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An Investigation of *Trypanosoma brucei* Hexokinases: Localization, Oligomerization, and Inhibition

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AN INVESTIGATION OF *TRYPANOSOMA BRUCEI* HEXOKINASES: LOCALIZATION, OLIGOMERIZATION, AND INHIBITION

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biochemistry and Molecular Biology

by
April Coley Joice
May 2012

Accepted by:
Dr. James Morris, Committee Chair
Dr. Lesly Temesvari
Dr. William Marcotte
Dr. Alex Feltus
ABSTRACT

Trypanosoma brucei is the causative agent of African sleeping sickness in humans and nagana in livestock. The parasite inhabits multiple environmental niches including the bloodstream of the mammalian host and the mid-gut of the tsetse fly vector. While in the bloodstream of its mammalian host, the organism depends solely on glycolysis for production of ATP. My studies focus on the first enzyme in glycolysis, hexokinase.

T. brucei has two hexokinases, TbHK1 and TbHK2 that are 98.5% identical at the nucleotide level. The hexokinases are expressed in the glycosomes of both procyclic form and bloodstream form parasites. Glycosomes are peroxisome-like organelles that house glycolysis, along with other functions, in trypanosomes. Here I present evidence that PEX7, a known glycosome import protein in other systems, may be responsible for the import of TbHKs into the glycosome. Further, I report that, in addition to the glycosome, TbHK2 also localizes to the flagellum of BSF parasites and to two punctate bodies proximal to the basal body in PF parasites.

TbHK1 has also been validated as a drug target for treatment of African trypanosomiasis. A high throughput screen has been conducted in which 220,233 compounds were tested against TbHK1 for inhibitory effects. In this screen, ebselen was identified as a potent inhibitor of TbHK1. Ebselen is a known cysteine-reactive seleno-organic compound. Here I show that while ebselen does indeed bind the Cys residues of TbHK1, this binding does not result in inhibition of the enzyme. Lastly, I show that three
Cys residues are necessary for the activity of TbHK1. These studies have improved our knowledge of the localization, inhibition, and oligomerization of TbHKs.
DEDICATION

To my husband and family:

Chad - I could not have done this without your love and support.

Dad, Ginny, and Dave - You have taught me that it takes hard work to get where you want to go in life, and you have supported me in my journey to get here.
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Most importantly, I would like to thank my husband, Chad Joice, my family, and friends, without whom this work would not have been possible.
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CHAPTER ONE
LITERATURE REVIEW

_Trypanosoma brucei_ is the protozoan parasite that is the causative agent of African sleeping sickness (also known as human African trypanosomiasis, or HAT) in humans and nagana in livestock. Together, HAT and nagana are a major cause of rural underdevelopment in mainly poor and remote regions of sub-Saharan Africa (1). HAT was almost eliminated in the mid-1960s due to an increase in surveillance and diagnosis in HAT-endemic areas (1). In the late 1990s, however, there was a resurgence of infections primarily due to civil war and social upheaval, which prevented access to the HAT-endemic areas and therefore disrupted surveillance and diagnosis programs (2). The World Health Organization recently reported that in 2009 the number of new cases reported dropped below 10,000 for the first time in 50 years. If one accounts for under-reporting, the actual number of cases probably approaches ~30,000. However, the lack of sensitive and specific diagnostic tests and a drug that is inexpensive, safe, and easily administrated remain the two main obstacles for elimination of HAT (3).

There are two clinical stages of HAT: the early hemolymphatic stage and the late encephalitic stage (4). The early hemolymphatic stage usually occurs 1-3 weeks after the bite of an infected tsetse fly. Symptoms include fever, adenopathies, malaise, headache, joint pain, generalized weakness, and weight loss. The late encephalitic stage has an inconsistent and gradual onset and patients present with a variety of symptoms including: irritability, general weakness, headache, apparent personality changes, and more severe
psychiatric problems such as violence, hallucinations, suicidal tendencies, and mania (4). Patients in the late encephalitic stage may also present with motor system, sensory, and sleep disturbances. If the patient is left untreated he/she will develop more severe symptoms including: seizures, more serious sleep issues, double incontinence, cerebral edema, systematic organ failure, coma, and inevitable death. The general symptoms listed above for the different stages will vary slightly depending on the subspecies of parasite with which a patient has been infected.

Even though the number of people tested for HAT has increased, there has not been much progress with regards to treatment or diagnosis of HAT (3), and the drugs currently used for treatment have severe side effects and/or are difficult to administer (1,4). Suramin was first used in the early 1920s and is still used to treat the Stage I disease caused by *T. b. rhodesiense*. There are several side effects associated with Suramin use including: anaphylactic shock, renal failure, skin lesions, and neurologic effects. If administered properly, the dosage schedule is complicated and may last up to 30 days (1,4). Pentamidine was first used in 1940 and is used to treat the Stage I disease for *T. b. gambiense* infections. It is given intramuscularly for a week and has few side effects.

Melarsoprol is an organoarsenic compound that was first used in 1949. It is used for treatment of Stage II Sleeping Sickness of *T. b. gambiense* infections where eflornithine is not available and is the only drug currently used for treatment of second-stage *T. b. rhodesiense* infections (1,4). Treatment with Melarsoprol requires a complex injection cycle over several consecutive days. The side effects include encephalopathic
syndrome, skin reactions, peripheral motor and sensorial neuropathies, and death. There are also recent treatment failures; which suggest that there is an emergence of resistance to melarsoprol (1,4).

Eflornithine was first used in 1981 and is the drug of choice for treat Stage II Sleeping Sickness caused by *T. b. gambiense*. The side effects include: bone marrow toxicity, gastrointestinal symptoms, and convulsions. The course of treatment lasts for 2 weeks, and 4 infusions per day are necessary. This coupled with the cost of the drug leads to the more toxic Melarsoprol being used in many areas of sub-Saharan Africa, despite Melarsoprol’s toxic side effects (1,4). Recently, combinations of nifurtimox with eflornithine and Melarsoprol have been researched and it appears that various combinations of the drugs were more effective than the monotherapies; however, the toxic side effects, difficulty of administration, and/or cost of the drugs remains as a boundary.

*Trypanosoma brucei gambiense* is found in western and central Africa and represents more than 90% of reported cases (3). *T. b. gambiense* causes a chronic infection. By the time symptoms (severe headaches, sustained fever, sleep disturbances, alteration of mental state, neurological disorders) are detected, patients often already have central nervous system involvement. *Trypanosoma brucei rhodesiense*, representing less than 10% of cases, is found in eastern and southern Africa and causes an acute infection. The first symptoms that emerge are typically a chancre, occasional headaches, irregular fevers, itching, and adenopathies. These symptoms usually persist for a few weeks or
months before the parasites cross the blood-brain barrier, invading the central nervous system causing the second stage of the disease.

Diagnosis of HAT also presents a problem to the poor rural areas where the disease is endemic. There is a card agglutination trypanosomiasis test that was developed in the 1970s to screen populations for *T. b. gambiense* infections, but this serological test must be confirmed by the detection of parasites in an infected individual (1,3). In order to diagnose an individual with Stage I Sleeping Sickness, the peripheral blood must be shown to contain trypanosomes. This is difficult with a *T. b. gambiense* infection, because there are very few parasites present in the peripheral blood other than at periods of cyclic parasitemia (4). In order to diagnose a patient with Stage II Sleeping Sickness, the cerebrospinal fluid must be assessed for the presence of the parasite. This requires a lumbar puncture, which is difficult to administer in the poor rural areas of sub-Saharan Africa.

I. THE LIFE CYCLE OF *TRYPANOSOMA BRUCEI*

As the trypanosome moves from the bloodstream of the mammalian host to the tsetse fly, it encounters environmental niches to which it must adapt. The life cycle changes that the parasite undergoes are highly regulated and interconnected (Figure 1.1).
Changes in cell structure, surface coat, and ATP production occur when the parasite transitions from the glucose-rich environment of the mammalian bloodstream to the tsetse fly vector. These changes are necessary to the survival of the parasite in its respective host. (5) Permission granted for use of the figure.

There are two main life cycle stages for *T. brucei*, the bloodstream form (BSF) and the procyclic form (PF). With the bite of an infected tsetse fly, the metacyclic parasites are released from the salivary glands of the fly into the bloodstream of the mammalian host at which time they differentiate into the slender form of the parasite (6). In the glucose-rich environment of the mammalian bloodstream, *T. brucei* possesses a
mitochondrion that is not fully functional and relies solely on glycolysis for production of ATP (7-10).

The surface of this stage of the parasite is covered with a coat of $\sim 10^7$ glycosyl phosphatidylinositol (GPI)-anchored variant surface glycoprotein (VSG) molecules per cell (11). The VSG genes are polycistronically transcribed from telomeric expression sites (ESs), only one of which is transcribed at a time (12). A switch changes transcription from one ES telomere to another. There is a repertoire of over 1000 VSG genes and pseudogenes that are recombined into active ESs periodically in order to protect the parasite from the host immune response, making recombination important for antigenic variation (13). When the parasitemia reaches a high level in the blood, the slender form begins to differentiate into the non-proliferative stumpy form that is pre-adapted for the midgut of the tsetse fly (6,14).

When a tsetse fly takes a bloodmeal from an infected host, the slender forms of the parasite are rapidly killed by proteases, while the stumpy form survives and differentiates into the procyclic form in the midgut of the fly (15). It is known that high concentrations (over 3 mM) of citrate or cis-aconitate (CCA) will induce this differentiation from the BSF to the PF, and that stumpy and procyclic forms express a family of proteins known as proteins associated with differentiation (PAD) (16). The PAD proteins have been shown to be required for CCA-induced differentiation of stumpy form parasites into procyclic forms at 20°C, the expected temperature of the tsetse fly midgut. Therefore, the PAD proteins are suggested to be involved in the parasite’s ability
to sense the change in environment from the bloodstream of the mammalian host to the midgut of the tsetse fly.

The procyclic form of *T. brucei* differs greatly from the BSF of the organism, a difference necessitated by a drastic change in environment (17). The cell has moved from the glucose-rich blood of its mammalian host to the glucose-deplete midgut of the vector, which requires the parasite to metabolize amino acids rather than glucose alone for ATP production (7-10). To meet its energy needs, the procyclic form possess a functioning mitochondria with all of the components required for an operating Krebs cycle and electron transport chain. A second result of the differentiation from BSF to procyclic form is the rearrangement of the kinetoplasts and nuclei along the anterior-posterior axis (18).

At this stage the parasite has also shed its VSG coat in favor of procyclic acidic repetitive proteins (PARPs; (19). These procyclins are actually synthesized and incorporated into the membrane on the surface of the cell prior to the VSG coat being shed (20). The genome contains four types of PARP genes. These genes encode three proteins with extensive glutamic acid-proline dipeptide repeats (EP1, EP2, and EP3 procyclin) and a protein with an internal pentapeptide repeat (GPEET procyclin).

After leaving the midgut, the parasites migrate to the salivary glands and undergo an asymmetric cell division that produces both long and short epimastigote forms (21). The short forms attach to the fly salivary glands and proliferate. This proliferation is also thought to be the point at which sexual reproduction can occur (22). In order to migrate to the proventriculus, the epimastigotes must differentiate into the non-proliferative
metacyclic form and detach from the salivary gland wall (21). It is not known whether or not this movement from the salivary gland to the proventriculus is active migration or is caused by passive diffusion due to an increase in cell number (18). The metacyclic form is pre-adapted for life in the mammalian host and has switched back to a VSG surface coat. These parasites are also infectious to the mammalian host and will be expelled during a tsetse fly bloodmeal.

II. THE TRYPANOSOME FLAGELLUM

The Trypanosma brucei flagellum has been studied extensively using various techniques including scanning electron microscopy, electron microscopy, and immunofluorescence assays. It has been determined that the flagellum and flagellar motility are necessary for survival and pathogenesis of the parasite (23). There is also a linear correlation between flagellar length and the size of the cell (24), indicating its importance in proper cell growth and division.
Figure 1.2 Structure of the Trypanosome Flagellum.

A schematic representation of the *Trypanosoma brucei* flagellum. It is important to note that the kinetoplast DNA has been left out of this schematic, but would be positioned attached to the TAC. Abbreviations: BB, basal body; TAC, tripartite attachment complex; FP, flagellar pocket; FAZ, flagellum attachment zone; FPC, flagellum pocket collar; PFR, paraflagellar rod; AX, axoneme. Adapted from: (25).

The Structure and Composition of the Flagellum

The flagellum of *T. brucei* contains a typical eukaryotic axoneme consisting of 9 outer doublet microtubules that surround a central pair of singlet microtubules and is connected to the cell body via the flagellum attachment zone. The paraflagellar rod (PFR) of the parasite runs along the length of the flagellum parallel to the axoneme (26); however, the PFR only begins from the point at which the axoneme exits the cell (27). Along with the axoneme, the PFR is necessary for trypanosome motility. Ablation of the PFR leads to cells that are so compromised in motility that they sediment rather than remaining in suspension (28).

Proper motility is important to the cell and is necessary for the rapid redistribution of variant surface glycoprotein (VSG) dimers on the cellular surface of the
trypanosome (29). The PFR, and therefore motility, are also required for an infection in mice (30), possibly due in part to the necessity for rapid redistribution of the VSG complexed with host immune factors like antibodies.

The basal body of the trypanosome nucleates the end of the axoneme (31) and is connected to the kinetoplast DNA (kDNA) through the tripartite attachment complex (TAC; (27,32). Segregation of the kDNA is dependent on the separation of the old and new basal bodies; however, the inability of kDNA to segregate does not affect the segregation of the basal bodies (32). The NIMA (never in mitosis gene A)-related kinase TbNRKC is a functional kinase that localizes to the basal body and is necessary for basal body separation during cell division (33). A reduction in basal body separation results in defects in construction of the new flagella attachment zone filament, which is present but does not extend to its full length (34).

The TAC consists of exclusion zone fibers, unilateral filaments, and differentiated mitochondrial membranes (27). The exclusion zone fibers run between the proximal end of the basal body and the outer mitochondrial membrane, which causes the exclusion of cytoplasmic ribosomes from the region. Unilateral filaments are present on only one side of the kinetoplast, and they link it with the inner mitochondrial membrane, which maintains the gap between the kDNA and the inner mitochondrial membrane. The differentiated mitochondrial membrane zone is located between the basal body and the kinetoplast, and there are no cristae observed in this zone of the mitochondria. The TAC serves to maintain the kinetoplast-basal body connection through all stages of the cell cycle.
There is also a fibrous connection between the basal body and the microtubule quartet, which is part of the flagellum attachment zone (35). The flagellum attachment zone runs the length of the cell body and is necessary for flagellar attachment to the cell body. More specifically, cells without FAZ1, a protein essential for flagellum attachment zone structure and function, possess detached flagella (36). In addition to detached flagella, these parasites also show a defect in cytokinesis, indicating the importance of flagellum attachment zone formation in cell division. The flagellum attachment zone is composed of the microtubule quartet; including its associated membrane, and a specific filament structure (35). The microtubule quartet is located at the proximal end of the flagellum between the basal bodies and is attached to the basal body and probasal body, as mentioned previously. Formation of a functioning flagellum attachment zone relies not only on the formation of a proper flagellum and basal body, but also on a functioning flagellar pocket (37).

The flagellar pocket is an invagination of the cell membrane located at the proximal end of the flagellum; where it emerges from the cytoplasm of the cell. It is the site of endocytosis in trypanosomes and is bordered by a collar and a neck region (38,39). The neck region is ~0.5 µm long, and plays a role in pinocytosis and uptake of microparticles through the flagellar pocket. This uptake is probably due to the movement of the flagellum and the body of the trypanosome, which causes the neck region to open and close thereby allowing microparticles to enter and exit the flagellar pocket (38). Clathrin-mediated endocytosis also occurs in the trypanosome flagellar pocket (39) and is essential in T. brucei. Defects in clathrin-mediated endocytosis result in a characteristic
“BigEye” cell in which the flagellar pocket is severely enlarged. Recently BILBO1 was identified as a part of the cytoskeletal framework of the flagellar pocket collar that is necessary for its biogenesis and the endocytotic activity of the new flagellar pocket (37).

Biogenesis of the Trypanosome Flagellum and Its Function in Cytokinesis

A functioning flagellum is necessary for cell division in *T. brucei*; however, non-flagellated cells can be produced (24). These non-flagellated cells undergo normal nuclear mitosis but have defects in flagellum attachment zone assembly and kinetoplast/basal body segregation and are unable to divide.

The first major event in cell division is the elongation of the pro-basal body (40). This elongated basal body lies in a posterior position in the cell, and it initiates the growth of the new daughter flagellum. After new flagellum synthesis is initiated, genesis of two new pro-basal bodies and the first phase of basal body separation begin, and the old pro-basal body becomes the basal body for the new flagellum (40).

The new flagellum is then extended, and PFR formation and extension is initiated (40). The PFR has two sites of elongation in which PFRA subunits are added to the flagellum: the major site and the minor site (41). The major site of addition is on the distal tip of the flagellum, and the minor site is along the length of the flagellum. As the new flagellum extends, it is linked to the old flagellum by the flagella connector (42), which extends ~400 nm (43). The flagella connector is only seen on the distal tip of the new flagellum and connects the tip of the axoneme of the new flagellum to the side of the axoneme, but not PFR, of the old flagellum (42). One result of the tips being bound is
that the old flagellum directs the position and orientation of the new flagellum as it elongates. This tethering lasts until detachment occurs (43).

Once flagellar extension is complete, kinetoplast division occurs, along with separation of the kinetoplast and basal body and mitosis (40). Proper segregation of the kDNA is mediated by the basal bodies; however, basal body segregation is not affected by aberrant segregation of the kDNA (32). The kDNA remains attached to the basal body by the TAC throughout replication (27). This TAC/kDNA connection must be remodeled at the S phase in order to adjust for the formation of the new TAC and replicated kNDA.

NIMA-related kinase, discussed previously, is involved in basal body separation, and overexpression of TbNRKC result in cells that are multinucleated and often possess a single large kinetoplast, abnormal numbers of basal bodies, and a disorganized exclusion zone; all of which eventually results in cell death (33). This second phase of basal body separation is also influenced by several factors: the elongation of the new flagellum, connection of the new flagellum to the old flagellum, and the base-to-tip wave propagation of the flagellum (34).

Finally cytokinesis occurs and the cell is divided along a plane that runs longitudinally through the cell in a manner in which each daughter cell receives a single nucleus, kinetoplast, and flagellum (40). Proper flagellar motility is required for cytokinesis in bloodstream form *T. brucei* (23). Cell division, however, can be completed even in the absence of nuclear segregation, forming zoid cells that are devoid of nuclear DNA (44).
III. GLYCOLYSIS AND GLYCOSOMES

*Trypanosoma brucei* relies solely on glycolysis for ATP production while residing in the glucose-rich environment of the mammalian host (7-10). One distinct feature of BSF trypanosomes is the localization of the first seven enzymes of glycolysis to a peroxisome-like organelle termed the glycosome (45,46).

**Figure 1.3 Glycolysis in the BSF of the African Trypanosome.**

The first seven enzymes of glycolysis are housed in the glycosome, a peroxisome-like organelle, in BSF trypanosomes. The abbreviations used are as follows: ALD: aldolase; DHAP: dihydroxyacetone phosphate; 1,3BPGA: 1,3-bisphosphoglycerate; ENO: enolase; F-6-P: fructose-6-phosphate; FBP: fructose 1,6-bisphosphate; G-3-P: glyceraldehyde 3-phosphate; G-6-P: glucose-6-phosphate; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; Glc: glucose; Gly-3-P: glycerol-3-phosphate; GPDH: glycerol 3-phosphate dehydrogenase; Mito: mitochondrial enzymes; PEP: phosphoenolpyruvate; 2-PGA: 2-phosphoglycerate; 3-PGA: 3-phosphoglycerate; PGI: glucose-6-phosphate isomerase; PGK:
phosphoglycerate kinase; PGM: phosphoglycerate mutase; PFK: phosphofructokinase; PK: pyruvate kinase; PYR: pyruvate; TbHK: *T. brucei* hexokinase 1 and/or 2; TPI: triose-phosphate isomerase. (47)

**The Glycosome**

Glycosomes are peroxisomal-like organelles, but glycosomes are functionally different from peroxisomes, which are also single-membrane organelles. One major difference is that glycosomes lack catalase which is a hallmark enzyme of peroxisomes. Catalase catalyzes the degradation of hydrogen peroxide into water and oxygen (48).

Glycosomes measure from 0.25-0.5 µm in diameter, and there are approximately 230 glycosomes in the average trypanosome (48). They are single-membrane organelles that contain 32% phospholipid and 68% protein by weight. There is also very little DNA purified with glycosomes, all of which could represent degradation products of nuclear or kDNA.

Glycosome-resident proteins are encoded by nuclear DNA and translated on cytosolic ribosomes. They are imported into the glycosome due to the presence of one of the peroxisomal targeting sequences (PTSs). There are three PTS sequences: PTS1, PTS2, and I-PTS. Import of the glycosome-resident proteins is essential to the survival of the parasite. In PF cells, RNAi to PEX14, a protein necessary for the formation of glycosomes, is only tolerated in glucose-free media (50). Addition of glucose back to the media is lethal; however, simultaneous RNAi of hexokinase (HK) rescues the lethal phenotype. These observations suggest that it is the unregulated activity of HK that results in lethality; though this may not be the only explanation as TbHK1 has been shown to be regulated by other mechanisms (51,52).
Peroxisomal matrix proteins do not use typical peroxisomal targeting sequences in order to be targeted to the glycosomal membrane (53). Briefly, peroxin 13 (PEX13), an integral membrane protein (54), along with peripheral membrane proteins PEX14 (55) and PEX17 form the docking complex of the glycosome. PEX2, PEX10, and PEX12 have been identified as integral peroxisomal membrane proteins containing RING fingers (53). These peroxins are important for the import of peroxisomal proteins, and probably function downstream of the docking complex. PEX3 contains a transmembrane domain and is suspected of being involved in peroxisomal membrane biogenesis. PEX1 and PEX6 are members of the AAA protein family and are thought to be involved in peroxisome biogenesis also (53). The AAA family of proteins function as ATPases and are associated with diverse cell activities. Importantly, some AAA+ proteins function in vesicular fusion events.

**Import of Glycosomal Proteins**

There are three different types of peroxisomal targeting sequences (PTS). They are PTS1, PTS2, and I-PTS (internal-PTS). The PTS1 and PTS2 targeting sequences have been well characterized, while less is known about the I-PTS.

The PTS1 is a C-terminal Ser-Lys-Leu motif originally characterized in firefly luciferase (56). This Ser-Lys-Leu sequence has been demonstrated to be sufficient to target proteins to the peroxisome (57). PTS1 proteins are known to localize to the glycosome through interaction with a peroxin known as PEX5.
The I-PTS (internal-PTS) proteins have an internal sequence that causes their localization to the peroxisome or glycosome membrane; however, there is no known I-PTS consensus sequence that results in glycosome/peroxisome localization. One example of a glycosomal protein that uses an I-PTS in trypanosomes is phosphoglycerate kinase (58).

The PTS2 sequence was first identified on the amino-terminus of rat peroxisomal 3-ketoacyl-CoA thiolase precursor (59). Further studies show that the N-terminal 11 amino acids of *Saccharomyces cerevisiae* thiolase are identical in 6 out of 11 residues with that of the rat thiolase isolated previously (60). Glover et al. analyzed the amino acids and determined that there were 3 identical amino acids in thiolases of mammals and yeast. Through mutagenesis, the N-terminal 16 amino acids of *S. cerevisiae* thiolase were demonstrated to be necessary and sufficient for protein targeting to the peroxisomes.

PEX7 (also known as PAS7 or PEB1) was first identified in yeast (61) as necessary for the localization of thiolase (a PTS2 protein), but not PTS1 proteins (61,62). More specifically, it was determined that PEX7 of *Saccharomyces cerevisiae* does not require a peroxisomal/glycosomal membrane for binding to thiolase, binds thiolase in a PTS2-dependent manner, and seems to bind thiolase that has already been folded (63). Coupled with the fact that PEX7 has no predicted transmembrane domain, the evidence suggests that PEX7 acts as a shuttle to transport PTS2 proteins into and out of the peroxisome/glycosome.
The *T. brucei* PEX7 was identified in 2007 and shown to have a molecular mass of 39.7 kDa, containing 361 amino acids (64). Using three trypanosomatid species, *T. brucei, Leishmania major, and Trypanosoma cruzi*, it was determined that the trypanosome PEX7 sequences share 65-76% identity with each other, while only sharing 32-34% identity with human PEX7. Interestingly, the trypanosomatid PEX7s contain an approximately 40 residue extension on their C-terminus, while the human and yeast have only 5 to 10 residues. This C-terminal region is proline-rich, but the importance of it has yet to be identified.

Mammalian PEX7 is also known to bind PTS2 proteins; however, it also interacts with the known PTS1-import protein PEX5, and this interaction is necessary for the import of PTS2 proteins (65). PEX5 is known to interact with PEX14, a glycosomal membrane protein, in yeast; however, it does not appear that PEX7 associates with PEX5 in *S. cerevisiae* (66).

In *T. brucei*, PEX5 is involved in the import of PTS1 proteins to the glycosome. PTS1 recognition by PEX5 is predicted to occur through seven predicted tetratricopeptide repeats (67). Tetratricopeptide repeats are units comprised of 34 amino acids and are present in proteins involved with diverse cellular functions. Similar to mammalian PEX5, there is a putative PEX7 binding box in the N-terminal half of the PEX5 of the three previously mentioned trypanosomatid species (64). This suggests that the trypanosomatid Pex7 proteins may also function by interacting with Pex5 protein; however, RNAi of PEX5 does not lead to mislocalization of PTS2 proteins, suggesting that the PEX7:PEX5 interaction may not be an absolute requirement in trypanosomes. In
L. major, PEX7 has been shown to bind to the PTS2 sequence as well as PEX5 and PEX14 (68).

**Figure 1.4 Glycosome Import in Trypanosoma brucei**

There are two options for PTS2 import into the glycosome. (1) A PTS2 protein is bound by PEX7, either before or after PEX7 binds to PEX5. The PTS2:PEX7 complex is then translocated into the glycosome via an interaction between PEX5 and PEX14. (2) The PTS2 protein binds PEX7 and the complex is imported into the glycosome via PEX14, with no interaction with PEX5. Abbreviations used: PFK: phosphofructokinase; PTS1: peroxisomal targeting signal 1; PTS2: peroxisomal targeting signal 2; TbHK: *Trypanosoma brucei* hexokinase. (47)

There are two models that would explain how PTS2 proteins are translocated into the glycosome. In the first model, a PTS2 protein binds PEX7, which may or may not already be bound to PEX5. After formation of a PTS2:PEX7:PEX5 complex, the entire complex would dock onto the glycosomal membrane. At this time the PEX7:PTS2 complex would be translocated into the glycosome, while the PEX5 remains in the cytosol. Once the PEX7:PTS2 complex is in the glycosome, the PTS2 protein is released, and PEX7 is transported back to the cytosol (69). In the second model, a PTS2
protein binds PEX7 and the complex is translocated into the glycosome through an interaction with PEX14, with no involvement from PEX5. The PEX7 would again be shuttled back out into the cytosol after release from the PTS2 protein.

**Glycolysis**

As mentioned previously, *T. brucei* BSF parasites rely solely on glycolysis for production of ATP (7-10). The first seven enzymes of glycolysis are localized to the glycosomes (45,46). In the glycosome, glucose is metabolized into 3-phosphoglycerate. The 3-phosphoglycerate is exported to the cytosol and further metabolized into pyruvate, which is then secreted from the cell (9,10). ATP production and consumption in the glycosome is balanced, and the net ATP is synthesized only in the cytosol. The enzymes involved in glycolysis are reviewed in Table 1 below.
### Table 1.1. Overview of the Glycolytic Enzymes of *T. brucei*

<table>
<thead>
<tr>
<th>Enzyme*</th>
<th>Molecular Mass (kDa)</th>
<th>Native Enzyme*</th>
<th>Subunit*</th>
<th>Number of Subunits*</th>
<th>BSF**</th>
<th>PF*</th>
<th>TS**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>295 ± 12</td>
<td>50.3 ± 0.8</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>PTS2</td>
<td></td>
</tr>
<tr>
<td>HK1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HK2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucosephosphate isomerase</td>
<td>105 ± 6</td>
<td>62.4 ± 1.6</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>PTS1</td>
<td></td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>196 ± 15</td>
<td>50.3 ± 0.8</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>PTS1</td>
<td></td>
</tr>
<tr>
<td>Aldolase</td>
<td>157 ± 3</td>
<td>40.5 ± 0.5</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>PTS2</td>
<td></td>
</tr>
<tr>
<td>Triosephosphate isomerase</td>
<td>55 ± 0</td>
<td>27.0 ± 0.5</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyceraldehyde-phosphate dehydrogenase</td>
<td>139</td>
<td>38.5 ± 0.5</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>PTS1</td>
<td></td>
</tr>
<tr>
<td>Phosphoglycerate kinase</td>
<td>48 ± 4</td>
<td>47.0 ± 0.7</td>
<td>1</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cPGK***</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gPGK***</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>56PGK***</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycerol-3-phosphate dehydrogenase</td>
<td>66 ± 6</td>
<td>37.2 ± 0.7</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>PTS1</td>
<td></td>
</tr>
<tr>
<td>Glycerol Kinase</td>
<td>82</td>
<td>52.5 ± 0.4</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>PTS1</td>
<td></td>
</tr>
</tbody>
</table>

*(70)

**(49)

*** (58)

**The Hexokinases of *T. brucei***

There are two hexokinases, TbHK1 and TbHK2, in *T. brucei* that are arranged in tandem on chromosome 10 (52). Both TbHK1 (71) and TbHK2 (71,72) are essential to BSF parasites and localize to the glycosome via a PTS2 sequence, as mentioned.
previously. The two hexokinases are 98% identical, with 7 of the 10 amino acid differences occurring at the C-terminus (52).

Despite their similarities, these two hexokinases have distinct characteristics. Recombinant protein for each hexokines has been produced, and rTbHK1 is active, while rTbHK2 alone is not. A result of the formation of a mixed hexamer is the activation of rTbHK2 by rTbHK1; which was demonstrated by mixing an inactivated rTbHK1 with rTbHK2, allowing hexamers to form, and testing for hexokinase activity (51). Also, if the C-terminal tail region of the enzymes is switched, the activity is switched as well (52). Another difference between the two enzymes is their essentiality in PF parasites; while TbHK1 is also essential in PF parasites, TbHK2 is not. A TbHK2 knockout cell line produced in PF parasites demonstrates altered cellular morphology, retarded growth, and increased cellular activity; however, they do survive. These TbHK2-/− cells also show an increase in cellular HK activity. It has been demonstrated that TbHKs are not regulated by their products, as most HKs are (73); however, more recent evidence suggests that when TbHK1 and TbHK2 form a mixed hexamer their activity may be regulated by pyrophosphate (51).

Most of the enzymatic assays done with TbHKs are done at physiological pH, but this does not take into account the environment of the glycosome under conditions of cell starvation. When cells are starved of nutrients, their glycosomes merge with lysosomes, thus lowering the pH of the glycosome (74). At a physiologically relevant lower pH, TbHK activity is diminished; however, the addition of glycerol 3-phosphate rescues the
activity (75). In the cell, glycerol 3-phosphate levels are increased, suggesting that it could impact or modulate TbHK1 activity.

The HKs of *T. brucei* have been identified as viable targets for treatments of HAT. Recently several inhibitors have been described including lonidamine (71), quercetin (76), and several small molecules (77). Most importantly, testing rTbHK1 for inhibitors using a high throughput screen has been validated, and analogues identified in this high throughput screen are still under investigation (77).
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CHAPTER TWO
EXTRA-GLYCOSOMAL LOCALIZATION OF TRYPANOSOMA BRUCEI HEXOKINASE 2

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ABSTRACT

The majority of the glycolytic enzymes in the African trypanosome are compartmentalized within peroxisome-like organelles, the glycosomes. Polypeptides harboring peroxisomal targeting sequences (PTS type 1 or 2) are targeted to these organelles. This targeting is essential to parasite viability, as compartmentalization of glycolytic enzymes prevents unregulated ATP-dependent phosphorylation of intermediate metabolites. Here, we report the surprising extra-glycosomal localization of a PTS-2 bearing trypanosomal hexokinase, TbHK2. In bloodstream form parasites, the protein localizes to both glycosomes and to the flagellum. Evidence for this includes fractionation and immunofluorescence studies using antisera generated against the authentic protein as well as detection of epitope-tagged recombinant versions of the protein. In the insect stage parasite, distribution is different, with the polypeptide localized to glycosomes and proximal to the basal bodies. The function of the extra-glycosomal protein remains unclear. While its association with the basal body suggests that it may have a role in locomotion in the insect stage parasite, no detectable defect in directional motility or velocity of cell movement were observed for TbHK2-deficient cells, suggesting that protein may have a different function in the cell.

Keywords: Trypanosoma brucei, Hexokinase, Flagellum, Glycosome, Glycolysis
INTRODUCTION

In the African trypanosome, *Trypanosoma brucei*, glycolytic enzymes have been localized to peroxisome-like organelles called glycosomes (Opperdoes and Borst, 1977). This organization, which is thought to serve as a means of regulating the pathway (Haanstra et al., 2008), is unusual as glycolysis is a cytosolic process in most cells. Even in systems with cytosolic glycolysis, glycolytic enzymes have been found in subcellular compartments distinct from the cytosol, although the function of these proteins in the alternative compartments may not be in the glycolytic pathway. These additional localizations include the sarcoplasmic reticulum in rabbit muscle tissue (Xu and Becker, 1998), chloroplasts (reviewed in Lunn (2007)), the *Toxoplasma gondii* apicoplast (Fleige et al., 2007), and sometimes (in some protists) in mitochondria (Liaud et al., 2000; Kroth et al., 2008). Glycolytic enzymes have also been found in the nucleus where, for example, GAPDH is involved in DNA repair or on the cell surface (e.g., aldolase serves as a plasminogen receptor in many pathogenic microorganisms (Kim and Dang, 2005; Avilan et al., 2011).

A number of species localize glycolytic and other metabolic enzymes near the flagella. *Chlamydomonas reinhardtii* has three glycolytic enzymes (phosphoglycerate mutase, enolase and pyruvate kinase) associated with the flagellum to produce ATP (Mitchell et al., 2005). In mammals, a hexokinase (HK\(^1\)) has been found attached to the fibrous sheath that surrounds the axoneme and outer dense fibers of sperm flagellum, suggesting a role in extra-mitochondrial energy production (Travis et al., 1998; Miki et al., 2004; Nakamura et al., 2008).
Kinetoplastid metabolic enzymes have been found proximal to the flagellum. For example, three isoforms of adenylate kinase localize to either the flagellar axoneme or paraflagellar rod (PFR) via an N-terminal extension in the proteins (Ginger et al., 2005). In *Leishmania*, a HK (which was characterized as a hemoglobin receptor) has been localized to the flagellar pocket, suggesting that it may serve multiple functions depending upon localization (Krishnamurthy et al., 2005).

The *T. brucei* genome encodes two 98% identical HK polypeptides (TbHK1 and TbHK2) that are expressed in both bloodstream form (BSF) and insect stage (procyclic stage, PF) parasites. These proteins form hexamers that in vitro have distinct biochemical properties depending on the ratio of TbHK1 and TbHK2 included in the oligomers (Chambers et al., 2008b). Proteomic analysis of purified glycosomes has revealed that both proteins are expressed in BSF and PF glycosomes (Colasante et al., 2006). While the function of these polypeptides is currently unresolved, genetics-based studies have confirmed that both are essential to BSF parasites (Albert et al., 2005; Chambers et al., 2008a).

The glycosomal localization of the TbHKs has been attributed to the presence of a N-terminal peroxisomal targeting sequence (PTS2), as this sequence has been shown to be responsible for the import of other glycosomally-targeted proteins (Blattner et al., 1995). Here, we report the unexpected life cycle-dependent dual localization of TbHK2. In BSF parasites, TbHK2 localizes to glycosomes and the flagellum, while in PF parasites TbHK2 localizes to glycosomes and regions proximal to basal bodies.
MATERIALS AND METHODS

Subcellular fractionation of trypanosomes

Subcellular fractionations were performed using BSF and PF line 449 of *Trypanosoma brucei brucei* (a genetically modified cell line derived from strain Lister 427) (Biebinger et al., 1997). BSF parasites were cultured in HMI-9 medium containing 10% heat-inactivated FCS (Invitrogen, USA) at 37°C under water-saturated air with 5% CO₂. PF trypanosomes were grown in SDM-79 medium (Brun and Shonenberger, 1979) supplemented with 15% FCS at 28°C with 5% CO₂. Cultures were always harvested prior to entering the stationary phase, i.e., at densities lower than 2 x 10⁶ cells/mL for BSFs or 2 x 10⁷ cells/mL for procyclic cells, by centrifugation at 700 g for 10 min.

Fractionation of cell compartments was performed using increasing concentrations of digitonin as follows: BSF parasites (10⁸ cells) and procyclic trypanosomes (2 x 10⁸ cells) were washed twice in ice-cold buffer (25 mM HEPES, pH 7.4, 250 mM sucrose and 1 mM EDTA) and then resuspended in 0.5 mL of the same buffer. The cell suspension was divided with each aliquot containing ~100 µg of protein. HBSS buffer (Gibco, USA) was added to adjust aliquot volume to 100 µL. Digitonin dissolved in dimethylformamide was then added followed by incubation (4 min at room temperature, RT). Untreated cells and those completely permeabilized (total release, the result of incubation in 0.5% Triton X-100) were used for comparison. After centrifugation of the suspensions (12,000 g for 2 min), the supernatant (released fraction) was probed by western blotting for cytosolic or glycosomal resident proteins. Western blotting was performed on samples resolved by 12% SDS-PAGE followed by transfer to
a nitrocellulose support. The membrane was incubated in block (1% non-fat milk, 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.05% Tween-20) and probed with appropriate antibodies. These included: rabbit polyclonal antisera raised against *T. brucei* pyruvate kinase (αPYK, antiserum used at a dilution of 1:100,000), *T. brucei* hexokinase (αTbHK, at 1:100,000), *T. brucei* enolase (αENO, at 1:150,000), or *T. brucei* glycerol kinase (αGK, at 1:100,000) as well as anti-haemagglutinin (αHA, 1:1,000, Rockland Immunochemicals, Gilbertsville PA, USA). Primary antibodies were detected with anti-rabbit IgG conjugated to horseradish peroxidase (Rockland Immunochemicals) and were visualized using the ECL Western Blotting System (Pierce, USA).

**Immunofluorescence microscopy on whole cell and cytoskeletal preparations**

Immunofluorescence (IF) assays were performed using a protocol modified from Field et al. (2004). In short, parasites were harvested by centrifugation (800 g, 5 min), washed with ice-cold Voorheis’s modified PBS (vPBS; 137 mM NaCl, 3 mM KCl, 16 mM Na₂HPO₄, 3 mM KH₂PO₄, 46 mM sucrose, 10 mM glucose, pH 7.6), and then fixed (10 min BSF; 1 h PF) in an equal volume of 6% paraformaldehyde and vPBS on ice. Cells were washed with vPBS, allowed to settle on poly-lysine slides, and then permeabilized with 0.1% Triton X-100 in PBS (137 mM NaCl, 3 mM KCl, 16 mM Na₂HPO₄, 3 mM KH₂PO₄) for 10 min. After washing in PBS, block (1% BSA and 0.25% Tween in PBS) was added (1 h, RT), followed by addition of the appropriate primary antibody. For localization of TbHKs, αTbHK (1:500) was used. For localization of TbHK2, affinity-purified TbHK2 polyclonal antisera (αTbHK2, 1:1 or 1:10 for BSF;
1:100 for PF), which was raised against a peptide corresponding to the C-terminal end of TbHK2 (CGVGAALISAIVADGK), was used (Morris et al., 2006). Endogenously tagged TbHK2 was localized using an affinity purified amyc polyclonal antibody (1:50, Rockland Immunochemicals, Gilbertsville PA, USA), while HA-tagged polypeptides were localized with an αHA antibody (1:100, Rockland Immunochemicals). The monoclonal antibody Mab 25 was used to detect the axoneme (Absalon et al., 2007), and Mab 22 was used to detect the tripartite attachment complex (TAC) fibers of the basal bodies (Bonhivers et al., 2008). Primary antibodies were detected with either FITC- or TexasRed-conjugated goat anti-mouse or goat anti-rabbit (1:100, Rockland Immunochemicals) and visualized on a Zeiss Axiovert 200M using Axiovision software version 4.6.3 for image analysis.

Cytoskeletons were isolated from cells washed in vPBS followed by incubation for 10 min with 0.5% Triton X-100 in MME (10 mM MOPS (3-(N-morpholino)propanesulfonic acid), 2 mM EGTA and 1 mM MgSO$_4$) buffer as previously described (Robinson et al., 1991). Samples were washed with vPBS, allowed to settle on poly-lysine coated slides and visualized as described (Robinson et al., 1991).

**Expression of tagged TbHK2**

To further explore localization, a constitutively expressed HA-tagged version of TbHK2 was generated using a pXS6plasmid for expression in trypanosomes. This vector drives expression with the *T. brucei* rRNA promoter, typically yielding robust expression
(Alexander et al., 2002). For cloning into this vector, *TbHK2* was amplified to yield a product with appropriate restriction sites for ligation.

In order to generate an endogenously tagged *TbHK2*, a linearized construct containing the 3’ end of the *TbHK2* open reading frame (ORF) fused to a myc epitope, an αβ tubulin linker, the phleomycin resistance gene, and the 3’ untranslated region of *TbHK2* was generated by PCR. Both constructs were used to transfect BSF strain Lister 427 cells (2.5 x 10^7 cells) using the Amaxa Human T Cell Nucleofector Kit (Lonza, Basel, Switzerland) as previously described (Burkard et al., 2007). For the PF transfections, linearized constructs were introduced by electroporation as previously described (Wang et al., 2000).

**Motility assays**

For motility assays, cells were imaged at maximum capture speed using the Zeiss AxioCam MRm camera and a Zeiss Axiovert 200M inverted microscope equipped with a 20x differential interference contrast (DIC) objective (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA) for 30 s. Enough fields were monitored so that at least 200 traces of individual cells could be generated yielding at least 10 productively motile cell traces available for analysis for each cell line. The position of the anterior end of each productively motile cell was plotted on each frame and the distance between each of these anterior positions was used to determine the incremental distance travelled. For each cell, total distance travelled was calculated by summing the incremental distances, and effective distance travelled was calculated by measuring the distance between the
initial and final position of the anterior end of the cell. The directional motility coefficient of each productively motile cell was calculated as:

$$ DMC = \frac{d_{\text{effective}}}{d_{\text{travelled}}} $$

where $d_{\text{travelled}}$ is defined as the sum of all incremental distances travelled for a given cell and $d_{\text{effective}}$ is defined as the linear distance between the initial and final position of the anterior end of the same cell.

All statistical analyses were performed using the KaleidaGraph 4.03 software package (Synergy Software, Reading, PA, USA). A box and whisker plot of incremental speed for each cell line was constructed where the 25th percentile, median and 75th percentile are represented. The limit of each whisker is defined by the smallest or largest value observed within a distance of 1.5 times the interquartile range from the end of each quartile with observations beyond this range indicated by open circles. DMC values generated for each cell line were used to generate a dot plot which was overlaid with a percentile plot with lines indicating the 5th percentile, 25th percentile, median, 75th percentile and 95th percentile. Statistical significance of differences in cell motility and directionality were determined using a Wilcoxon-Mann-Whitney rank sum test for unpaired data. The shape of the DMC distributions was assessed by calculation of skewness and kurtosis. Briefly, for a given distribution, skewness indicates the relationship of the mean and median values such that a skewness of zero indicates equality of the values while negative skewness indicates a median greater than the mean and positive skewness indicates a median less than the mean. For the same distribution,
kurtosis can be calculated as a measure of the source of the variance. Leptokurtic distributions (represented by positive kurtosis values) are centrally clustered with a few extreme values contributing the majority of observed variance, while the variance of platykurtic distributions (negative kurtosis) is derived from many values.

RESULTS

Fractionation studies of BSF parasites suggest extra-glycosomal TbHK localization

Initial studies on the organization of glycolytic enzymes in T. brucei revealed that most TbHK activity was associated with glycosomes (Misset et al., 1986). Following completion of genome sequencing, it was recognized that T. brucei harbors two 98% identical HK genes (TbHK1 and TbHK2) that are predicted to yield polypeptides with N-terminal peroxisomal targeting sequences (PTS2) that would localize expressed proteins to glycosomes. Indeed, proteomic analysis of glycosomes has confirmed that both polypeptides are components of the organelles in PF and BSF parasites (Colasante et al., 2006).

Earlier fractionation studies that scored TbHK activity may have described only the compartmentalization of TbHK1, as the function (and activity) of TbHK2 in vivo is not clear. In vitro, recombinant TbHK1 has HK activity while recombinant TbHK2 lacks detectable enzyme activity, behavior that has been experimentally attributed to protein sequence difference in the C-termini of the proteins (Morris et al., 2006). To further explore TbHK distribution, parasite lysates were fractionated and analyzed by western blotting using antibodies to glycosomal- or cytosolic-resident proteins. These included
an antiserum raised against native TbHK purified from BSF trypanosomes that is likely a mixture of both TbHK1 and TbHK2 (αTbHK) (Misset et al., 1986) (Fig. 2.1A).

Interestingly, TbHK was released at ~0.1 mg digitonin/mg protein, a concentration that was found to liberate cytosolic proteins such as enolase (ENO) and pyruvate kinase (PYK) but not the glycosome-resident protein glycerol kinase (GK). To release GK, ~0.5 mg digitonin/mg protein was required, a value consistent with the reported concentration required for compromising the glycosomal membrane (Hannaert et al., 2003).

![Figure 2.1](image)

**Figure 2.1. Subcellular distribution of glycolytic enzymes in bloodstream form (BSF) *Trypanosoma brucei brucei* parasites analyzed by digitonin fractionation and immunofluorescence (IF) microscopy.**

(A) BSF trypanosomes were incubated with increasing concentrations of digitonin and release of enolase (ENO), glycerol kinase (GK), hexokinase (TbHK) and pyruvate kinase (PYK) monitored by western blots using enzyme-specific antisera including an αTbHK antiserum. Total release (T.R.) lanes correspond to cells incubated with 0.5% Triton X-100 to permeabilize all membranes. (B) Fixed BSF parasites were visualized by IF microscopy using an αTbHK antiserum (b) and a monoclonal antibody to Axoneme.
the axoneme (c). DAPI (a) was added with anti-fade reagent to stain the nucleus and kinetoplast DNA (kDNA) in all samples. All images are an extended focus produced from Z-stack layers. Scale bar = 5 µm.

The extra-glycosomal distribution of the TbHK in the fractionation warranted further investigation to identify the compartment(s) that the proteins occupy. Using IF microscopy to resolve localization, the αTbHK serum yielded a largely punctate signal consistent with the expected glycosomal localization of the TbHKs (Fig. 2.1B). These apparently conflicting results led us to consider distribution of the individual TbHKs, with a focus on TbHK2.

**IF microscopy using TbHK2-specific antisera reveals flagellar localization in BSF parasites**

Because the αTbHK antisera likely detects both TbHK1 and TbHK2, an affinity-purified antibody generated against the C-terminal tail of TbHK2 (αTbHK2) was used to resolve the contribution of TbHK2 to the unanticipated fractionation results. (Note: the different affinity-pure antisera used lack sufficient sensitivity for use in the western blots used in the fractionation studies (Morris et al., 2006).) By IF microscopy using the αTbHK2 antisera, TbHK2 localized to the flagellum in BSF parasites (Fig. 2.2A), with faint labeling of putative glycosomes. These findings were reproducible using different blocking schemes (either 20% FBS or 1% BSA supplemented with 0.25% Tween-20) or antisera raised in different organisms (mice and rabbit, Fig. 2.2A, a and b, respectively). While flagellar staining was intense, glycosomal staining was not as readily obvious, although a single image from a Z stack layer revealed punctate staining consistent with
glycosomal staining (Fig. 2.2A, d, white arrows). Additional experimental controls were pursued to confirm the specificity of the antisera. First, flagellar signals were eliminated when the primary antibodies were incubated with the peptide used to generate the antibody (prior to addition of the sample) (Fig. 2.2A, e and f).

**Figure 2.2 Immunofluorescence (IF) microscopy of bloodstream form (BSF)**

*Trypanosoma brucei brucei* parasites reveals localization proximal to the flagellum.

(A) IF microscopy was performed using affinity-purified polyclonal sera raised against the C-terminus of *T. brucei* hexokinase 2 (TbHK2) (aTbHK2 antisera, from mouse (a) or rabbit (b) detected with a species-specific FITC conjugated secondary antibody. A Z-stack layer of aTbHK2 antisera stained BSF 90-13 cells (d) and the extended focus image that included the stack (c) harbor glycosome-like bodies (arrows). Pre-incubation of aTbHK2 antisera with peptide (4°C, 1 h) reduced the staining pattern in BSF parasites (e and f). (B) Analyses of two examples of cytoskeletal preparations by IF microscopy using aTbHK antisera (a and d) and axoneme (b and e) monoclonal antibodies. Scale bar = 5 µm.
We speculated that the discrepancies between the two IF assays (using αTbHK, Fig. 2.1B, versus αTbHK2 antisera, Fig. 2.2A) could be the result of the faint extraglycosomal signal in the former experiment being obscured by the intensity of the signal emanating from reactions with both TbHK1 and TbHK2 in the glycosomes. In order to eliminate the signal resulting from the TbHK (namely TbHK1) that is highly expressed in the BSF glycosomes, cells were fractionated to enrich for cytoskeletal components. This procedure eliminates glycosome-resident proteins and was followed by IF microscopy using αTbHK. Under these conditions, TbHK co-localized with the axoneme (Fig. 2.2B), indicating that both sera react with an antigen found to be associated with the flagellum.

Flagellar localization of tagged TbHK2

While three different antisera (mouse and rabbit αTbHK2 and rabbit αTbHK) indicated that TbHK2 localized to the flagellum, these observations could be the result of the three antisera cross-reacting with a flagellar antigen that is immunologically similar to the TbHKs. To rule out this possibility, recombinant TbHK2 bearing an antigenic tag was expressed in the parasites and localized. First, a TbHK2 chimera bearing a C-terminal HA tag was expressed from an integrated copy of the constitutively expressed vector pXS6:HK2HA. As with the αTbHK, the over-expressed TbHK2:HA localized predominantly to glycosomes (Fig. A-1). Longer integration time revealed flagellar staining (white arrows), again suggesting that the intense glycosomal foci that result from over-expression were masking the less intense flagellar signal. Supporting this
possibility, cytoskeletal preparations demonstrated that TbHK2:HA co-localized with an axonemal component (Fig. A-1).

Because over-expression could potentially alter localization we next introduced, through allelic exchange, an endogenous tag (myc) in frame with the 3’ end of the TbHK2 ORF. IF microscopy using an amyc mAb to probe TbHK2 localization in the endogenous tag cell line yielded findings consistent with those from the αTbHK2 antibody, with co-localization of TbHK2:myc and the axonemal-reactive antibody (Fig. 2.3).

Figure 2.3. Immunofluorescence (IF) microscopy of endogenously tagged Trypanosoma brucei hexokinase 2 TbHK2 bloodstream form (BSF) parasites. BSF strain Lister 427 parasites expressing TbHK2 bearing a myc tag from an authentic allele probed for both myc (b, f, and j) and axoneme (c, g, and k) antigens. DAPI (a, e, and i) signal was also included for all samples.
Lifecycle stage differences in the distribution of TbHK2

PF parasites differ from BSF parasites in a number of ways, including having the capacity to use both glycolysis and amino acid metabolism for ATP production. Digitonin fractionation of PF parasites followed by western blotting using the pan-specific αTbHK revealed a TbHK distribution distinct from BSF parasites, with signal limited to fractions requiring ~0.5 mg digitonin/mg protein for release (Fig. 2.4A), consistent with the concentration required for compromising the glycosomal membrane (Hannaert et al., 2003).
Figure 2.4. Subcellular distribution of glycolytic enzymes in procyclic form

(PF) *Trypanosoma brucei brucei* parasites suggest altered extra-glycosomal localization of *T. brucei* hexokinase 2 (TbHK2). (A) Enolase (ENO), glycerol kinase (GK), hexokinase (TbHK) release was monitored by western blotting using aENO, aGK and aTbHK antisera, respectively, after treating PF 29-13 trypanosomes with increasing digitonin concentrations. (B) Fixed parasites (a, b, c, and d) and cytoskeletons (e, f, and g) were stained with aTbHK antiserum (b and e) and either basal body (c) or axoneme (f) monoclonal antibodies. Scale bar = 5 μm.

To confirm the difference in distribution of the TbHKs in PF parasites, fixed parasites and PF cytoskeletal preparations were probed with the αTbHK antisera (Fig. 2.4B). In whole cells, the distribution of signal is punctate, consistent with glycosomal
localization. Unlike the BSF parasites, extraction of the cytoskeleton did not yield flagellar-associated signal.

Probing fixed parasites and cytoskeletal preparations with the αTbHK2 antisera again yielded localization that differed from the BSF parasites. In whole cells, signal was detected in foci, consistent with glycosomes in PF parasites. However, two intense areas of staining proximal to the mtDNA (the kDNA) were unanticipated. When focusing on a basal body marker (which partially obscures the glycosomal staining), there was close association of the TbHK2 signal with the basal body marker (Fig. 2.5B). In this case, cells harboring newly divided kDNA having both basal bodies and TbHK2 associated with the new structure. Probing cytoskeletal preparations with both the TbHK2 antisera and the basal body mAb suggested that the signals were very close to one another.
Figure 2.5. Immunofluorescence (IF) microscopy of procyclic form (PF) 

*Trypanosoma brucei* brucei parasites using a *T. brucei* hexokinase 2 (TbHK2)-specific antibody. (A) PF 29-13 cells probed with the TbHK2-specific affinity-purified antisera (aTbHK2, b). This image is an extended focus of Z-stack layers. (B) PF 29-13 parasites assayed with both aTbHK2 antisera (b and f) and basal body monoclonal antibody (c and g) with the Z-stack optimized for depth of the basal bodies within the cell. (C) PF 29-13 cytoskeleton preparations stained with aTbHK2 antisera (a and d) and basal body antibody (b and e). Scale bars = 5 µm.

Using PF parasites expressing an endogenously myc-tagged TbHK2, we corroborated these findings. First, distribution of the myc signal in whole cells differs from what was found with BSF parasites, with no detectable flagellar labeling but foci consistent with glycosomes evident in fixed parasites (Fig. 2.6A). Extraction of cytoskeletons followed by IF microscopy using basal body-specific antisera indicated close localization of the anti-myc signal and the basal body (Fig. 2.6B.)
Figure 2.6 Immunofluorescence (IF) microscopy of endogenously-tagged *T. brucei* hexokinase 2 (TbHK2) in procyclic form (PF) *Trypanosoma brucei brucei* parasites.

Parasites expressing an endogenously myc tagged TbHK2 were fixed (A) or used to prepare cytoskeletons (B) and then probed for the myc epitope (A, b and f; B, a, d, and g) for localization with the axoneme (A, c and g; or basal bodies (B, b, e, and h). Scale bars = 5 µm.

**TbHK2**−/− PF parasites are as motile and directional as the parental parasites

The role of extra-glycosomal TbHK2 is not clear but its extra-glycosomal localization to either the flagellum or the basal bodies suggested that the protein could have some role in the biology of the flagellum. While TbHK2 is essential to BSF
parasites (limiting our ability to score the impact of knockout on this life-cycle stage), PF cells lacking TbHK2 have been generated (Morris et al., 2006). The PF TbHK2-deficient cells are slightly larger, have increased cellular HK activity and have a modest delay in doubling time (Morris et al., 2006). The new finding of TbHK2 proximal to the base of the flagellum in the PF parasites led us to consider that the protein could participate in one of the functions of the flagellum, possibly (but not limited to) motility.

To explore this, the motility of parental PF 29-13 cells and TbHK2−/− PF parasites was observed through time-lapse microscopy and cell velocity and directional motility were scored. Trypanosomes from both populations displayed a range of motility, including cells that were nearly immobile, cells that were non-productively motile (moving in a circular motion), and cells that moved linearly over the length of the time lapse capture (Fig. 2.7A). Cell speed analysis of the motile portion of the population revealed that both the parental cells and TbHK2−/− parasites were similarly motile. The cells exhibited maximum speeds of 16.3 and 13.2 mm/s, mean speeds of 6.2 and 4.9 mm/s, and median speeds of 4.9 and 4.7 mm/s, for parental and knockout cells, respectively (Fig. 2.7B). Although all values observed for parental parasites marginally exceeded those observed for TbHK2−/− parasites, the populations did not have significantly different motility ($P = 0.8768$), with values similar to those previously observed for PF parasites (Hutchings et al., 2002; Rodriguez et al., 2009).
Figure 2.7. Impact of *T. brucei* hexokinase 2 (TbHK2) knockout on single cell motility. Cell motility and direction of parental procyclic form (PF) 29-13 and TbHK2−/− trypanosomes were assessed based on 30 s time-lapse capture sequences. (A) Representative images used to assess 29-13 and TbHK2−/− cell line motile properties. Position of the anterior end of actively motile trypanosomes at each time point over the entire time course is indicated by the white dots in each of the five images. The directional motility of all trypanosomes in these images was high (directional motility coefficient (DMC ~1) with the exception of the TbHK2−/− cell indicated in the lower right corner of each image that moved in an “L”-shaped pattern (DMC = 0.572). Black circles enclose non-productively motile cells in both images. Black scale bar equals 50 mm. (B) Incremental speed of productively motile cells represented by a box and whisker plot. (C) Directional motility of each cell line was assessed by plotting the DMCs observed for each actively motile cell. A dot plot of these values is overlaid with a percentile plot.

While the knockout cell line displayed similar speed to the parental line, motion without direction could lead to a phenotype distinct from the parental line. To assess this, the directional tendency of
individual cells was assessed by calculation of the directional motility coefficient (DMC) (Fig. 7C). While the mean and median DMC of the parental and knockout parasites were not statistically different (means of 0.883 and 0.776 and medians of 0.948 and 0.776, for parental 29-13 and TbHK2\textsuperscript{-/-} parasites, respectively; \( P = 0.2225 \)), the data sets displayed considerable differences in shape. While both data sets are similarly skewed (\(-1.2851\) and \(-0.43748\), respectively, for parental 29-13 and TbHK2\textsuperscript{-/-}), the nature of the variation observed among the DMC distributions differs (29-13 kurtosis of 0.68861, TbHK2\textsuperscript{-/-} kurtosis of -1.1514). Taken together, these results suggest that no differences in directional motility exist between the two cell lines, but greater variation in individual cell directional motility occurs in TbHK2 deficient parasites.

**DISCUSSION**

The association of glycolytic proteins with flagellum is not without precedence, as enzymes involved in glucose metabolism have been found in the flagellum from a range of organisms, including green algae and mammalian spermatozoa (Travis et al., 1998; Mitchell et al., 2005). The role of these proteins has primarily been postulated to be in the production of ATP for the flagellum. Is TbHK2 serving a similar role in the African trypanosome flagellum? TbHK2 is competent for HK activity when organized with TbHK1 into oligomers (Chambers et al., 2008b), and TbHK1 has been identified in the flagellar proteome (Oberholzer et al., 2011), suggesting that an extra-glycosomal interaction of the two proteins may occur.

It is important to emphasize that the observations described here regarding TbHK2 localization do not rule out the possibility of TbHK1 also localizing to the flagellum. The two proteins, however, may have different levels of distribution between the flagellum and glycosome. This suggestion is supported by the intense staining of the flagellum and weak labeling of glycosomes using the aTbHK2 antisera compared with
the largely glycosomal signal observed with the aTbHK antisera. Seven of the 10 amino acid differences between TbHK1 and TbHK2 reside in the C-terminal tails of the proteins, suggesting that this region may in part be responsible for this difference in distribution.

If TbHK2 is indeed oligomerized with TbHK1 in the flagellum, it may be playing some role in the regulation of TbHK activity (as seen in vitro, (Chambers et al., 2008b)). To yield ATP, this model would require that other components of the glycolytic pathway reside near the flagellum. Consistent with that, pyruvate phosphate dikinase, glycerol-3-phosphate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase have all been found associated with the trypanosome flagellum (Broadhead et al., 2006; Oberholzer et al., 2007), but no indications exist to date for a functional flagellar glycolytic pathway capable of ATP production.

The reported lack of regulation of several glycolytic enzymes (including TbHK, phosphofructokinase (PFK), and glycerol kinase (GK) has been demonstrated to be incompatible with cytosolic localization (Bakker et al., 2000; Haanstra et al., 2008). It has been proposed that compartmentalization of the majority of the enzymes inside of glycosomes overcomes the potentially toxic effects of this lack of regulation (Bakker et al., 2000; Haanstra et al., 2008). If indeed the enzymes function in the flagellum, then there should exist a compensatory mechanism in that cellular compartment, particularly if pyruvate kinase (PYK) is present, as any net ATP synthesized could be consumed by the unregulated glycolytic kinases in the production of phosphorylated metabolites. By connecting ATP synthesis directly to ATP-consuming cellular processes (such as
flagellar movement), the parasite may avoid the consequences of unregulated kinase activity. Alternatively, flagellar TbHK2 may participate in the metabolism of sugar nucleotides to generate ATP – an activity that has been demonstrated in vitro with other HKs (Gamble and Najjar, 1955), but which remains undetected with recombinant TbHKs.

The mechanism behind the delivery of TbHK2 to the flagellum remains to be resolved. A comparison of known and predicted flagellar-resident T. brucei protein sequences with the TbHK2 sequence does not reveal an obvious conserved sequence that could direct localization. Additionally, comparison with sequences from flagellar-associated glycolytic proteins from other organisms such as green algae and mammals does not yield any notable conserved sequences (Travis et al., 1998; Mitchell et al., 2005). However, boar GAPDH exists as a hexamer when associated with sperm flagellum but not in muscle tissue, suggesting oligomerization may play a role in targeting (Westhoff and Kamp, 1997).

The first indication that TbHK2 might have an extra-glycosomal localization was based on the finding that TbHKs were released by concentrations of digitonin sufficient to liberate cytosolic proteins (Fig. 1A). In contrast, some TbHK2 signal remained associated with the flagellum in detergent-extracted cytoskeletons, suggesting that within a life-cycle there exist distinct inter-flagellar pools of the protein. In BSF parasites, for example, a portion of the protein resides in the flagellar compartment but is subject to liberation by low concentrations of detergent, while the remaining fraction is more tightly associated with the cytoskeleton, based on sensitivity of the signal to detergent extraction.
The life-cycle stage-dependent distribution of TbHK2 suggests that the protein may have different functions during distinct developmental phases. In PF parasites, TbHK2 basal body association could result from docking and binding via transitional fibers prior to entry into the flagellum in a relationship similar to that which has been observed in *Chlamydomonas* between IFT52 proteins and transitional fibers of basal bodies (Deane et al., 2001). The association with the basal body in PF parasites could position the protein for mobilization down the length of the BSF flagellum. Because the PF cell has a dynamic metabolism that is not entirely dependent on glycolysis for ATP generation, it is possible that TbHK2 localization along the length of the flagellum in BSF parasites reflects the demand of the organelle for ATP, which in that life-cycle stage can only be provided by glucose metabolism.

The lack of a detectable phenotype in TbHK2-deficient cells, however, suggests TbHK2 may not be limited to a metabolic function in the flagellum, but rather the protein may play a role in sensing of glucose and other hexoses. Glucokinases and HKs from other systems including yeast, plants and animals, have all been shown to be central in conveying information to the cell regarding environmental glucose availability that allows the cell to respond (Rolland et al., 2001). In other kinetoplastids, hexose transporters have been found associated with the flagellum (Snapp and Landfear, 1999), suggesting that uptake of glucose may occur proximal to the flagellar positioning of TbHK2, positioning it to serve in an environmental glucose sensing role. In *T. brucei*, signaling enzymes associated with the flagellum are not without precedence, as an
adenylate cyclase (ESAG4) has been found associated with the flagellum in both PF and BSF parasites (Paindavoine et al., 1992).

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ABSTRACT

In *Trypanosoma brucei*, the bulk of the glycolytic enzymes are located in a peroxisome-like organelle called the glycosome. Glycolytic enzymes are translated on cytosolic ribosomes and localized to the glycosome via a peroxisomal targeting sequence (PTS), of which there are three: PTS1, PTS2, and internal-PTS. It has also been previously demonstrated that the localization of these enzymes to the glycosome is necessary for cell survival. The first enzyme in glycolysis, hexokinase (HK), is known to localize to the glycosome via PTS2-induced targeting. PTS2-containing proteins in other systems have been shown to interact with the PTS2 receptor PEX7 (also called peroxin 7), which interacts with PEX14 (peroxin 14), a glycosomal matrix protein, allowing import of the PTS2-protein:PEX7 complex. Here we demonstrate that PEX7 co-localizes
with glycosomes and present preliminary data suggests that recombinant PEX7 interacts with recombinant HK1.

INTRODUCTION

*Trypanosoma brucei* is the causative agent of African sleeping sickness in humans and nagana in cattle and other livestock. One distinct difference between trypanosomes and their mammalian hosts is the localization of the first seven enzymes of glycolysis to the glycosome in the bloodstream form (BSF) of the parasite (1,2). Glycosomes are single-membrane peroxisomal-like organelles; however, glycosomes are functionally distinct from peroxisomes. For example, catalase, a hallmark enzyme of peroxisomes, is absent from glycosomes (3). Glycosome-resident proteins are encoded by nuclear DNA and translated on cytosolic ribosomes. These proteins are then imported to the glycosome as a consequence of harboring a peroxisomal targeting sequence (PTS).

There are three known PTSs: PTS1, PTS2, and I-PTS. The PTS1 and PTS2 sequences are located on the protein C-terminus (4) and N-terminus (5), respectively. The third PTS sequence is more variable and is termed an Internal-PTS (I-PTS).

Proper import of glycosome-resident proteins is essential to the survival of the parasite. In procyclic form (PF) parasites, RNAi of PEX14, a protein necessary for the formation of glycosomes, is only tolerated when cells are first pre-adapted to growth in glucose-free media, unless there is a coincident silencing of HK (6). This suggests that compartmentalization plays a role in regulation of the HK; however, this may not be the only explanation as other mechanisms have been shown to regulate TbHK activity (7,8).
Additionally, localization of glycolytic proteins to the glycosome, providing proper organization of the pathway, is likely essential in the BSF of the parasite, as it depends solely on glycolysis for ATP production.

*T. brucei* expresses two hexokinases, TbHK1 and TbHK2, both of which possess a PTS2 in their N-terminus (9). Peroxin 7 (PEX7) has been shown in other systems to be necessary for localization of PTS2 proteins (10-12). A *T. brucei* PEX7 has also been identified. This protein is predicted to be 39.7kDa (13) and share 65-76% identity with PEX7 homologs from other trypanosomatids; however, the trypanosomatid PEX7s only share 32-34% identity with human protein.

Here we explore the function of TbPEX7 with particular focus on localization of the protein and studies to explore its ability to bind TbHK. We have found that the majority of the TbPEX7 co-localizes to glycosomes suggesting most TbPEX7 is bound by cargo destined for the glycosome and is not found in the cytoplasm. Additionally, we have preliminary evidence suggesting that TbPEX7 binds to TbHK *in vitro*.

**MATERIALS AND METHODS**

**Production of Recombinant TbPEX7 Protein**

The open reading frame of TbPEX7 was amplified by PCR from genomic DNA isolated from PF 29-13 (427 strain) parasites and cloned into the pMAL-TEV vector (a gift from Dr. Michael Sehorn). The sequence was inserted in-frame upstream from the maltose-binding protein (MBP) tagging sequence, and constructs were confirmed with sequencing. This plasmid was transformed into *E. coli* M15(pREP), and cultures were
grown at 37°C to an OD$_{600}$ of ~0.5 and induced with 0.3 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). The culture was grown at RT overnight and harvested at 2900 xg at 4°C. The pellet was resuspended in column buffer [(20 mM Tris-Cl, pH 7.4, 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)] with 1 cOmplete Mini, EDTA-free protease tablet (# 11 836 170 001, Roche, Mannheim, Germany) and frozen. The thawed pellet was sonicated and centrifuged (10,000 x g, 30 min, at 4°C). Soluble proteins were run over an amylose resin (#E8021S, New England Biolabs, Ipswich, MA) and washed with column buffer. The protein was eluted with column buffer containing 10 mM maltose, and the elutions were analyzed using SDS-PAGE.

**Production of Recombinant TbHK1 Protein**

The open reading frame of TbHK1 was amplified by PCR from pQE30:HK1 (7) and cloned into pQE70 (Qiagen, Valencia, CA) in-frame upstream of a His$_6$ tagging sequence. The purification of the C-terminally-tagged TbHK1 was performed using a protocol developed for purification of a N-terminally His$_6$ tagged TbHK1 (7) and products resolved by SDS-PAGE analysis.

**In Vitro Pull-down Experiments**

PEX7:MBP (21 µg in column buffer) and HK1:His$_6$ (45 µg in 20 mM Tris-HCl pH 7.4) were mixed by rotation at 4°C for 1-2 hours. Ni-NTA beads (#R10-22-40-42/43, Qiagen, Valencia, CA) were then added to the mixture and rotation continued at 4°C for 1-2 hours. After the incubations, the beads were centrifuged (1000 x g; 2 min) and the
unbound fraction was removed. The beads were then washed in buffer (20 mM Na$_2$HPO$_4$, 5 mM glucose, 0.4 M (NH$_2$)$_2$SO$_4$), and the proteins were eluted with buffer containing 250 mM imidazole. Samples were resolved using SDS-PAGE, transferred to nitrocellulose, and analyzed via Western. For recognition of TbHK1, a mouse monoclonal anti-His$_6$ antibody (1:2000; Cell Signaling Technology, Danvers MA) was used, and for recognition of PEX7, a mouse monoclonal anti-MBP antibody (1:100,000; New England Biolabs, Ipswich MA) was used. Relative intensity of bands was determined using Image J software.

**Expression of Tagged TbPEX7 and Variants In Vivo**

A constitutively expressed GFP-tagged TbPEX7 was generated using pXS2 (a gift from Dr. Jay Bangs). In order to generate this construct, TbPEX7 was amplified from purified genomic DNA with the appropriate sites for ligation and cloned into the vector, and the plasmid construct confirmed through sequencing.

TbPEX7 variants were generated using the pXS2:PEX7 as a template and the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). The primers used were as follows: D132N-5’CTTTT CCTGCAGTTGGAATGGGGCTGTGAAGCTC3’ and H241L-5’ACAGTGTGCCCCGGCCTCGATAACGCGTGCCGA3’.

For the transfection of PF 29-13 parasites (strain 427), linearized constructs (for stable transfection) or circular plasmid (for transient transfection) were introduced by electroporation as described previously (14).
**Fluorescence Microscopy**

Live cells were harvested (800 x g, 5 min) and washed twice with Voorheis’s modified PBS (vPBS; 137 mM NaCl, 3 mM KCl, 16 mM Na$_2$HPO$_4$, 3 mM KH$_2$PO$_4$, 46 mM sucrose, 10 mM glucose, pH 7.6). The cells were then applied to a slide and visualized on a Zeiss Axiovert 200M using Axiovision software version 4.6.3 for image analysis.

**Immunofluorescence Microscopy**

Immunofluorescence assays (IFA) were performed as reported (15). Briefly, cells were harvested (800 x g for 5 min) and washed in vPBS. Next, cells were fixed in 3% paraformaldehyde on ice for 1 hour, and washed with vPBS. The parasites were then allowed to settle on a poly-L-lysine slide and permeabilized with 0.1% Triton X-100 in PBS (137 mM NaCl, 3 mM KCl, 16 mM Na$_2$HPO$_4$, 3 mM KH$_2$PO$_4$) for 10 min and washed in PBS. Blocking solution (1% BSA and 0.25% Tween in PBS) was then added to each well for 1 hour at RT. The primary antibodies, anti-GFP3E6 (1:400; Molecular Probes, Eugene OR) and αHK (1:500) (16) were incubated with both 29-13 parental cells and the overexpressing cell line pXS2:PEX7eYFP. After washing with PBS, the cells were then incubated with appropriate secondary antibody, FITC conjugated goat anti-mouse (1:100, Rockland, Gillbertsville PA) or TexasRed conjugated goat anti-rabbit (1:100, Rockland, Gillbertsville PA), and visualized on a Zeiss Axiovert 200M using Axiovision software version 4.6.3 for image analysis.
RESULTS

Recombinant TbPEX7 and TbHK1

Recombinant TbPEX7 was partially purified from bacteria (Figure 3.1A). SDS-PAGE analysis revealed a species at the approximate size of the PEX7:MBP fusion protein, 70 kDa. The smaller proteins are likely degradation products of the PEX7:MBP fusion protein. A C-terminally tagged TbHK1 was also produced for use in this study (Figure 3.1B). Having the His$_6$ tag on the C-terminus, rather than the N-terminus is important because the PTS2, thought to be responsible for binding to TbPEX7, is located on the N-terminus of the protein, and a tag there may interfere with binding to TbPEX7. In order to verify that the recombinant, C-terminally tagged protein is functional, we determined its specific activity (0.60 ± 0.03 mmol•min$^{-1}$•µg$^{-1}$), which is comparable to that of the N-terminally tagged protein (0.79 ± 0.01 mmol•min$^{-1}$•µg$^{-1}$, respectively; (7).

Figure 3.1. Purification of rTbPEX7 and rTbHK1. (A) SDS-PAGE analysis of recombinant TbPEX7:MBP. (B) SDS-PAGE analysis of rTbHK1 produced with a C-terminal His$_6$ tag.
Pull-down of PEX7MBP with HK

In order to determine whether or not PEX7 was binding to HK in vitro, we used the aforementioned protein preparations in pull-down assays. The His$_6$ tag fused to TbHK1 should bind the Ni-NTA resin, as demonstrated in Fig. 3.2 (HK/PEX7, anti-His$_6$) by the appearance of HK in the elution fractions alone. If the TbPEX7:MBP fusion protein binds TbHK1, it would appear in the elution along with TbHK1. TbPEX7:MBP (Fig. 3.2, HK/PEX7, anti-MBP) does appear in the elution fractions, suggesting binding to TbHK1. However, in the control incubation of TbHK with MBP alone (Fig. 3.2, HK/MBP), a small amount of MBP appears in the elution fractions as well (Fig. 3.2, HK/MBP, anti-MBP). This suggests that MBP may have a slight interaction with the Ni-NTA resin or with TbHK. It is apparent that TbPEX7:MBP does not interact with resin alone, as evidenced by the absence of signal detected in the elution fractions (Fig. 3.2, PEX7, anti-MBP). Further analysis will be required to determine if rTbPEX7:MBP is binding rTBHK1.
Figure 3.2. Pull-down of PEX7:MBP using TbHK1:His\textsubscript{6} that is bound to Ni-NTA resin. For the HK/PEX7 pull-down, rTbHK1 (45 µg) was incubated with rTbPEX7 (21 µg). The αHis\textsubscript{6} antibody is used to detect rTbHK1, and the anti-MBP antibody is used to detect rTbPEX7. For the HK/MBP pull-down control, rTbHK1 (45 µg) was incubated with MBP (1 µg). The anti-His\textsubscript{6} antibody is used to detect rTbHK1, and the anti-MBP antibody is used to detect rMBP. For the PEX7 pull-down control, rTbPEX7 (21 µg) was incubated alone with Ni-NTA resin, and the anti-MBP antibody is used to detect rTbPEX7.

**Localization of TbPEX7**

In order to determine whether TbPEX7 localizes to the glycosomes of *T. brucei*, an overexpression cell line was made in which the cells overexpress TbPEX7:eYFP. Using immunofluorescence assays (IFA), TbPEX7:eYFP overexpressed in PF trypanosomes localizes to punctate bodies throughout the cell (Figure 3.3, green). In order to determine if these bodies were glycosomes, the slides were probed for a
glycosome-resident protein, TbHK (Figure 3.3, red). The merge demonstrates that TbPEX7:eYFP co-localizes with TbHK, and therefore the glycosomes.

**Figure 3.3. PEX7eYFP co-localizes with TbHK.** IFA of PF29-13 and pXS2:PEX7eYFP cells performed using both anti-GFP3E6 antisera (green; detected with FITC conjugated secondary antibody) and anti-HK antiserum (red; detected with a TR conjugated secondary antibody). DAPI (blue) was used to detect DNA in all samples. Scale bar = 5 µm.

Rhizomelic chondrodysplasia punctata (RCDP) is classified as a peroxisomal biogenesis disorder in humans (17). A diversity of human PEX7 mutations cause mislocalization of peroxisomal proteins, and some of these mutations have been found to be responsible for RCDP in patients (Fig. 3.4A, top panel).
Figure 3.4. TbPEX7 variant production and localization. (A) In a study of RCDP in humans (17), PEX7 mutations were identified at the indicated residues and determined to be responsible for the syndrome (top panel). Residues D134, H241, and H285 are conserved among humans, mice, yeast, and trypanosomes. The TbPEX7 D132 and H241 were altered using site-directed mutagenesis (bottom panel) and the impact on localization scored (B). Scale bar = 5 µm.
These mutant human PEX7 proteins fail to recognize receptors on the peroxisomes and therefore do not localize to peroxisomes correctly. A comparison of the sequences of PEX7 from a number of species and *T. brucei* PEX7 (Fig. 3.4A) revealed that residues D134, H241, and H285 are conserved in all species examined. Notably, these same residues are found to be mutated in patients with human peroxisome import defects, suggesting these conserved amino acids may be central to peroxisome/glycosome localization. To explore the role of these residues on TbPEX7 localization, site-directed mutagenesis was used to alter TbPEX7, yielding D132N and H241L variants. Overexpression constructs (pXS2:PEX7eYFP, pXS2:PEX7D132NeYFP, and pXS2:PEX7H241LeYFP) were then generated and localization analyzed by fluorescence microscopy (Figure 3.4B). The TbPEX7 D132N and H241L variants yielded localization to the glycosomes similar to that of wild-type TbPEX7; however, there does appear to be increased cytosolic distribution as well. Further analysis would be needed to determine if this difference is significant.

**DISCUSSION**

While the mechanism for peroxisome/glycosome import of PTS1 and PTS2 proteins has been elucidated in other systems, to date it has not been definitively shown in *T. brucei*. In mammalian cells, PEX7 is known to bind PTS2-containing proteins and is necessary for their translocation to the peroxisome; however, interaction with PEX5 is also necessary for import of PTS2 proteins (17). Interestingly, in yeast, it does not appear
that PEX7 associates with PEX5 for import of PTS2-containing proteins (18). After import of the PTS2:PEX7 complex, PEX7 is shuttled back to the cytosol, in yeast (19).

In trypanosomes, PEX5 is responsible for import of PTS1 proteins to the glycosome (20). In *T. brucei*, PEX5 contains a putative PEX7 binding box (13). In a related trypanosomatid, *Leishmania major*, PEX7 binds PTS2 proteins as well as PEX5, and PEX14 (21). This suggests that PEX5 and PEX7 may interact prior to import into the glycosome via PEX14 in *L. major*.

**Figure 3.5. Model for PTS2 protein localization to the glycosome.** There are two models for PTS2 protein import into the glycosome. (1) A PTS2 protein is bound by PEX7, either before or after PEX7 binds PEX5. The PTS2:PEX7:PEX5 complex is then imported into the glycosome through an interaction with PEX14. (2) A PTS2 protein is bound by PEX7, and that complex is imported via PEX14 into the glycosome. In both models, PEX7 would be shuttled back into the cytosol after release of the PTS2 protein in the glycosome.

Here we have demonstrated that overexpressed TbPEX7eYFP co-localizes almost exclusively with glycosomes in PF trypanosomes. We also present preliminary evidence
that suggests rTbPEX7 may be binding rTbHK1 \textit{in vitro}. Based on our findings here and reports in other systems, we propose two models that would explain PTS2 protein import into the glycosome (Fig. 3.5). First, a PTS2 protein could bind PEX7, which may or may not already be bound to PEX5. After the PTS2:PEX7:PEX5 complex forms, interaction with PEX14 would allow translocation into the glycosome. Second, PEX7 binds the PTS2 protein, and this complex is translocated into the glycosome via an interaction with PEX14. For both models, PEX7 is shuttled back into the cytosol after release of the PTS2 protein.
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   3293
    4908-4918


CHAPTER FOUR

TRYPANOSOMA BRUCEI HEXOKINASE 1 CYSTEINE RESIDUES PARTICIPATE IN INHIBITOR BINDING AND OLIBOMERIZATION

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ABSTRACT

Glycolysis is essential to Trypanosoma brucei, the causative agent of African sleeping sickness in humans, suggesting enzymes in the pathway could be targets for drug development. Recently, ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one, Eb) was identified as a potent inhibitor of the first enzyme in glycolysis, T. brucei hexokinase 1 (TbHK1), in both a screen of a library of pharmaceutically active compounds and in a high throughput screen of a 220,223 compound library. Eb has a history of promiscuity as an enzyme inhibitor, inactivating proteins through formation of selenyl-sulfide conjugates with Cys residues. Cys residues are important for enzymatic activity, as the irreversible disulfide-reactive reagent tris (2-carboxyethyl)phosphine inhibited TbHK1 (IC₅₀ of 6.6 ± 0.4 mM). Additionally, three (C212, C327, and C386) of the nine Cys residues were required for enzyme activity, as determined by site-directed mutagenesis. The remaining six active variants remained as sensitive to Eb as the unaltered enzyme.
Pre-incubation of TbHK1 with Eb prevented 3-(N-maleimidopropionyl)-biocytin (MPB) modification of free TbHK1 Cys residues in a dose dependent fashion. While Eb binds to TbHK1 Cys residues, inhibition likely works through a distinct mechanism, as SID 17387000, a TbHK1 inhibitor that is a structural analog of Eb, did not alter MPB labeling. Last, blocking free Cys residues with MPB did not disrupt Eb inhibition, suggesting that Eb interaction with TbHK1 Cys is distinct from mode of inhibition.

**INTRODUCTION**

*Trypanosoma brucei* is the causative agent of African sleeping sickness in humans and nagana in livestock, both of which are major causes of underdevelopment in poor and remote regions of sub-Saharan Africa (1). Bloodstream form (BSF) *T. brucei*, the lifecycle stage that grows rapidly in the blood of the mammalian host, depends solely on glycolysis for ATP production. As a consequence, glycolytic enzymes from the parasite have been considered as potential targets for therapeutic design. Hexokinase (TbHK), the first enzyme in glycolysis, has previously been genetically and chemically validated as a potential target. Further, the enzyme has been the subject of both structure-based approaches and high throughput screening (HTS) campaigns to identify compounds with potential as leads in therapeutic development.

*T. brucei* harbors two hexokinase genes, *TbHK1* and *TbHK2*, and both are essential to BSF parasites (2,3). The TbHKs, whether purified from parasites or heterologously expressed in *E. coli*, oligomerize into hexamers. Due to the 98% identity of TbHK1 and TbHK2, the contribution of both to hexamer formation *in vivo* has
remained elusive. However, recombinant heterohexamers generated in vitro have kinetic properties more similar to those reported for T. brucei-derived TbHK than recombinant TbHK homohexamers (4), suggesting that in the parasite, the oligomers are most likely heterohexamers. Notably, the composition of the heterohexamers in vivo is regulated in response to the nutritional environment in which the cells are cultured (4). The mechanisms behind this dynamic hexamerization, including the enzyme protein domains that participate in oligomerization, remain to be elucidated.

Recently, a high throughput screen has been completed with 220,233 compounds assessed as inhibitors of TbHK1 (5). From this screen, six structurally related compounds were identified. Ebselen (2-phenyl-1,2-benzisoselenazol-3(2H)-one, Eb) was the most potent TbHK1 inhibitor found through the HTS campaign, with an IC$_{50}$ of 0.05 ± 0.03 µM (5). Notably, 2-phenyl-1,2-benzisothiazol-3(2H)-one (SID 17387000), which differs from Eb by replacement of the selenium with sulfur, was also identified in the HTS as a potent TbHK1 inhibitor (IC$_{50}$ of 2.0 ± 0.5 µM).

Eb can form selenyl-sulfide adducts with Cys residues of proteins. Studies of the Eb mechanism of inhibition of human indoleamine 2,3-dioxygenase (IDO) indicated that Eb reacts with multiple IDO Cys residues causing a change in enzyme conformation that leads to inactivation of the protein (6). Here, we explore the role Cys residues have in Eb inhibition of TbHK1. Through these efforts, we have found that Eb indeed does interact with TbHK1 Cys residues, but this interaction does not account entirely for the observed inhibition. Additionally, we have characterized the role of the Cys residues in enzyme
activity and the impact of their modification on oligomerization, finding that several of the Cys residues are essential for catalysis and can influence hexamerization.

MATERIALS AND METHODS

Reagents

Tris (2-carboxyethyl)phosphine (TCEP), glucose-6-phosphate dehydrogenase, \( \beta \)-nicotinamide adenine dinucleotide (NAD\(^+\)), adenosine triphosphate (ATP), and glucose were purchased from Sigma (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburgh, PA), while phosphoenol pyruvate (PEP), 2-phenyl-1,2-benzisoselenazol-3(2H)-one (ebselen, Eb, PubChem SID 856002) and glucosamine were obtained from VWR International (West Chester, PA). 3-(N-maleimidopropionyl)-biocytin was purchased from Cayman Chemical (Ann Arbor, MI).

Recombinant Enzyme Purification and Assay Conditions

Recombinant TbHK1 was purified as described from a culture of \textit{E. coli} M15(pREP) harboring pQE30 (Qiagen, Valencia, CA) with TbHK1 cloned in frame of a His\(_6\) tagging sequence (7). Briefly, a 10 mL culture was used to inoculate a 1 L culture which was grown to an OD of ~1 and then induced for 24 hr at room temperature with 0.250 mM isopropyl \( \beta \)-D-1-thiogalactopyranoside (IPTG).

TbHK1 Cys variants were generated using the wildtype pQE30 TbHK1 construct and a QuikChange II Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). Primers used for the mutagenesis are listed in Supplementary Table 1 (Table S1) and generation
of all variants was confirmed by sequencing. Protein expression and purification were performed as described above, with fractions from the purification probed by Western blotting using an anti-RGS-His$_6$ antibody (Qiagen, Valencia, CA) to identify those harboring the recombinant protein. All variants were ~90% pure based on coomassie blue staining of proteins resolved by SDS-PAGE.

HK assays were performed in triplicate as described using a coupled reaction to measure enzyme activity (Misset 1984 and Morris 2006). In short, the coupled assay uses glucose-6-phosphate dehydrogenase (G6PDH) to convert glucose-6-phosphate (G6-P) generated by HK to 6-phosphogluconate with coincident reduction of NADP to NADPH, which is monitored spectrophotometrically at 340 nm. Kinetic analyses were performed using KaleidaGraph 4.1 (Synergy Software, Reading, PA).

Native Gel Analysis

For native gels, protein samples were diluted in native gel loading buffer (10% (v/v) glycerol, 2.7 mM Tris-HCl, pH 6.8, 0.1% bromphenol blue) and resolved on a 4% polyacrylamide gel (4% bis acrylamide, 375 mM Tris-Cl, pH 8.8, 0.05% (v/v) TEMED, and 0.05% ammonium persulfate) using a Tris/glycine buffer (2.7 mM Tris-HCl and 192 mM glycine, pH 6.9). Proteins were detected by silver staining (4).

3-(N-Maleimidopropionyl)-biocytin (MPB) Assays

TbHK1 and variants (equal amounts as determined by coomassie staining of a SDS-PAGE) were incubated (30 min, RT) with MPB (50 µM) in buffer [(20 mM
Na$_2$HPO$_4$, 5 mM glucose, 0.4 M (NH$_4$)$_2$SO$_4$], resolved by 4% native gel electrophoresis, and monomer analyzed following transfer to nitrocellulose by western blotting using an anti-biotin antibody (1:10,000; Cell Signaling Technology Danvers, MA). Band intensity was quantitated using ImageJ software. To score the consequences of inhibitor on MPB labeling, TbHK1 was incubated with increasing concentrations of inhibitor (15 min, RT) followed by addition of MPB (50 µM), and the mixture incubated for an additional 30 min at RT.

RESULTS

We found previously in a screen of a library of pharmacologically active compounds (LOPAC) screen that the thiol-reactive reagent 4-chloromercuribenzoic acid was a potent inhibitor of TbHK1 (84.7 ± 0.4% inhibition at 10 µM; 5). Because the role of thiols in TbHK1 activity was unclear, we tested the irreversible disulfide-reactive reagent tris (2-carboxyethyl)phosphine (TCEP) as an inhibitor of TbHK1. This small molecule is hydrophilic, suggesting it would have access to residues near the active site that may participate in binding to hydrophilic substrates. TCEP inhibited TbHK1, with an IC$_{50}$ of 6.6 ± 0.4 µM, supporting the notion that Cys residues may play a role in enzyme catalysis.

Eb, which has been shown to modify active site Cys residues leading to enzyme inactivation, was identified as a TbHK1 inhibitor in two independent screens. In the first, a LOPAC screen, Eb inhibited TbHK1 88.1 ± 0.6% inhibition at 10 µM. In the second, Eb and five other structurally related isobenzothiazolinones were identified as TbHK1
inhibitors in a high throughput screen of 220,233 small molecules. While Eb was the most potent isobenzothiazolinone (IC$_{50}$ of 0.05 ± 0.03 µM), the finding that it can form covalent selenyl-sulfide conjugates with Cys residues suggested it may not be an ideal lead for further development. Supporting this supposition, Eb has been identified as an inhibitor of a number of different enzymes in multiple validated HTS campaigns, possibly because of its Cys-reactive nature (5) (Fig. 4.1A). The observed covalent modification of Cys residues in other enzymes suggests that Eb potentially interacts with TbHK1 through one of the nine Cys residues in the enzyme (Fig. 4.1C).

Fig. 4.1. Ebselen, a known Cys-reactive compound, inhibits TbHK1 activity. (A)

Chemistry involving the Se of Ebselen (Eb) can lead to covalent modification of Cys residues via a selenyl-sulfide conjugate. (B) SID 17387000 is a structural analog of Eb and also a potent TbHK1 inhibitor. (C) The predicted distribution of TbHK1 Cys residues, with the catalytic base (D214) included to indicate the active site. [The structure is based on the yeast Hxk structure, as described (13)].
DTT can block, but not reverse, Eb inhibition of TbHK1

Eb inhibition of IDO has been reported to be reversible by inclusion of the reducing reagent dithiothreitol (DTT) in the assays. To determine if Eb inhibition of TbHK1 was reversible, the enzyme was incubated with the inhibitor either before or after treatment with DTT and the enzyme activity scored. Alone, DTT (1 mM) had little impact on enzyme activity. Addition of DTT after Eb incubation failed to rescue enzyme activity, while co-incubation (hatched bars) or pre-incubation (solid bars) of TbHK1 with DTT prevented Eb inhibition, even at a concentration of inhibitor that repressed enzyme activity more than 80% when reducing agent was not included (Fig. 4.2). One possible explanation of these observations is that DTT prevented TbHK1 inhibition by interacting directly with Eb to block interaction of the small molecule with the enzyme. A report describing the formation of DTT/Eb adducts supports this possibility (8).
Fig. 4.2. DTT can block but not reverse TbHK1 inhibition by Eb. TbHK1 (32 ng) was incubated with Eb alone (white bars) or co-incubated with DTT and Eb (hatched bars) for 15 minutes prior to assay. For the gray and solid bars, TbHK1 (32 ng) was pre-incubated with Eb or DTT, respectively, for 15 minutes and then the other was added to the mixture and allowed to incubate for an additional 15 minutes.

Mutagenesis of individual Cys residues in TbHK1

The importance of Cys residues in both enzyme catalysis and inhibitor binding remained unclear, so to further characterize their role, variants harboring individual changes of the nine Cys residues were generated (Fig. 4.1C). Upon the change to Ala, three of the variants (C212, C327, and C386) lacked detectable enzyme activity (Table 1), while three other variants (C369A, C401A, and C402A) had subtly reduced specific activities. The remaining two variants (C242A and C445A) had activities similar to the unaltered enzyme.
Table 4.1. Specific Activity and Sensitivity to of TbHK1 Cys Variants

<table>
<thead>
<tr>
<th>Variant</th>
<th>Specific Activity $^{\text{a}}$ (mmol$^{-1}$•min$^{-1}$•µg$^{-1}$)</th>
<th>Eb IC$_{50}$ (µM)</th>
<th>TCEP IC$_{50}$ (mM)</th>
<th>Eb/MPB EC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.60 ± 0.03</td>
<td>0.35 ± 0.07</td>
<td>6.6 ± 0.4</td>
<td>0.36</td>
</tr>
<tr>
<td>C212A</td>
<td>ND$^a$</td>
<td>ND</td>
<td>ND</td>
<td>0.53</td>
</tr>
<tr>
<td>C242A</td>
<td>1.1 ± 0.12</td>
<td>0.55 ± 0.02</td>
<td>4.1 ± 0.3</td>
<td>0.31</td>
</tr>
<tr>
<td>C327A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.62</td>
</tr>
<tr>
<td>C369A</td>
<td>0.24 ± 0.02</td>
<td>0.26 ± 0.02</td>
<td>7.5 ± 0.4</td>
<td>0.56</td>
</tr>
<tr>
<td>C386A</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.40</td>
</tr>
<tr>
<td>C401A</td>
<td>0.17 ± 0.03</td>
<td>0.29 ± 0.04</td>
<td>10.1 ± 0.7</td>
<td>0.48</td>
</tr>
<tr>
<td>C402A</td>
<td>0.23 ± 0.01</td>
<td>0.43 ± 0.11</td>
<td>7.2 ± 0.2</td>
<td>0.40</td>
</tr>
<tr>
<td>C445A</td>
<td>0.79 ± 0.01</td>
<td>0.52 ± 0.05</td>
<td>5.4 ± 0.1</td>
<td>0.09</td>
</tr>
</tbody>
</table>

$^a$ND, Not determined, as activity was not detected

Eb and TCEP were tested against the active variants. Both compounds were potent inhibitors of all of the variants, with no detectable difference in sensitivity compared to unmodified TbHK1 (Table 4.1). These observations could be explained in several ways: First, the inhibitors may interact with more than one Cys residue on the protein. Alternatively, the critical Cys that interacts with the Cys-reactive reagent leading to inactivation of the enzyme may be one of the three required for activity (C212, C327, or C386). Last, the compounds may interact with residues other than (or in addition to) Cys to inhibit the enzyme.

To further elucidate the role of Cys residues in Eb inhibition, the ability of Eb to prevent MPB labeling of Cys residues was scored by pre-incubating the enzyme with Eb prior to MPB addition. In the absence of Eb, MPB modified TbHK1 Cys residues, though up to the concentrations used in these studies (80 µM), it does not inhibit the enzyme. With increasing concentrations of Eb, however, MPB biotinylation of free TbHK1 Cys residues was reduced, with complete ablation of detectable signal at 2 µM.
Eb (Fig. 4.3A, top). The effective concentration of Eb that caused a 50% reduction in MPB labeling (EC\textsubscript{50}) was 0.36 \(\mu\)M for TbHK1 (Table 4.1). Most of the TbHK1 variants had similar EC\textsubscript{50} values, with the exception being C445A (Table 1), which had a 4-fold lower EC\textsubscript{50} value than unmodified TbHK1, however; the experiment must be repeated in order to confirm this result. Also, MPB-labeled protein remained sensitive to Eb inhibition (Fig. 4.3C).

**Fig. 4.3. Ebselen prevents MPB modification of TbHK1; however, MPB modification does not block Ebselen inhibition.** (A) TbHK1 was incubated with increasing concentrations of Eb (top) or compound 7000 (bottom) for 15 min. MPB (50 \(\mu\)M), which biotinylates free Cys residues, was then added to the mixture and incubated for an additional 30 min. The blot was probed
with anti-biotin antibody. (B) Graphical representation of data in (A). (C) TbHK1 (32 ng) was pre-incubated with MPB (50 µM) to determine the effects of the modifying agent on Eb inhibition.

These findings suggested that Eb is indeed interacting with TbHK1 Cys residues, similar to its behavior when inactivating human IDO (6). Notably, a minor contaminant in the TbHK1 preparation remained MPB labeled, even in the presence of 1 µM Eb (not shown), indicating that the reduction of TbHK1 labeling is not simply the result of excess Eb interacting with the MPB and preventing labeling.

Pre-incubation of TbHK1 with SID 17387000 (7000), a structural analog of Eb (Fig. 4.1B), only modestly altered MPB biotinylation of Cys residues, with ~50% reduction of labeling at the highest concentration of 7000 tested (Fig. 4.3A and Fig. 4.3B).

**Cys variants differ in hexamer abundance**

The impact of Cys mutations on the hexamerization of the protein was also variable. Several variants (C369A, C386A, C401A, and C445A) had a slight increase in hexamer abundance when compared to recombinant TbHK1, while C212A, C242A, C327A, and C402A were not impacted (Fig. 4.4A). These findings were generally in good agreement with the relative monomer abundance. That is, as hexamer concentration increased, the same sample had a corresponding relative decrease in monomer abundance, though resolving this correlation is confounded by the inability to score the contribution of higher ordered oligomers like dimers and trimers.
Figure. 4.4. Relative hexamer, monomer, and free Cys abundance for TbHK1 and variants. (A.) Relative hexamer and monomer abundance of TbHK1 and Cys variants. Relative hexamer abundance (hatched bars) was determined by silver staining a 4% Native gel and analyzing the appropriately sized band. Relative monomer abundance (black bars) was determined through Western analysis (with anti-His antibody) of a 4% native gel of each of the variants. (B.) MPB modification of the monomer was determined by incubating each of the samples with MPB (50 µM) for 30 minutes, running the samples on a 4% native gel, transferring protein to nitrocellulose, and probing with anti-biotin.

Notably, we found that C445A had a ~10-fold reduction in monomer abundance, suggesting this residue influences oligomerization. C445A also has an increased number of free Cys residues available for biotinylation by MPB per monomer as compared to TbHK1 (Fig. 4.4B). Potentially this alteration may lead to a very unstable monomer structure that, as a result of the modification, has many of the Cys residues exposed.
DISCUSSION

Currently there are four drugs approved for treatment of African trypanosomiasis: suramin, pentamidine, melarsoprol, and eflornithine. Suramin and pentamidine are only effective against the initial blood-stage of the disease and are not useful for treatment once the parasite has crossed the blood-brain barrier, the second stage of the disease (9). For the second stage of the disease, melarsoprol, an arsenic derivative first used in 1949, is the main-line treatment; unfortunately, it has many side effects, including death. Eflornithine is also used to treat the second stage of the disease in *T. gambiense* infections, but requires a very complex dosage scheme. Therefore, it is imperative that new effective anti-trypanosomal compounds be discovered, with a focus on understanding the targets of the treatments and how the drugs are interacting with these targets in order to anticipate possible resistance mechanisms.

TbHK1 has previously been validated as a target for therapeutic treatment of African trypanosomiasis (3,5,10) with Eb identified as a potent inhibitor of TbHK1 in a recent HTS (5), having an IC$_{50}$ of 0.05 ± 0.03 µM. Compound 7000 is also a potent inhibitor with an IC$_{50}$ of 2.0 ± 0.5 µM. In order to establish toxicity to human hexokinases, the compounds were screened against human glucokinase (hGlk). Ebselen almost completely inhibited hGlk (97.8%), while compound 7000 was weakly inhibitory at 10 µM (6.7%). Interestingly, Eb and compound 7000 are structurally very similar, with the only difference being a sulfur in compound 7000 that replaces the selenium of Eb. As a consequence of the similarities (but the lack of the reactive Se group), SID 7000 is useful as a control compound for Eb.
Here we show that Eb interacts with TbHK1 Cys residues; however, the inhibition of Eb is not a consequence of this interaction. This leads us to speculate that the IC\textsubscript{50} and EC\textsubscript{50} calculations made for Eb were overestimating the amount of Eb necessary for its inhibitory function in part because Cys residues were likely binding the Eb in the solution and thus the Eb was not contributing to the inactivation of the enzyme.

During our investigation of the affects of specific Cys residues on the binding of Eb, we found that certain Cys residues are necessary for activity of the enzymes. We also observed that C445 has an increase in the number of free Cys residues available for biotinylation by MPB as compared to unmodified TbHK1 and the remainder of the variants. This finding, along with the impact on hexamerization and minimal impact on enzyme specific activity, suggests that the monomeric C445A may be improperly folded, with all of the properly folded protein in the hexameric form.

While Eb is a potent inhibitor of rTbHK1, it is also promiscuous in its activities. Eb is a known antioxidant. One of its antioxidant functions is to mimic glutathione peroxidase, utilizing reduced glutathione to reduce hydrogen peroxide and lipid hydroperoxides. Eb has also been identified as a potent electrophile, facilitating inter- and intramolecular disulfide linkages (11). Previously, Eb has been found to react with up to 8 Cys residues of human IDO, causing a change in conformation that leads to inactivation (6). Despite its promiscuous nature, Eb has been used in phase II clinical trials for treatment of ischemic stroke and was found to improve the outcome of patients (12). This usefulness also occurs in spite of the noted potent \textit{in vitro} inhibition of hGlk, suggesting the compound does not have access to this protein \textit{in vivo} or that inhibition of
hGlk can be tolerated during the therapeutic implementation of Eb. While we do not believe that Eb would make an efficacious treatment for HAT, gathering more information about the modes of inhibition of compounds as well as the structures of the enzymes they inhibit is important in the development of new compounds for the treatment of African trypanosomiasis.

**Footnotes**

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The abbreviations used are: BSF, bloodstream form; G6-P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; Gly3P, glycerol-3-phosphate; GK, glycerol kinase; HK, hexokinase; PF, procyclic form; rTbHK1, recombinant *Trypanosoma brucei* hexokinase 1; TbHK, *T. brucei* hexokinase; Eb, Ebselen.
REFERENCES


CHAPTER FIVE

SUMMARY

*Trypanosoma brucei* is the protozoan parasite that causes African sleeping sickness in humans and nagana in livestock. There are two main life cycle stages for *T. brucei*, the bloodstream form (BSF) and the procyclic form (PF), inhabiting the mammalian bloodstream and tsetse fly vector, respectively. The environment in each of these organisms is vastly different in terms of the fuel sources available to the trypanosome. In the bloodstream of the mammalian host, glucose is abundant; however, when the parasite is ingested by the tsetse fly it experiences a drastic drop in glucose concentration (1).

The BSF of the organism depends solely on glycolysis for production of ATP, and the focus of this study has been the first enzyme in the glycolytic pathway, hexokinase. *T. brucei* has two hexokinases, TbHK1 and TbHK2, that are 98% identical, and both of which are necessary for survival of the BSF of the parasite (2,3).

Localization Of TbHK1 And TbHK2 To The Glycosome

One distinct feature of BSF trypanosomes is the localization of the first 7 enzymes of glycolysis to a peroxisome-like organelle termed the glycosome (4,5). These glycosome-resident proteins are encoded by nuclear DNA, translated on cytosolic ribosomes, and transported into the glycosome. It has been shown in other systems that there are three peroxisomal (or glycosomal) targeting sequences (PTS): PTS1, PTS2, and
I-PTS, and these sequences are necessary for proper import into the glycosome. TbHKs possess a type 2 PTS, and mislocalization to the cytosol is lethal to the parasite (6).

PEX7, a known PTS2 protein chaperone (7-9), has been identified in trypanosomes (10). In order to further explore the possibility of a TbHK-TbPEX7 interaction, we produced a trypanosome cell line that overexpresses GFP-tagged TbPEX7. Using this cell line, we have determined that TbPEX7 does localize to the glycosomes and cytosol of *T. brucei*. We have also cloned the TbPEX7 gene into a bacterial expression vector and produced a recombinant, tagged PEX7. Preliminary evidence suggests that rTbPEX7 does interact with rTbHK1.

The TbPEX7:TbHK interaction is important because of its potential as a drug target. If a compound was produced that inhibited this interaction, it could potentially be lethal to the parasite. The recombinant proteins produced could be used for a high throughput screening approach to identify inhibitors of the TbPEX7:TbHK interaction.

The Extra-Glycosomal Localization Of TbHK2

While TbHK1 and TbHK2 are 98% identical, the recombinant proteins have distinct biochemical characteristics (11). Because glycolytic enzymes in other systems are known to have functions outside of glycolysis, we were interested in the possible extra-glycosomal localization of TbHK2.

In order to examine this, antibodies (specific to the C-terminal region in which the two TbHKs are different) to TbHK2 were produced in rabbit and mice. In BSF parasites, both antibodies localized to the flagellum, with a lesser signal seen in the glycosome.
Digitonin fractionations of BSF parasites also demonstrate TbHK release consistent with that of known cytosolic proteins. In order to confirm the extra-glycosomal localization, we produced a *T. brucei* cell line in that overexpresses tagged TbHK2 and a cell line with an endogenously tagged TbHK2 in BSF parasites. Both cell lines produced TbHK2 that localized to the flagellum, confirming the previous results.

All of the above-mentioned work was done in BSF parasites; however, we were also interested in determining whether the flagellar localization was stage specific. In fact, the anti-TbHK2 antibodies detected foci consistent with glycosomes, and no flagellar signal was detected. Digitonin fractionations of the PF parasites also demonstrated TbHK release consistent with that of glycosome-resident proteins. However, in addition to the glycosomal staining with the anti-TbHK2 antibody, two foci proximal to the kDNA (mitochondrial DNA) were identified, and an endogenously tagged TbHK2 PF cell line confirmed these results.

This study identifies a novel localization for TbHK2, which could lead to future studies in the identification of its role in the flagellum. While it has not been demonstrated to date, the life stage specificity of flagellar localization of TbHK2 suggests that it may be playing a role in environmental sensing. It is also important to note that while it is not expected, TbHK1 localization to the flagellum was not ruled out.

**TbHK1 Cysteine Residues Participate In Inhibitor Binding And Oligomerization**

Recently, ebselen, a known cysteine-reactive reagent, was identified as a potent mixed inhibitor of TbHK1 in a LOPAC screen and subsequent HTS of 220,233
compounds (12). This led us to further explore the role that cysteines have inhibitor binding, as it is unclear how ebselen functions. However, we anticipate that it may form covalent adducts with one or more of the nine cysteine residues found in TbHK1.

Mutation of individual cysteine residues of TbHK1 led to the discovery that three residues (C212, C327, and C386) are necessary for enzyme activity, and four cysteine residues (C369, C386, C401, and C445) affected the relative amounts of hexamer and monomer. Interestingly, there was no marked difference in inhibition of the active variants by ebselen. In order to determine whether or not ebselen was indeed binding TbHK1 cysteine residues, we used 3-(N-maleimidopropionyl)-biocytin (MPB) to biotinylate free cysteine residues after pre-incubation with ebselen. Through these studies, we have found that ebselen does bind cysteine residues, but that the inhibitory effects of ebselen are not caused by this interaction. Developing a greater understanding of the modes of inhibition of compounds as well as the structures of the enzymes they inhibit is important in the development of new compounds for the treatment of African trypanosomiasis.
REFERENCES


Appendix A

Supplementary Information for Chapter 2

Figure A-1: Immunofluorescence (IF) microscopy of *Trypanosoma brucei brucei* bloodstream form (BSF) parasites over-expressing *T. brucei* hexokinase 2 with a C-terminal hemagglutinin fusion (HK2HA). Both an HK2 over-expressing cell line
(pXS6:HK2HA) with a hemagglutinin tag and the parent 427 cell line were assayed with an antibody for HA (A, b, g, and l; B, a, d, and g) and an antibody for the axoneme. Fixed parasites (A) and cytoskeletons (B) of each are shown. The white arrows point out distinct areas of co-localization. The over-exposure panels demonstrate the faint signal detected in the whole-cell preparations. All images (A and B) are an extended focus produced from Z-stack layers, and DAPI counterstain is shown for the whole-cell preparations. Scale bars = 5 µm.
**Appendix B**

**Supplementary Information for Chapter 4**

### Table B-1. Primers used for Cys Variant Production.

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<th>Variant</th>
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<td>C212A</td>
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<tr>
<td>C242A</td>
<td>ACTGGTTCCAATGCGGCTACTTTGAGACGGA</td>
</tr>
<tr>
<td>C327A</td>
<td>CTGTCGTCTATTAACGCCCTTCTCTGCAGCACTG</td>
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<td>C369A</td>
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<td>C386A</td>
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<tr>
<td>C445A</td>
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