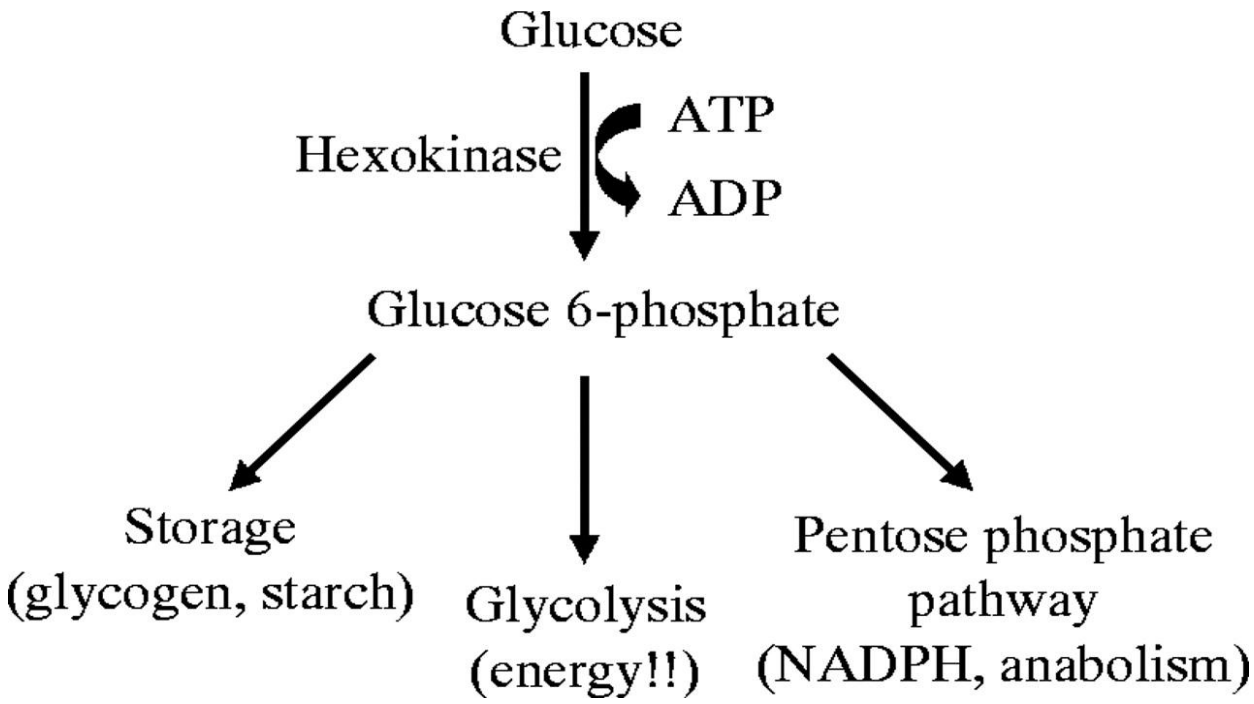

Fig. 1. Phosphorylation catalyzed by hexokinase, is the first step in common pathways of glucose metabolism (Ref: Wilson J.E., *Isozymes of mammalian hexokinase: structure, subcellular localization and metabolic function*)

Formation of the Glc-6-P by hexokinase targets glucose to alternative metabolic pathways: glycolysis, glycogen synthesis and the pentose phosphate pathway (1) (Fig. 1). These metabolic pathways are associated to different extents with various organs of the body and with the different isozymes of mammalian hexokinase. Type I hexokinase binds to mitochondria through interaction with porin, the protein that forms channels through which metabolites traverse the outer mitochondrial membrane (OMM). The Type II isozyme also binds to the mitochondria. Hexokinase Type III and Type IV do not bind to the mitochondria

due to the absence of hydrophobic N-terminal residues. Thus these isozymes might be associated with metabolic pathways other than glycolysis (1). Table 1.1 summarizes the similarities and differences between the different types of isozymes.

Table1.1 Summary of comparison of different isozymes of mammalian hexokinase.

Properties	**HKI**	**HKII**	**HKIII**	**HKIV**
Size (kDa)	100	100	100	50
Tissues	Brain, Kidney, Red Blood Cell	Skeletal Muscle, Fat	Liver, Intestine, Kidney	Liver, Pancreas
K_mGlc (mM)	0.03	0.3	0.003	6
K_mATP(mM)	0.5	0.7	1.0	0.6
Function of N-terminal half	Regulatory	Catalytic	Regulatory	-
Function of C-terminal half	Catalytic	Catalytic	Catalytic	-
Inhibition by Glc-6-P	Yes	Yes	Yes	No
Pi Relief	Yes	No	No	-
Subcellular localization	Mitochondria	Mitochondria	Nuclear Periphery	-

Structure of Hexokinase

The structure of recombinant human brain hexokinase (Fig. 2) has been solved by methods of X-ray crystallography (13-19). Brain hexokinase is a functional monomeric enzyme in

solution; however it exists as a dimer in the crystal, with Glc/Glc-6-P or Glc/Pi binding in both N- and C-halves (15, 16). In the dimer, HKI subunits align head-to-tail; with two fold rotational symmetry such that the N-terminal domain of one subunit interacts with C-terminal domain of other subunit (13, 15-18). The monomeric structure of an engineered monomer of hexokinase I adopts a rod-like conformation similar to that for a subunit in other crystalline dimers (14, 19).

Hexokinase in its monomeric form has two structurally similar halves: N-terminal and C-terminal domains which are separated by a transition helix which extends from residues 448 to 475 (14-20). Each half has a large domain (residues 13-74, 210-447 in N-terminal half and residues 466-522, 658-895 in the C-terminal half); and small domain (residues 75-209, 448-465 in N-terminal half and residues 523-657, 896-913 in C-terminal half) (19). The relative position of the two domains in the C-terminal half in HKI defines two conformational states for HKI, open and closed. The N-terminal half however has been observed in only a closed conformation. Moreover, the N-terminal half evidently is more stable than the C-terminal half, as the C-terminal half folds before the N-terminal half (20, 21).

Although resembling the catalytically-active C-terminal half, the N-terminal half of the HKI is inactive. Nonetheless, the N-terminal half has a binding pocket for the Glc and Glc-6-P which is nearly identical to that of the C-terminal half (16). Crystal structures reveal the high affinity binding site for Pi, which overlaps the binding site of the 6-phosphoryl group of the Glc-6-P, is at the N-terminal half (15, 16). The conformation of the N-terminal half in its Pi and Glc-6-P complexed structures differs only in the conformation of loop 445-450 at the N-

terminal end of the transition helix (19). Aleshin *et al*. (16, 19) engineered a monomeric form of HKI and crystallized it as a Glc/Glc-6-P complex and as a Glc/ADP complex, finding two binding sites for Glc-6-P and two binding sites for ADP.

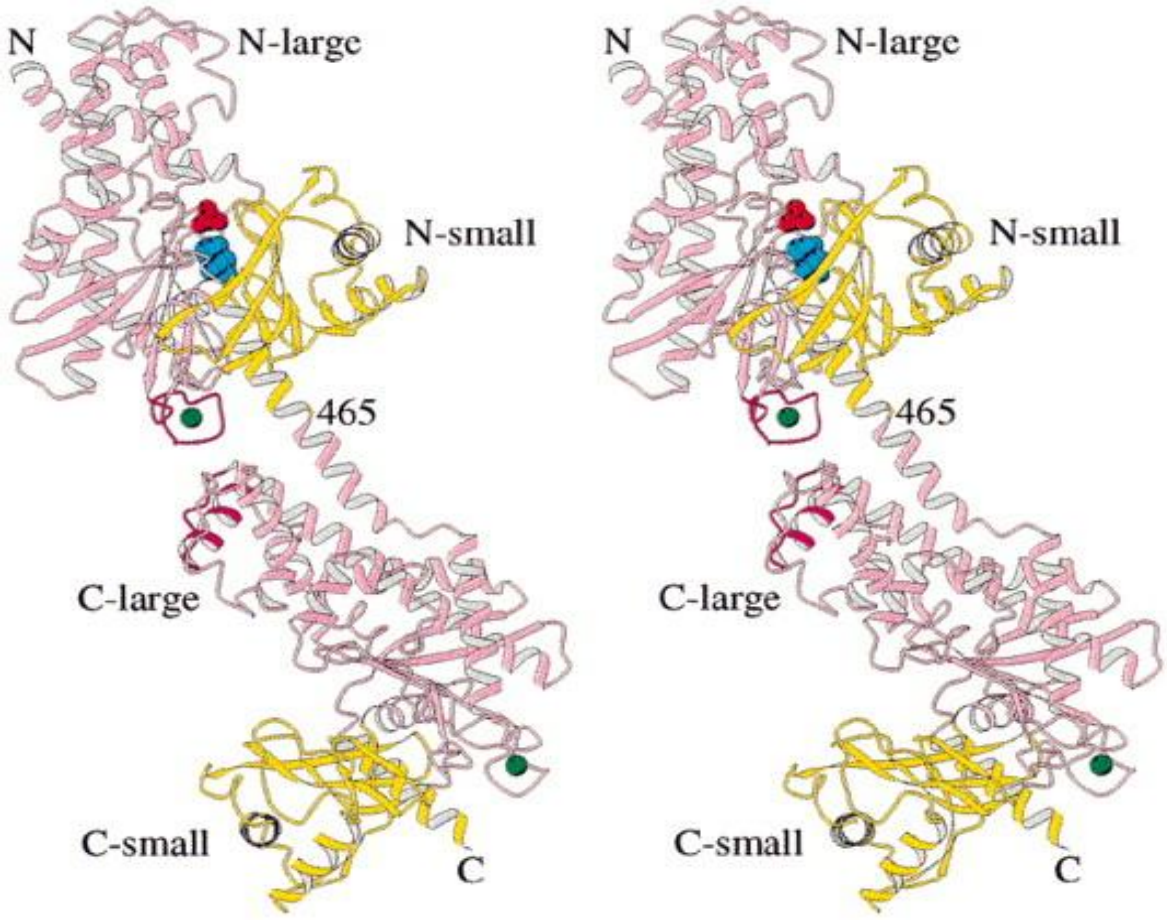

Fig 2. Crystal structure of hexokinase complexed with Glc and Pi. The color code is as follows: yellow, small domains; light purple, large domains; dark purple, segments participating in intrachain salt links; blue, bound glucose; red, bound phosphate; green, bound metal ions (15).

Mitochondrial Binding of Hexokinase I

Hexokinase I and II have the ability to bind to the outer mitochondrial membrane (OMM), a property which requires the first 15 residues of its N-terminus (22-25). Xie and Wilson (25) proposed that the hydrophobic tail is helical and Mulichak *et al.* (17) confirmed a helical structure by a crystal structure determination of the native rat-brain enzyme. Hexokinase I association with the OMM allows preferential access to newly formed mitochondrial ATP (22), but perhaps more importantly the interaction with the voltage-dependent anion channel (VDAC) putatively is arrests mitochondrially-linked apoptosis (26, 27).

The N-terminal sequence of the rat brain hexokinase was determined to be

X-*NH-Met-Ile-(Ala, Gln)-Ala-Leu-Leu-Ala-Tyr*-

where X is blocking group at the N-terminal methionine. The chemical identity of the blocking group is unknown, but generally assumed an N-acetyl group. Elimination of the N-terminal segment by endogenous proteases in crude brain extracts eliminates mitochondrial binding (28). N-acetylation of the methionine is the norm rather than the exception for many cellular proteins and hence, finding a blocked N-terminus on the brain hexokinase is not unexpected (29, 30). The N-terminal segment of the hexokinase is readily accessible at the surface of the hexokinase. Hence, it is highly susceptible to proteolytic modification (28). Kurakawa *et al.* (31) discovered that the treatment of brain hexokinase with chymotrypsin resulted in the loss mitochondrial binding. Polakis and Wilson (28) later found that chymotrypsin removed a limited hydrophobic segment at the N-terminus. Hence, in an intact enzyme, this segment must be located at the surface of the hexokinase molecule, permitting

interaction with the mitochondria. Xie and Wilson (32) have reported through their crosslinking study that binding of hexokinase is accompanied by the formation of an oligomeric structure (tetramer). Hence hexokinase associated with the mitochondrial membrane might be the mixture of monomer and tetramer. Wicker *et al*. later showed that the binding of the hexokinase to the mitochondria is a cooperative process with a Hill coefficient of approximately 3 (45); however, Rose and Warms found to cooperativity in the re-association of HKI with rat-brain mitochondria (22).

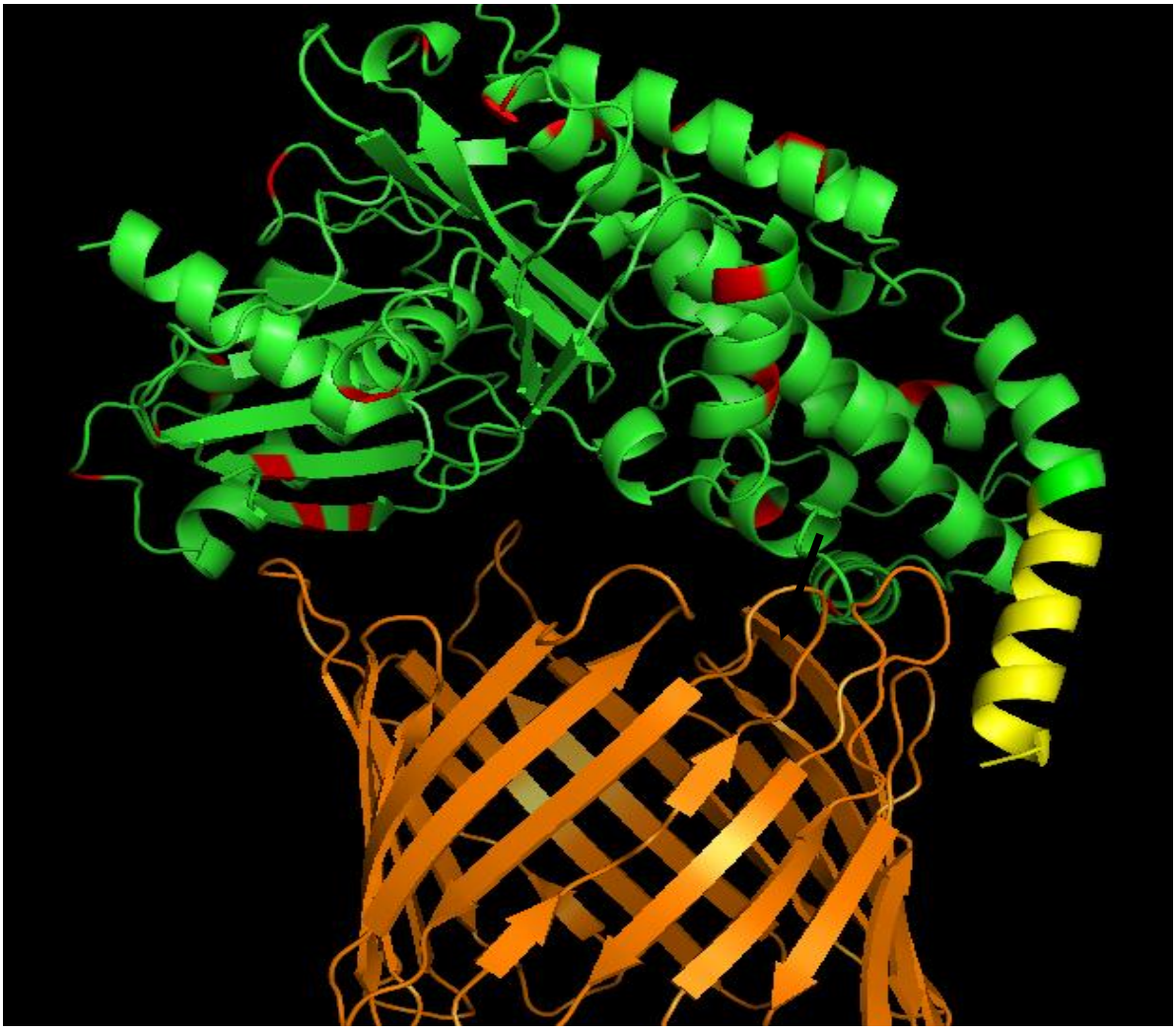

Fig. 3a. HKI N-terminal half and VDAC interaction by modeling (Yang Gao, unpublished). Orange represent the β-barrel structure of VDAC, yellow represents first 15 residues of the hydrophobic N-terminal, while green represent the rest of the N-terminal half.

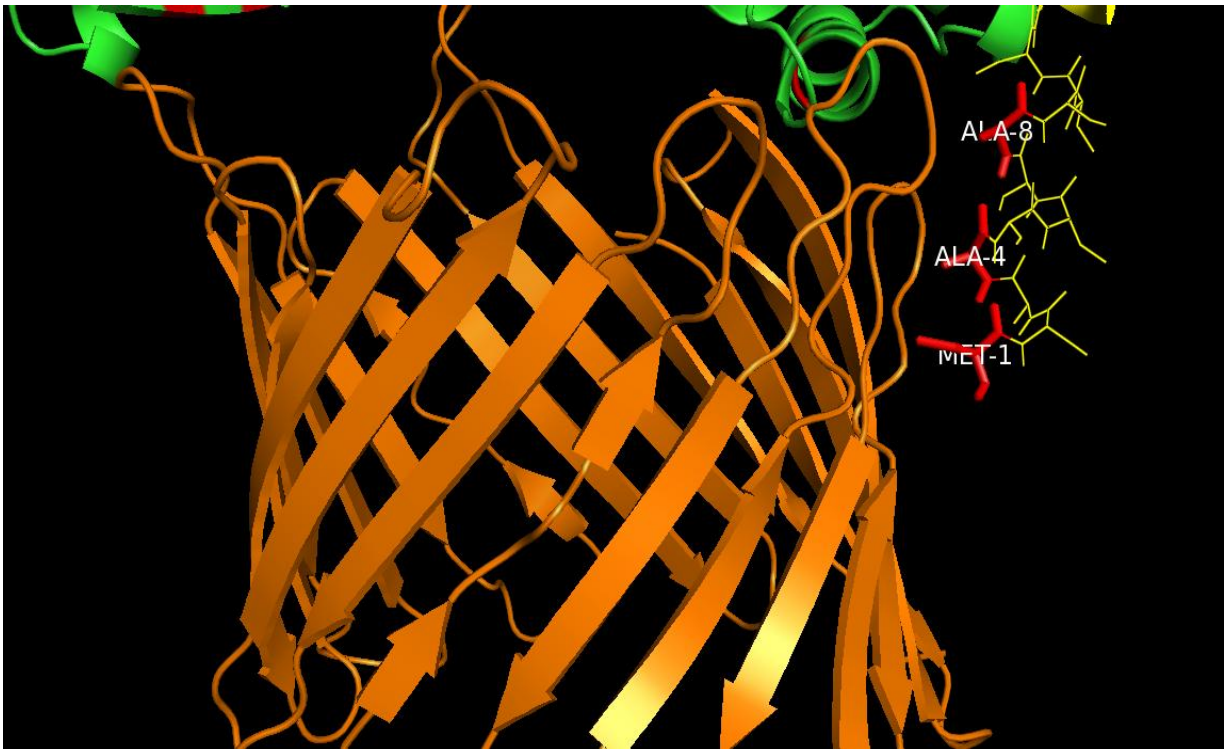

Fig 3b. Closer look at the residues of hexokinase interacting with a particular side of the VDAC. Residues colored red are Met1, Ala4, and Ala8.

Glucose-6-phosphate Release of Mitochondrially Bound Hexokinase

Glc-6-P is not only a potent inhibitor of brain hexokinase at physiological concentrations, but it can also release the enzyme from the brain mitochondria (22). HKI-release of Glc-6-P from mitochondria may be the first step in an enzyme degradation pathway (22) and/or a process linked to apoptosis (33-36). Fromm and Zewe (37) demonstrated that Glc-6-P is a competitive inhibitor of ATP and linear noncompetitive inhibitor of glucose. Skaff, *et al.* (38) in 2005 showed that Glc-6-P binding to the N-terminal half is responsible for the release

of recombinant human brain hexokinase from the mitochondrion. It has been shown that Glc-6-P induces conformational change in the N-terminal half of the HKI that consequently releases the enzyme from the mitochondrial membrane. The exact understanding of the presumed conformational change has been elusive, as crystal structure reveal Glc-6-P and Pi bound N-terminal halves have the same conformation but for a span of five residues preceding the transition helix. Mehyar (39) in 2011 found the residues of the N-terminal helix critical to binding with the mitochondrial. Mutations A4L, A8L and Q5P individually caused ten-fold decreases in HKI binding to mitochondria relative to the wild-type enzyme (39). Residues A4 and A8, which define a contiguous surface, does not tolerate large hydrophobic side chains (39).

Hypothesis

Some studies suggest that hexokinase binding to the mitochondria is determined by the hydrophobicity of the N-terminal tail (40, 41). Co-localization of brain hexokinase with the VDAC at the OMM suggests more complex and specific interactions than the general hydrophobic interactions with the lipid (26). Chymotrypsin removes the hydrophobic tail on the N-terminal domain and thus prevents the binding of hexokinase to the mitochondria (22, 28, 31) implying the importance of the first 12-residues in the N-terminal domain. It was also discovered that certain residues among those first 12 residues are critical for hexokinase binding to the mitochondria (39). Leucine replacements of Ala4 and Ala8 abolish mitochondrial binding, suggesting these residues come in contact with VDAC. Met1 could be a part of the surface defined by Ala4 and Ala8 that is critical to the interaction of the N-terminal helix of HKI with the membrane-buried surface of VDAC.

Due to the blocked N-terminus, our lab has been unsuccessful in obtaining the N-terminal sequence of recombinant HKI. Methionine aminopeptidase (MAP) from *Escherichia coli* specifically cleaves methionine at position 1 provided the residue at position 2 has a small side chain (42-44). In fact, approximately 40% of proteins expressed in *Escherichia coli* begin with threonine (42, 43). The first five residues of the HKI are **Met-Ile-Ala-Ala-Gln**. The presence of isoleucine does not allow MAP to cleave the methionine from position 1, but mutating isoleucine to threonine should enable the action of MAP. The resulting construct should begin with threonine, be amenable to N-terminal sequencing, and be unable to bind to mitochondria; however, the loss of binding properties can arise from the presence of a

charged N-terminus, the loss of methionine from position 1, and/or the change in position 2 from isoleucine to threonine.

In order to determine the factors responsible for the loss of mitochondrial binding properties, the first four residues of the N-terminal helix are repeated, defining new sequence positions -3 to 0. The extended helix was again blocked and resisted N-terminal sequencing, but bound to mitochondria. Mutation of Ile(-2) to threonine resulted in a construct amenable to N-terminal sequencing, and without the loss of binding properties. In the extended and blocked construct, the mutation of Ile2 to threonine had little effect on mitochondrial binding, whereas the mutation of Met1 to leucine greatly diminished mitochondrial binding. From the observations, the side chain of Met1 is a critical factor in interaction of HKI with the mitochondrion, and that positions 1, 4 and 8 define a contiguous surface that recognizes VDAC.

Thesis Organization

This thesis has three chapters. Chapter 1 is a general introduction of HKI providing background information related to structure, properties of metabolite regulation and mitochondrial binding, as well as the goal of research, and a brief summary of the outcome of experimental work. Chapter 2 presents the experimental work in detail, and represents a manuscript for publication in a peer-reviewed journal. Chapter 3 provides a conclusion and possible directions of future work.

References:

1. Wilson J.E. (2003) J. Exp. Biol. **206**, 2049-2057

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

3. Katzenm, H. M., Schimke, R.T. (1965) Proc. Natl. Acad. Sci. USA **54**, 1218-1225

4. Schwab, D.A., Wilson J.E. (1989) Proc. Natl Acad. Sci. USA **86** 2563-2567

5. Wilson, J.E. (2003) J. Exp. Biol. **206,** 2049-2057

6. Crane, R.K., Sols, A. (1954) J. Biol. Chem. **210**, 597-606

7. Ureta, T. (1975) in *Isozymes III* (Markert, C L,ed) pp 575-601, Academic Press Inc., New York, NY

8. Fromm, H J. (1981) in *The Regulation of Carbohydrate Formation and Utilization in Mammals* (Veneziale, C M,ed) pp 45-68, University Park Press, Baltimore, MD

9. Rose, I.A., Warms, J.V., O'Connell, E.L. (1964) Biochem. Biophys. Res. Commun. **15**, 33-37

10. Tsai, H.J., Wilson, J.E. (1995) Arch. Biochem. Biophys. **316**, 206-214

11. Aleshin, A.E., Zeng, C., Bartunik, H.D., Fromm, H. J., Honzatko, R.B. (1998) J. Mol. Biol. **282,** 345-357

12. Ellison, W.R., Lueck, J.D., Fromm, H.J. (1975) J. Biol. Chem. **250**. 1864-1871

13. Rosano, C., Sabini, E., Rizzi, M., Deriu, D., Murshudov, G. Bianchu, M., Serafini, G., Magnani, M., Bolognesi, M. (1999) Structure. **7**, 1427-1437

14. Aleshin, A.E., Malfois, M., Liu, X., Kim, C., Fromm, H.J., Honzatko, R.B., Koch, M.H., Svergun, D.I. (1999) Biochemistry. **38**, 8359-8366

15.Aleshin, A., Zeng, C., Bartunik, H.D., Fromm, H.J. Honzatko, R.B. (1998) J. Mol. Biol. **282**, 345-357

16. Aleshin, A., Zeng, C., Bourenkov, G.P., Bartunik, H.D., Fromm, H.J., Honzatko, R.B. (1998) Structure. **6**, 39-50

17. Mulichak, A.M., Wilson, J.E. Padmanabhan, K., Garavito, R.M.(1998) Nat. Struct. Biol. **5**, 555-560

18. Aleshin, A.E., Fromm, H.J., Honzatko, R.B. (1998) FEBS Lett. **434**,42-46

19. Aleshin, A.E., Kirby, C., Liu, X., Bourenkov, G.P., Bartunik, H.D., Fromm, H.J., Honzatko, R.B. (2000) J. Mol. Biol. **296**, 1001-1015

20. Smith, A.D., Wilson, J.E. (1991) Arch. Biochem. Biophys. **291**, 59-68

21. White, T.K., Kum, J.Y., Wilson, J.E. (1990) Arch. Biochem. Biophys. **276**, 510-517

22. Rose, I., Warms, J.V.B. (1967) J. Biol. Chem. **242**, 1635-1645

23. Kropp, E.S., Wilson, J.E. (1970) Biochem. Biophys. Res. Commun. **38**, 74-79

24. Felgner, P.L., Messer, J.L., Wilson, J.E. (1979) J. Biol. Chem. **254**, 4946-4949

25. Xie, G., Wilson, J.E. (1988) Biochem. Biophys. Arch. **267**, 803-810

26. Linden, M., Gellerfors, P., Nelson, B.D. (1982) FEBS Lett. 141. 189-192

27. Fiek, C., Benz, R., Roos N., Brdiczka, D. (1982) Biochim. Biophys. Arch. **688**, 429-440

28. Polakis, P.G., Wilson, J.E. (1985) Biochem. Biophys. Arch. **236**, 328-337

29. Brown, J.L., Roberts, W.K. (1976) J. Biol. Chem. **251**, 1009-1014

30. Lebherz, H.G., Bates, O.J., Bradshaw, R.A. (1984) J. Biol.Chem. **259**, 1132-1135

31. Kurakawa, M., Yokoyama, K., Kaneko, M., Ishibashi, S. (1983) Biochem. Biophys. Res. Commun. **115**, 1101-1107

32. Xie, G., Wilson, J.E. (1990) Biochem. Biophys. Arch. **276**, 285-293

33. Gottlob, K., Majewski, N., Kennedy, S., Kandel, E., Robey, R.B., Hay, N. (2001) Genes Dev. **15**, 1406-1418

34. Vyssokikh, M.Y., Zorova, L., Zorov, D., Heimlich, G., Jurgensmeier, J.J., Brdiczka, D. (2002) Mol. Biol. Rep. **29**, 93-96

35. Azoulay-Zohar, H., Israelson, A., Abu-Hamad, S., Shoshan-Barmatz, V. (2004) Biochem. J. **377**, 347-355

36. Birnbaum, M.J. (2004) Dev. Cell. **7**, 781-782

37. Fromm, H.J., Zewe, V. (1962) J. Biol. Chem. **237**, 1661-1667

38.Skaff, D.A., Kim, C.S., Tsai, H.J., Honzatko, R.B., Fromm, H.J. (2005) J. Biol. Chem **280**, 38403-38409

39. Mehyar, N., "Human brain hexokinase : Determinants of mitochondrial binding and mechanism of nucleotide release" (2011). Graduate Theses and Dissertations. Paper 12226.

40. Gelb, B.D., Adam, V., Jones, S.N., Griffin, L.D., MacGregor, G.R., McCabee, E.R. (1992) Proc. Natl. Acad. Sci. USA. **89**, 202-206

41. Ma, Y., Taylor, S. (2002) J. Biol. Chem. **277**, 27328-27336

42. Sherman, F., Stewart, J.W., Tsunasawa, S. (1985) BioEssays. **3**, 27-31

43. Ben-Bassat, A., Bauer, K., Chang, S.K., Ambo, K.M., Boosman, A., Chang, S. (1987) J. Bacteriol. **169**, 751-757

44. Hirel, P.H., Schmitter, J.M., Dessen, P., Fayat, G., Blanquet, S. (1989) Proc. Natl. Acad.

Sci. USA. **86**, 8247-8251.

45. Wicker, U., Bucheler, K., Gellerich, F.N., Wagner, M., Kapische, M., Brdiczka, D.

(1993) Biochimica et Biophysica Acta, **1142**, 228-239.

CHAPTER 2: ROLE OF THE N-TERMINUS OF RECOMBINANT HUMAN BRAIN HEXOKINASE IN MITOCHONDRIAL BINDING

Manjit Maskey and Richard Honzatko

Abstract

The association of hexokinase I and II (hereafter, HKI and HKII, respectively) with the mitochondrion arguably stands as a checkpoint in some pathways of apoptosis. HKI (often called brain hexokinase) requires N-terminal residues 1 through 15 for mitochondrial binding. Ala4 and Ala8 of HKI play critical roles in mitochondrial binding, whereas Thr12 is far less important. Met1 would define a contiguous surface with Ala4 and Ala8, assuming an α-helical structure for these N-terminal residues. Recombinant human HKI, as is the case for the native enzyme from rat, cannot be sequenced due to a blocked amino terminus. Mutation of Ile2 to threonine, enables an *Escherichia coli* expression system to cleave the Met1 from the N-terminus. The resulting enzyme (Ile2Thr HKI) does not bind to the mitochondrion. The addition of four residues to the N-terminus by the duplication of residues 1-4, results in a protein with a blocked N-terminus and wild-type properties of mitochondrial binding. The mutation of Ile(position –2) to threonine again causes cleavage of methionine (position –3)from the N-terminus, but does not reduce mitochondrial association. The mutation of Ile2 to threonine in the extended construct has no effect, but the mutation of Met1 to leucine greatly reduces mitochondrial association. The results indicate an important and perhaps an essential role for the side chain of position 1 (methionine) in

mitochondrial association. Blockage of the amino terminus does not appear to be a critical factor in mitochondrial association of HKI.

Introduction

Hexokinase I (ATP: D-hexokose 6-phosphotransferase, EC2.7.1.1) catalyzes the initial crucial step of glycolysis, phosphorylating glucose (Glc) to produce glucose-6-phosphate (Glc-6-P), using $[ATP\text{-}Mg]^{2-}$ as the phosphoryl donor (1-3).

$$Glucose + [ATP\text{-}Mg]^{2-} \longrightarrow Glc\text{-}6\text{-}P^{2-} + [ADP\text{-}Mg]^{1-} + H^{+}$$

Four different kinds of hexokinase have been identified in mammalian tissues (4) – types I, II, III, and IV. Except Type IV hexokinase which has molecular weight of 50 kDa, other mammalian hexokinases have molecular weights of 100 kDa (5). Mammalian hexokinase types share approximately 70% of sequence identity (5). Even N- and C-terminal halves of isozymes from I to III show extensive sequence similarity of about 50% and structure similarity (5). These sequence similarities between mammalian isoenzymes evidently originate from the duplication and fusion of a primordial hexokinase gene similar to that of Type IV hexokinase and yeast hexokinase (6). The properties of a hexokinase type does not differ vary from one tissue to another, but the amount each isozyme differs depending on age and nutritional factors (7). Hexokinase I (HKI), also known as brain hexokinase, can be inhibited by the number of ligands including Glc-6-P which is also the product of the reaction (4). Glc-6-P inhibits brain hexokinase catalysis by binding to either the C- or N-terminal halves with high affinity (8, 9). Inorganic orthophosphate (Pi) under normal

physiological conditions can decrease the inhibition caused by the Glc-6-P (10) through an allosteric mechanism that couples both halves of the enzyme (11, 12).

Even though HKI is a dimer in crystals (13, 14), the monomer is the dominant form in solution. HKI has two structurally similar halves (N-terminal and C-terminal halves), separated by a transition helix (residues 448 to 475) (14-20). The N-terminal half of the brain hexokinase is catalytically inactive, but contains a binding pocket for Glc and Glc-6-P which is nearly identical to that of the C-terminal half (14). The crystal structure also reveals that N-terminal half has a high-affinity binding site for the Pi which overlaps the binding site for the 6-phosphoryl group of Glc-6-P (14, 16). Glc-6-P, however, inhibits brain hexokinase by binding to either half of the enzyme (21).

Hexokinases I and II bind to mitochondria. The binding property requires the first 15 residues of the polypeptide. None of the residues have formal electrostatic charge, and the amino terminus is blocked for HKI isolated from rat brain (22-25). Xie and Wilson (25) proposed that the N-terminal segment is in an α-helical conformation, and Mulichak *et al.* (17) revealed that segment as a helix in a crystal structure. Mitochondrial association may give HKI a competitive advantage over other cytosolic transphosphorylases for mitochondrially synthesized ATP (18); however, more recent work suggests that the mitochondrial-hexokinase complex acts as a checkpoint in apoptosis (42-44). Targeting of HKI to the mitochondrion may be the result of specific interactions between HKI and the voltage-dependent anion channel (VDAC) of outer mitochondrial membrane (45, 46).

N-terminal sequence of brain hexokinase is

X-*NH-Met-Ile-Ala-Ala-Gln-Leu-Leu-Ala-Tyr-,*

where X is a blocking group (most probably an acetyl group) on the amino terminus of Met 1. Modification of this hydrophobic N-terminal segment by the endogenous proteases in crude brain extracts results in the loss HKI-mitochondrial binding properties (26). Mehyar (27) has determined critical roles for Ala4 and Ala8. Mutations here individually to leucine cause a 10-fold decrease in HKI binding affinity to mitochondria (27). Ala4 and Ala8 as part of an α-helix form a contiguous surface that does not tolerate large hydrophobic side chains (27), and presumably is in contact with the membrane buried surface of VDAC.

N-terminal sequencing of HKI (native or recombinant) by Edman degradation has always been unsuccessful because of blocking groups at the amino terminus. N-terminus of the recombinant human enzyme from *Escherichia coli* expression systems is blocked (presumably by the *N*-formyl group); however, methionine aminopeptidase (MAP) in *E. coli* removes methionine from position 1 depending on the residue type at position 2 of the polypeptide chain (28, 29). The mutation of Ile2 to threonine should enable MAP modification of wild-type HKI. The resulting enzyme should begin with Thr2, and be amendable to sequencing by Edman degradation. In fact, this hypothesis is confirmed here by experiment. Ile2Thr HKI can be sequenced by Edman degradation, and moreover, does not bind to the mitochondrion. The loss of mitochondrial binding can be due to the presence of a free amino terminus, the mutation of Ile2 to threonine, or to the loss of methionine from position 1. Shown here by the properties of various constructs of HKI, the change in Ile2 to threonine and the presence of a free amino terminus are not responsible for the loss of the mitochondrial binding property. Instead, the side chain of methionine at position 1 is critical

to wild-type binding properties. Hence, Met1, along with Ala4 and Ala8 define a contiguous surface on the N-terminal helix of HKI that is critical to mitochondrial association.

Experimental Procedures

Materials— Adenosine triphosphate (ATP), Nicotinamide adenine dinucleotide phosphate (NADP), ampicillin, deoxyribonuclease I (DNase I), bovine serum albumin (BSA), leupeptin, protease cocktail inhibitor and phenylmethylsulfonyl fluoride (PMSF) was purchased from Sigma. Diethylaminoethyl (DEAE) high performance liquid chromatography (HPLC) resin was from Tosohaas. *E. coli* strain BL21 (DE3) competent cells were from Novagen. Glc-6-P dehydrogenase was bought from Roche Molecular Biochemicals. I sopropyl-1-thio-β-D-galactopyranoside (IPTG) came from Anatrace. Sequencing polyvinylidene fluoride (PVDF) membranes were purchased from Applied Biosystems.

Construction of Wild-type Hexokinase and Mutant Plasmids— Human brain hexokinase (HKI) has been cloned into pET-11a as reported in a previous study (31). Hexokinase mutants were created through PCR modification with oligonucleotide primers synthesized at DNA Sequencing and Synthesis Facility of Iowa State University. Mutants Ile2Thr, MIA-HKI, MIAA-HKI, MTAA-HKI, MIAA-Met1Leu, and MIAA-Ile2Thr were created using following forward primers (and their reverse complements), respectively:

5’-GGAGATATACATATGACCGCCGCGCAGCTCCTG-3’;

5’-GGAGATATACATATGATCGCCATGATCGCCGCGCAGCTCC-3’;

5’-GGAGATATACATATGATCGCCGCGATGATCGCCGCGCAGCTCC-3’;

5’-GGAGATATACATATGACCGCCGCGATGATCGCCGCGCAGCTCCTG-3’;

5’-GGAGATATACATATGATCGCCGCGCTGATCGCCGCGCAGCTCCTG-3’; and

5’-GGAGATATACATATGATCGCCGCGATGACCGCCGCGCAGCTCCTG-3’

All constructs were confirmed through sequencing of the entire gene by the DNA Sequencing and Synthesis Facility at Iowa State University.

Expression and Purification of Wild-type and Mutant Hexokinase— Wild-type or mutant pET-211a-HKI plasmids were transformed into *E. coli* strain BL21 (DE3). Expression and purification of HKI constructs here followed protocols in the literature (31). The concentration of the protein was measure by the Bradford method with bovine serum albumin as a standard. Protein purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Pig Liver Mitochondria Isolation— Fresh pig livers were obtained from the Meat Laboratory of Iowa State University. Mitochondrial purification is described (32) with modifications as discussed in (1). Both outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) integrities were measured as described previously (33, 34). Mitochondria were stored at –80°C in the presence of storage buffer.

The HKI Activity Assay— Hexokianse I activity was measured using the glucose-6-phosphate dehydrogenase (G6PDH)-coupled spectrophotometric assay at pH 8.0. Assay solutions have 3 mM $MgCl_2$, 3 mM DTT, 0.3 mM NADP and 10 µg/ml G6PDH in 50 mM Tris-HCl (pH 8.0) and concentrations of Glc and $[ATP\text{-}Mg]^{2-}$ that varied from 1/5 x K_m to 5 x K_m.

NADPH production was monitored at wavelength of 340 nm. Assays were initiated by the addition of enzyme and linear progress curves monitored for 2 minutes. Slopes of progress curves determined initial rates in µmole/min. The capacity of the coupling enzymes was verified by observing two-fold increase in velocity with a doubling of added hexokinase. Fits of the Michaelis-Menten model to initial velocity data used the software GraFit (35).

Hexokinase Mitochondrial Binding— Purified HKI was dialyzed twice against mitochondrial-binding buffer (250 mM sucrose, 5 mM Glc, 50 mM NaCl, 5 mM $MgCl_2$ 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 11000 x *g* for 5 minutes at 4°C. 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 30 minutes on ice, the mixture of HKI and mitochondria was centrifuged at 11000 x *g* for 5 minutes. Unbound HKI remained in the supernatant fluid and was separated from the pellet. Pelleted mitochondria were suspended in mitochondrial-binding buffer, less the $MgCl_2$, NaCl, and glucose, and centrifuged again. This was step was twice-repeated.

N-terminal Sequencing— A thin piece of polyvinylidene fluoride (PVDF) was activated using 100% methanol. PVDF membrane was washed a couple of times with dionized-distilled water and dipped into 2 mg/ml hexokinase overnight at 4°C. The PVDF was taken out of the protein solution and rinsed sparingly with water before submitting to the Protein Facility at Iowa State University.

Results

Rationale for HKI mutants— The N-terminal 15 residues forms an α-helix. As shown previously by Mehyar (27), mutations individually of Ala4 and Ala8 to leucine eliminate hexokinase binding to the mitochondrion. Ala4 and Ala8 lie on the same face of the helix, and in a recent model that face is in contact with the outer surface of the β-barrel of VDAC (27). Met1 extends the surface defined by Ala4 and Ala8.

The mutation of Ile2 to threonine should allow *E. coli* methionine aminopeptidase (MAP) to cleave methionine from the N-terminus, putting Thr2 at the lead position of the polypeptide chain. Hence, the N-terminal sequence of Ile2Thr should be *Thr-Ala-Ala-Gln-Leu-* rather than *Met-Thr-Ala-Ala-Gln-*. Thr2-HKI should have an unblocked N-terminal residue and be amenable to Edman degradation and sequencing.

As subsequent experiments revealed the loss of mitochondrial binding due to the Ile2Thr mutation further constructs were designed to determine the cause of property-loss (free N-terminus, the mutation of position 2 to threonine and/or the elimination of methionine at position 1). Constructs MIA-HKI, MIAA-HKI, MTAA-HKI, MIAA-Ile2Thr, and MIAA-Met1Leu, where the leading letters represent the single-letter code of additional amino acids, are simply the duplication of the first three residues of wild-type HKI (MIA), first four residues of wild-type HKI (MIAA) or the first four residues of Ile2Thr HKI (MTAA). Assuming either or both MIA-HKI and MIAA-HKI would have wild-type binding properties, MTAA-HKI would introduce a free amino terminus and retain methionine and isoleucine at positions 1 and 2, respectively. MIAA-Ile2Thr would test whether the mutation at position 2

influences binding properties. MIAA-Met1Leu would test whether the mutation a position 1 influences binding properties.

Protein Purity and N-terminal Sequencing— Protein is at least 95% as on the basis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). As the exact status of the N-terminus is an important aspect of work here, each purified construct was subjected to Edman degradation reactions. N-terminal sequencing of the purified wild-type- HKI, MIA-HKI, MIAA-HKI, MIAA-Met1Leu and MIAA-Lle2Thr provide no sequence information even with samples delivery 100 pmoles of protein. Ile2Thr and MTAA-HKI gave intense chromatographic peaks clearly indicating a sequence of *Thr-Ala-Ala-Gln-Leu-* and *Thr-Ala-Ala-Met-Ile-*, respectively.

Kinetics of Wild-type and Mutant Hexokinases— The kinetic parameters of the different mutants are comparable to those of wild-type HKI (Table 1). As the N-terminal segment can be remove entirely without influencing catalysis or metabolite regulatory properties of HKI, the lack of any effect on basic kinetic properties is an expected outcome.

Table 1.**Kinetic parameters for wild-type and mutant HKI constructs**

HKI Construct	**k_{cat}(sec^{-1})**	**K_m^{ATP}(mM)**	**K_m^{Glc}(µM)**
wild-type	73.00	0.80	120
Ile2Thr	74.33	0.8436	126.38
MIA-HKI	71.16	0.777	79
MIAA-HKI	65.26	0.884	85.37
MTAA-HKI	70.43	1.09	126.39
MIAA-Ile2Thr	74.00	0.948	76.21
MIAA-Met1Leu	68.42	0.805	90

Table 1. Footnote.

Determination of k_{cat} employed concentration of Glc and ATP of 1.6 mM and 9 mM. Determination of K_m^{ATP} employed a Glc concentration of 1.6 µM and concentrations of ATP from 0.3–5 mM. Determination of K_m^{Glc} employed an ATP concentration of 9 mM and concentrations of Glc of 10–600 µM.

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

Mitochondrial Binding of Wild-type and Mutant Hexokinases— The isolated mitochondria are devoid of native hexokinase activity. Fig. 1 shows that the binding response curve of wild-type hexokinase from my result is comparable to the wild-type hexokinase binding experiment performed by Mehyar (27). Fig.1 and Fig. 2 shows that with the increase in the hexokinase concentration, its binding to the mitochondria increase in most of the purified proteins.

Scheme I represents the simplest equilibrium model that accounts for the binding of the wild-type and mutant hexokinase to the mitochondria.

$$E + Q \leftrightarrow EQ$$

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

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 the previous work, non-specific binding to the mitochondrion by HKI does not occur at the significant levels. The ratio of enzyme bound to the total enzyme, $E_{o,}$ is as follows

$$R([E]) = (K_1[E]) / (1+K_1[E]) \qquad \text{Eq. 1}$$

The concentration of the free specific mitochondrial sites Q is itself a function of the concentration of the enzyme. Algebraic manipulation of the equilibrium expression in the Scheme I and the relationship for mass conservation of total enzyme E_o and total specific sites Q_o gives 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]:

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

Substitution of Eq. 2 into the Eq. 1provides 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

$$V = sR(E_o, Q_o, K_1) \qquad \text{Eq. 3}$$

where 's' is a proportionality constant that relates the fraction of bound enzymes R to the velocity V in µmoles/min. Undefined quantities s, E_o, Q_o, and K_1 cannot be determined by a nonlinear least square fit of the data from a single experiment. Thus, values for some of these quantities must come from other determinations. Firstly, one assumes that the HKI-VDAC complex has an equal number of HKI and VDAC subunits. The value for Q_o (7.1×10^{-8} M) then is an estimate based on 42000 VDAC molecules per mitochondrion (36), 7.2×10^9 mitochondria per mg of total mitochondrial protein (37), and 0.013 mg of mitochondria in each 100 µl assay. Moreover, $s = Q_o$ x (specific activity) x (assay volume), where the specific

activity for HKI is $6x10^9$ µmole $min^{-1}mg^{-1}$ the assay volume is in liters. The data are fit to Eq. 3 using K_1 as the sole adjustable parameter.

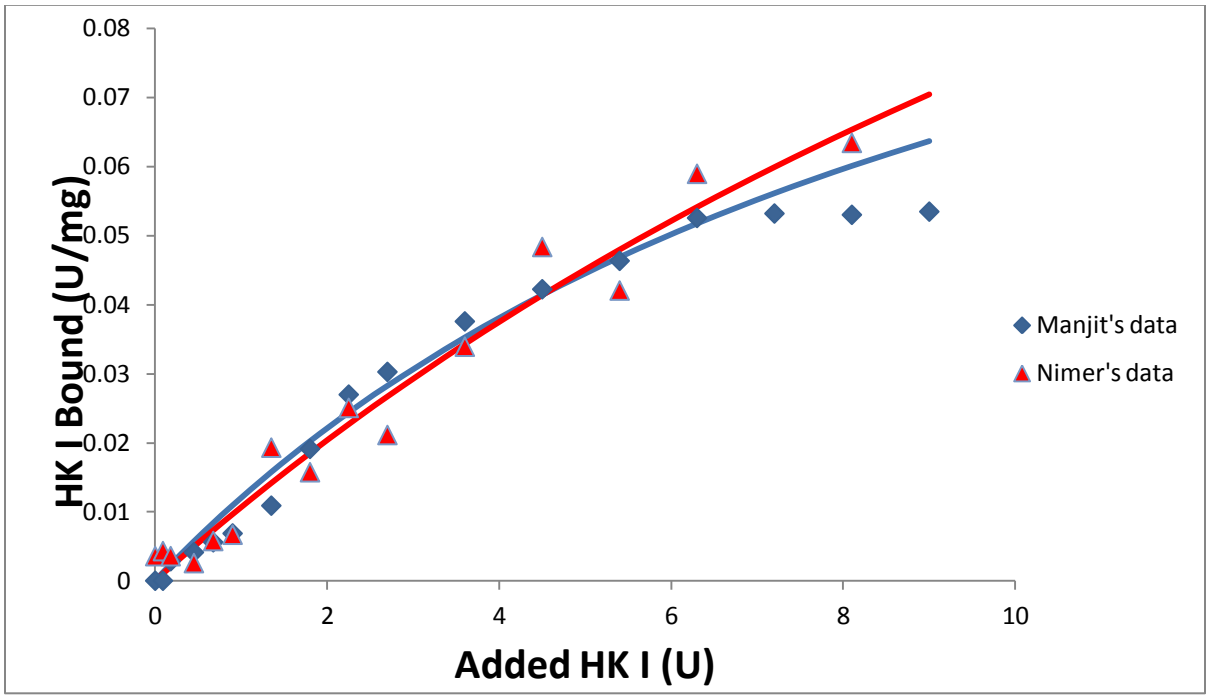

Fig. 1. **HKI binding to the pig liver mitochondria.** Plot represents the rate from varying concentration of wild-type HKI, from Mehyar's (27) (▲) data and my data (♦), bound to 1.7 mg, wet weight mitochondria in each fraction in 250 mM sucrose and 50 mM HEPES, pH 7.4. The solid lines represent fitted curves using Eq. 3 with the parameters mentioned in the Result section and Table 2.

Table 2**. Parameters for wild-type and mutant HKI binding to the pig liver mitochondria.**

HKI construct	**Association constant K_1 (M^{-1})**
Wild-type	1.8×10^4
Ile2Thr	$.21 \times 10^4$
MIA-HKI	2.35×10^4
MIAA-HKI	2.9×10^4
MTAA-HKI	1.95×10^4
MIAA-Ile2Thr	0.94×10^4
MIAA-Met1Leu	0.32×10^4

Table 2. Footnote.

Parameter K_1 is defined in the Results section. HKI bound to 1.7 mg, wet weight mitochondria in each fraction in 250 mM sucrose and 50 mM HEPES, pH 7.5 was measured by activity.

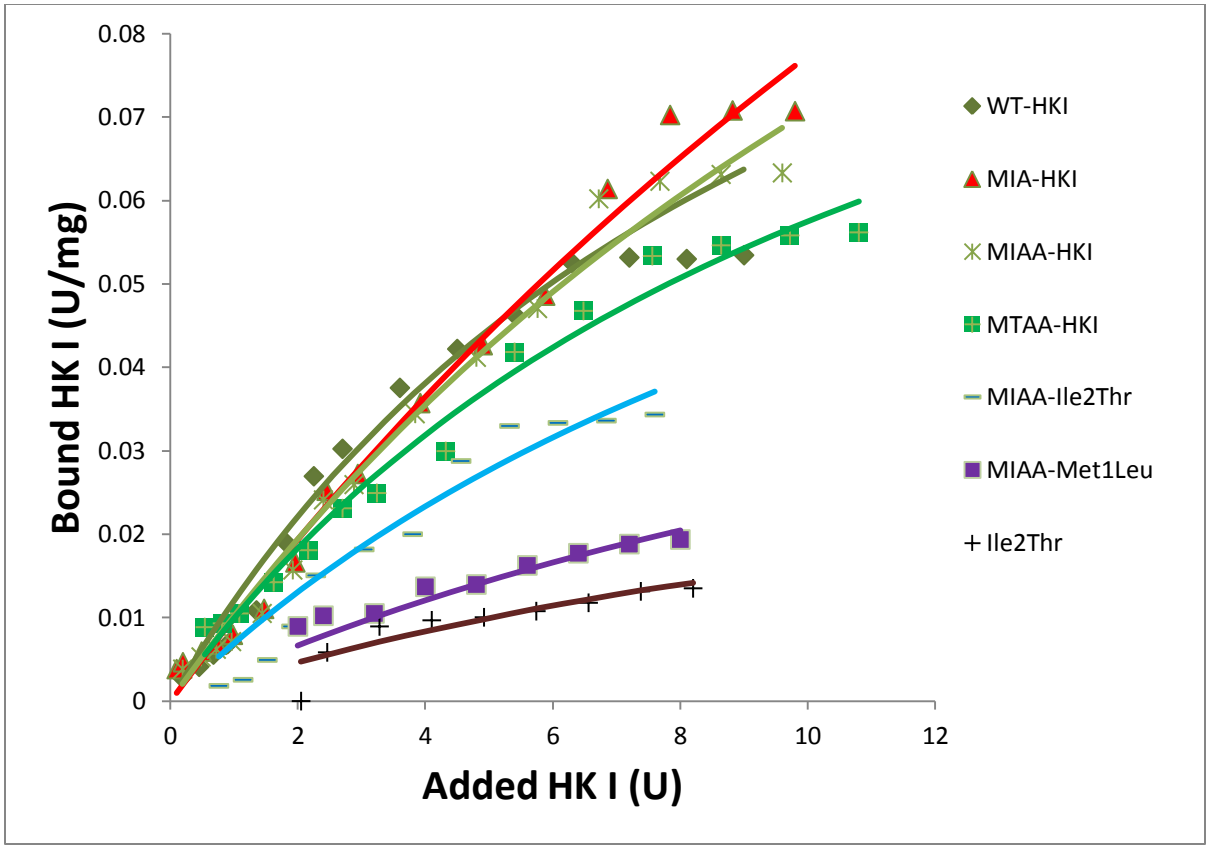

Fig. 2. **HKI binding to pig liver mitochondria.** Plot represent vrlocities from varying concentrations of wild-type (♦), MIA-HKI (▲), MIAA-HKI (Ж), MTAA-HKI (■), MIAA-Ile2Thr (–), MIAA-Met1Leu (■), and Ile2Thr (+) bound to 1.7 mg, wet weight mitochondria in each fraction in 250 mM sucrose and 50 mM HEPES, pH 7.5. The solid lines represent fits using Eq. 3 with the parameters given in the Results section and Table 2.

Discussion

Various mechanisms can be responsible for the disruption of HKI-mitochondrial binding. Mutations might cause a global conformational change in HKI, promoting aggregation and conformational states not conducive to mitochondrial association. Parameters from kinetics of the mutant constructs employed here are indistinguishable from those of wild-type HKI, ruling out global conformational changes. Similar results were obtained for mutations of other residues of the N-terminal segment (27).

Pittler *et al.* (38) proposed that an N-acetyl group is present at the N-terminus of native rat brain hexokinase. Wild-type recombinant HKI resists Edman degradation due to the absence of a free amino group at the N-terminus. This has been demonstrated indirectly by the Ile2Thr enzyme, which lacks methionine from position 1 and is amenable to N-terminal chemical sequencing. The N-terminal blocking moiety could be the N-formyl group; however, repeated attempts to deblock by standard chemical protocols failed. There are reports of recombinant proteins expressed in *E. coli* with an N-acetyl group at the N-terminus (39). An acetyl group at the N-terminus cannot be removed chemically without extensive hydrolysis of the polypeptide chain, rendering the modification irreversible. N-terminal acetylation of the methionine is of great significance to many proteins, especially to the cytosolic proteins which need to escape the transmembrane localization during their translocation process (47). The status of the N-terminus could greatly influence the affinity of HKI for the mitochondria, and hence constructs were designed and tested for the effect of a free terminal amino group on binding properties. Although Ile2Thr has relatively low binding affinity for the mitochondrion, MTAA-HKI has a free amino terminus and yet binds

to mitochondria with affinity comparable to that of wild-type enzyme and MIAA-HKI. There is no indication that HKI requires a blocked N-terminus in order to bind to the mitochondrion.

The acetyl-linked N-terminal methionine is critical for some protein-protein interactions (40). In such cases, N-acetyl methionine can be without formal charge in a hydrophobic environment, whereas a positively charged free amino group would be disfavored (40). Evidently a blocked N-terminus for HKI is not essential for mitochondrial association, but instead could eliminate uncontrolled chemistry. A membrane-buried amino group would necessarily be neutral (unprotonated) and reactive as a nucleophile. Over time, an unblocked N-terminus could react indiscriminately with electrophilic centers in other membrane-associated proteins. The resulting cross-linked proteins would contribute to a pool of damaged proteins that could interfere with normal regulatory processes.

Hydrophobic surfaces contribute to the interaction of HKI with the mitochondrion (41), but the specific binding of the brain hexokinase to only mitochondria cannot be explained by a nonspecific hydrophobic interaction with the lipid bilayer (25). Indeed, mutations of Ala4 and Ala8 to leucine eliminate HKI binding to mitochondria, indicating a specific protein-protein interaction that targets HKI to the outer membrane of the mitochondrion (27). Coarse grain modeling (Yang Gao, and Richard Honzatko, unpublished) indicates that the surface defined by Ala4 and Ala8 is spatially contiguous and has a preferred (low energy) placement on the surface of the β-barrel of VDAC. In that coarse-grain model of a VDAC-HKI complex, the center of mass of the N-terminal half of HKI is over the pore of VDAC, in proximity to residues that are significant to high affinity HKI-mitochondrial binding. The

model indicates that Met1 and to a lesser extent Ile2 could also participate in hexokinase-mitochondrial interactions.

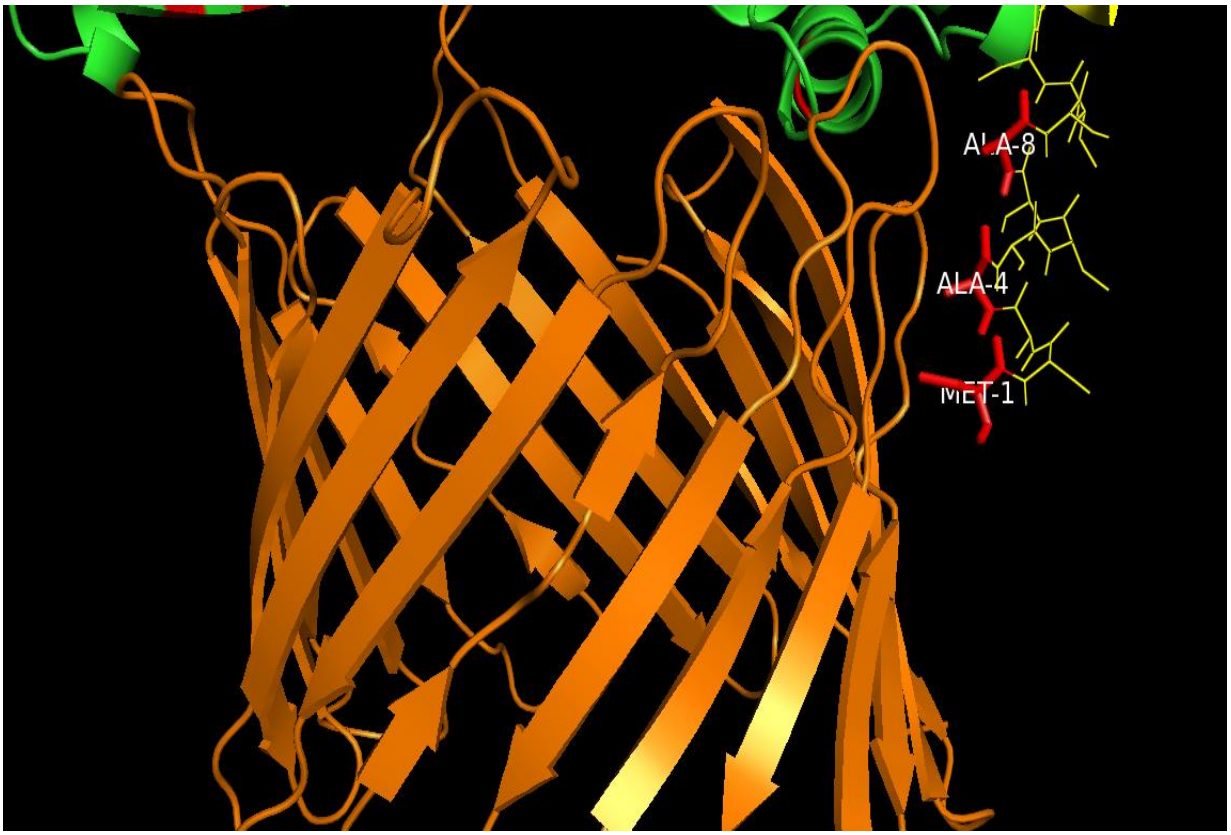

Fig 3. Closer look at the residues of hexokinase interacting with a particular side of the VDAC. Residues colored red are Met1, Ala4, and Ala8

To investigate the role of Ile2 and Met1 in binding interactions, residues (Met-Ile-Ala-Ala) were introduced at the N-terminus, duplicating the first four residues of wild-type HKI. The addition of four residues did not alter binding affinity for the mitochondrion relative to wild-type enzyme. The mutation of Ile2 to threonine of this elongated construct (MIAA-Ile2Thr) has little effect on HKI-mitochondrial binding; however, the mutation of Met1 to leucine

(MIAA-Met1Leu) causes a significant reduction to binding affinity. This result is consistent with the current course-grain model in that Met1 extends the contiguous surface defined by Ala4 and Ala8 (Fig. 5). Moreover, as leucine is a conservative replacement for methionine that often does not cause a significant change in protein conformation, the observation of a significant effect here indicates a highly specific level of recognition of the side chain of methionine by the interacting partner.

References

1. Gonzalez, C., Ureta, T., Sanchez, R., Niemeyer, H. (1964) Biochem. Biophys. Res. Commun. **16**, 347-352

2. Grossbard, L., Schimke, R.T. (1996) J. Biol. Chem. **241**, 3546-3560

3. Katzen, H.M. (1967) Adv. Enzyme Regul. **5**, 335-356

4. Crane, R.K., Sols, A. (1954) J. Biol. Chem. **210**, 597-606

5. Wilson, J.E. (1995) Rev Physiol Biochem. Pharmacol. **126**, 65-198

6. Wilson, J.E. (2003) J. Exp. Biol. **206**, 2049-2057

7. Katzenm, H. M., Schimke, R.T. (1965) Proc. Natl. Acad. Sci. USA **54**, 1218-1225

8. Ureta, T. (1975) in *Isozymes III* (Markert, C L,ed) pp 575-601, Academic Press Inc., New York, NY

9. Fromm, H J. (1981) in *The Regulation of Carbohydrate Formation and Utilization in Mammals* (Veneziale, C M,ed) pp 45-68, University Park Press, Baltimore, MD

10. Rose, I.A., Warms, J.V., O'Connell, E.L. (1964) Biochem. Biophys. Res. Commun. **15**, 33-37

11. Tsai, H.J., Wilson, J.E. (1995) Arch. Biochem. Biophys. **316**, 206-214

12. Aleshin, A.E., Zeng, C., Bartunik, H.D., Fromm, H. J., Honzatko, R.B. (1998) J. Mol. Biol. **282,** 345-357

13.Aleshin, A., Zeng, C., Bartunik, H.D., Fromm, H.J. Honzatko, R.B. (1998) J. Mol. Biol. **282**, 345-357

14. Aleshin, A., Zeng, C., Bourenkov, G.P., Bartunik, H.D., Fromm, H.J., Honzatko, R.B. (1998) Structure. **6**, 39-50

15. Aleshin, A.E., Malfois, M., Liu, X., Kim, C., Fromm, H.J., Honzatko, R.B., Koch, M.H., Svergun, D.I. (1999) Biochemistry. **38**, 8359-8366

16.Aleshin, A., Zeng, C., Bartunik, H.D., Fromm, H.J. Honzatko, R.B. (1998) J. Mol. Biol. **282**, 345-357

17. Mulichak, A.M., Wilson, J.E. Padmanabhan, K., Garavito, R.M.(1998) Nat. Struct. Biol. **5**, 555-560

18. Aleshin, A.E., Fromm, H.J., Honzatko, R.B. (1998) FEBS Lett. **434**,42-46

19. Aleshin, A.E., Kirby, C., Liu, X., Bourenkov, G.P., Bartunik, H.D., Fromm, H.J., Honzatko, R.B. (2000) J. Mol. Biol. **296**, 1001-1015

20. Smith, A.D., Wilson, J.E. (1991) Arch. Biochem. Biophys. **291**, 59-68

21. Liu, X., Kim, C.S., Kurbanov, F.T., Honzatko, R.B., Fromm H.J. (1999) J. Biol. Chem.

274, 31155-31159

22. Rose, I., Warms, J.V.B. (1967) J. Biol. Chem. **242**, 1635-1645

23. Kropp, E.S., Wilson, J.E. (1970) Biochem. Biophys. Res. Commun. **38**, 74-79

24. Felgner, P.L., Messer, J.L., Wilson, J.E. (1979) J. Biol. Chem. **254**, 4946-4949

25. Xie, G., Wilson, J.E. (1988) Biochem. Biophys. Arch. **267**, 803-810

26. Polakis, P.G., Wilson, J.E. (1985) Biochem. Biophys. Arch. **236**, 328-337

27. Mehyar, N., "Human brain hexokinase : Determinants of mitochondrial binding and mechanism of nucleotide release" (2011). Graduate Theses and Dissertations. Paper 12226.

28. Sherman, F., Stewart, J.W., Tsunasawa, S. (1985) BioEssays. **3**, 27-31

29. Ben-Bassat, A., Bauer, K., Chang, S.K., Ambo, K.M., Boosman, A., Chang, S. (1987) J. Bacteriol. **169**, 751-757

30. Hirel, P.H., Schmitter, J.M., Dessen, P., Fayat, G., Blanquet, S. (1989) Proc. Natl. Acad.

Sci. USA. **86**, 8247-8251

31. Skaff, D.A., Kim, C.S., Tsai, H.J. Honzatko, R.B., Fromm H.J. (2005) J. Biol. Chem.

280, 38403-38409

32. Graham, J.M. (1993) Methods. Mol. Biol. **19**, 29-40

33. Wojtczak, L., Zaluska, H., Wroniszewska, A., Wojtczak, A.B. (1972) Acta. Biochim. Pol. **19**, 227-234

34.Rice, J.E., Lindsay, J.G. (1997) in Subcellular Fractionation, A Practical Approach (Graham, J.M., Rickwood, D., et al) pp 107-142, Oxford University Press, New York, NY

35. Leatherbarrow,, R.J. (2001) GraFit, Version 5, Erithacus Software Ltd., Horley, UK

36. Aleshin, A.E., Fromm H.J., Honzatko, R.B. (1998) FEBS Lett. **434**, 42-46

37. Estabrook, R.W., Holowinsky, A. (1961) J. Biophys. Biochem. Cytol. **9**, 19-28

38. Pittler, S.J., Kozak, L.P., Wilson, J.E. (1985) Biochim. Biophys. Acta. **843**, 186-192

39. Charbaut, E., Redeker, V., Rossier, J., Sobel, A. (2002) FEBS. **529**, 341-345

40. Scott, D.C., Monda, J.K., Bennett, E.J., Harper, J.W., Schulman, B.A. (2011) Science. **334**, 674-678

41. Ehsani-Zonouz, A., Golestani, A., Nemat-Gorgani, M. (2001) Mol. Cell. Biochem. **223**, 81-87

42. Pastorino, J.G., Hoek, J., Shulga, N. (2005) Cancer Res. **65**, 10545-10554

43. Pastorino, J.G., Hoek, J., Shulga, N. (2002) J. Biol. Chem. **277**, 7610-7618

44. Robey, R., Hay, N. (2005) Cell Cycle. **4,** 654-658

45. Linden, M., Gellerfors, P., Nelson, B.D. (1982) FEBS Lett. **141**, 189-192

46. Fiek, C., Benz, R., Roos, N., Brdiczka, D. (1982) Biochim. Biophys. **688**, 429-440

47. Forte, G.M.A., Pool, M.R., Stirling, C.J. (2011) PLoS Biol. **9** (5)

CHAPTER 3: GENERAL CONCLUSIONS

The hydrophobicity of 15 N-terminal residues does not explain the specificity of HKI binding to the outer membrane of the mitochondrion. Selective mutations among the first 15 residues in past investigations indicated the significance of Ala4 and Ala8 in the interaction, but the significance of Met1, which is adjacent to Ala4 assuming a helical structure, was untested. Experiments here clearly indicate the importance of the side chain of Met1 in the HKI-mitochondrion interaction, rather than the presence of a blocked amino terminus. The surface defined by positions 1, 4 and 8 is contiguous and must couple tightly with a target protein as even relatively conservative changes abolish interactions with the mitochondrion. Mutations here represent the first test of a recent model that putatively represents the atomic-resolution interaction of the voltage-dependent anion channel (VDAC) with HKI, and happily the model has survived. Additional mutations of HKI in regions other than the N-terminal segment will represent more stringent tests of the current model for the VDAC-HKI complex.

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