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Reductive Evolution of Mitochondria - Related Organelles in Anaerobic Protist

Ph.D. Thesis

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I declare that I substantially contributed to the results presented in this thesis.

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1. Introduction

Contemporary eukaryotes emerged most likely from a massive radiation that resulted in a complexity and diversity of their structure and metabolism. Some unicellular eukaryotes were grouped into a paraphyletic taxon called the Archezoa. These anaerobic or intracellular organisms were considered to be primarily amitochondriates as no organelles of typical mitochondrial morphology were found and it was proposed that they separated from the main eukaryotic lineages before the acquisition of mitochondria (Cavalier-Smith 1986, 1989). However, in the last decade, archezoans including parasitic and free-living members of Parabasalia, Diplomonadida, Amoebozoa and Microsporidia groups were shown to possess a double-membrane bound organelle related to mitochondria. These organelles most likely evolved from more complex ancestral mitochondria and according to the extent of their modification and reduction they were classified into two types: (i) hydrogenosomes that produce hydrogen and ATP by substrate level phosphorylation and (ii) mitosomes that do not participate in cellular energy metabolism and ATP generation. The only common feature for these two types of organelles and mitochondria seems to be the formation of Fe-S clusters.

Nowadays, mitosomes and hydrogenosomes are found among all 6 supergroups of eukaryotes and most likely represent only a peak of a very large diversity of mitochondria relatives (Keeling et al., 2005; Hampl and Simpson, 2008). It is more than likely that all extant eukaryotes have or had organelles of mitochondrial ancestry. One also cannot exclude the possibility that in some eukaryotes mitochondria were completely lost and only a minority of nuclear encoded genes of α-proteobacterial origin remain their common history. The study of mitochondria relatives can help us to better understand the early evolution of eukaryotes and elucidate which metabolic processes

are ancestral or adaptive as a response to various habitats. This thesis is in particular focused on the hydrogenosome and the mitosome, organelles that are best characterized in two common worldwide parasites of men, the parabasalid *Trichomonas vaginalis* and the diplomonad *Giardia intestinalis*.

2. Hydrogenosomes

The first hydrogenosomes were discovered in parabasalids *Trichomonas vaginalis* and *Tritrichomonas foetus*, the causative agents of urogenital trichomonosis of men and cattle (Lindmark and Müller 1973; Cerkasovova et al., 1976). More recently, hydrogenosomes have been found in various parasitic and free-living protists, such as marine free-living ciliates *Plagiopyla frontata* (Fenchel and Finlay, 1991), *Metopus concordus* (van Bruggen et al., 1984), *Sonderia sp*. (Dyer, 1989), fresh water free-living ciliates *Trimyema sp*. (Broers et al., 1991), rumen dwelling ciliates *Dasytrichia ruminantium* (Yarlett et al., 1981), *Isotricha sp*., *Dasytricha sp*., *Entodinium sp*. (Lloyd et al., 1989; Paul et al., 1990), ciliate *Nyctotherus ovalis* (Hackstein et al., 1999; Boxma et al., 2005) from the alimentary tract of *Blattodea americana*, rumen dwelling chytridiomycota *Neocallimastix frontalis* (O'Fallon et al., 1991; Marvin-Sikkema et al., 1994), and *Piromyces sp.* (Akhmanova et al., 1999), heteroloboseans *Psalteriomonas lanterna* (Brul et al., 1994) and *Sawyeria marylandensis* (Barbera et al., 2010).

T. vaginalis hydrogenosomes are organelles 0.2-1μm in diameter which are surrounded by closely apposed membranes that form multiple peripheral intramembranous vesicles with a high concentration of cations such as calcium and magnesium (Benchimol, 2009). In *Trichomonas*, hydrogenosomes are located in close proximity to cytoskeletal structures including axostyle and costa. It has been proposed that, similarly to mitochondria, vesicles budding from the ER provide hydrogenosomes

with new membranes and membrane lipids for growth and biogenesis (Benchimol, 2009).

Under anaerobic conditions, hydrogenosomes oxidize pyruvate to acetate, CO_2 and molecular hydrogen. This metabolic activity is coupled with production of ATP by substrate level phosphorylation. Oxidative decarboxylation of pyruvate is catalyzed by an Fe-S protein PFO (pyruvate:ferredoxin oxidoreductase), an enzyme typical for anaerobic and nitrogen-fixing bacteria (Müller, 1993; Hrdy et al., 2008). The key features distinguishing hydrogenosomes from mitosomes and mitochondria are the production of hydrogen by [FeFe] hydrogenase and the activity of PFO. Mitochondria use the pyruvate dehydrogenase complex for oxidative decarboxylation of pyruvate, while in organisms with mitosomes hydrogenase and PFO activities occur in the cytosol.

The maturation of Fe-S proteins, which are present within hydrogenosomes, cytoplasm and nucleus requires Fe-S cluster biosynthesis. Immunolocalization and phylogenetic analysis of key-enzymes involved in Fe-S cluster formation revealed that Fe-S cluster biosynthesis is localized within hydrogenosomes and is homologous to the mitochondrial ISC biosynthesis pathway (Tachezy et al., 2001; Sutak et al., 2004). The central component cysteine desulfurase IscS catalyzes the production of sulfur while frataxin serve as a donor of iron for the formation of transient Fe-S clusters. The formation occurs on the IscU and IscA, which serve as scaffolds from where the Fe-S clusters are transferred to apoproteins by the assistance of chaperone Hsp70 and the J-type cochaperone Jac1. Fe-S cluster biosynthesis requires a source of reducing power that is facilitated by the [2Fe2S] ferredoxin and its redox partners. (Lill and Müllenhoff, 2005; Hrdy et al., 2004). Almost all proteins and accessory components of the ISC pathway have been found in the *Trichomonas* genome and the activity of

crucial components was detected in hydrogenosomes (Tachezy and Smid, 2008; Hrdy et al., 2008).

Cytosolic or nuclear Fe-S apoproteins require the product of ISC biosynthesis from mitochondria. The ISC export machinery consists of the ABC half-transporter Atm1 and the sulfhydryloxidase Erv1. However, it is still unclear which component is exported from mitochodria to the cytosol (Lill and Müllenhoff, 2008). Surprisingly, no sequences homologous to the Atm1 or Erv1 have been found in *Trichomonas* hydrogenosomes, while components of cytosolic ISC assembly system are present. In mitochondria, a P-loop NTPase Ind1 is required for maturation of Fe-S proteins of the multi-subunit respiratory complex I (Bych et al., 2008). *Trichomonas* hydrogenosomes contain two FeS subunits of a highly reduced form of complex I, called NuoE and NuoF (Hrdy et al., 2004). Three P-loop NTPases homologuous to the Ind1 with a hydrogenosomal targeting signal at their N-terminus were identified in *Trichomonas* proteome. Subsequently, the representative homolog TVAG_027170 was localized in hydrogenosomes (Rada et al, 2011).

Hydrogenosomes found in various lineages of protist and fungi differ in shape, size, membrane composition and metabolic pathways, suggesting that hydrogenosomes evolved from a mitochondrial ancestor multiple times independently as a response to oxygen-poor environment. Examples of hydrogenosomal diversity can be found among some anaerobic ciliates. Ciliates found in fresh water and marine sediments such as *Trimyema sp.* and *Plagiopyla frontata* harbour intracellular methanogenous archebacteria that use hydrogen produced by hydrogenosomes for reduction of CO_2 and production of ATP (Embley et al., 1993-4). Organelles of another anaerobic ciliate *Nyctotherus ovalis* provide the missing link between the representative hydrogenosome as described in some parabasalids and mitochondria. Inner membranes of

hydrogenosomes from *Nyctotherus* form cristae and contain the mitochondrial-type lipid cardiolipin. *Nyctotherus* hydrogenosomes also posses DNA encoding ribosomal proteins, ribosomal RNA and tRNA for organellar proteosynthesis as well as some components of mitochondrial complex I and II. Components of this partial electron transport chain are probably capable of producing detectable membrane potential (Boxma et al., 2005; de Graaf et al., 2011). An important feature linking *Nyctotherus* hydrogenosomes to mitochondria is the process of decarboxylation of pyruvate, where *Nyctotherus* use pyruvate dehydrogenase complex instead of PFO. Hydrogenosomes in *Nyctotherus* most likely also contain some enzymes of the tricarboxylic acid cycle, homologues of mitochondrial import and processing machinery, and various mitochondrial-type metabolite transporters (de Graaf et al., 2011).

2.1. Origin of mitochondrial and hydrogenosomal proteins

Nowadays, there is no doubt that hydrogenosomes are relatives of mitochondria which are believed to have evolved from a common α-proteobacterial endosymbiont (most likely related to Rickettsiases) about 2 bilion years ago. The transition of the endosymbiont to protomitochondria was coupled with the transfer of the endosymbiont genes to the host nucleus or their loss. However, this event did not diminish the complexity of the organelle proteome. Recent mitochondria code for relatively few genes, ranging from 3 in *P. falciparum*, 13 in humans, 28 in yeast up to 85 in *A. thaliana* (Richly et al., 2003). However, hundreds of proteins were identified in mitochondrial proteomes, and of these only about 14% in human and 16% in yeast have predicted α-proteobacterial origin (Gabaldon and Huynen et al., 2004). Numerous new proteins were recruited from host organisms, through lateral gene transfer from other prokaryotes, or evolved as a new eukaryotic inventions. Thus, about 99% of mitochondrial proteins are

nuclearly encoded, imported by the mitochondria after their synthesis in the cytosol. However, to function within the eukaryotic cell, the mitochondrial ancestor had to establish the protein import machinery, as well as the transport of substrates, exchange of nucleotides and various metabolic products. Some of the later eukaryotic acquisitions are common to all mitochondria, including the major components of outer and inner translocase complexes (Tom40, Tim23 and Tim22), the most abundant inner membrane carrier for ADP/ATP exchange, and some proteins of the α-proteobacterial origin such as mitochondrial processing peptidase, soluble chaperones and Sam50 subunit of the outer membrane protein assembly machinery. These proteins were probably acquired before the divergence of the major eukaryotic lineages (Dolezal et al., 2006; Shiflett and Johnson 2010). The representative mitochondria found in yeast and human possess typical function in aerobic energy conversion, such as oxidative decarboxylation of pyruvate, tricarboxylic acid cycle, electron transport chain and ATP synthesis by oxidative phosphorylation. Mitochondria also harbour oxidation and synthesis of fatty acid, synthesis of heme groups, steroids, amino acids, iron sulphur clusters and biotin as well as their own genome replication, transcription and translation including post-translational modifications in protein synthesis.

Hydrogenosomes share with mitochondria a number of similarities, such as conserved mechanisms of iron sulphur cluster assembly, conserved mechanisms of NAD^+ regeneration and a common mode of protein targeting (Tachezy et al., 2001; Hrdy et al., 2004; Dolezal et al., 2005). These functions require components of α-proteobacterial origin, new eukaryotic inventions and some proteins of bacterial origin that were acquired by lateral gene transfer. Hydrogenosomal proteins that originated from α-proteobacteria represent in particular both subunits of trichomonad NADH dehydrogenase, succinyl CoA synthase (the only remaining enzyme of the tricarboxylic

acid cycle in trichomonad hydrogenosome), mitochondrial type chaperones Hsp70 and Cpn60, components of the mitochondrial-type iron sulphur cluster assembly machinery (ISC) and putative Sam50 subunit of the outer membrane protein assembly machinery (SAM). New eukaryotic inventions represent putative subunits of the TIM complex (Tim23 and Pam18) and in particular several genes homologues to the mitochondrial carrier family (MCF).

Unlike mitochondria, hydrogenosomes harbour some proteins that are found mostly in obligate or facultatively anaerobic bacteria. These typical hydrogenosomal proteins include key enzymes involved in pyruvate conversion such as PFO and [FeFe] hydrogenase and some proteins involved in oxidative stress protection. The hydrogen hypothesis suggests that hydrogenase and PFO were present in a common α-proteobacterial endosymbiont but lost from mitochondria. However, neither eukaryotic hydrogenase nor PFO have been shown to cluster with other α-proteobacterial homologues. The more probable mitochondrion-first hypothesis suggests that mitochondria irreversibly lost their capacity for oxidative phosphorylation and acquired hydrogenase and PFO by lateral gene transfer from anaerobic bacteria (Embley et al., 2003). Although hydrogenase and PFO homologues were most likely present in early eukaryotes, their exact phylogenetic position still remains unclear (Embley et al., 2003).

Specific maturases of [FeFe] hydrogenase HydG, E and F were most likely inherited by lateral gene transfer. These maturases can be found also in chloroplast of green algae but are missing in other excavate *Giardia intestinalis* and archamoebae *Entamoeba histolytica* (Pütz et al., 2006; Henze, 2008). Other hydrogenosomal proteins that were acquired by lateral gene transfer are involved in oxidative stress protection. Anaerobic protists have to cope with a low oxygen concentration in their natural environment, because their organelles contain highly oxygen sensitive enzymes such as

PFO and [FeFe] hydrogenase (Smutna et al., 2009). These proteins require protection against molecular oxygen and ROS. ROS, like hydrogen peroxide, are detoxified in hydrogenosomes by proteins that belong to the thioredoxin-linked peroxiredoxin system. These include thioredoxin reductase, thioredoxin and thioredoxin peroxidase (Mentel et al., 2008).

In addition to the hydrogenosomal thioredoxin-linked peroxiredoxin system, two functionally related antioxidant proteins, the ruberythrin and peroxiredoxin thiol peroxidase OsmC were identified in the proteome of the *T. vaginalis* hydrogenosome (Henze, 2008). Ruberythrin and OsmC are most probably related to prokaryotic peroxidases and together with components of the thioredoxin-linked peroxiredoxin system are found among anaerobic and facultatively anaerobic eubacteria. Except for the presence of a ruberythrin homologue in *E. histolytica*, none of these proteins have been found in other eukaryotes to date (Henze, 2008; Mentel et al., 2008). In *Trichomonas* hydrogenosomes, oxygen is most likely reduced by four electrons to water by flavodiiron proteins. These were acquired by lateral gene transfer from anaerobic bacteria and their homologues can be found only in other few anaerobic protists such as *Giardia, Entamoeba* or *Mastigamoeba* (Smutna et al., 2009).

2.2. The proteome of the *T. vaginalis* hydrogenosome

The majority of proteins destined for the mitochondria contain N-terminal targeting presequences. Mitochondrial and hydrogenosomal presequences exhibit a similar hydrophobic pattern, and some are actually interchangeable. However, hydrogenosomal presequences are generally shorter and consequently many of them are not capable of forming amphipathic α-helixes. Analyses of the *T. vaginalis* genome revealed the presence of 147 genes coding for putative hydrogenosomal proteins based

on identification of N-terminal targeting presequences (Smid et al., 2008). However, the actual size of the hydrogenosomal proteome is likely larger when matrix proteins, which could be targeted to the organelle independently on N-terminal presequences, are taken into consideration. These include proteins with noncleavable signals or those containing internal signals, such as hydrophobic membrane proteins and proteins targeted to intermembrane space (Mentel et al., 2008). The internal targeting signals that usher proteins into mitochondria are still poorly understood. Nothing is known about their structure in hydrogenosomes and consequently no algorithms are available to predict hydrogenosomal proteins targeted to the organelles by means of N-terminal independent mechanisms.

To evaluate the actual size of the hydrogenosomal proteome and to confirm previous *in silico* predictions, 2D SDS page analysis of *T. vaginalis* and *T. foetus* hydrogenosomes were performed (Henze, 2008). Together with *in silico* predictions, hydrogenosomes are estimated to harbour nearly 200 proteins (Henze, 2008; Smid et al., 2008). Surprisingly, from these only 61 proteins were identified by 2D SDS page - mass spectrometry analysis, including a small percentage of false positives. The partial proteome of the *T. vaginalis* hydrogenosome consists particularly of proteins that are involved in pyruvate fermentative metabolism, iron sulphur cluster assembly machinery, amino acid metabolism and oxidative stress protection (Henze, 2008). Hydrophobic proteins are difficult to solubilize for isoelectric focusing. This may explain why, with one exception, none of predicted or putative components of membrane machinery required for biogenesis of hydrogenosome and metabolic exchange were identified by 2D SDS gel analysis. Only one membrane protein, an ADP/ATP carrier Hmp31 was identified by mass spectrometry, although components of protein import machinery and various metabolite transporters are expected to function in membranes of

hydrogenosomes (Henze, 2008). Therefore, novel approaches based on suitable protein extraction and more sensitive mass spectrometry analysis should be performed to better analyze the membrane proteins in hydrogenosome and to evaluate the actual size of hydrogenosomal proteome.

2.3. Biogenesis of hydrogenosomes

The hydrogenosomal genome was relinquished during the mitochondrion-to-hydrogenosome transition. The loss of respiratory complexes that are partially encoded by mitochondrial DNA most likely resulted in the loss of hydrogenosomal genome. Consequently, all hydrogenosomal proteins are encoded in the nucleus and imported postranslationally. As previously mentioned, to be imported the proteins must possess targeting signals that are recognized at the organelle surface. Subsequently, they are transported across membranes, and upon translocation they undergo processing to become active (Shiflett and Johnson, 2010).

In mitochondria, protein translocation is mediated by specific multiple heterooligomeric protein complexes called translocase of the outer membrane (TOM), protein sorting and assembly machinery of the outer membrane (SAM) and translocase of the inner membrane (TIM) (Chacinska et al., 2009). This process requires the action of membrane receptors, β-barrel pores, motor proteins, chaperones, some associated accessory proteins as well as small Tims. The general machinery of protein import is best exemplified by yeast (Chacinska et al., 2009). The majority of proteins directed into mitochondria are transported through the outer membrane TOM complex, which serves as the general gate into the organelle. Imported proteins are subsequently sorted according to their final destination to TIM23 complex that facilitates import into the matrix or to Tim22 translocase, which imports proteins into the inner membrane. β-

barrels are integrated into the outer membrane by the *β*-barrel sorting and assembly SAM complex (Chacinska et al., 2009).

Tom40 is a *β*-barrel protein that is a core component of TOM complex. Like VDAC (Voltage Dependent Anion Channel), it has a porin-3 motif, suggesting that Tom40 and VDACs represent ancient paralogues, and belong to a common mitochondrial porin family (Pfam family PF01459). Surprisingly, no mitochondrial porins have been identified in the completed *Trichomonas* genome to date, although a Tom40 homologue is reported for the *Trichomonas* relative *Giardia intestinalis* (Dagley et al., 2009) and other organisms harbouring mitosomes such as *Entamoeba*, *Cryptosporidium* and *Encephalitozoon* (Dyall and Dolezal., 2008). However, hydrogenosomes have to import proteins and exchange metabolites with the cytosol and thus distant homologues of mitochondrial porins most likely function in the outer membrane of hydrogenosome. These homologues may be discovered using more sensitive searches such as the hidden Markov models.

Hydrogenosomes and mitochondria produce various metabolites including ATP and hereby supply other cellular compartments with energy for various metabolic activities. Consequently, ATP synthesized within the organelle must be transported across its double membranes to the cytosol. The mitochondrial inner membrane contains up to 55 distinct carriers of the large MCF family (Mitochondrial Carrier protein Family) including ATP/ADP carriers (ACCs), amino acid carriers, transporters of inorganic ions and cofactors linking organelle metabolism with the cytosol (Kunji and Crichton, 2010). In the *Trichomonas* genome, five genes were found with homology to MCF, but none of them are closely related to typical ACCs. One of these called Hmp31 represents one of the most abundant hydrogenosomal proteins. This protein was successfully targeted to the inner membrane of yeast mitochondria (Dyall et al., 2000).

Despite the distant sequence homology, Hmp31 and all other hydrogenosomal MCF proteins contain structural and sequence patterns suggesting ATP/ADP exchange activity. This activity was experimentally confirmed for the Hmp31 homologue in *T. gallinae* (Tjaden et al., 2004). Mitochondrial-type ACCs were also described in the ciliate *N. ovalis* and the fungus *N. frontalis* (de Graaf et al et al., 2011; Voncken et al., 2002).

Multiple spanning proteins such as MCF carriers are imported into the inner membrane of the organelle by the activity of Tim22 translocase. Import of proteins that are addressed for the matrix, as well as insertion of single spanning proteins into the inner membrane is facilitated by a heterooligomeric complex TIM23. The core components of TIM23 complex are the Tim23 channel and its associated subunit Tim17. Genes related to Tim17 and Tim23 sequences were identified in *Trichomonas* genome and thus the TIM23 complex is expected to function in *Trichomonas* hydrogenosome (Dolezal et al., 2006).

Import of proteins into the matrix of the organelle is cofacilitated by the TIM23 associated molecular motor, called the PAM complex. In yeast, the PAM complex consist of three membrane associated components Tim44, Pam18, Pam16, a soluble matrix chaperone mtHsp70, and a nucleotide exchange factor Mge1. Except of Pam16, these components were identified in *Trichomonas* genome, suggesting that a PAM-like system may function in protein import into the hydrogenosome (Dyall and Dolezal., 2008).

2.4. Chaperones of the intermembrane space

Considering that mitochondria and mitochondria-related organelles evolved from a common intracellular gram-negative bacteria, analogous behaviour should be expected in the intermembrane space of the organelle and periplasmic space in bacteria.

Precursors of mitochondrial and bacterial proteins are sorted within the intermembrane or periplasmic space by the assistance of small chaperones that prevent their misfolding and non-specific aggregation. During the transition of endosymbiont to mitochondria, bacterial type chaperones were replaced by ~10kDa chaperones called small Tims (TIMs). In yeast and human, five small Tims Tim8, Tim9, Tim10, Tim12 and Tim13 form two functional pairs Tim9/10 and Tim8/13 that assemble into a hexameric complex ($\alpha_3\beta_3$). Tim12 act as a transient peripheral subunit of the Tim22 inner membrane translocase and participates in unloading precursor proteins delivered by the Tim9/10 complex. The Tim9/10 complex ferries hydrophobic precursors to the inner membrane and guides *β*-barrels to the outer membrane of the organelle. The Tim8/13 complex is functionally homologous to the Tim9/10 complex. However, Tim8/13 subunits, unlike Tim9/10 subunits, are dispensable for growth in yeast mutants (Chacinska et al., 2009).

Among anaerobic protist, small Tims homologues have surprisingly only been identified in *Cryptosporidium parvum* (Alcock, unpublished data). However, a similar or analogous small Tims-like system is expected in *Trichomonas* hydrogenosomes. Multi-spanning membrane protein Hmp31 most likely depends on small Tims-like chaperones to assist its insertion into the inner membranes of hydrogenosomes. Therefore, similar chaperones may function in *Trichomonas* hydrogenosome (Dyall and Dolezal., 2008).

3. Mitosomes

Mitosomes represent the most highly reduced form of mitochondria. Similarly to hydrogenosomes, mitosomes have been found in organisms inhabiting oxygen limited niches, such as the parasite of human intestine *Giardia intestinalis* (Diplomonads) (Tovar et al., 2003) and *Entamoeba histolytica* (Amoebozoa) (Tovar et al., 1999). They

are also found in intracellular parasites including *Trachipleistophora hominis* (Wiliams et al., 2002) and *Encephalitozoon cuniculi* (Microsporidia) (Katinka et al., 2001) and *Cryptosporidium parvum* (Apicomplexa) (Riordan et al., 2003). Mitosomes lost the majority of typical mitochondrial function such as the tricarboxylic acid cycle, electron transport chain and oxidative phosphorylation. Unlike hydrogenosomes, mitosomes do not produce any ATP. PFO and [Fe-Fe] hydrogenase are localized in some organisms in the cytosol. There are only two known mitosomal pathways. The sulfate activation pathway was identified in mitosomes of *E. histolytica* (Mi-ichi et al., 2009; Maralikova et al., 2010) and related free-living *Mastigamoeba* balamuthi (Nyvltova et al., unpublished data). All other known mitosomes possess machinery for FeS cluster assembly. No DNA has been found in any mitosome to date and it follows that mitosomal proteomes are most likely reduced.

Mitosomes are tiny ovoid organelles limited by two apposed membranes enclosing a granular matrix. Similarly to hydrogenosomes, membranes do not form cristae. The size and quantity of mitosomes within a cell varies between different species. The earliest studies of *E. histolytica* observed a single organelle up to three per cell (Tovar et al., 1999). However, later studies revealed that in *Entamoeba* trophozoites, mitosomes are highly abundant organelles, with over 150 per cell (Leon-Avila and Tovar, 2004). Considerably less mitosomes were observed in *Giardia* (25 to 50 mitosomes per cell) (Dolezal et al., 2005) and only 10 mitosomes or less were found in *Encephalitozoon cuniculi* (Katinka et al., 2001; Goldberg et al., 2008). In comparison to mitochondria and hydrogenosomes, mitosomes are substantially smaller organelles, with the size ranging from less than 0,2μm in *Giardia* up to 0,5μm in *Entamoeba* (Tovar et al., 2003; Leon-Avila and Tovar, 2004). In *Giardia*, mitosomes are distributed within a cell with higher occurrence in the lateral and posterior region of the trophozoites. One

of the peculiarities of their distribution in *Giardia* is their close proximity to the basal bodies between the two *Giardia* nuclei (Regoes et al., 2005).

3.1. Fe-S cluster assembly

Bacteria possess at least three independent systems that are crucial for Fe-S cluster assembly. The ISC machinery is required for housekeeping Fe-S proteins, the NIF system is used especially for Fe-S cluster formation in the nitrogenase of nitrogen-fixing bacteria and the SUF system most probably repairs damaged Fe-S clusters under oxidative stress or iron limited conditions (Tachezy and Smid, 2008). With two exceptions (discussed later), mitosomes inherited the ISC system. The level of its complexity differs in various organisms (Tachezy and Smid, 2008). Representative mitosomes in *Giardia* most likely contain a nearly complete set of proteins required for formation of transient Fe-S clusters on the molecular scaffold (IscS, IscU, Nfu, IscA-2) and a set of proteins for transfer of transient Fe-S clusters to apoproteins (IscA-2, glutaredoxin 5, chaperone mtHsp70, cochaperone HscB and the nucleotide exchange factor GrpE) (Tachezy and Smid, 2008). Surprisingly, frataxin, which is present in all mitochondria and in the *Trichomonas* hydrogenosome, is absent in the *Giardia* mitosome. However, frataxin was detected in the genome of a close relative of *Giardia*, the diplomonad *Spironucleus vortens,* and localized in their mitosomes (Zubacova, unpublished results). Frataxin was also found in the genome of *C. parvum* and localized in mitosomes of *E. cuniculi* (Goldberg et al., 2008). Ind1 is another member of ISC machinery that is absent in *Giardia* mitosomes. This could be explained by the absence of complex I that requires Ind1 for FeS cluster formation. Similarly to *Trichomonas*, no homologues of Atm1 or Erv1 have been found in *Giardia* mitosomes, although the cytosolic Fe-S cluster assembly system is present. Fe-S cluster biosynthesis requires the

source of reducing power that is facilitated by [2Fe2S] ferredoxin and its ferredoxin:$NADP^+$ reductase. Based on genomic studies, these components are predicted in mitosomes of *C. parvum* and *E. cuniculi* (Tachezy and Smid, 2008). However, ferredoxin:$NADP^+$ reductase was not detected in the proteomes of the *Trichomonas* hydrogenosome or the *Giardia* mitosome. In *Trichomonas* hydrogenosomes, [2Fe2S] ferredoxin is most probably reduced by PFO or rudimental complex I, both of which are not present in *Giardia* mitosomes.

Two archamoebae, pathogenic *Entamoeba histolytica* and free living *Mastigamoeba balamuthi,* are the only known eukaryotes that replaced ISC machinery with the NIF system (Nyvltova, unpublished data). Interestingly, *E. histolytica* NifS (EhNifS) and NifU (EhNifu) are most probably localized in the cytosol (Ali et al., 2004; Dolezal et al., 2010), although its mitosomal localization was also suggested (Maralikova et al., 2010). Unlike *Entamoeba*, *Mastigamoeba* possesses two forms of NifS and NifU. One form contains a mitochondrial targeting sequence that directs NifS and NifU into the organelle, while the second form of NifS and NifU is present in the cytosol (Nyvltova, unpublished data). Interestingly, genome of *Acantamoeba castellani* (a relative of *Entamoeba* and *Mastigamoeba*) contain annotated genes for both ISC and NIF components, but nothing is yet known about their localization within a cell (van der Giezen, 2009).

3.2. Biogenesis of the mitosome - similarities to hydrogenosomes and mitochondria

As with hydrogenosomes, all mitosomal proteins are encoded in the host nucleus and posttranslationally imported into the organelle using either N-terminal or inner targeting signals. N-terminal signals that address imported proteins into mitochondria, hydrogenosomes, and mitosomes have a similar pattern. However, like proteins destined

to hydrogenosomes, mitosomal precursors possess the shorter presequences capable for targeting into the organelle. Mitosomal and hydrogenosomal presequences also possess less positively charged amino acid residues than mitochondrial precursors. Consequently, by contrast to mitochondrial presequences, a noticeable proportion of mitosomal and hydrogenosomal N-terminal extensions are most likely unable to form amphipathic α-helixes (Dyall and Dolezal, 2008; Smid et al., 2008). N-terminal signals contain a cleavage site that is recognized and processed by a processing peptidase in the matrix of the organelle. Each cleavage site possesses a specific motif that determines which peptide bond is cleaved. The cleavage motif in mitochondrial presequences typically contains arginine at the position -2 (P_2) or -3 (P_3) proximal from the cleavage site and positively charged residues are also present distal from the cleavage site. All identified mitosomal presequences contain arginine at the position P_2, but unlike mitochondrial presequences, the distal positively charged residues are absent. Many hydrogenosomal and mitosomal presequences have similar patterns, but most hydrogenosomal ones do contain arginine or other positively charged amino acid residues distal from the cleavage site as shown in mitochondrial N-terminal extensions (Smid et al., 2008). To date, N-terminal targeting signals have been reported for mitosomal precursors in *E. histolytica* (Tovar et al., 1999), *C. parvum* (Riordan et al., 2003) and *G. intestinalis* (Smid et al., 2008). In mitochondria, N-terminal and internal targeting signals are recognized by receptors of the heterooligomeric complex TOM. Amphipathic presequences recognize receptors Tom20 and Tom22 while precursors with internal signals are recognized by the receptor Tom70. Mitochondrial precursors are subsequently passed through the Tom40 and Tim23 channels into the matrix, where their N-terminal presequences are cleaved by processing peptidase.

Tom70 has been found in animals and fungi and among anaerobic eukaryotes. It was functionally characterized in *Blastocystis* and its shortened version was recognized in *E. cuniculi*. All mitosomal proteins in *E. cuniculi* lack N-terminal extensions and contain internal signals for delivery into the organelle. This observation is consistent with the presence of Tom70 and absence of MPP (Tsasousis et al., 2011; Goldberg et al., 2008). Tom20 and Tom22 receptors have not been found in any anaerobic eukaryote so far, probably because their sequences are too diverse, which is why searches for their homologues are based on less sensitive detection of their structure similarities rather than on sequence homology (Perry et al., 2006, 2008). Another possibility could be convergent evolution that resulted in a speciation of novel unknown systems.

A distant homologue for Tom40 was characterized in *Giardia* (Dagley et al., 2009) and Tom40 candidates were also identified in genomes of *C.parvum* (Abrahamsen et al*.,* 2004), *E. cuniculi* (Katinka et al., 2001) and *E. histolytica* (Loftus et al., 2005). Considering that mitochondrial porins can be found in other anaerobic eukaryotes possessing mitochondria-related organelles*,* it is likely that Tom40 related sequences can be found in all species containing mitochondria relatives (Hjort et al., 2010). Sam50 and mtHsp70 are the only components of translocation machinery that are homologous with α-proteobacteria. Sam50 has been reported for *Entamoeba* (Dolezal et al., 2010), *Cryptosporidium* and microsporidia, but surprisingly it was not identified in *Giardia* (Shiflett and Johnson, 2010).

Homologues of the Tim17/22/23 protein family can be found in the genomes of *E. cuniculi* and *C. parvum* (Shiflett and Johnson, 2010). *Giardia* and *Entamoeba* do not contain any of these (Dolezal et al., 2006). Their organelles represent the most reduced predicted translocation machinery consisting only of distant homologues of Tom40, Pam16/18, mtHsp70 and processing peptidase (*Giardia*) or of Tom40, Sam50 and

mtHsp70 (*Entamoeba*). It is unclear how *Giardia* and *Entamoeba* mitosomes facilitate import of matrix and multispanning inner membrane proteins, although another translocase mediating protein import across/into inner membrane of the organelle is expected.

In mitochondria, processing peptidase (MPP) consists of 2 subunits: an α-subunit is required for presequence recognition, binding and release, while a β-subunit provides the catalytic activity. *Giardia* MPP, so called GPP, is exceptional, because it is active only as a single β-subunit monomer, while the α-subunit is missing. *Trichomonas* MPP, called HPP, functions as a heterodimer, but the α-subunit is very divergent and in an initial study was not recognized (Brown et al., 2007; Smid et al., 2008). Mitochondrial α-MPP interacts with positively charged residues distal from the cleavage site. However, mitosomal precursors contain shorter presequences that do not possess any of the distal positively charged residues while some of the shortened hydrogenosomal presequences contain these residues (Smid et al., 2008). Maybe this is one of the reasons why GPP is unable to process any mitochondrial presequences while HPP is capable for processing at least some shorter ones.

Imported and processed precursors need to be properly folded and assembled. This process usually requires assistance of molecular chaperones and ATP. MtHsp70 functions as a part of the PAM complex while Cpn60 and Cpn10 together refold imported matrix proteins. All three mtHsp70, Cpn60 and Cpn10 originated from α-proteobacteria and their homologues are found in most eukaryotes including anaerobic protist. An mtHsp70 has been localized in mitosomes of *Entamoeba* (Tovar et al., 2007), *Cryptosporidium* (Slapeta and Keithly, 2004), *Trachipleistophora* (Williams et al., 2002) and *Encephalitozoon* (Tsaousis et al., 2008) while Cpn10 and Cpn60 have been

localized in mitosomes of *Giardia* (Regoes et al., 2005), *Entamoeba* (Tovar et al., 1999) and *Cryptosporidium* (Riordan et al., 2003).

3.3. Mitosomes require import of ATP

The only metabolic activity of mitosomes is the formation of Fe-S clusters and the sulfate activation pathway. Both activities, together with protein import, refolding, and assembly, require a source of ATP. Hydrogenosomes and mitochondria-like organelle in *Blastocystis* produce ATP by substrate level phosphorylation, while mitosomes have to rely on host ATP. A single ADP/ATP carrier and one phosphate carrier were characterized in *Entamoeba* mitosomes (Chan et al., 2005; Dolezal et al., 2010) and an MCF carrier with specificity for ATP and ADP was characterized in *A. locustae* (Williams et al., 2008). Intracellular microsporidians that do not contain any homologue of mitochondrial type ADP/ATP carrier could possibly import host ATP as described in some intracellular bacteria such as *Rickettsia* and *Chlamydia*. The *E. cuniculi* genome contains four genes homologous to bacterial type nucleotide transporters. When expressed in parasites, one of them colocalizes with the mitosomal marker Hsp70. Thus, it is possible that *E. cuniculi* mitosomes import ATP from the cytosol similarly to intracellular bacteria (Tsaousis et al., 2008).

4. Mitochondria-like organelles in *Blastocystis*

Blastocystis hominis is a unicellular strict anaerobe that inhabits the gastrointestinal tract of men. It belongs to a large group of eukaryotes called Stramenopila that includes various algae, diatoms and water molds. Although *Blastocystis* is a strict anaerobe, it contains organelles with metabolic characteristics of aerobic and anaerobic mitochondria and hydrogenosomes. Blastocystis mitochondria-

like organelles most likely contain homologues for two canonical hydrogenosomal enzymes PFO and a "long type" [FeFe] hydrogenase, and also probably contain homologues for the mitochondrial PDH complex. Thus, *Blastocystis* organelles can probably decarboxylate pyruvate either by the activity of PDH complex like *Nyctotherus* or by using PFO (Stechmann et al., 2008). The only other known eukaryotes that contain PDH complex together with PFO/PNO are *Euglena* and *Chlamydomonas*. *Blastocystis* mitochondrial like organelles also contain mitochondrial genome coding for some components of the electron transport chain as well as some ribosomal proteins, ribosomal RNA and tRNA for organellar proteosynthesis. *Blastocystis* probably pass electrons from complex I via rhodoquinone with fumarate as the terminal electron acceptor, and thus be capable for anaerobic respiration as described in *Euglena*. Blastocystis organelles also most likely contain some of the proteins required for the tricarboxylic acid cycle, urea cycle, and fatty acid metabolism, as well as some components of mitochondrial protein import machinery and various mitochondrial-type carriers (Stechmann et al., 2008). However, neither Tom40 nor Sam50 were identified among Blastocystis ESTs (Stechmann et al., 2008).

5. Aims of the thesis

Giardia and *Trichomonas* genome sequences have been recently completed. However, previous bioinformatic analyses of the *Trichomonas* genome yielded only limited information about machineries in hydrogenosomal membranes that are required for exchange of metabolites and protein import into the organelles. In the case of mitosomes, only few proteins are known to reside within these organelles.

The aims:

- **To develop protocol for isolation of mitosome-rich fraction from *Giardia.***
- **To analyse proteome of the *Giardia* mitosome and partial analysis of selected proteins.**
- **To analyse proteome of hydrogenosomal membranes and characterize selected membrane proteins.**

6. Publications

- The Core Components of Organelle Biogenesis and Membrane Transport in the Hydrogenosomes of *Trichomonas vaginalis*.

- The Minimal Proteome in the Reduced Mitochondrion of the Parasitic Protist *Giardia intestinalis*.

- The Monothiol Single-domain Glutaredoxin is Conserved in the Highly Reduced Mitochondria of *Giardia intestinalis*.

7. Unpublished results

C-tail anchored proteins in the *Trichomonas* hydrogenosome

The α-helical C-tail anchored proteins represent a heterogenous group of membrane proteins with a large functional N-terminal domain exposed to the cytosol and a short membrane insertion at their C-terminus that is flanked at both ends by positively charged residues. These include components of the outer membrane of the organelle such as mitochondria, chloroplast and peroxisomes as well as some membrane proteins of the endoplasmatic reticulum. In mitochondria, C-tail anchored proteins include subunits of TOM complex Tom5, Tom6, Tom7, a component of the electron transport chain cytochrome b_5, Fis1 protein (involved in mitochondrial maintanance and morphology), and the isoform of vesicle associated membrane protein VAMP-1B (Borgese and Fasana, 2011).

Bioinformatic analysis of identified protein sequences from *Trichomonas* hydrogenosomes revealed the presence of 12 integral monotopic proteins with transmembrane domains (TMD) close to the C-terminus. These 12 protein sequences have characteristics of C-tail-anchored proteins (TA). TMDs of the C-tail anchored proteins in hydrogenosomes are 19-23 amino acid residues in length, somewhat longer than their mitochondrial counterparts. This feature may reflect the thickness and variable composition of the outer membrane present in hydrogenosome and mitochondria. The C-terminal segment that follows the transmembrane domain is 2-16 amino acid residues in length and contains 2-7 positively charged residues.

It is generally accepted that TA proteins are integrated into biomembranes in a post-translation manner. The hydrophobic C-terminal TMD emerges from the ribosome only after termination of translation, therefore the short polar C-terminus must be translocated through membranes post-translationally (Borgese et al., 2007). The general

model of C-tail protein import is based on rapid insertion of newly synthesized TA proteins into target membranes of ER and organelles such as peroxisomes, chloroplasts and mitochondria, whereas TA proteins destined for vesicles of the secretory pathway must reach their membranes by vesicular transport (Borgese et al., 2007). TA proteins with moderately hydrophobic TMDs, such as cytochrome *b5*, are capable of spontaneous integration into the ER membrane in the absence of energy and without the requirement of receptors or cytosolic factors. The free energy released during insertion of a TMD into a biomembranes is most likely required to overcome the kinetic energy barrier of lipid bilayer by the polar C-terminal domain (Brambillasca et al., 2006). This mechanism could possibly represent the most ancient insertion pathway of hydrophobic proteins into biomembranes. The final step of unassisted insertion seems to be common for all TA proteins with moderate or more hydrophobic TMDs.

TA proteins targeted to the ER with more hydrophobic TMDs require constant chaperoning to prevent misfolding and non-specific aggregation in the cytosol (Mariappan et al., 2010). Hydrophobic TMDs of newly synthesized proteins interact with the chaperon-type Bat3 complex on free ribosomes. This protein complex facilitates binding of native TA proteins and their transfer to the TA-interacting factor TRC40. The TRC40-TA complex is recruited for ER insertion by the TRC40 receptors (Get1/2). Recognition of TA proteins by TRC40 is considered to be a key determining step in targeting to the ER. Its disruption leads to mistargeting of TA proteins to mitochondria instead of the ER (Schuldiner et al., 2008; Mariappan et al., 2010). The analogous system consisting of chaperones and TA protein receptors and/or translocase might be present to address synthesized TA proteins from the cytosol to the organelle. However, the majority of TA proteins do not require any cytosolic factors or TOM

translocase for their import into the outer membrane of mitochondria (Setoguchi et al., 2006).

Targeting signals that discriminate between insertion of TA proteins into organelles and ER are poorly understood. It is believed that targeting signals are present at the C-tail segment consisting of TMD and its flanking region with a high presence of charged residues. Hydrophobicity and length of TMD together with the level of charged residues in TMD flanking regions define the subcellular targeting (Borgese et al., 2007; Byers et al., 2009). Proteins with less hydrophobic C-tail segments were targeted rather to mitochondria than to the ER. When positively charged residues were substituted in TMD flanking regions by serine, mitochondrial TA proteins were misstargeted to the ER (Byers et al., 2009; Horie et al., 2002). It is also possible, that different composition of the outer membrane of each organelle and ER can be critical for insertion of various TA proteins.

To investigate the topology of representative C-tail-anchored proteins in the hydrogenosome, TVAG_277930, TVAG_272350, TVAG_192370, and TVAG_283120 genes were epitopically expressed in *T. vaginalis* T1 strain with HA tag at their C-terminus. First, it was confirmed by immunofluorescence microscopy that all five recombinant proteins were targeted into hydrogenosomes. Subsequently, hydrogenosomes from each transfected strain were isolated and treated with trypsin as described in Fig. 1. Addition of trypsin to isolated hydrogenosomes resulted in cleavage of the N-terminal domain, whereas the C-terminal domain remained intact, because it was protected by the outer membrane of hydrogenosome. Proteolysis of the exposed domain resulted in a shift in mobility on SDS-PAGE from the size of the complete non-cleaved recombinant protein to the smaller size of the protected C-terminal domain consisting of the TMD, C-terminal segment and HA tag (Fig. 1). The C-terminal domain

was degraded only when Triton X-100 was added to solubilize the lipid bilayers of hydrogenosomes. The matrix protein of the hydrogenosome PFO was used as a control to monitor how intact membranes of the organelle were after trypsinolysis.

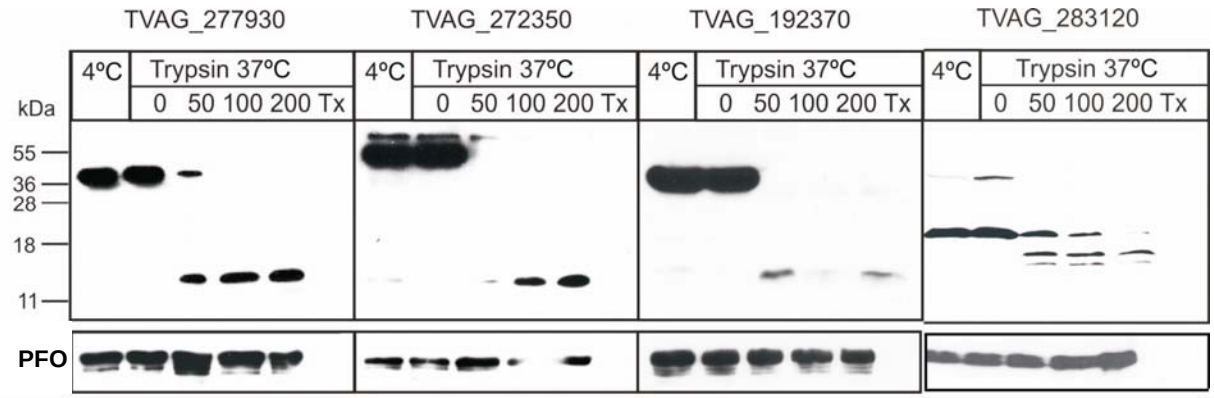

Fig. 1. Expression of C-tail anchored proteins in the outer membrane of hydrogenosome. Hydrogenosomes were isolated from a *T. vaginalis* strain expressing recombinant proteins with a C-terminal hemagglutinin tag and incubated for 30 min at 4°C and 37°C with 0, 50, 100 or 200 µg/ml trypsin or 200 µg/ml trypsin plus 0.5% Triton X-100 (Tx). Samples were analyzed by SDS-PAGE and immunoblotting using a mouse monoclonal anti-HA tag and anti-PFO antibodies. Pyruvate:ferredoxin oxidoreductase (PFO) was used as a control for matrix proteins.

8. Conclusions

8.1. The hydrogenosome

Our study of the hydrogenosomal membrane proteome revealed the presence of functional homologues of the mitochondrial membrane transporters and core components required for the organelle biogenesis. Hereby we extended support for the common origin for hydrogenosomes and mitochondria from an ancestral endosymbiont. However, the adaptation of hydrogenosomes to low-level oxygen or anaerobic habitats resulted in substantial differences between the core metabolism and membrane machineries in hydrogenosomes and those functioning in mitochondria. Hydrogenosomes contain up to 58% unknown membrane proteins. These possibly represent highly divergent homologues of known systems that are difficult to detect by any bioinformatic approaches or represent novel analogous systems that may be typical for *Trichomonas* hydrogenosomes or other Excavata.

Outer membrane proteins. In the outer membrane of the hydrogenosome, we identified: (i) monotopic C-tail anchored proteins, (ii) proteins of the mitoporin family (Tom40), (iii) the Sam50 subunit of the complex SAM, and (iv) four isoforms of the *β*-barrel proteins Hmp35/Hmp36. We showed that *Trichomonas* Tom40-3 is a part of a complex of ~230 and ~590 kDa. This observation is similar to features of mitochondria, where Tom40 porin is a part of multi-subunit complex of similar size. However, none of the hydrogenosomal membrane proteins contain N-terminal signal anchors as described in many mitochondrial proteins, including receptors of the TOM complex, Tom20 and Tom70. Because we did not identify in the hydrogenosome any significant homologues of the VDAC protein family, Tom40 could possibly function in metabolite exchange across the outer membrane instead. The outer hydrogenosomal membrane contains at least 12 C-tail anchored proteins that we identified in the proteome. Surprisingly, the

hydrogenosomal C-tail anchored proteins do not reveal any homology to those described in mitochondria and thus their possible function in biogenesis of the hydrogenosome remains unknown. Hmp35/Hmp36 are unique proteins of the *Trichomonas* hydrogenosome. Their homologues cannot be detected among any of other available genome sequences. Although their function is unknown, Hmp35 possesses at its C-terminus a cystein motif with a predicted function in metal ion transport. Thus, these unique hydrogenosomal proteins may represent iron transporters to support hydrogenosomal iron sulphur cluster formation.

The inner membrane proteins. Proteome of the inner membrane of the hydrogenosome seems to be less complex than in mitochondria. We identified candidates for the core subunits of (i) the complex TIM, (ii) PAM and (iii) multiple homologues of the MCF carriers. However, hydrogenosomal homologues of the Tim17/22/23 family are too divergent to distinguish whether these correspond to the Tim17, Tim22 or Tim23 core subunits of the TIM complex. Hydrogenosomes contain several homologues of the multispanning membrane carriers and thus one of the homologue of the Tim17/22/23 protein family is expected to function as the Tim22 translocase. It is also possible that *Trichomonas* hydrogenosomes contain only a single inner membrane translocase that imports matrix proteins and also integrates proteins into the inner membrane. This mechanism is suggested for *Trypanosoma*, which contains only one gene for a Tim17/22/23 protein (Schneider et al., 2008). Hydrogenosomes reduced or lost many of the typical mitochondrial metabolic activities. This could explain the limited set of MCF homologues. All 5 hydrogenosomal MCF carriers are predicted to transport ADP/ATP across its membranes. Therefore, it is not clear how other substrates and metabolites are exchanged such as pyruvate, malate and amino acids, eg.

Hydrogenosomes produce considerably smaller transmembrane electrochemical potential than mitochondria (Dyall et al., 2000). Thus, it is unlikely that this potential plays an important role in protein import into these organelles. This observation could explain an unusual diversity of the Tim17/22/23 protein family homologues in *Trichomonas* hydrogenosomes.

The intermembrane space proteins. We identified two putative small Tims (TvTim9-10a,b). Surprisingly, these small Tims contain only one of four cysteines that are hallmarks of mitochondrial paralogues. It is possible that the single cysteine in TvTim9-10a,b is important for their import into the intermembrane space as shown in small Tims in mitochondria. Neither Mia40 nor Erv1 have been found in the hydrogenosomal membrane fraction and also have not been detected in the *Trichomonas* genome so far. These proteins facilitate formation of intra- and inter-molecular disulfide bonds in small Tim complexes. The hypothesis described in Allen et al., 2008 suggests that during the early evolution of eukaryotes the import of cysteine rich proteins occurred in the absence of Mia40 and no Mia40 paralogues have been found in Excavates so far. However, Erv1 is usually present in all eukaryotes. The absence of Erv1 in *T. vaginalis* hydrogenosomes may reflect rather low redox environment within the hydrogenosomal intermembrane space. The absence of Erv1 was also observed in other anaerobic protists such as diplomonads and *E. histolytica*.

8.2. The Mitosome

We demonstrated that the proteome of the *Giardia* mitosome was dramatically reduced when compared with mitochondria. After extensive mass spectrometry and bioinformatic analysis we identified 139 putative mitosomal proteins. From these, 20 were localized to mitosomes. We identified: (i) a set of enzymes required for iron

sulphur cluster assembly machinery including monothiol glutaredoxin 5, (ii) a limited set of components for protein import and molecular chaperones, (iii) a novel diflavin protein with NADPH reductase activity, and (iv) a VAP homologue which likely interacts with a cell compartments and cytoskeletal structures.

FeS cluster assembly. Formation of FeS clusters is the only known function of *Giardia* mitosomes. We found all key components including IscS, IscA, IscU, and Nfu in proteome of mitosomes. Moreover, we identified and partially characterized glutaredoxin 5. We showed that the GiGrx5 contains unusually long N-terminal extension of 77 amino acids, which is absent in the mature protein. The mechanism of processing of glutaredoxin precursor protein is unclear, because the mitosomal processing peptidase is unable to process this extension. We showed that recombinant GiGrx5 is able to form a homodimeric structure, which coordinates labile [4Fe-4S] cluster. Although its function is not clear, it is likely that GiGrx5 is involved in transfer of FeS clusters to apoproteins in mitosomes.

Protein import machinery. The only components of import machinery that we identified are Tom40, Pam 18/16 and molecular chaperones. A putative Sam50 homologue is most likely common to all eukaryotes including anaerobic protists and its presence is predicted in the earliest mitochondria. The absence of Sam50 in *Giardia* may be explained either by its secondary loss or by the function of an analogous system. It is elusive how *Giardia* import and assemble Tom40 channel into the outer membrane in the absence of Sam50 homologue. It is also unclear how mitosomes transport proteins into the matrix of the organelle as we did not find any homologue of Tim17/22/23 family. We have not identified any homologue of the VDAC protein family. This observation is consistent with majority of metabolic pathways that require various transporters for exchange of metabolites and ions. We proposed that *Giardia* Tom40 ,in

addition to protein import, facilitates exchange of small metabolites and ions across the outer membrane. Despite of intensive searches for homologues of MCF carriers we have not identified any protein sequences homologues to ADP/ATP carriers. It rises the question how *Giardia* mitosomes import ATP and thus support iron sulphur proteins maturation, protein import and folding and organelle division. The major function of the *Giardia* mitosome could be the export of a component that is required for iron sulphur cluster assembly in the cytosol. Surprisingly, mitosomes do not contain any of the half-transporter Atm1 or the sulfhydryl oxidase Erv1. Thus it is a puzzle, if and how mitosomes support iron sulphur proteins maturation in other cellular compartments. Among molecular chaperones, we identified another two novel mitosomal proteins, an orthologue of yeast DNA J protein Jac1 with a predicted function in Fe-S cluster biogenesis, and DNA J protein of type III family which role in *Giardia* mitosomes is unclear.

The minimal proteome of the *Giardia* mitosome and the reduced proteome of the *Trichomonas* hydrogenosome reflect a loss of typical mitochondrial functions as a response to living strategy of anaerobic protist under oxygen limited conditions. Our findings contributed to new insights into the reductive evolution of mitochondria related organelles present in anaerobic protists and extend our previous knowledge on their biogenesis and metabolism.

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