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Localization of the Fe-S cluster biosynthesis in the bloodstream stage of *Trypanosoma brucei*

Master thesis

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Annotation

Localization of two key components of the Fe-S cluster biosynthesis, Nfs and Isu, was studied in the bloodstream stage of *Trypanosoma brucei*. Immunofluorescent microscopy, digitonin fractionation followed by Western blot analysis and cysteine desulfurase activity assay were applied. Both the enzymes were detected in the mitochondrion.

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Contents

1. Introduction

1.1 Significance of Trypanosoma brucei

Trypanosoma brucei is widely used as a model organism for molecular biology research. There are several reasons why this organism deserves a special attention. It is a causing agent of African sleeping sickness, a severe illness affecting millions of people directly as a disease or indirectly by killing their livestock. In order to be transmitted among mammalian hosts via an insect vector, trypanosomes undergo a complex life cycle with several diverse cell types adapted to specific circumstances in different hosts. The prevailing forms are the bloodstream stage (BS), which infects mammals, and the procyclic stage (PS), known to be the main form found in the tse-tse fly. Moreover, trypanosomes are very interesting model organisms, as they contain several unusual molecular mechanisms, such as alternative *trans*-splicing, polycistronic transcription, mitochondrial DNA organized into a kinetoplast disk (kDNA) and RNA editing in mitochondria. Trypanosomatids belong to Kinetoplastida, considered to be one of the earliest branches of the eukaryotic tree (Hampl *et al*., 2009).

The species *T. brucei* is subdivided into five subspecies, two of which are causative agents of human sleeping sickness and the remaining ones invade mammals. *T. b. gambiense* is spread across western and central Africa and causes a chronic form of the human trypanosomiasis. In contrast, *T. b. rhodesiense* is responsible for the acute form of this disease and is restricted to eastern Africa. Next, *T. b. brucei* causes a form of African trypanosomiasis called nagana affecting livestock and cattle. All these subspecies are among mammalian hosts transmitted via various species of tse-tse fly from the genus *Glossina*. Finally, the two geographically most widespread subspecies are *T. b. equiperdum* and *T. b. evansi*, which are responsible for dourine and surra, respectively, most affecting horses, camels and water buffalos (Lai *et al*., 2008). These two subspecies, however, use other means of transmission, including mechanical transmission by tabanid flies or are spread directly by exchange of blood (Schnaufer *et al*., 2002).

In total, African human trypanosomiasis occur in 36 countries of sub-Saharan Africa, where 60 million people are exposed to risk and hundreds of thousands of them may be infected. According to the World Health Organization, number of newly reported cases significantly dropped to 7000 in year 2010. On the other hand, in 1995 only 10% of all

newly infected people were diagnosed and treated. 95% of these cases are caused by the chronic infection and 5% by the acute form of trypanosomiasis (WHO).

The disease usually develops in two stages: the first haemolymphatic phase is caused by trypanosomes dividing in subcutaneous tissues, blood and lymph. If this first stage of the disease goes unrecognized and untreated, parasites will overcome haemoencephalytic barrier and invade the central nervous system. This second neurologic phase manifests itself by changes in behavior, confusion and disorders in sleeping cycle, which gave the disease its name. Without medication trypanosomiasis is fatal. Unfortunately, currently available treatment is difficult and unreliable, due to toxicity of widely used drugs, but also to emerging resistance. In addition, medication and health care is limited or even non-existent in substantial parts of sub-Saharan Africa (Barrett *et al*., 2003; WHO).

1.2 Life cycle

In order to divide up to maximum numbers in the host, thus increasing the chance of successful transmission via the vector and to evade a new vertebrate host, trypanosomes go through a complex life cycle, during which they dramatically change the cell shape and surface, rebuild their organelles as well as several aspects of their cell metabolism (Matthews, 2005). Repositioning of the kDNA disk relative to the nucleus, observed during the life cycle, allowed the description of separate cell stages (Van den Abbeele *et al*., 1999).

Together with host blood trypanosomes are sucked into the midgut of *Glossina* spp., where they persist in the form of the PS. At this stage of the life cycle, they are usually attached to the midgut surface via a flagellum, with only its most distal part freely emerging from the anterior end (Hammarton *et al*., 2007). The kDNA is positioned posterior to the nucleus, in the middle between the nucleus and the posterior end of the cell. On the molecular level, distinguishing features of this stage are i.e. high expression of the respiratory chain complexes and the surface glycoprotein procyclin (Timms *et al*., 2002; Urwyler *et al*., 2005). Parasites circulate through the vector´s digestive system and in the process part of them undergoes a form of sexual exchange. It has been shown recently that this is a very fast and localized process (Peacock *et al*., 2011), although its molecular aspects remain elusive. Finally, the flagellates emerge in salivary glands of the vector, where they become attached via their flagellum to the salivary gland epithelium. Movement to other compartment within the host is associated with morphological changes, including

repositioning of the kDNA in respect to the nucleus. The salivary stage has its kDNA located in front of the nucleus, and the cell is hence termed the epimastigote (Hammarton *et al*., 2007). Subsequently, shorter cells termed the metacyclic form are produced. The cell cycle is arrested in these shorter cells, which are apparently pre-adapted for the entry into the mammalian host (Matthews, 2011). One of them is the expression of variant surface glycoproteins (VSG), which enable trypanosomes to completely and frequently change their surface proteins and thus escape the mammalian immune response (Pays *et al*., 2007).

When transmitted into the blood, the flagellum serves for motion again and the kDNA becomes relocated to the posterior end. Thus, the bloodstream cell type is known as the trypomastigote, called long slender bloodstream form due to the cell shape. In addition, the flagellum is connected with the cytoplasmic membrane creating an undulating membrane, structure expected to facilitate motility in blood and subcutaneous tissues (Hammarton *et al*., 2007). Morphologically and metabolically reduced mitochondrion is another characteristic feature of this stage. Trypanosomes keep dividing within the host until a very high density is reached, at which stage of the disease a switch into the short stumpy form is induced. These cells do not undergo mitosis and become arrested (Matthews, 2011). This is advantageous for the parasite as its primary aim is not to kill the host. Furthermore, all cells are arrested in the same point of the cell cycle and start preparing for next transition. They initiate expression of respiratory complexes and other genes in order to pre-adapt for another entry into the insect vector (Matthews, 2005).

1.3 The procyclic stage

In order to briefly describe the trypanosome cell, I will focus on the procyclic stage. According to the "insect-host first" hypothesis (Maslov *et al*., 2001), it is regarded as the ancestral cell type and the BS as the derived one, adapted to favorable and extensive niche of its mammalian host. Later on, I will describe unique features and adaptations of the BS.

The organization of DNA in the diploid nucleus of trypanosomes is quite unique, as three types of chromosomes can be distinguished based on their size. First, there are 11 pairs of large megabase-sized chromosomes harboring housekeeping genes. The genes are very dense, organized into clusters and transcribed into polycistrons (Hendriks and Matthews, 2007). Second, there are a few intermediate-sized chromosomes and, finally, about 100 minichromosomes ranging from 50 to 150 kb in size complete the chromosomal set (Hertz-

Fowler *et al*., 2007). The latter genetic elements are responsible for variations in composition of surface glycoproteins in the BS, as they carry genes for VSG and are responsible for DNA recombination by transferring of different VSG genes into sites of expression. Often transpositions and mutations lead to fast changes in expression of different VSG genes (Pays et al., 2007).

Instead of *cis*-splicing of pre-mRNA transcripts, found in majority of eukaryotes, *trans*-splicing is known to be very widespread and the predominant form of splicing there (Sutton and Boothroyd, 1986). It proceeds via the attachment of a common highly conserved 39-bp long leader sequence being attached to the 5′-terminus of each nuclear encoded pre-mRNA (Sutton and Boothroyd, 1986). A significant portion of genes has two or more alternative splice leader acceptor sites, which can be preferentially used in different life stages (Nilsson *et al*., 2010). Until present, almost nothing is known about the regulation and the real significance of the alternative *trans*-splicing. Still, it seems that virtually all possible and conceivable mechanisms are used. In general, gene expression is stage regulated and controlled at post-transcriptional level by RNA stability, *trans*-splicing, translation, mRNA and protein stability (Nilsson *et al*., 2010; Rettig *et al*., 2011).

Presence of acidocalcisomes is another trait typical for trypanosomatids. These are single membrane organelles characteristic by acidic environment and high concentration of calcium, according to their name (Docampo and Moreno, 1999). In their lumen, high concentration of pyrophosphate and polyphosphates with bound calcium, magnesium, sodium and other ions are present. The main function of these organelles appears to be the storage of these compounds in a high concentration. For example, pyrophosphate can be utilized for instant ATP synthesis and calcium is an important signal mediator in many pathways. Taken together, acidocalcisomes may represent a suitable adaptation to the parasitic life style, which allows the cell to handle different environmental circumstances and their instant changes (Docampo and Moreno, 2001).

Another specialized organelle is the glycosome, a single membrane, peroxisome-derived organelle present in high number per cell. Its main function is to harbor first six steps of glycolysis, with the produced 1,3–bisphosphoglycerate being exported into the cytosol, where it is converted to phosphoenolpyruvate and re-imported into the glycosome (Bringaud *et al*., 2006). What may seem to be an unnecessarily complex pathway is actually another ingenious adaptation to the parasitic life style. The ratios of ATP/ADP or NADH/NAD$^+$ differ inside the glycosome from the cytosol, and the metabolic pathways inside are thus independent of the circumstances in the latter compartment. As a

consequence, this mechanism helps parasites to better handle deficiency in glucose or its overload and very high osmotic pressure (Michels *et al.*, 2000). Glycerol-3-phosphate produced in the glycosomes is converted to dihydroxyacetone phosphate using glycerol-3-phosphate dehydrogenase located in the inner mitochondrial membrane, supplying electrons into the respiratory chain and maintaining the redox balance in the glycosome at the same time (Bringaud *et al.*, 2006).

The mitochondrion is likely the most interesting organelle in the trypanosome cell. In its PS form it is a single large and reticulated structure spreading across the whole cell. As mentioned above, its extensive mitochondrial DNA called kDNA is in members of the genus *Trypanosoma* composed of thousands of mutually intercalated circles of two types: maxicircles present in about two dozens of identical copies, each 23 kb long and ~5000 1 kb-long minicircles (Liu *et al.*, 2005). Maxicircles encode typical mitochondrial genes, however, some of them present are in an encrypted form (Stuart *et al.*, 2005 and see below). Most of these genes specify subunits of complexes of the respiratory chain (six subunits of complex I [NADH dehydrogenase], apocytochrome b of complex III [cytochrome c reductase], three subunits of complex IV [cytochrome c oxidase], subunit 6 of complex V [ATP synthase], ribosomal protein RPS12 and finally two ribosomal RNA genes) (Lukeš *et al.*, 2005). As in other eukaryotes, numerous subunits of these respiratory complexes, as well as all subunits of complex II are encoded in the nucleus and produced proteins are imported into the mitochondrion, which demands coordinated expression of both mitochondrial and nuclear genomes (Timms *et al.*, 2002).

The encrypted mitochondrial genes are originally called cryptogenes, as in parts of their coding sequence they do not resemble their homologues in other eukaryotes. However, extensive research revealed that these "many mistakes" are in fact "repaired" on the transcriptional level by a unique and extremely sophisticated process coined the term RNA editing (Benne *et al.*, 1986). The numerous kDNA minicircles provide information for editing of the maxicircle-derived transcript, since they encode hundreds of small genes termed guide RNAs (gRNAs). Transcripts of these gRNAs hybridize with the pre-edited maxicircle transcripts and allow, editing via the additions or deletions of uridine residues (Aphasizhev and Aphasizheva, 2010). During this process, 5´end of a gRNA serves as an anchor and pairs with a 3´end of a complementary pre-mRNA. Subsequently, single uridines are one by one inserted or deleted from the pre-mRNA sequence according to the complementary gRNA. Since pre-edited mRNAs are much longer than individual gRNAs, each mRNA has to undergo multiple rounds of gRNA-mediated editing, until it is corrected

along its entire reading frame and hence ready for translation. The whole machinery utilizes numerous enzymatic activities, ATP as a source of energy and UTP as a substrate. The extent of RNA editing differs significantly not only in term of a given transcript but also among different species (Maslov *et al*., 1994). The non-canonical direction from the 3´ end forwards prevents start of translation of unedited or only partially edited transcripts. Finally, the fully edited mRNAs become almost homologous to typical non-edited mitochondrial mRNAs in other eukaryotes and undergo translation into proteins (Horváth *et al*., 2000).

Curiously, even the less known mitochondrial translation of trypanosomes possesses a host of unusual features. Ribosomes have extremely short rRNAs that are probably compensated for by unusual protein composition (Zíková *et al*., 2008b), since these are the most protein-rich ribosomes known (Lukeš *et al*., 2011). Moreover, mitochondrial ribosomes have to cooperate solely with eukaryotic nuclear encoded tRNAs, since no tRNA is encoded within the kDNA, and all have consequently to be imported from the cytosol (Tan *et al*., 2002). Alternative *trans*-splicing can be the mechanism enabling the dual localization of a single tRNA, at least this was experimetally established in the case of isoleucyl-tRNA (Rettig *et al*., 2011). Which signals determine the transport of tRNAs and how the machinery works still remains a mystery, although it was shown recently that it does not depend on respiratory complexes as implied earlier (Paris *et al*., 2010). It is also worth mentioning that another form of RNA editing, although mechanistically different from the uridine insertion/deletion type, was described in trypanosome tRNAs (Wolgamuth-Benedum *et al*., 2009).

Trypanosomes utilize at least three different pathways to produce ATP. First of them is standard oxidative phosphorylation via the respiratory chain. While the presence of the complex I or multi-subunit NADH dehydrogenase has been questioned for a long time (Opperdoes and Michels, 2008), it has been shown recently that an unusual complex I is indeed present in both the PS (Verner *et al*., 2011) and the BS of *T. brucei* (Surve *et al*., 2012), although its activity and composition remain largely obscure. Besides NADH dehydrogenase and succinate dehydrogenase (complex II), electrons are supplied to ubiquinone also from membrane bound glycerol-3-phosphate dehydrogenase and rotenone-insensitive NADH dehydrogenase (Bringaud *et al*., 2006). From ubiquinone, electrons pass into two directions. Part of them goes into standard respiratory complexes III and IV, which create membrane potential by pumping hydrogen ions into the inter-membrane space (Horváth *et al*., 2005). A fraction of the electrons bypasses complexes III and IV, as it is directed to the trypanosome alternative oxidase located in the inner mitochondrial

membrane. This pathway is unable to produce ATP at ATP synthase, as well as cannot upkeep membrane potential and seems to be utilized mainly in the excess of glucose (Bringaud *et al*., 2006). Second source of ATP are the glycosomes, where glycolysis takes place (Michels *et al*. 2006). Third, ATP is also produced by acetate:succinate CoA transferase (Bochud-Alleman and Schneider, 2002).

The mostly utilized source of energy is of course glucose, but in its shortage, amino acids can be used as well, in particular proline, which is supposed to be present in high concentration in the vector´s midgut (Bringaud *et al*., 2006). The prevalence of one ATP producing pathway over the others depends on the availability of metabolisable resources. If glucose is available at high concentration for the trypanosome cell, it will suppress its respiratory chain activity and will use glycolysis as its main energy-producing pathway. However, under glucose-poor conditions, respiratory chain will again become up-regulated and will be utilized as the main source of energy (Bringaud *et al*., 2006).

1.4 The bloodstream stage

Despite being the true causative agent of the African sleeping sickness, the mitochondrion of the BS is much less known as compared to its counterpart in the PS. Some methods and approaches standardly used for PS have yet not been optimized for the BS. Moreover, the mito-proteome is so far available only for the PS (Panigrahi *et al*., 2009), which makes this organelle much more amenable for functional studies.

The mitochondrion of the BS is characteristic by its reduced and elongated shape, lack of cristae and position on the periphery of the cell. While this putatively inactive organelle has been recently shown to retain some function, its contribution to the cell metabolism remains rather elusive. Since these parasites live in a glucose rich environment, canonical oxidative phosphorylation is not necessary for their survival, with substrate phosphorylation fully covering their energetic requirements (Hannaert *et al*., 2003). Interestingly, maxicircle encoded genes are transcribed and their mRNAs are edited, processed and translated, with all proteins participating on any of these processes hence being essential for cell viability (Schnaufer *et al*., 2002; Stuart *et al*., 2005; Cristodero *et al*., 2010). Although, RNA editing proceeds in a stage-dependent manner, it is very much the same process as in the PS (Schnaufer *et al*., 2002; Timms *et al*., 2002). It is virtually counterintuitive that even the akinetoplastic *T. b. evansi* (= BS lacking any kDNA),

mitochondrial DNA polymerase was found to be indispensable for survival (Paris *et al*., 2011). In general, subunits of the respiratory chain are edited in the PS at higher rate, except for several subunits of complex I, which are predominantly expressed and edited in the BS (Schnaufer *et al*., 2002; Surve *et al*., 2012). Similar to the PS, import of tRNAs into the mitochondrion is essential for translation of the BS (Cristodero *et al*., 2010).

Considering cell metabolism and ATP production in the BS, oxidative phosphorylation is not active and respiratory chain is significantly reduced (no cytochrome-dependent complexes are active) (Bringaud *et al*., 2006). NADH dehydrogenase together with glycerol-3-phosphate dehydrogenase supply electrons via ubiquinone to trypanosome alternative oxidase as the terminal electron acceptor (Chaudhuri *et al*., 2006). Membrane potential across the mitochondrial inner membrane, which was postulated as a requirement for protein import, is maintained by the activity of ATP synthase that works in reverse as an ATPase (Schnaufer *et al*., 2005). Indeed, in the BS complex V does not fulfill its conventional function, as its F1 part rotates in reverse at the expense of ATP consumption. The produced energy is utilized for export of hydrogen ions out of the mitochondrial matrix in order to create membrane potential, as was experimentally proven by the depletion of the F1 domain by RNA interference (Schnaufer *et al*., 2005).

Because of the adaptations mentioned above, glycosome-located glycolysis remains the prevailing source of energy for the BS, but contrary to the PS, one more enzyme is present there, with 3-phosphoglycerate being produced. As a consequence, the redox and ATP production and consumption are balanced within the glycosomes. Most ATP is produced during the last three steps of glycolysis, located in the cytosol (Bringaud *et al*., 2006).

Mitochondria are essential components of all eukaryotic cells, mostly as a source of energy. All known eukaryotic cells contain a mitochondrion or at least a remnant of it (named hydrogenosomes or mitosomes). However, it was proposed that the crucial function of mitochondrion-derived organelles is not oxidative phosphorylation, but iron-sulfur cluster biosynthesis (Lill and Kispal, 2000; Tovar *et al.,* 2003; Šuťák *et al*., 2004).

Iron sulfur (Fe-S) clusters are ubiquitous and generally essential cofactors for a wide range of enzymes. The most widespread clusters are those with the [2Fe-2S] or [4Fe-4S], with types such as [3Fe-4S] less frequent. Their main beneficial property is the capability to transfer electrons. Clusters are not only part of the respiratory chain complexes but are also present in numerous other metabolic enzymes such as aconitase, fumarase and lipoate synthase. Furthermore, they are used for iron metabolism and uptake regulation, for example in iron responsible protein (IRP), they may have simply a structural function or serve as ligands in DNA repair (DNA primase, helicase BACH1, Nth endonuclease III-like). The crucial function of Fe-S clusters can be illustrated by several human diseases caused by improper Fe-S biosynthesis, namely: Friedrich´s ataxia, Glrx5-deficient sideroblastic anemia, ISCU myopathy and multiple mitochondrial dysfunctions syndrome (Lill *et al.*, 2006; Lill, 2009; Sheftel *et al.*, 2010; Rouault, 2012).

Fe-S clusters are very ancient cofactors since they are widespread among all known living organisms. In total, three different Fe-S clusters biosynthetic pathways are known: ISC, NIF and SUF. In eukaryotic cells there is the ISC pathway, which has a mitochondrial location and α-proteobacterial origin. Plants usually possess also the SUF biosynthetic pathway in plastids, due to their descent from cyanobacteria. The NIF pathway is characteristic for nitrogen fixing bacteria (Lill *et al.*, 2006).

1.5.1 The ISC assembly pathway

Despite being relatively simple molecules composed of just few atoms, Fe-S clusters are assembled by highly complex assembly machinery, composed of more than twenty currently known components. This pathway seems to be highly conserved in all living organisms (Lill, 2009). Indeed, the general mechanism of Fe-S biosynthesis is conserved, although in certain organisms some of its components can be omitted, duplicated, added or highly diverged. Rouault (2012) has recently suggested even duplication of the whole assembly pathway in different compartments of mammalian cells.

Contemporary view on Fe-S biosynthetic pathway in *T. brucei* is based on its comparision with the homologous pathway in *Saccharomyces cerevisiae* (Šmíd *et al.*, 2006; Paris *et al.*, 2010; Long *et al.*, 2011). The available functional data in combination with

information from the trypanosomal genome database (Nilsson *et al*., 2010) indicate rather canonical version of the pathway in this derived protist.

One of the key enzymes is the cysteine desulfurase (Nfs), which provides the sulfur atoms (Mühlenhoff *et al*., 2004). The Nfs enzyme needs for its proper function a small protein Isd11, since its depletion disables desulfurization and Fe-S assembly (Adam *et al*., 2006; Paris *et al*., 2010). The sulfur is further transferred to the scaffold protein Isu, which binds the whole Fe-S cluster and stabilizes it. Surprisingly, it is still not clear which enzyme provides the iron atom. Traditionally, this role was attributed to frataxin, but this has never been proved experimentally beyond reasonable doubt (Stemmler et al., 2010). Recently, Rouault (2012) suggested that a complex of glutathion and glutaredoxin is the donor of iron, mainly based on the work of Qi and Cowan (2011) who showed that Isu and the glutathion-glutaredoxin complex can exchange whole Fe-S clusters. Considering trypanosomal frataxin, Long and colleagues (2008b) proofed it is essential for the Fe-S assembly but contrary to the mammalian frataxin, processing of the protein is not required in trypanosomes (Long *et al*., 2008a). Another component of the machinery is ferredoxin that provides electrons obtained by ferredoxin reductase from NADH. Surprisingly, there are two ferredoxin genes in *T. brucei*, but just one ferredoxin reductase was identified (Piya Changmai, personal communication). The newly assembled Fe-S clusters are likely transferred further via scaffold proteins, which assure their proper conformation. In trypanosomes, homologues of Ssq, Mge, Nfu, Iba57, Isa1 and Isa2 have been identified. For Nfu was recently suggested a specific role in maturation of succinate dehydrogenase in human cells (Navarro-Sastre *et al*., 2011). Interestingly, three different genes were identified in *T. brucei* genome (Shaojun Long, Zuzana Vávrová, unpublished data), but their precise function is not known yet. Isa1 and Isa2 seem to complement for each other to some extent, since depletion of both of the proteins caused the most severe growth phenotype. Moreover, after this RNAi, activities of Fe-S containing enzymes decreased only in the mitochondrion (Long *et al*, 2011). Altogether, it seems that the scaffold proteins are specific for a given set of target proteins (Sheftel *et al*., 2010; Long *et al*., 2011). By cooperation of these scaffold proteins, the newly assembled Fe-S cluster are directed to a specific apoprotein in order to form a holoprotein, or it is exported outside of the mitochondrion (Fig. 1).

The ISC assembly pathway is followed by a transport system exporting the newly formed Fe-S clusters outside of the organelle. In *T. brucei* candidate proteins for this activity are Atm1 and Erv1, whereas the Mia40 protein, highly conserved in eukaryotes, seems to be missing from its genome (Julius Lukeš, personal communication). Atm1 is a membrane

bound transporter belonging into a large family of ABCB transmembrane proteins. Interestingly, it is still not clear what is the substrate being exported out of the mitochondrion but recent opinions side with the theory that it is a form of sulfur (Rouault, 2012). In the cytosol, the ISC export pathway is followed by the cytosolic iron-sulfur protein assembly pathway (CIA), which mediates transfer and incorporation of Fe-S clusters into cytosolic proteins. It remains to be solved whether CIA pathway is responsible only for repair and regulation of the clusters or is capable of their mitochondrion-independent *de novo* synthesis (Lill, 2009; Sheftel *et al*., 2010; Rouault, 2012).
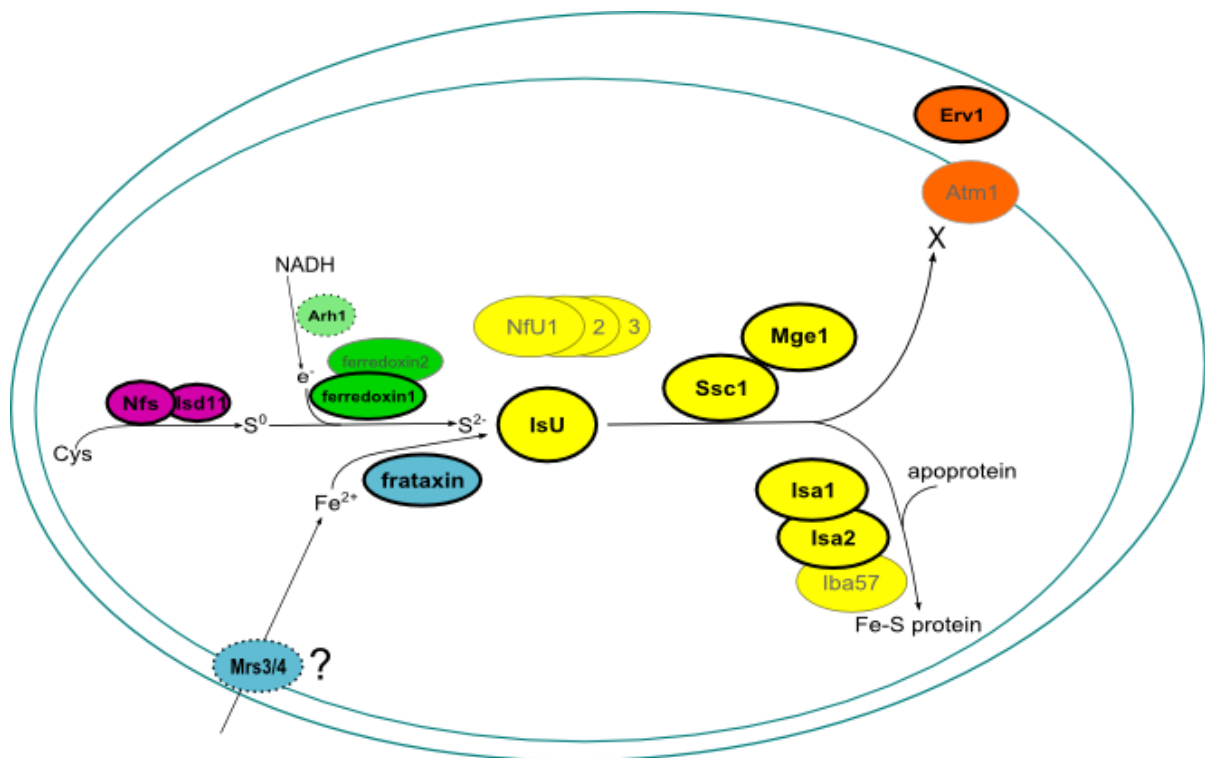

Figure 1: Model of the ISC pathway in the PS of *T. brucei*.

I have focused on the cysteine desulfurase Nfs and the scaffold protein Isu. Nfs belongs among pyridoxal 5´-phosphate-dependent enzymes and it abstracts sulfur from cysteine, concomitantly producing alanine. Because free sulfur would be toxic for the cell, it is not released but forms a persulfide with cysteine residues of the enzyme (Mühlenhoff *et al*., 2004). Nfs has dual localization in yeast, since it was detected in mitochondria and in the nucleus. These two proteins are derived from a single translation product which contains both targeting sequences, being imported primarily into the mitochondrion, with a minor fraction being further transported into the nucleus (Naamati *et al*., 2009). The mitochondrial

form of Nfs is crucial for maturation of both mitochondrial and cytosolic Fe-S proteins, whereas the nuclear Nfs is responsible for thiomodification of tRNAs (Mühlenhoff *et al*., 2004; Naamati *et al*., 2009). The cytosolic and mitochondrial forms of Nfs (ISCS) were detected also in mammalian cells (Biederbick *et al*., 2006). Contrary to the yeast Nfs, these are produced by differential translation from an alternative start codon which bypasses the mitochondrial targeting sequence (Land and Rouault, 1998). Furthermore, the same dual localization was also reported for the mammalian Isu homologue. In contrast to the Nfs protein, the two forms of Isu are created due to alternative *cis*-splicing of pre-mRNA, thus they share most of their coding sequence but differ in their 5´ends (Tong and Rouault, 2000). The cytosolic isoforms are most probably involved in an alternative cytosolic Fe-S clusters biosynthetic pathway (Rouault, 2012). Moreover, the cytosolic Nfs plays role in molybdenum cofactor biosynthesis in humans and interestingly, Isd11 seems to be dispensable in this case (Marelja *et al*., 2008).

The identification and functional analysis of trypanosomal Nfs and Isu was reported by Šmíd *et al*. (2006). Ablation by RNA interference of each of the given transcripts caused severe decrease of Fe-S clusters biosynthesis manifested in the decrease of aconitase activity, metabolic changes and finally resulted in severe growth phenotype. *In vivo* a complex of Nfs, Isd11 and Isu proteins is formed, as was shown by Paris and colleagues (2010) using the TAP tagging strategy followed by mass spectrometry analysis. In mammalian cells, frataxin was detected in this complex as well (Schmucker *et al*., 2011). Similar to other organisms, Isd11 is required for proper activity of trypanosomal Nfs and both these proteins are indispensable not only for Fe-S assembly but also for thiolation of cytoplasmic tRNAs (Paris *et al*., 2010).

Moreover, some data about Fe-S clusters biosynthesis are available from various parasitic protists which are phylogenetically distant from Kinetoplastida. These parasites possess mitosomes or hydrogenosomes, which are mitochondrial remnants lacking DNA and most typical mitochondrial metabolic pathways. Both Nfs and Isu homologues were detected in the mitosomes of *Giardia intestinalis* (Tovar *et al*., 2003). Furthermore, a homologue of Nfs was located in the hydrogenosomes of *Trichomonas vaginalis* and capacity for Fe-S cluster assembly was verified in this compartment. Contrary to yeast, the protein is absent from the nucleus and even lacks the mitochondrial targeting motif conserved among wide range of cysteine desulfurases (Šuťák *et al*., 2004). Finally, microsporidia deserve special attention. Whereas in *Encephalitozoon cuniculi* Nfs and Isu are present in mitosomes, in the related species *Trachipleistophora hominis*, Nfs was located in the mitosome but Isu and

frataxin in the cytosol (Goldberg *et al*., 2008). Considering all these results, a hypothesis was suggested that Fe-S assembly is the only function common for all the mitochondrion-derived organelles and retained in these highly adapted and peculiar parasites (Lill and Kispal, 2000; Tovar *et* al., 2003; Šuťák *et al*., 2004;).

Almost all the core components of the ISC pathway have been detected and described in the PS of *T. brucei*. However, only very scarce data are known considering the BS. Thus, I have focused on the two key proteins of the assembly pathway, Nfs and Isu, and addressed their localization within the cell. For this purpose, I prepared constructs containing these genes fused with a v5 tag. These vectors were electroporated into both stages and the obtained cell lines were scrutinized by immunofluorescent assay (IFA) and digitonin fractionation followed by Western blot analysis. I verified the mitochondrial localization of the Nfs and Isu proteins in BS.

2. Objectives

- To localize Nfs and Isu in the BS of *T. brucei*.
- To establish appropriate methods for subcellular localization of proteins in the BS.
- To establish the Nfs activity assay in *T. brucei*.

3. Methods

3.1 Cultivation of Trypanosoma brucei

All experiments were performed using the procyclic *T. brucei* 29-13 strain and the bloodstream single-marker strain as wild-types (Wirtz *et al*., 1999). Procyclic cells were grown in the SDM-79 medium supplemented with 10 % fetal bovine serum at 27° C (Brun and Schoenberger, 1979) in the presence of hygromycin (50 μg/ml) and G418 (15 μg/ml) (Wirtz *et al*., 1999). The Nfs and Isu procyclic knockdown cell lines were prepared by Šmíd *et al*. (2006) and grown in medium supplemented with phleomycin (2.5 μg/ml) and tetracycline (1 μg/ml) for induction of the RNA interference, respectively. Bloodstream cells were grown in the HMI-9 medium supplemented with 10 % fetal bovine serum at 37° C, in 5 % CO_2 in the presence of G418 (2.5 μg/ml) (Wirtz *et al*., 1999). The bloodstream cell line with the HA-tagged Isu protein was prepared by Shaojun Long and was grown in the HMI-9 medium in the presence of G418 (2.5 μg/ml) and puromycin (0.1 μg/ml). The newly prepared v5-tagged cell lines were grown in the presence of drugs appropriate for each parental cell line plus puromycin (1 μg/ml for PS; 0.1 μg/ml for BS) and tetracycline (1 μg/ml) was added in order to induce expression of the tagged proteins. Cell densities were measured using the Beckman Coultier Z2 Particle Counter®. Cells were washed with 1 x PBS prior to all experiments.

3.2 Digitonin fractionation

The digitonin fractionation method was used in order to prepare samples of subcellular fractions for SDS-PAGE. I used protocol kindly provided by Dr. Michael Ginger (Lancaster University). Cells were centrifuged (1 300 g, 16° C, 10 min), washed with 1 x PBS and STE-NaCl buffer afterwards. Suspension of 6.5 x 10^8 cells/ml in STE-NaCl buffer was prepared. This cell suspension was aliquoted and mixed with STE-NaCl buffer and different amounts of digitonin (Sigma) according to the Table I. Samples were incubated at 27° C for 4 minutes. Afterwards, samples were centrifuged (12 500 g, room temperature = RT, 2 min) and put straight onto ice. 150 μl of the obtained supernatants was used and mixed with 250 μl of hot 2 x SDS loading buffer and 100 μl of 1M DTT solution. The obtained samples were boiled for 5 minutes and stored at -20° C until use.

Table I: Preparation of separate samples for digitonin fractionation.

sample number	1	2	3	4	5	6	7	8	9
final concentration of digitonin [mM]	0	0.05	0.1	0.2	0.3	0.4	0.5	1	1.5
cell suspension [µl]	150	150	150	150	150	150	150	150	150
STE-NaCl buffer [µl]	150	142.5	135	120	105	90	75	0	0
STE-NaCl buffer with digitonin, concentration 1.6mM [µl]	0	7.5	15	30	45	60	75	150	300

3.3 Western blot analysis

Samples obtained by digitonin fractionation or whole cell lysates were separated by SDS-PAGE and subsequently examined by Western blot analysis. Whole cell lysate samples were prepared by dissolving of 5×10^7 cells in 50 µl of 2 x PBS and 50 µl of 2 x SDS loading buffer. 15 – 30 µl of samples was loaded onto 12 % SDS polyacrylamide gels (Tab. II) and separated by electrophoresis (120 V, 1.5 h). Subsequently, proteins were electroblotted onto polyvinylidene difluoride membranes (Amersham Hybond-P, GE Healthcare), 65 V, 4° C, 1.5 h. In order to avoid nonspecific signal staining, membranes were blocked in 5 % non-fat milk in PBS with 0.05 % Tween for at least 1 hour at RT. Incubation with primary antibodies was carried out in a milk solution at 4° C overnight. All primary antibodies were *T. brucei* specific polyclonal antibodies used in 1:1 000 dilution, except for enolase which was in 1:10 000 dilution, for more details see Tab. III. Anti-v5 tag antibody was a commercial monoclonal antibody (Invitrogen) used in 1:2 000 dilution. Secondary anti-rabbit, -chicken and -mouse antibodies (Sigma-Aldrich, Molecular Probes), coupled to horseradish peroxidase, were applied in 1:1 000 dilution (RT, 1 h). In between separate steps, membranes were washed in 1 x PBS with 0.05 % Tween five times for 5 minutes. Western blots were visualized using Pierce ECL Western Blotting Substrate (Thermo Scientific) and a Fujifilm LAS-3000 camera.

If a polyvinylidene difluoride membrane was used repeatedly for incubation with different antibodies, it was stripped in between. The membrane was incubated in 50 ml of stripping buffer at 50° C for 45 minutes. Afterwards, it was washed in 1 x PBS with 0.05 % Tween five times for 5 minutes, blocked in the milk solution (1 h) and standard antibody incubation followed.

Table II: Gels for SDS-PAGE.

[ml]	12 % separating gel	5 % stacking gel
30 % acrylamide	4	0.67
1 M Tris-HCl pH 8.8	3.75	-
1 M Tris-HCl pH 6.8	-	0.5
MiliQ H_2O	2.05	2.7
10 % sodium dodecyl sulfate	0.1	0.04
10 % ammonium persulfate	0.1	0.04
TEMED	0.004	0.004

Table III: Proteins used as controls for Western blot analysis.

Antibody	Protein	Size [kDa]	Localization	Reference
trCOXIV	subunit of complex IV	37	inner mitochondrial membrane	Zíková et al., 2008c
VDAC	voltage dependent anion channel	30	outer mitochondrial membrane	Pusnik et al., 2009
MRP2	mitochondrial RNA-binding protein	22	mitochondrial matrix	Zíková et al., 2008a
Erv1	sulfhydryl oxidase	30	mitochondrial intermembrane space	Michael Ginger, Lancaster University
TDP1	tyrosyl-DNA phosphodiesterase	30	nucleus	Das et al., 2010
aldo	aldolase	40	glycosome	Paul A.M. Michels University of Louvain
TI	triosephosphate isomerase	27	glycosome	Paul A.M. Michels University of Louvain
enolase		50	cytosol	Paul A.M. Michels University of Louvain
Hsp70	heat shock protein, chaperone	70	mitochondrial matrix	Guler et al., 2008

3.4 Preparation of pT7v5-Nfs and pT7v5-Isu constructs

In order to obtain cell lines expressing tagged proteins, gene constructs were prepared containing Nfs (Tb11.55.0013) and Isu (Tb09.211.2830) genes followed by v5 tags and electroporated into cells. The Nfs gene was amplified from the trypanosome genome DNA by PCR using the following primers:

5´- GGAAGCTTATGTTTAGTGGTGTTCGCGTAC -3´ and 5´- TTAGATCTCCGCCACTCCACGTCTTTAAGG -3´ (added restriction sites for BglII and HindIII are underlined). The *Isu* gene was amplified from primers 5´- AGGAAGCTTATGCGGCGACTGATATCATC- 3´and 5´- AGGGATCCGCTTGACACCTCACC- 3´ (added restriction sites for BamHI and HindIII are underlined).

The obtained products were cloned into pGEM-T Easy vector, cleaved out using specific endonucleases and ligated into pT7v5 vector, which was verified by DNA sequencing. The validated pT7v5-Nfs and pT7v5-Isu constructs were linearized by restriction with NotI and electroporated into PS and BS wild-type cells. The transfection cell lines were selected based on puromycin resistance and expression of the tagged protein constructs was induced by tetracycline. Expression of the tagged construct was verified by Western blot analysis using a commercial anti-v5 antibody (Invitrogen).

3.5 Immunofluorescent assay (IFA)

Transfection cell lines were analyzed by immunofluorescent microscopy in order to visualize the tagged proteins. Cells were centrifuged (1 300 g, 16° C, 10 min) and resuspended in fresh media to final concentration 10^7 cells/ml. MitoTracker Red (20 nM for BS, 100 nM for PS, Molecular Probes) was added and incubated with cells for 30 minutes (37° C for BS, 27° C for PS). Subsequently, cells were centrifuged (1 300 g, 16° C, 10 min), washed in 1 x PBS and resuspended in 1 ml of 4 % paraformaldehyde in 1 x PBS and 1.25 mM NaOH. 250 µl of the solution was applied on a microscope slide and incubated for 10 minutes at RT. Slides were washed with 1 x PBS and incubated in 100 % ice cold methanol (20 min) to permeabilize the cells. Slides were washed three times with 1 x PBS. Unspecific binding of antibodies was prevented by blocking in 5.5 % FBS in PBS-Tween (0.05 %), RT, 1 hour. Slides were washed two times in 1 x PBS. Primary anti-v5 antibody (Invitrogen) was

added in 1:100 dilution in 5 % milk in PBS-Tween (0.05 %) and incubated at 4° C overnight. Slides were washed three times with 1 x PBS. Secondary antibody (anti-mouse, Molecular Probes) was applied in 1:1000 dilution in 5 % milk in PBS-Tween for 1 hour at room temperature in dark. Slides were washed three times with 1 x PBS. To stain DNA, VectaShield solution (containing DAPI, Molecular Probes) was covered onto slides. Cover slides were added and samples were incubated for 30 minutes in dark. Subsequently, samples were visualized using a fluorescent microscope (Axioplan2 imaging, ZEISS).

3.6 Nfs activity assay

Nfs activity assay enables to measure concentration of sulfur released by cysteine desulfurase. The reaction includes L-cysteine as a substrate, dithiotreitol as an oxidizing agent of sulfur and pyridoxal-5´-phosphate as a cofactor. After addition of N,N-dimethyl-*p*-phenylenediamine sulfate and $FeCl_3$, methylene blue is formed in amount quantitatively proportional to the sulfur released due to the cysteine desulfurase activity. The protocol for this method was kindly provided by Eva Nývltová (Charles University, Prague). I performed measurement of Nfs cysteine desulfurase activity using bloodstream single-marker, procyclic 29-13 and procyclic Nfs knockdown cell lines (Šmíd *et al*., 2006). Cells were centrifuged (1 300 g, 16° C, 10 min), washed with 1 x PBS, centrifuged again and resuspended in 20 mM HEPES/NaOH buffer up to final concentration 10^8 cells/ml. Cells were sonicated at 0.5 cycle, amplitude 0.6 for 30 s, put onto ice for 30 s and sonicated again. Cell suspension was centrifuged (1 300 g, 16° C, 10 min) and the obtained supernatant was used. Samples were prepared from cell suspension (0 − 1 ml), 0.5 mM L-cysteine, 1 mM dithiotreitol, 10 µl pyridoxal-5´-phosphate monohydrate and 20 mM HEPES/NaOH buffer up to the total volume 1 ml. Samples were incubated at 27° C for 1 − 2 hours. Then, the reaction was stopped by addition of 100 µl 20 mM N,N-dimethyl-*p*-phenylenediamine sulfate in 7.2 N HCl and 100 µl 30 mM $FeCl_3$ in 1.2 N HCl. Samples were incubated at room temperature in dark for 30 minutes and afterwards concentration of formed methylene blue was measured at $\lambda = 650$ nm using a Shimadzu UV-1601 spectrophotometer. Standard curve was prepared using 0 − 100 µM Na_2S in 20 mM HEPES/NaOH buffer.

3.7 Buffers and solutions

1 x Phosphate buffered saline (PBS), 1 l
  8 g NaCl
  0.2 g KCl
  3.58 g $Na_2HPO_4 . 12 H_2O$
  0.24 g KH_2PO_4
  pH 7.4

STE-NaCl buffer, 20 ml
  1.711 g sacharose
  0.175 g NaCl
  40 µl 0.5 M EDTA
  0.5 ml 1 M Tris-HCl pH 7.4

2 x SDS loading buffer, 10 ml
  0.24 ml 1 M Tris-HCl pH 6.8
  2 ml 50 % glycerol
  0.8 ml 10 % sodium dodecyl sulfate
  0.2 ml 2-mercaptoethanol
  0.4 ml 1 % bromphenol blue
  6.36 ml MilliQ water
  store at -20° C

5 x Running buffer for SDS-PAGE, 1 l
  15 g Tris
  72 g glycine
  5 g SDS

10 x Transfer buffer, 1 l
  29 g glycine
  58 g Tris
  3.7 g SDS

Stripping buffer, 50 ml
  3.125 ml 1 M Tris-HCl pH 6.7
  0.35 ml mercaptoethanol
  10 ml 10 % sodium dodecyl-sulfate
  MilliQ water up to 50 ml

20 mM HEPES/NaOH buffer, 200 ml
  0.953 g HEPES
  NaOH up to pH 8

4. Results

The main aim of my work was to determine the subcellular localization of the Nfs and Isu proteins in the bloodstream stage of *T. brucei*. Since in this life cycle stage the proteins are almost undetectable using specific antibodies (Shaojun Long, unpublished data) we decided to create cell lines with tagged forms of these proteins. Afterwards, I took advantage of two methods: digitonin fractionation and immunofluorescent microscopy. Considering possible difficulties and artifacts caused by the added tags, I introduced the same gene constructs also in the procyclic cells, where Nfs and Isu are supposed to be present in the mitochondrial matrix (Šmíd *et al*., 2006). In total, I prepared four transfectant cell lines (two genes in both of the cell stages) that all were examined with immunofluorescent microscopy and digitonin fractionation followed by Western blot analysis. Moreover, I have made use of a bloodstream cell line with HA-tagged Isu prepared by Shaojun Long and examined it using immunofluorescent microscopy. I also intended to compare the cysteine desulfurase activity of Nfs in separate cell fractions, but because of difficulties with the activity assay and unreliability of preliminary results, this aim was eventually abandoned.

4.1 Wild-type procyclic cells

First, I performed digitonin gradient fractionation using wild-type (WT, 29-13 strain) procyclic cells and the obtained samples were used for Western blot analysis (Fig. 2). Both the Nfs and Isu proteins are detectable only in the 9th fraction corresponding to the presence of mitochondrial control proteins. All the controls used demonstrate that plasmatic membrane is disrupted and cytosolic proteins are released at a very low concentration of digitonin (0.1 mM). Later on, glycosomal and nuclear membranes are damaged and content of these organelles is released. At last, mitochondrial membranes are perforated and mitochondrial proteins become detectable.
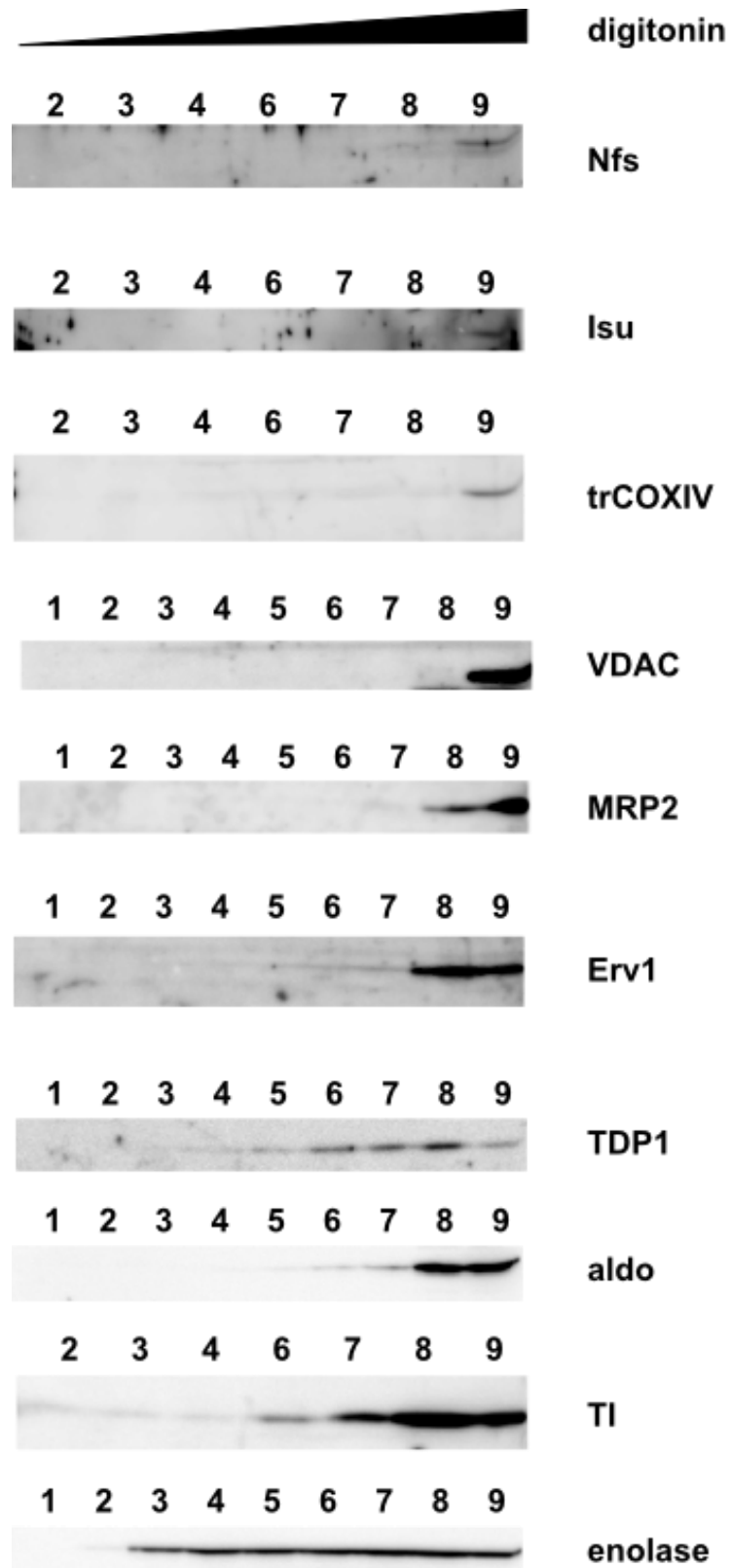
Figure 2: Western blot analysis of digitonin gradient fractionation, PS WT cells. Nfs, Isu – specific polyclonal antibodies against the trypanosomal proteins. Control proteins used: trCOXIV – mitochondrial inner membrane; VDAC – mitochondrial outer membrane; MRP2 – mitochondrial matrix; Erv1 – mitochondrial intermembrane space; TDP1- nucleus; aldo – glycosome; TI – glycosome; enolase – cytosol. For more details see Tab. III.

4.2 Nfs-v5 tagged procyclic cells

Figure 3 depicts Western blot analysis of samples from digitonin gradient fractionation performed with procyclic cells containing v5-tagged Nfs. The anti-v5 antibody recognizes a single specific band, which corresponds to the size of Nfs (48 kDa). These bands are detectable only in the 8th and 9th fractions which precisely corresponds to the pattern of Hsp70, a control protein from mitochondrial matrix. However, Erv1, a small soluble protein located in the mitochondrial intermembrane space, is detected already in the 6th fraction.

The outcome from immunofluorescent microscopy (Fig. 4) accurately corresponds with the results obtained with digitonin fractionation. The anti-v5 signal clearly co-localizes with MitoTracker in the mitochondrion.
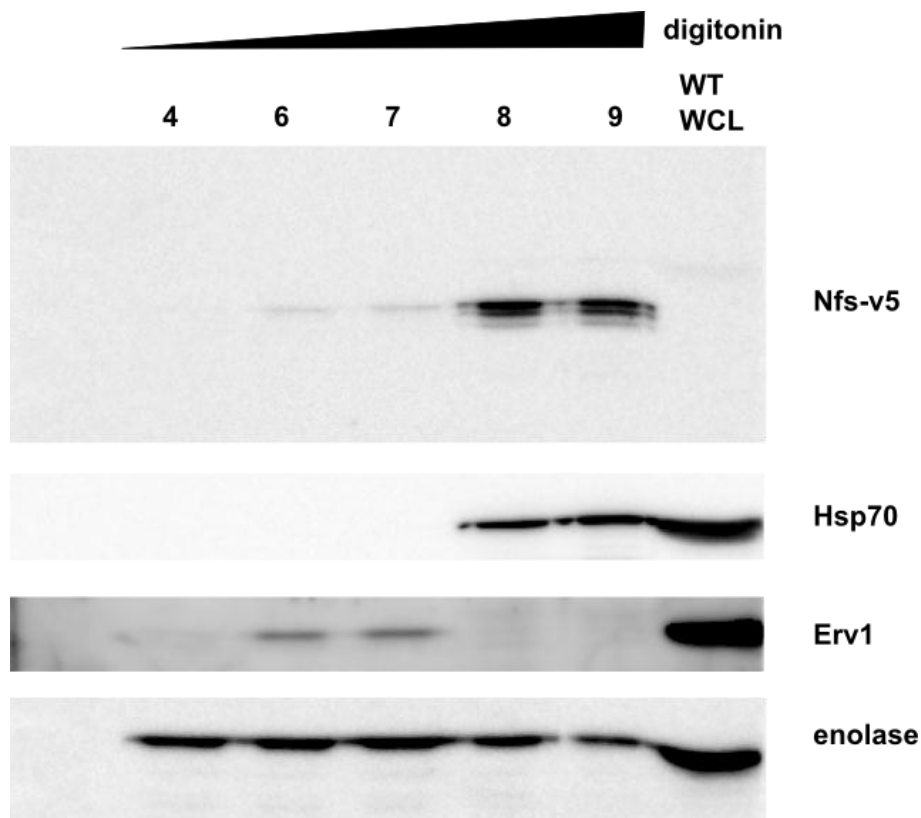

Figure 3: Western blot analysis of digitonin gradient fractionation, Nfs-v5 tagged PS cells. WT WCL – whole cell lysate from WT cells; Hsp70 – mitochondrial matrix; Erv1 – mitochondrial intermembrane space; enolase – cytosol.
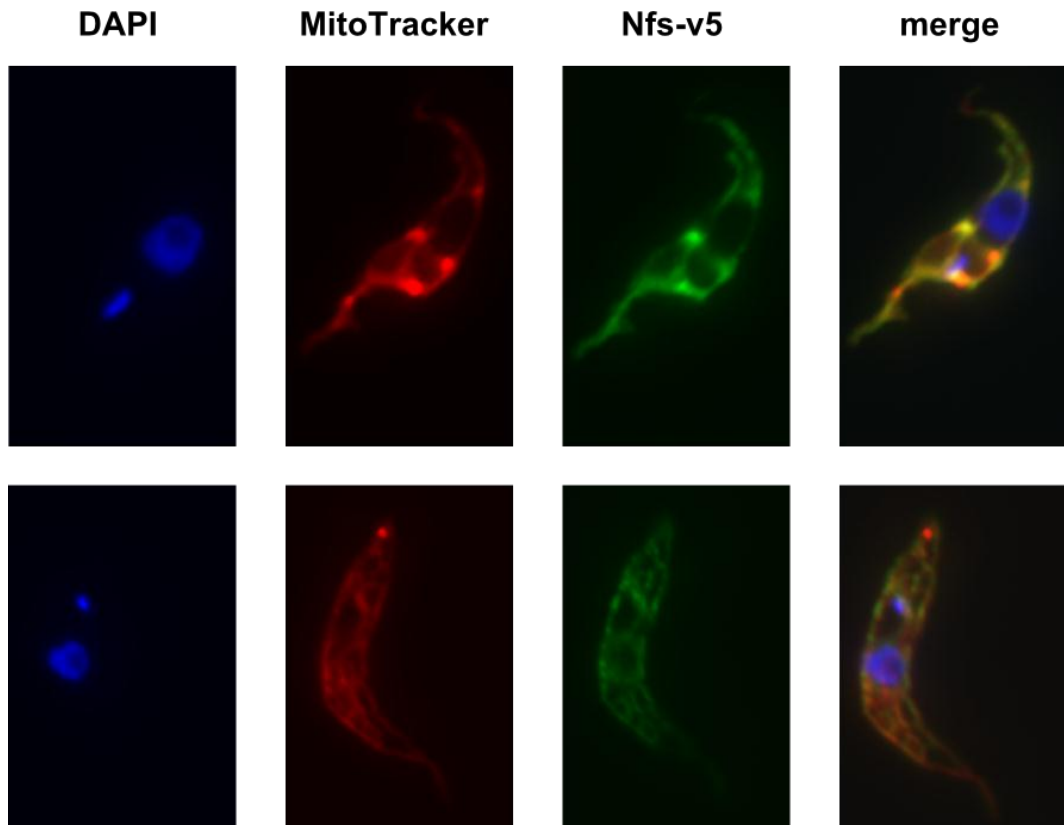
Figure 4: Immunofluorescent microscopy of Nfs-v5 tagged PS cells. DAPI stains specifically the nucleus and the kinetoplast, MitoTracker Red the mitochondrion, Nfs-v5 is visualized using anti-v5 monoclonal antibody.

4.3 Isu-v5 tagged procyclic cells

The results from the same Western blot analysis but using Isu-v5 procyclic cells instead of Nfs-v5 looks quite different (Fig. 5, compare to Fig. 3). Although the controls display the same pattern as in the previous experiment, the Isu-v5 protein is most abundant in the 7th fraction and the signal disappears in later fractions. The intermembrane protein Erv1 revealed the same pattern as Isu-v5, whereas the mitochondrial matrix protein Hsp70 is stained only in the 8th and 9th fractions.

Using immunofluorescent assay, the result from the Isu-v5 cells precisely corresponds to Nfs-v5 cells (Fig. 6), namely the Isu-v5 protein is present unequivocally in the mitochondrion and fully overlaps with the MitoTracker.
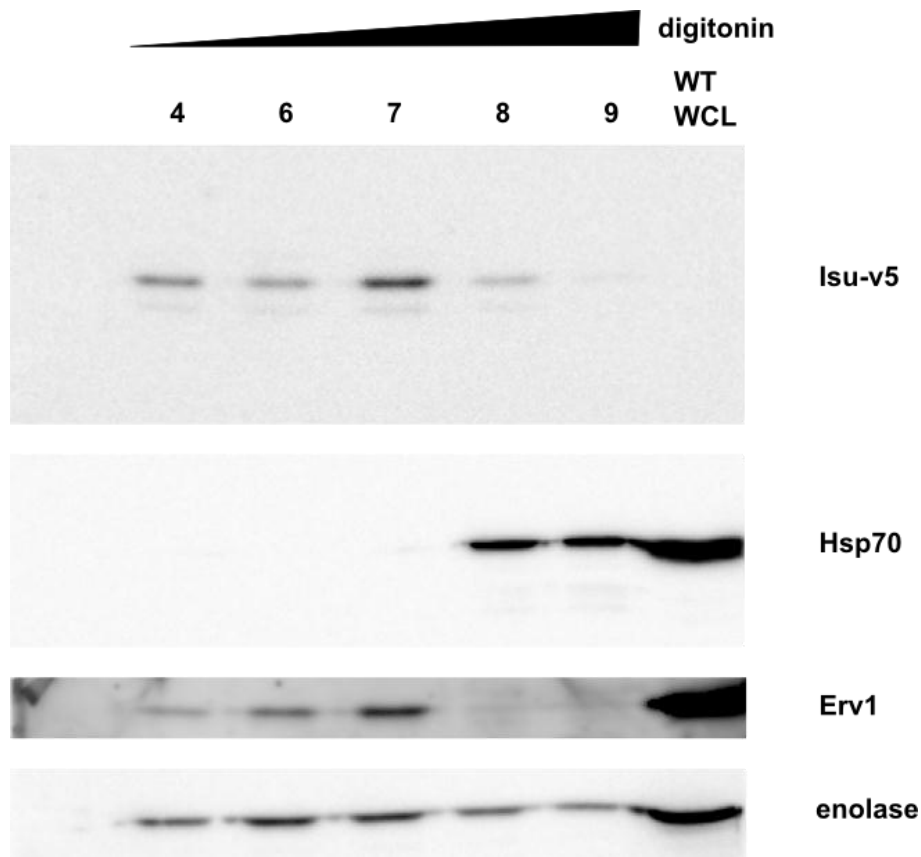

Figure 5: Western blot analysis of digitonin gradient fractionation, Isu-v5 tagged PS cells. WT WCL – whole cell lysate from WT cells; Hsp70 – mitochondrial matrix; Erv1 – mitochondrial intermembrane space; enolase – cytosol.
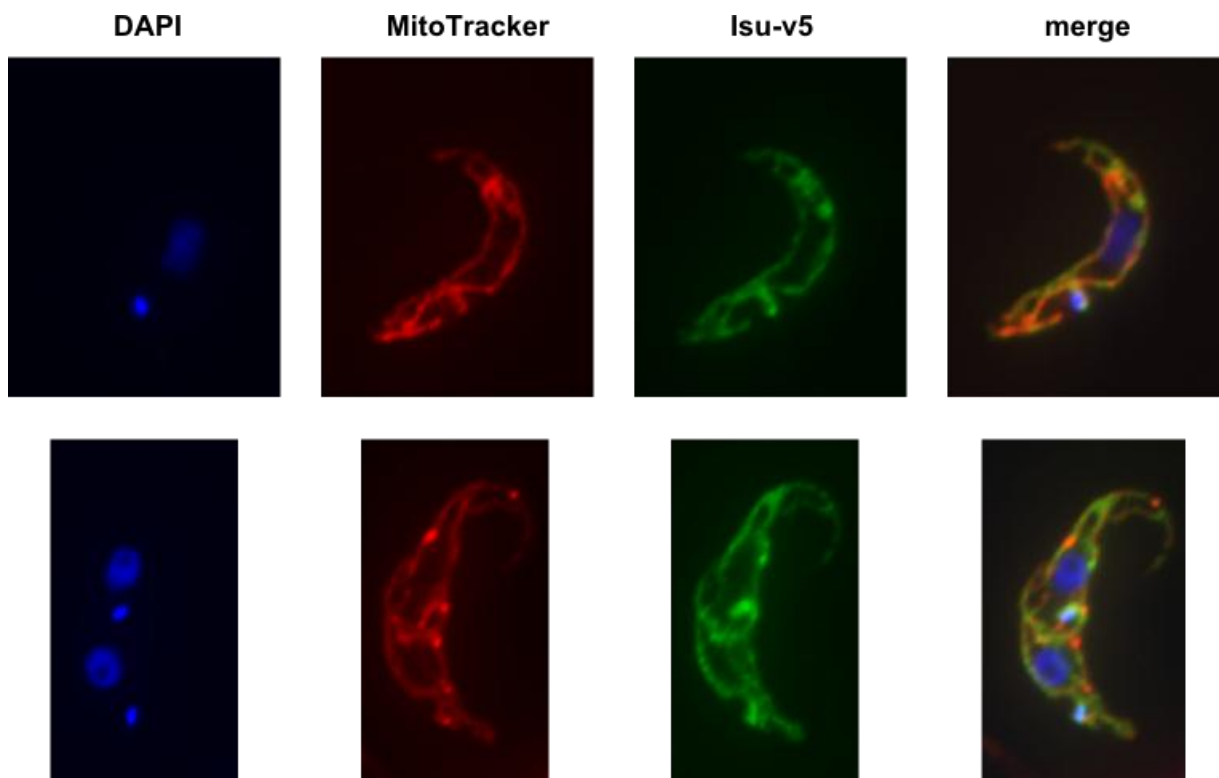
Figure 6: Immunofluorescent microscopy of Isu-v5 tagged PS cells. DAPI stains specifically the nucleus and the kinetoplast, MitoTracker Red the mitochondrion, Isu-v5 is visualized using anti-v5 monoclonal antibody.

First step in order to determine the subcellular localization of the studied proteins in the bloodstream stage of *T. brucei* was to perform digitonin gradient fractionation with WT bloodstream cells. According to Fig. 7, Nfs is present in the cytosol because the specific band appeared already in the 3rd fraction, same as enolase, whereas mitochondrial controls were detectable only in the 8th and 9th fractions. Unfortunately, my efforts to detect Isu in bloodstream cell fractions failed. The Isu protein was undetectable probably because it is present only in a very low amount in this cell stage.
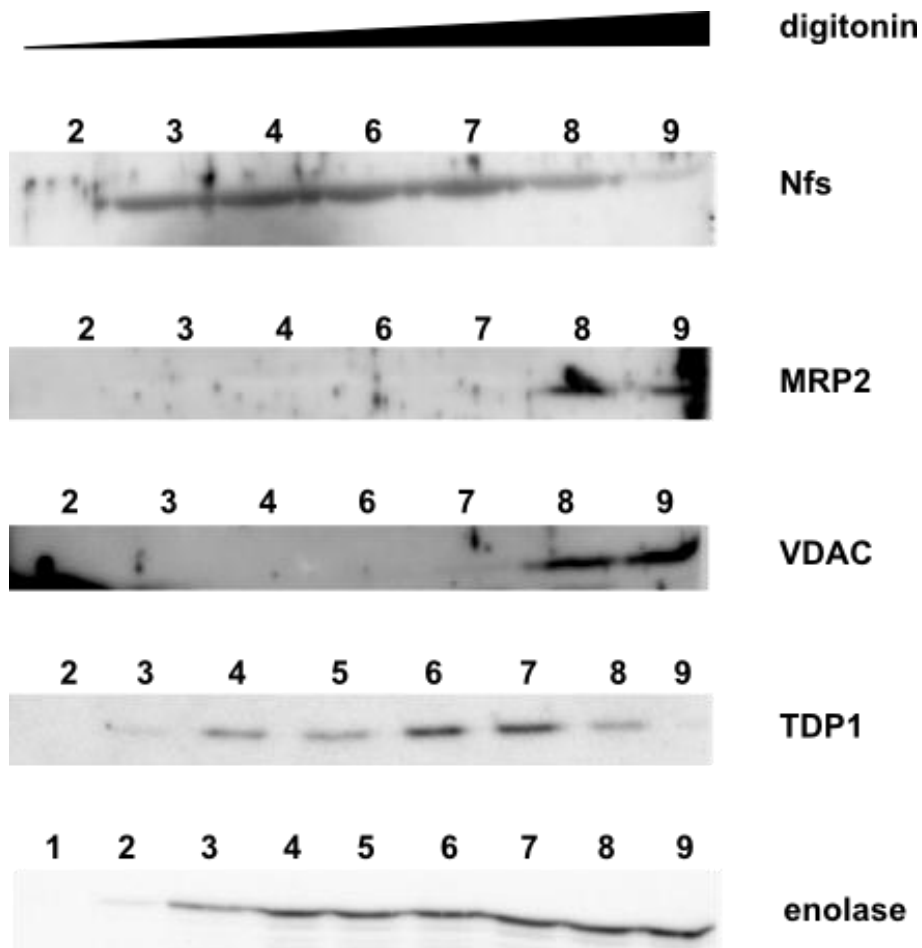

Figure 7: Western blot analysis of digitonin gradient fractionation from WT BS cells. Control proteins used: MRP2 – mitochondrial matrix; VDAC – mitochondrial outer membrane; TDP1- nucleus; enolase – cytosol.

4.5 Nfs-v5 tagged bloodstream cells

In the Nfs-v5 tagged bloodstream cells, Nfs corresponds to Hsp70 based on the digitonin fractionation. Both the proteins appeared already in the 7th fraction and were most abundant in the 8th and 9th fraction (Fig. 8). This result was verified with IFA, since the Nfs protein localizes again to the mitochondrion (Fig. 9). This localization correlates with the procyclic stage, where Nfs was detected in the mitochondrion as well.
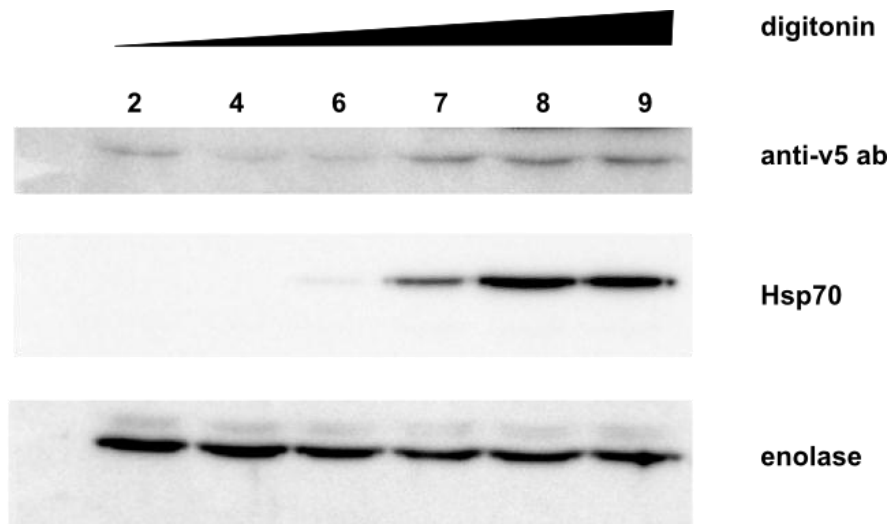

Figure 8: Western blot analysis of digitonin gradient fractionation, Nfs-v5 tagged BS cells. Hsp70 – mitochondrial matrix; enolase - cytosol.
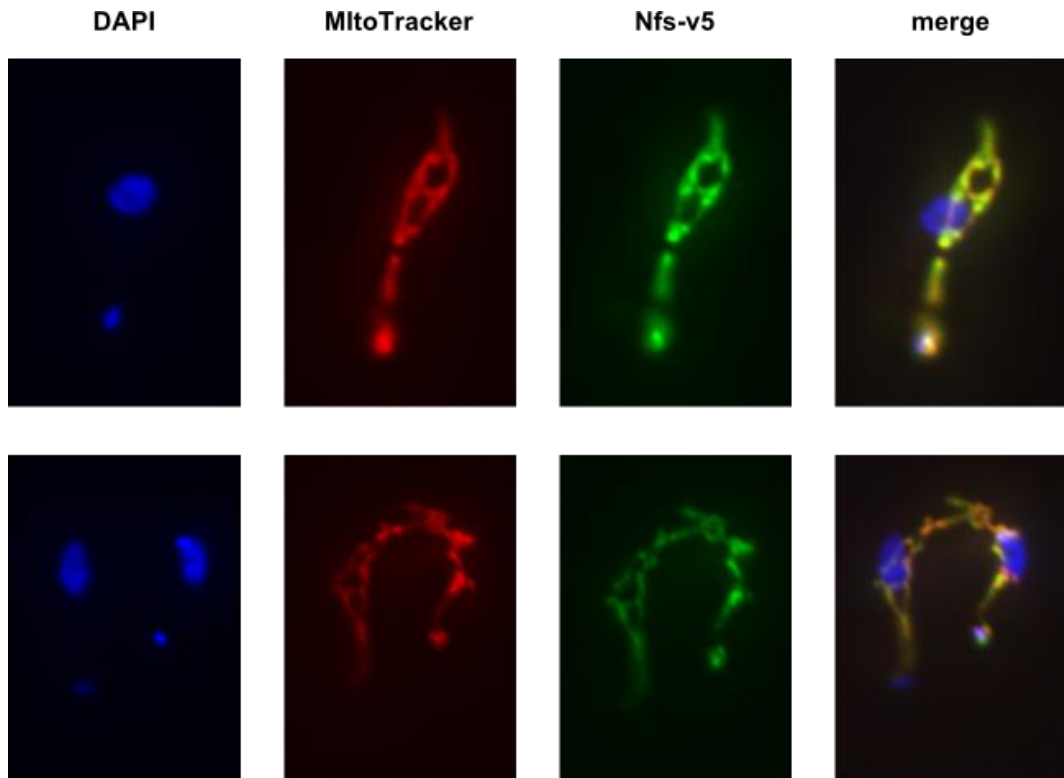
Figure 9: Immunofluorescent microscopy of Nfs-v5 tagged BS cells. DAPI stains specifically the nucleus and the kinetoplast, MitoTracker Red the mitochondrion, Nfs-v5 is visualized using anti-v5 monoclonal antibody.

4.6 Isu-v5 and Isu-HA tagged bloodstream cells

Detection of the Isu-v5 tagged protein in the bloodstream stage is parallel to those from the procyclic stage. As seen on Fig. 10, only a very weak signal was detected in the 6th fraction, although the pT7-v5 vector is supposed to induce strong overexpression in the cells. In contrast, the mitochondrial matrix protein Hsp70 is present only in the 8th and 9th fractions. The cytosolic protein enolase indicates that the loading was accurate and sufficient in all fractions.

Same results were acquired from immunofluorescent assay using either the v5-tagged or HA-tagged bloodstream cell lines (Figs. 11, 12). The Isu protein was detected solely in the mitochondrion and this method did not indicate its minor presence in other cell compartments.
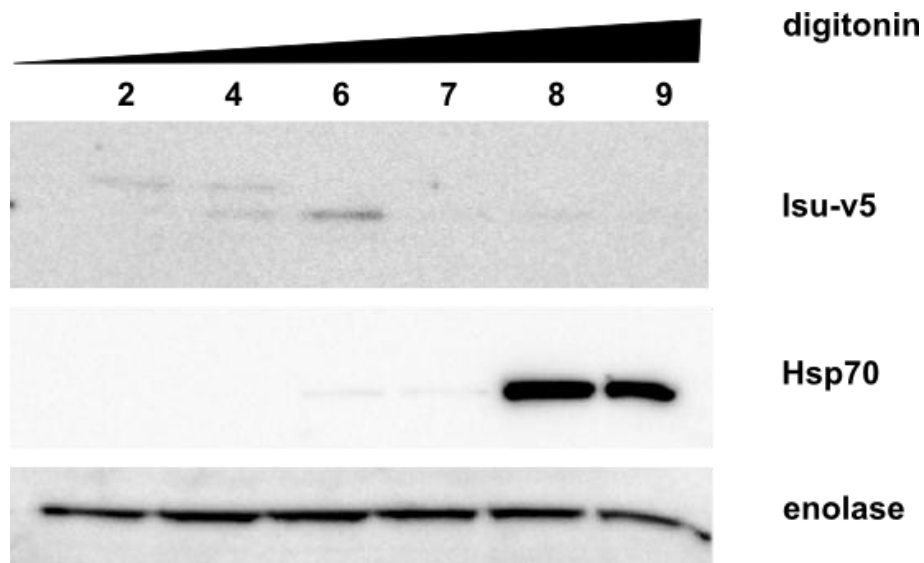
Figure 10: Western blot analysis of digitonin gradient fractionation, Isu-v5 tagged BS cells. WT WCL – whole cell lysate from WT cells; Hsp70 – mitochondrial matrix; enolase - cytosol.

Figure 11: Immunofluorescent microscopy of Isu-v5 tagged BS cells. DAPI stains specifically the nucleus and the kinetoplast, MitoTracker Red the mitochondrion, Isu-v5 is visualized using anti-v5 monoclonal antibody.
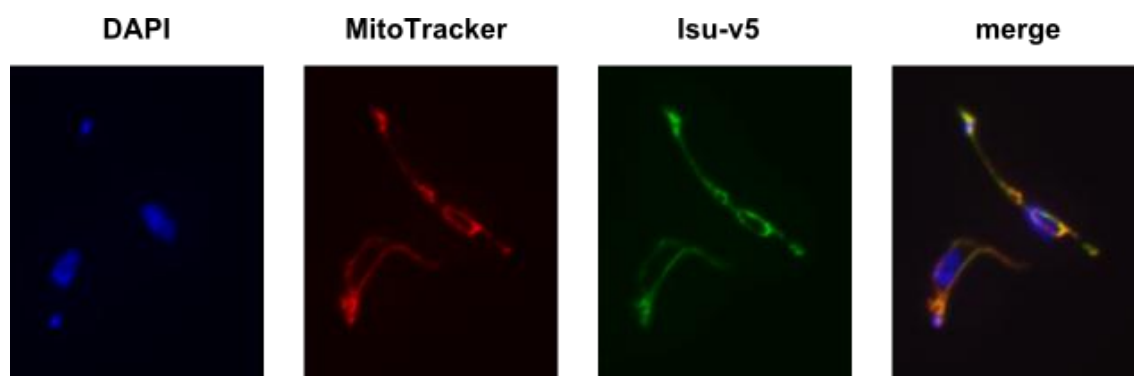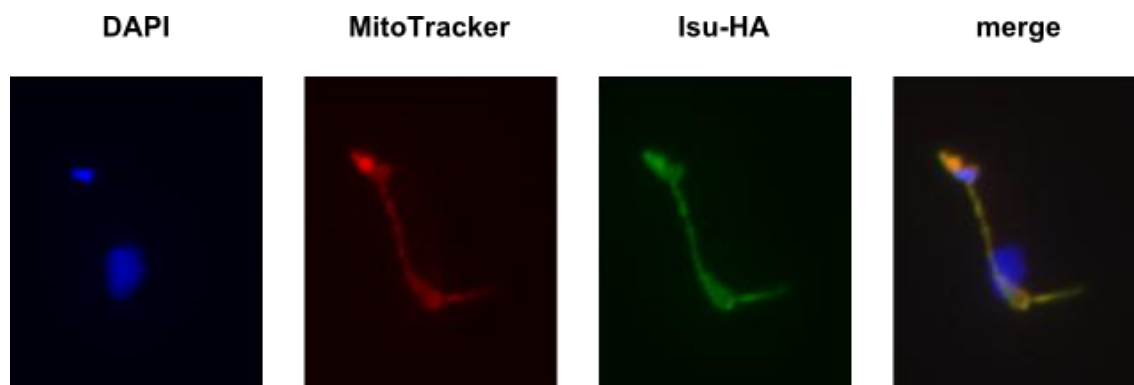

Figure 12: Immunofluorescent microscopy of BS Isu-HA tagged cells. DAPI stains specifically the nucleus and the kinetoplast, MitoTracker Red the mitochondrion, Isu-HA is visualized using anti-HA monoclonal antibody.

30

4.7 Nfs activity assay

The Nfs protein is a cysteine desulfurase, which abstracts sulfur from cysteine producing alanine. This enzymatic activity can be measured spectrophotometricaly owing to methylene blue formed during the assay in amount precisely proportional to the sulfur released from cysteine (Urbina *et al*., 2001).

The activity was measured in the PS and BS WT cells (Fig. 13) and lysates from the PS cells, in which Nfs was inducibly ablated by RNAi. Non-induced cells were used as a control (Fig. 14). The obtained values increased with the higher amounts of used cells, but the correlation was not proportional. The cysteine desulfurase activity is diminished in the BS as compared to the PS. However, there was no decrease in the Nfs activity in the knock-down cells after RNAi induction. Moreover, the error bars indicated very high standard deviation.
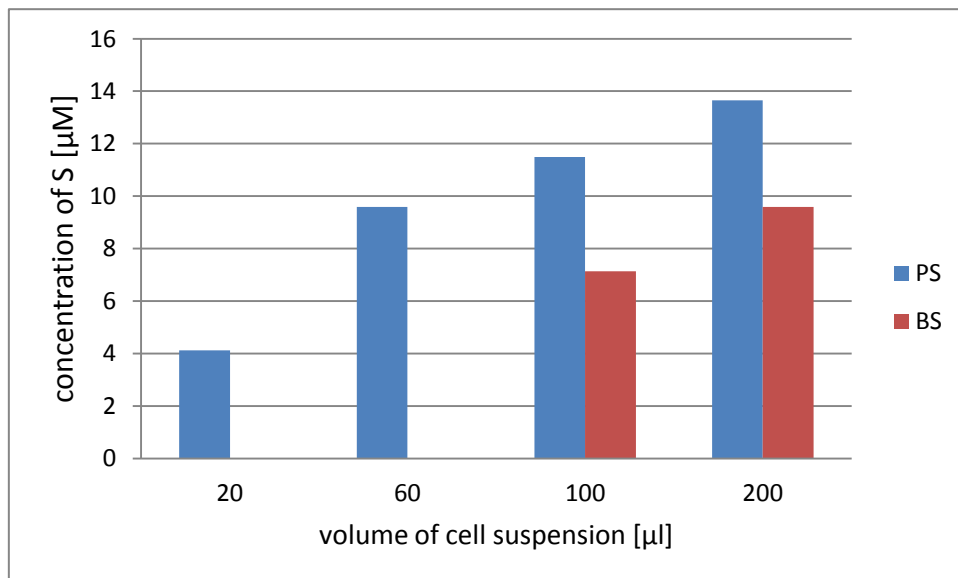

Figure 13: Nfs activity assay, procyclic and bloodstream WT cells. A representative result from repeated measurements. PS – procyclic stage, BS – bloodstream stage.
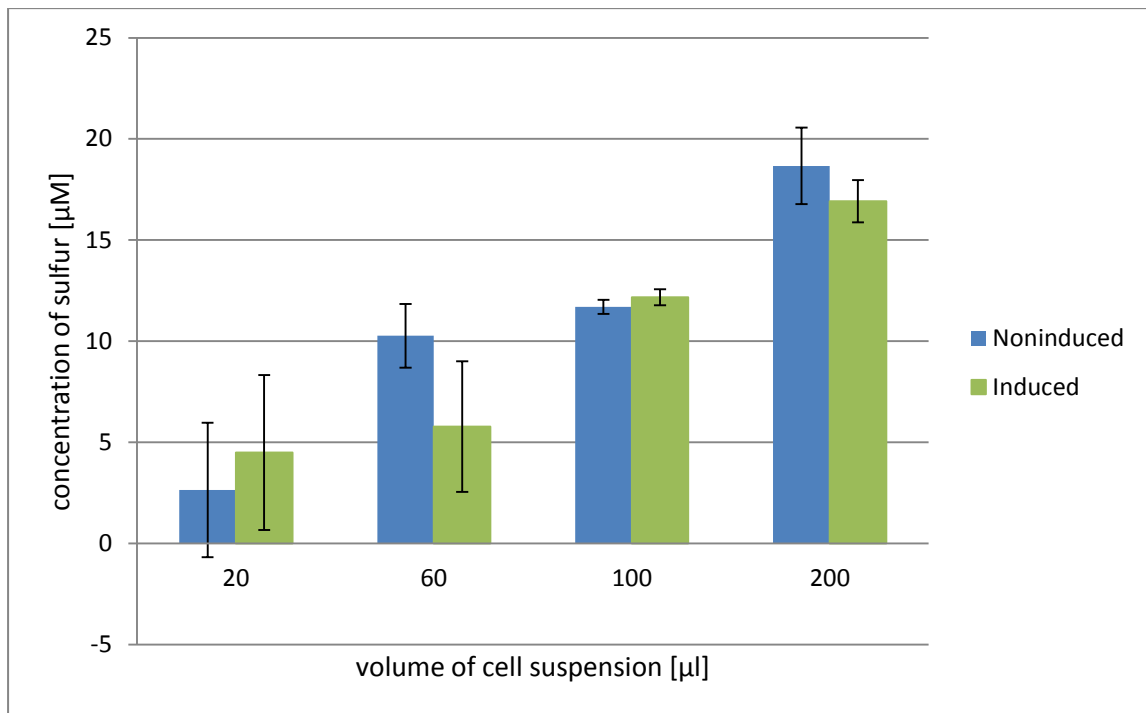
Figure 14: Nfs activity assay, PS Nfs knock-down cells. Average values from two independent measurements, error bars indicate standard deviation

5. Discussion

5.1 The procyclic stage

Two independent experimental approaches showed that in the PS both the Nfs and Isu proteins are present in the mitochondrion (Figs. 2, 3). This outcome is in agreement with a previous study on *T. brucei* (Šmíd *et al*., 2006) and also with data obtained from yeast (Mühlenhoff *et al*., 2004) and mammalian cells (Land and Rouault, 1998). Such a conclusion is straightforward from experiments with the wild-type and Nfs-v5 tagged cells.

The results obtained from Isu-v5 PS cells are slightly confusing, since the results from digitonin fractionation do not completely correlate with the previous observations. Contrary to Nfs-v5, the Isu-v5 protein has on the Western blot the same pattern as Erv1 rather than Hsp70. Still, the Erv1 protein is located in the mitochondrion, althogh not in the matrix. While digitonin fractionation indicated the presence of Isu in the intermembrane space of the organelle, from IFA one can conclude localization of the protein in the mitochondrion, without being able to distinguish among different organellar compartments.

In order to evaluate the two approaches used, I can deduce that IFA is much more trustworthy for the following reasons. First, despite being performed in a consistent manner, the only result which is ambiguous and do not correlate with the others is the digitonin fractionation of the Isu-v5 cells. Second, Nfs and Isu in general colocalize and cooperate together forming a complex, which has been verified in different organisms including *T. brucei* (Li *et al*., 2006; Paris *et al*., 2010). Indeed, in the mammalian tissues a homologue to Nfs is in both mitochondria and cytosol followed by its interacting partner Isu (Li *et al*., 2006; Tong and Rouault, 2006).

A unique situation has been described in microsporidia. Goldberg and co-authors (2008) reported different results from two studied microsporidian species. Whereas in *Encephalitozoon cuniculi* both Nfs and Isu were detected in mitosome, surprisingly, in *Trachipleistophora hominis* Isu was confined to the cytosol. While no explanation was provided, it is worth noting that these highly reduced fungi are well known for a number of unusual adaptations and reductions (Keeling and Fast, 2002). Another point to consider is that Nfs is active as cysteine desulfurase and hence also fulfills functions independent of Isu. For instance, Naamati *et al*. (2009) detected Nfs in *Saccharomyces cerevisiae* both in the mitochondrion and nucleus. They also showed that the protein possesses two separate targeting sequences directing it into these two compartments, with both of these forms being

essential for the cell. The nuclear isoform of Nfs is supposed to be involved in thio-modification of tRNAs (Mühlenhoff *et al*., 2004; Naamati *et al*., 2009). In addition, one more function was reported for human Nfs in the cytosol, where it serves as a sulfur donor for molybdenum cofactor biosynthesis (Marelja *et al*., 2008).

Third, while in IFA we follow whole cells and hence can evaluate their integrity, we do not know exactly what is going on during digitonin fractionation when the mitochondrion may at some stage disintegrate into many small vesicles (Hassan Hashimi, personal communication). Fourth, digitonin fractionation is very difficult to be standardized and repeated in exactly the same fashion. Even though I was conscious of these obstacles and was trying to keep the protocol as standardized as possible, my efforts obviously failed. Every time I maintained a constant volume of buffers, concentration of digitonin, time and temperature for incubation of cells with the detergent. The only variable I can imagine is that the digitonin was heated in order to be completely dissolved, and I admit not to have checked the temperature of the digitonin solution when being added to the cells. Still, due to a very small volume of the digitonin solution I can hardly imagine that this would explain significant differences among individual experiments.

In any case, the important outcome of the digitonin fractionation is that the Isu-v5 protein shows exactly the same pattern as Erv1. For some reason, Erv1 was released by varying concentrations of digitonin (Figs. 2 and 5) and the same was the case for Isu-v5. While Erv1 is present in the mitochondrial intermembrane space, Isu is supposedly localized in the mitochondrial matrix, where the whole Fe-S clusters biosynthetic pathway is situated. One possible explanation is that this unexpected localization is an artifact caused due to the added tag. While Nfs is a large protein (48 kDa), Isu is more than half the size (19 kDa) and it is therefore possible that even a small 5 kDa tag influences its localization within the organelle. It may have interacted with the mitochondrial targeting sequence, disabled proper import and resulted in the miss-localization in the intermembrane space of the mitochondrion.

Another conundrum is that both Isu-v5 and Erv1 disappeared in the last two fractions of the digitonin fractionation, although Erv1 was detected there in the wild-type PS. During preparation of the 9th fraction, twice the amount of buffer with digitonin was used as compared to all other samples with the volume of cell suspension kept equal. This may explain a lower signal in this fraction, but it is still not a satisfying explanation for the whole result. One would expect that the last two fractions will contain the whole cell lysate, because all membranes within the cells are disrupted and the whole cell content is released.

Control antibodies against Hsp70 and enolase used on the same membrane clearly validated that these fractions were not empty, although they may have been more diluted.

If we take a close look at Fig. 2 and consider all the control proteins used, we may find some discrepancies. In general, it corresponds to other studies using the same method (Lai *et al*., 2012) but not all the mitochondrial controls displayed precisely the same pattern. Instead, some of them (trCOXIV, VDAC) are detectable only in the last fraction, while others (MRP2, Erv1) are present in the last two fractions. Nfs and Isu belong to the latter group. Common features of trCOXIV and VDAC are that they are subunits of large protein complexes located in the mitochondrial membranes, complex IV in the inner membrane and VDAC in the outer one. There are two possible explanations for their behavior in the digitonin fractionation. First, the large size of their complexes may matter, as it was shown that Nfs and Isu form a large 440 kDa tetramere complex in mammalian cells (Li *et al*., 2006). Second, soluble proteins are released in lower concentration of digitonin than membrane bound and we cannot distinguish whether Nfs and Isu are bound directly or indirectly to the mitochondrial membrane and a high concentration of detergent is needed to release them.

Altogether, the digitonin fractionation does not seem to be an accurate or absolutely reliable method and should be used only cautiously and combined with other approaches. Alternatively, we could assess the results from an opposite point and fully trust the digitonin fractionation. From this perspective it shows the intermembrane localization of Isu, which was not revealed by IFA, because resolution of the used microscope cannot distinguish between individual compartments of the mitochondrion. However, Isu is not supposed to be present in the intermembrane space of the mitochondrion and such localization contradicts its proposed function as a scaffold protein in the Fe-S clusters biosynthesis (Agar *et al*., 2000).

5.2 The bloodstream stage

The Nfs and Isu proteins were detected in the mitochondrion of the BS of *T. brucei*. These results are not unexpected and correspond to those obtained in the PS. However, given major differences in mitochondrial metabolism between both stages, it was worth the efforts to establish them experimentally. My results nicely correspond with a study of Paris and colleagues (2010), who detected Isd11 in the BS mitochondrion and simultaneously

confirmed interactions among Nfs, Isd11 and Isu in the PS by TAP tag purification followed by mass spectrometry analysis. Furthermore, thiolation of tRNAs was detected in the BS mitochondrion, which serves as an indirect proof for the presence of Nfs in the same compartment (Paris *et al*., 2011).

Based on IFA, both the Nfs and Isu proteins are present in the mitochondrion. The outcomes from digitonin fractionation and Western blot analysis are less straightforward. Yet their ambiguity is of the same kind as observed in the Isu-v5 tagged PS. In particular, using this method the Nfs protein appeared to be in the cytosol of the wild-type BS cells.

To the best of my knowledge, the biosynthesis of Fe-S clusters is most likely an important function of the mitochondrion in the BS. A hypothesis was postulated that the Fe-S cluster biosynthetic pathway is the most ancient, common and absolutely ubiquitous function of all mitochondria (Lill and Kispal, 2000; Tovar *et al*., 2003). Several protist parasites carrying reduced forms of mitochondria termed mitosomes or hydrogenosomes are known to have lost to varying degree many functions associated with the organelle which have, however, invariably retained the Fe-S clusters biosynthesis (Tovar *et al*., 2003; Šuťák *et al*.,2004; Goldberg *et al*., 2008; Tachezy, 2008). The situation is different and quite unique in the BS of *T. brucei*, the mitochondrion of which has many functions reduced as compared to its counterpart in the PS. Here I bring a brief recapitulation of what is known about this morphologically and functionally reduced organelle.

The kinetoplast is not essential for viability of this stage, as is exemplified by the existence of dyskinetoplastic species *T. b. equiperdum* and akinetoplastic *T. b. evansii* (Lai *et al*., 2008). Moreover, cells lacking the kinetoplast can be induced artificially (Schnaufer *et al*., 2002). These cells proliferate as wild-type BS of *T. brucei*, but are arrested in this stage and cannot be transmitted by the tse-tse fly (Schnaufer *et al*., 2002; Timms *et al*., 2002). Still, transcription, RNA editing, RNA processing and translation of mitochondrial transcripts and tRNAs import are essential for viability of the BS (Schnaufer *et al*., 2001; Hashimi *et al*., 2010; Cristodero *et al*., 2010), with the latter one being recently also demonstrated for the mitochondrion of *T. b. evansi* (Paris *et al*., 2011). Still, all these processes seem to be essential just to ensure the synthesis of a single protein in the mitochondrion of the BS of *T. brucei*, namely subunit 6 of the ATP synthase, which utilizes ATP to maintain membrane potential (Schnaufer *et al*., 2005). Since membrane potential is required for import of nuclear-encoded tRNAs and proteins needed for translation, hundreds of proteins involved in the processing, editing and translation are required, and thus

indispensable for the cell, just to produce a single mitochondrial protein. Hence, the net benefit for the cell is highly questionable, and can be best explained by the "irremediable complexity" theory (Gray *et al*., 2010; Lukes *et al*., 2011).

It is well known that oxidative phosphorylation is not active in the BS. Recently, it has been shown that although complex I is expressed in the BS, it does not contribute to the NADH dehydrogenase activity and its real function remains elusive (Surve *et al*., 2012). Instead, glycerol-3-phosphate dehydrogenase and trypanosomatid alternative oxidase enable oxidation of NADH to NAD^+ necessary for glycolysis (Hannaert *et al*., 2003).

Next to energy generation, mitochondria are involved in other essential metabolic processes such as Ca^{2+} storage, fatty acids synthesis and degradation. In most eukaryotes, fatty acids are synthesized in mitochondria by so-called type II synthases. However, these enzymes are working only at a very low level in trypanosomes (Lee *et al*., 2006), their products remain within the mitochondrion and in the PS they are tightly associated with respiration (Guler *et al*., 2008), whereas depletion of the pathway disrupted kinetoplast segregation in the BS (Stephens *et al*., 2007; Clayton *et al*., 2011). Therefore, the bulk of fatty acids is synthesized by elongation in the endoplasmic reticulum (Lee *et al*., 2006). Furthermore, degradation of fatty acids was transferred to glycosomes in all Trypanosomatida and consequently is not an essential mitochondrial function (Hannaert *et al*., 2003).

Interestingly, although trypanosomes possess organelles called acidocalcisomes, the storage of Ca^{2+} likely belongs among key mitochondrial functions. Ca^{2+} is supposed to be involved in three separate pathways within the organelle. First, it stimulates activity of several dehydrogenases, however, which are most likely not expressed in the BS (Docampo and Lukeš, 2012). Second, Ca^{2+} activates metabolite carriers on the inner mitochondrial membrane but again such function may not be utilized in the BS, since almost no metabolic activity exists in its mitochondrion (Hannaert *et al*., 2003). Third, Ca^{2+} triggers apoptosis but the starting mechanism for an apoptosis-like cell death is different in trypanosomes (Docampo and Lukeš, 2012). Actually, the single piece of information known about Ca^{2+} homeostasis in the BS mitochondrion is that a mitochondrial calcium uniporter is present and the import of Ca^{2+} is enabled due to membrane potential (Docampo and Lukeš, 2012). Moreno and co-workers (1992) reported a very low amount of endogenous calcium in the BS, with the main pool being probably located in the endoplasmic reticulum and acidocalcisomes. To the best of my knowledge, there is no explanation for the import of

calcium into the mitochondrion which is devoid of citric acid cycle and classical respiratory chain.

Altogether, scrutinizing the functions of the mitochondrion in the BS, their essentiality seems to be rather elusive. Data presented in this work indicate that the Fe-S cluster biosynthesis remains potentially functional in this reduced and downregulated organelle, retaining its essentiality for the cell. This anticipation is in a good agreement with the hypothesis about the ancient and tight association of this pathway with the ancestral mitochondrion (Tovar *et al.*, 2003; Šuťák *et al.*, 2004).

5.3 Nfs activity assay

As far as I know, this is the first time that Nfs activity assay was applied on trypanosomes, yet it does not seem to be useful and applicable in its present form. In the wild-type cells, activity did not increase proportionally with the volume of samples. The obtained values were lower for the BS compared to the PS, which can be explained by smaller cell size and lower metabolic activity of the former stage. More importantly, there was no decrease of Nfs activity in the PS Nfs knock-downs although the protein was significantly ablated (Šmíd *et al.*, 2006).

There are several reasons possibly explaining the failure. Since, there are more cysteine desulfurase enzymes that are undistinguishable by this method, we may not detect solely and specifically the Nfs activity. Furthermore, the amount of material used may be under the detection limit of the assay, although 10 x increased amount of cells did not improve the outcome (data not shown) and the 100 µl volume displayed the lowest difference in standard deviation (Fig. 14). This method was described by Urbina *et al.* (2001) in an *in vitro* design and was later implemented for *Escherichia coli* cells co-overexpressing Nfs together with Isd11 (Mühlenhoff *et al.*, 2004). However, the results published for yeast (Adam *et al.*, 2006) seem to have been contradicted by a later study (Goldberg *et al.*, 2008). It is not surprising that I was not able to measure any endogenous Nfs activity in trypanosomes.

5.4 ISC pathway in the bloodstream stage

Altogether, I bring here the first direct evidence for the presence of the ISC pathway in the BS mitochondrion. Even though most components are undetectable in the BS using specific antibodies (Shaojun Long, unpublished data), the pathway seems to be present and active. Evidence provided herein and elsewhere (Paris *et al*., 2010) strongly indicates that the whole complex of Nfs, Isd11 and Isu is present in the studied mitochondrion. The cysteine desulfurase is most probably utilized not only for Fe-S assembly, but also for tRNA thiolation (Paris *et al*., 2011). Even the Isu protein is essential in the BS, since its depletion by RNAi was lethal (DeHua Lai, unpublished data). *T. brucei* possesses two ferredoxin genes and at least one of them is indispensable in the BS (Eva Stříbrná, Shaojun Long, Piya Changmai & Julius Lukeš, unpublished data). Regarding the subsequent scaffolds in the Fe-S pathway, only Isa1 and Isa2 were studied in the BS so far, and since their parallel depletion by RNAi caused just a very minor growth phenotype, they appear not to be essential for the BS stage, but are indispensable for the PS (Long *et al*., 2011). Indirect yet strong evidence for functionality of the Fe-S cluster biosynthesis is based on an experiment in which Fe-S dependent enzymes aconitase and fumarase have been overexpressed and found to be active in the BS (Shaojun Long, Piya Changmai, personal communication). Altogether, the presence of all key components of the ISC pathway has been verified in the mitochondrion of the BS of *T. brucei,* where it fulfills so far the most important function of this highly down-regulated mitochondrion, a situation reminiscent of mitosomes (Tovar *et al*., 2003; Šuťák *et al*., 2004; Goldberg *et al*., 2008).

6. Conclusions

- Both Nfs and Isu are present in the mitochondrion of the BS.
- The ISC pathway is the crucial mitochondrial function in the BS.
- In order to establish localization of mitochondrial proteins in the BS, IFA is much more reliable method than digitonin fractionation followed by Western blot analysis.
- The Nfs activity assay is not applicable in the described design.

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