Iron-sulfur proteins and the role of iron in the gene expression of *Trichomonas vaginalis*

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PhD. Thesis

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“Study of the rare and curious...often brings to light general phenomena which may be exaggerated in the rare but overlooked in the commonplace.”

(Henrici 1940)

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Data presented in this thesis are results of collaboration within the team of Laboratory of Biochemical and Molecular Parasitology and with our Czech and foreign colleagues. I declare that the involvement of Mgr. Lenka Horváthová (maiden name Boháčová) in this work was substantial and that she contributed significantly to obtaining these results.

Prof. RNDr. Jan Tachezy, PhD.
Thesis supervisor

CONTENTS:

ABSTRACT 5

REVIEW 6

1. Introduction 6
2. FeS clusters and FeS proteins 8
3. FeS proteins in anaerobic parasitic protozoa 9
    3.1. *Trichomonas vaginalis* 9
    3.2. *Giardia intestinalis* and *Entamoeba histolytica* 11
4. FeS proteins biogenesis 13
5. FeS proteins biogenesis in anaerobic parazitic protozoa 17
    5.1. *Trichomonas vaginalis* 17
    5.2. *Giardia intestinalis* 21
    5.3. *Entamoeba histolytica* 21
6. The impact of iron on trichomonad gene expression 22
7. References 25

AIMS OF THE THESIS 37

PUBLICATIONS 38

CONCLUSIONS 39

ABSTRACT

Iron is an essential nutrient for the parasitic protist *Trichomonas vaginalis* as a component of iron-sulfur (FeS) proteins that are indispensable for energy metabolism of the parasite. The FeS clusters are formed by FeS cluster (ISC) assembly machinery that resides, together with a number of FeS proteins, in *Trichomonas* hydrogenosomes. These double-membrane bound organelles, which are related to mitochondria, metabolize malate and pyruvate and produce ATP and molecular hydrogen. To obtain more complete information about hydrogenosomal pathways with particular focus on FeS proteins and ISC machinery, we participated on analysis of *T. vaginalis* genome sequence. To predict hydrogenosomal localization of putative gene products, we developed an application "Hunter" for the *in silico* searching for N-terminal presequences that are required for protein targeting into the hydrogenosomes. This approach substantially contributed to annotate genes coding for hydrogenosomal proteins that provided base for construction of novel map of hydrogenosomal metabolism as well as for following proteomic studies.

Investigation of hydrogenosomal proteins led to identification of three members of Hyd machinery that is required for the maturation of the specific FeS cluster of hydrogenases named H cluster. *T. vaginalis* is, thus far, the second eukaryotic organism in which the complete set of Hyd proteins have been described. We further provided evidence that the acquisition of [FeFe] hydrogenases and Hyd maturases occurred once during eukaryotic evolution.

Iron availability modulates expression of a number of proteins that are important for the establishment of *Trichomonas* infections, proliferation and virulence. We utilized transcriptomic as well as proteomic approach to compare changes between *T. vaginalis* cultivated under iron-rich and iron-restricted conditions. The transcriptome analysis was based on the combination of two powerful methods: oligonucleotide microarrays and comparative expressed sequence tag (EST) sequencing of cDNA libraries. In the proteomic analysis we focused specifically on hydrogenosomes, because the main changes associated with iron availability occur in these organelles. Both surveys revealed important changes in the physiology of the pathogen; mainly proteins of energy metabolism and ISC assembly system were affected by iron availability. One of the most striking observations from these investigations was the differential regulation of individual copies of the expanded gene groups, with only some of the copies showing iron-dependent regulation. This finding might reflect the functional diversification of individual gene copies after gene duplication.

REVIEW

1. Introduction

Among the inorganic elements that play a significant role in biology, iron is of fundamental importance. This metal is crucial for the survival and replication of almost all living organisms because of its unparalleled versatility as a biological catalyst. Its unique features originate from the extreme flexibility of the Fe^{2+}/Fe^{3+} redox potential, which is determined by the character of ligands and the adjacent protein domains; thus, iron sites can encompass almost the entire biologically significant range of redox potentials. However, these properties also make iron potentially toxic; therefore, it must be constantly chaperoned. To achieve iron homeostasis, specialised molecules for the acquisition, transport and storage of iron have evolved (Richardson et al., 2010).

Iron is a cofactor of a variety of proteins that can be classified according to the coordination chemistry of the metal: (i) haemoproteins, which function as O_2 carriers, O_2 activators or electron-transfer proteins; (ii) iron-sulfur (FeS) proteins, which are mainly involved in electron transfer; and (iii) non-haem, non-iron-sulfur, iron-containing proteins (Crichton, 2001). Important examples of the latter are proteins involved in iron transport and storage, such as ferritin and transferrin, or ribonucleotide reductase, which is a key enzyme in DNA synthesis (Uhlin and Eklund, 1994). An example that is exclusively found in anaerobes and facultative aerobes is the superfamily of flavodiiron proteins (Smutna et al., 2009).

One of the explanations for the unusually high iron requirement of anaerobic parasitic protists such as *Trichomonas vaginalis*, *Giardia intestinalis* and *Entamoeba histolytica* is the requirement of iron for the formation of FeS proteins that play a key role in the energy metabolism of these protists. Because they do not possess conventional mitochondria, these organisms were at first believed to be descendants of protoeukaryotic cells as they have existed prior to the endosymbiotic acquisition of mitochondria. However, the "primitively amitochondrial" view was later overturned by the discovery of genes encoding the mitochondrial proteins Cpn60 and pyridine nucleotide transhydrogenase (PNT) in *E. histolytica* (Clark and Roger, 1995), followed by similar discoveries in other organisms such as *T. vaginalis* and *G. intestinalis* (Horner et al., 1996; Roger et al.,1996, 1998). In addition to trichomonads, hydrogenosomes were also found in various ciliates, chytrid fungi and heterolobosean flagellates (reviewed in van der Giezen et al., 2005). Based on the presence of two bounding membranes (Henze and Martin, 2003), the results of phylogenetic studies (Embley et al., 1995), and the similarity of hydrogenosomal and mitochondrial targeting signals (Bradley et al., 1997), it was widely accepted that hydrogenosomes

are modified mitochondria. Subsequently, organelles of mitochondrial origin were discovered in all remaining "amitochondrial" lineages. The first to be discovered was the mitosome in *E. histolytica* (Tovar et al., 1999, 2003; Mai et al., 1999), followed by the discovery of mitosomes in the microsporidians *Encephalitozoon cuniculi* (Katinka et al., 2001) and *Trachipleistophora hominis* (Williams et al., 2002); finally, *G. intestinalis* was also found to contain mitosomes (Tovar et al., 2003).

Hydrogenosomes are double-membrane-bound organelles that generate ATP by substrate-level phosphorylation with the concomitant production of molecular hydrogen. In contrast, mitosomes have lost their capacity to synthesise ATP. Both hydrogenosomes and mitosomes possess homologous machineries required for the formation of FeS clusters; in fact, this is the only common and essential function that these organelles share with mitochondria (Lill et al., 1999; Hjort et al., 2010). However, one striking exception exists: the mitosomes of *E. histolytica* have lost the iron sulphur cluster assembly (ISC) machinery, and instead, *Entamoeba* has adopted a bacterial-type nitrogen fixation (NIF) machinery that is localised to the cytosol (Ali et al., 2004; van der Giezen et al., 2004)

In addition to housekeeping functions, iron affects specific host-pathogen interactions associated with the virulence of pathogenic microorganisms. Host iron is mostly intracellular, and the limited amount of extracellular iron is tightly bound to proteins such as transferrin and lactoferrin (Dunn et al., 2007). Therefore, invading pathogens have evolved effective iron-acquisition mechanisms to fulfil their needs for iron (Sutak et al., 2008). Over the past decade, numerous experiments have focused on the iron-dependent change in expression of various *Trichomonas* proteins. The cytoadherence of *T. vaginalis* to host cells, the apoptosis of host cells mediated by cysteine proteases secreted by the parasite, and resistance to complement-mediated lysis were shown to be affected by iron availability (Alderete et al., 1995; Mundodi et al., 2006; Kummer et al., 2008). The most significant changes were revealed in the expression of hydrogenosomal proteins, including the FeS and non-FeS proteins involved in both energy metabolism and FeS-cluster assembly (Vanácová et al., 2001; Horváthová et al., in press; Beltrán et al., in preparation).

The following text summarises the current knowledge regarding FeS clusters and their assembly, with particular emphasis on anaerobic parasitic protozoa and the overall impact of iron on gene expression in *T. vaginalis*.

2. FeS clusters and FeS proteins

Proteins that contain iron-sulfur (FeS) clusters as a prosthetic group are widely utilised in all living organisms for a variety of cellular processes. Inorganic FeS metal compounds were already present in hydrothermal vents in the anaerobic atmosphere of the ancient earth and were most likely the catalysts mediating the biological formation of C-C bonds, contributing to the success of early life forms (Huber and Wächtershäuser, 1997; Martin et al., 2008).

FeS clusters consist of iron cations (Fe^{2+} or Fe^{3+}) and inorganic sulfide anions (S^{2-}) and can be classified into simple and complex clusters. Simple clusters are of two types: (i) clusters of bacterial rubredoxins, in which a single iron ion is liganded to four cysteine residues and no acid-labile sulphur is present, and (ii) conventional clusters (the basic units of which are rhombic [2Fe2S] and cubane [4Fe4S]) that contain acid-labile bridging sulfur (Crichton, 2001). These basic units can be further combined in clusters that contain up to eight iron ions. Simple clusters are coordinated in proteins through iron bound by cysteinyl ligands or, less often, by the mixture of cysteinyl and histidyl ligands. In complex clusters, which can be derived from simple precursors by chemical modifications, unique ligands or ions of other elements such as molybdenum or oxygen can be present. An important example of a complex cluster is the active site of [FeFe] hydrogenases named the H cluster. The H cluster is composed of a standard [4Fe4S] cluster bridged to a [2Fe] subcluster through a non-protein dithiolate ligand. The coordination of the cubane cluster is typical; however, the 2Fe subcluster is coordinated to the protein only through the dithiolate ligand and binds two CN- and three CO ligands (Nicolet et al., 2000). Properties similar to that of the H cluster are found in the heterometalic cluster of Mo-nitrogenases (FeMo-co) (Shepard et al., 2011). Hybrid cluster protein (HCP) accommodates the mixed cluster [4Fe-2S-2O], which is a hybrid between an iron sulphur and an iron oxo cluster (Macedo et al., 2002). The particular character of ligands and the protein domains involved in the cluster coordination determines its reduction potentials, ranging approximately from -700 mV to 400 mV (Beinert, 2000). This versatility allows FeS proteins to fulfil a broad variety of functions, such as electron transport, enzyme catalysis, DNA replication or gene-expression regulation (Lill and Mühlenhoff, 2005).

In typical eukaryotes, mitochondria are the major iron-consuming subcellular organelle. The majority of cellular FeS proteins are present in mitochondria, participating in pathways that lead to ATP production by oxidative phosphorylation. These proteins include FeS proteins identified in complex I, II and III of the respiratory chain and the citric-acid-cycle component aconitase. Mitochondrial [2Fe2S] ferredoxin plays the role of electron mediator in the biosynthesis

of steroids and haem and in the formation of FeS clusters. In the cytosol, FeS proteins are necessary for amino acid metabolism (glutamate dehydrogenase, isopropylmalate isomerase, sulfite reductase), the regulation of iron homeostasis (cytosolic aconitase homologue IRP-1), ribosome biogenesis (Rli1) and cytosolic FeS cluster assembly (Cfd1p/Nbp35, Narf/Nar1, Tah18 and Dre2) (Sharma et al., 2010).

Finally, FeS proteins, such as base-excision-repair glycosylases (Boal et al., 2007), DNA-repair helicases (Rudolf et al., 2006) or DNA primases (Klinge et al., 2007), are essential in the nucleus. In photosynthetic eukaryotes, FeS proteins are present in the chloroplast, in which [4Fe4S] clusters serve as the terminal electron-accepting cofactors in photosystem I and act as electron-transfer cofactors in the cytochrome-b_6f complex and in soluble ferredoxin (Jin et al., 2008). [2Fe2S] ferredoxin has also been found in the apicoplast, which is a remnant, non-photosynthetic, secondary plastid present in apicomplexan parasitic protists such as *Plasmodium falciparum* (Vollmer et al., 2001).

3. FeS proteins in anaerobic parasitic protozoa

The common feature of the unicellular parasites *T. vaginalis, E. histolytica* and *G. intestinalis* is that they inhabit oxygen-poor environments. Their energy metabolism is fermentative, producing pyruvate via a classic Embden-Meyerhof pathway. Further processing of pyruvate, a key intermediate product that is linked to ATP production by substrate-level phosphorylation, is accomplished through reactions catalysed by two FeS proteins: pyruvate:ferredoxin oxidoreductase (PFOR) and [FeFe] hydrogenase. In *Entamoeba* and *Giardia*, these enzymes are known to function in the cytosol, whereas in *Trichomonas,* they are compartmentalised into the hydrogenosome.

3.1 *Trichomonas vaginalis*

In *T. vaginalis* hydrogenosomes, oxidative decarboxylation of pyruvate is catalysed by [4Fe4S] PFOR (Williams et al., 1987). Released electrons are then transferred via [2Fe2S] ferredoxin to hydrogenase, which is responsible for the synthesis of molecular hydrogen (Marczak et al., 1983; Payne et al., 1993). In addition to pyruvate, malate is another hydrogenosomal substrate that is oxidatively decarboxylated to pyruvate by NAD-dependent malic enzyme (ME) (Drmota et al., 1996). The transfer of electrons from NADH to ferredoxin is mediated by the mitochondrial homologue of the catalytic module of complex I. Two subunits of

the complex identified in *T. vaginalis* are the FeS proteins TvH-47 (homologue of NuoF) and TvH-22 (homologue of NuoE) (Hrdy et al., 2004). Interestingly, virtually all key enzymes of carbohydrate metabolism are encoded by multiple paralogous gene copies, only a subset of which are expressed under particular environmental conditions (Horváthová et al., in press).

Hydrogenases of *T. vaginalis* belong to the broad class of [FeFe] hydrogenases, which are modular proteins consisting of a conserved structural core that is indispensable for stability and activity (H domain), complemented by a variable number of accessory domains. The H domain, as mentioned above, accommodates the H cluster, an active site of hydrogenase that is responsible for the production of molecular hydrogen. The accessory domains of [FeFe] hydrogenases are predominantly [4Fe4S] and [2Fe2S] ferredoxin-like clusters that function as electron-transfer centres, connecting the active site to the protein surface (Nicolet et al., 2000). *T. vaginalis* possesses an unusually diverse group of hydrogenases that can be divided into four distinct families based on the presence of four different sets of accessory domains (Carlton et al., 2007) (Figure 1). The first three families differ in the number and type of N-terminal FeS clusters, indicating differences in electron transport between the electron donor and H-cluster. The type-IV hydrogenase is a fusion protein composed of a complete N-terminal hydrogenase domain that corresponds to type-II hydrogenase and a C-terminal diflavin reductase that contains motifs for the binding of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) prosthetic groups. Interestingly, this C-terminal diflavin domain is homologous to NADPH-cytochrome P450 (CPR) and to the CPR domain of the pyruvate:NADP oxidoreductase (PNO) of *Euglena gracilis* and *Cryptosporidium parvum* (Rotte et al., 2001).

Hydrogenases are present in *T. vaginalis* hydrogenosomes and accompanied by three specific maturases (Hyd proteins), which are required for the maturation of the H cluster and are consequently indispensable for the hydrogenase activity (Putz et al., 2006). Numerous studies focusing on Hyd maturases, including the crystal structures of HydF and HydE, are available, albeit mainly for bacteria (Rubach et al., 2005; Nicolet et al., 2008; Cendron et al., 2011). HydE and HydG are S-adenosylmethionine (SAM)-dependent enzymes, possessing the typical SAM domain that accommodates a [4Fe4S] cluster; one additional [2Fe2S] cluster is present in HydE (Nicolet et al., 2008). Although the crystal structure of HydF was published, the precise nature of its FeS cluster(s) was not clearly established, which most likely reflects the dynamic nature of HydF in the maturation process. However, it is highly probable that one [4Fe4S] cluster and one [2Fe2S] cluster are bound to HydF before interaction with the other maturases HydE and HydG (Cendron et al., 2011).

Another interesting protein of the trichomonad hydrogenosome is the hybrid cluster protein (HCP; originally called "prismane") that is common in prokaryotes and was initially isolated from the strictly anaerobic sulfate-reducing bacterium *Desulfovibrio vulgaris* (Pierik et al., 1992). HCP coordinates two FeS clusters, one of which may be either a [2Fe2S] or a [4Fe4S] in different types of the enzyme, whereas the other is a unique [4Fe-2S-2O] cluster (van den Berg et al., 2000; Macedo et al., 2002). Although widely examined at the biophysical level, little is known about the function of HCP *in vivo*. In prokaryotes, it was reported to be associated with nitrate and nitrite metabolism (Cabello et al., 2004), to function as a hydroxylamine reductase (Wolfe et al., 2002), and to be involved in oxidative stress protection (Briolat and Reysset, 2002; Almeida et al., 2006). HCP-coding genes are also present in several unrelated lineages of eukaryotic protists: aerotolerant anaerobes *G. intestinalis*, *Spironucleus barkhanus*, *E. hystolytica* and *Mastigamoeba balamuthi*; heterolobosean *Naegleria gruberi*; chromalveolate *Thalassiosira pseudonana*; and green alga *Chlamydomonas reinhardtii* (Andersson et al., 2006). It is noteworthy that all of these organisms possess [FeFe] hydrogenases. It was proposed that HCP might oxidise reduced ferredoxin and therefore compete with hydrogenase for electrons from reduced ferredoxin (Mus et al., 2007). However, the exact physiological role of HCP in eukaryotes remains unknown, and its further investigation might yield intriguing results.

A homologue of Rli1, which is an essential protein that coordinates two [4Fe4S] clusters, is present in the cytosol of *T. vaginalis*. It was recently demonstrated that Rli1 is indispensable for rRNA processing and ribosome formation, therefore representing a functional link between the biogenesis of FeS proteins and the biogenesis of ribosomes (Yarunin et al., 2005; Lill and Mühlenhoff, 2008).

3.2 *Giardia intestinalis* and *Entamoeba histolytica*

In the mitosome-harbouring organisms *G. intestinalis* and *E. histolytica*, PFOR and [FeFe] hydrogenase occur in the cytosol, and the acetyl-CoA generated by these enzymes is converted to acetate via an acetyl-CoA synthetase (ADP-forming) that directly phosphorylates ADP to ATP (Townson et al., 1996; Emelyanov and Goldberg, 2011; Sánchez et al., 2000). Cytosolic ferredoxins of these two parasites contain two [4Fe4S] clusters and belong to a different ferredoxin type than do the [2Fe2S] ferredoxins from mitochondria or trichomonad hydrogenosomes. However, the mitochondrial-type [2Fe2S] ferredoxin was localised within the mitosomes of *G. intestinalis* (Dolezal et al., 2005).

E. histolytica and *G. intestinalis* are also known to produce dihydrogen; nevertheless, their

cytosolic hydrogenase activity is at least one order of magnitude lower than in *T. vaginalis* (Lloyd et al., 2002; Emelyanov and Goldberg, 2011). In the genome of *G. intestinalis*, a single gene coding for the type-I hydrogenase was identified; in the genome of *E. histolytica*, three putative hydrogenase genes were found (Meyer, 2007). Two hydrogenases of the latter belong to type I, whereas the N-terminus of the third one contains a unique domain with eight Cys residues forming a yet unknown structure (Nixon et al., 2003) (Figure 1).

Genes coding for homologues of Rli1 are also present in the genomes of *Giardia* and *Entamoeba*.

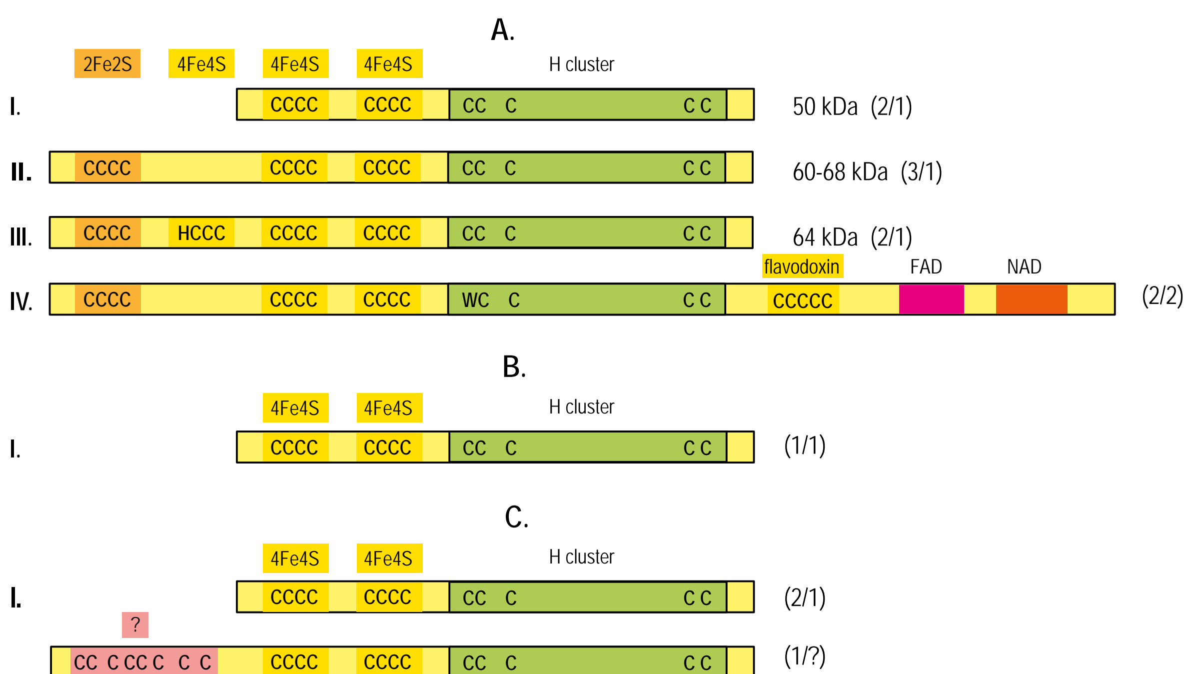

Figure 1. [FeFe] hydrogenases of anaerobic parasitic protozoa. Primary sequence features of the [FeFe] hydrogenases found in *T. vaginalis* (A), *G. intestinalis* (B), and *E. histolytica* (C). Conserved cysteine residues (C) implicated in the coordination of FeS clusters are emphasized. Numbers in brackets indicate the number of genes in the genome/expressed genes.

4. FeS proteins biogenesis

Assembly of FeS clusters and their insertion into apoproteins depends on an incredibly complex set of biogenesis proteins. In bacteria, three different systems for FeS-protein maturation are recognised: the ISC assembly machinery, the SUF (sulfur mobilisation) system, and the NIF system. The former two ensure the synthesis of housekeeping FeS proteins under normal and oxidative-stress conditions, respectively; the latter serves in the specific maturation of nitrogenases in azototrophic bacteria (Johnson et al., 2005; Fontecave and Ollagnier-de-Choudens, 2008). During evolution, ISC and SUF machineries were transferred by endosymbiosis to eukaryotes; mitochondria have retained proteins homologous to the components of the bacterial ISC assembly machinery, whereas plastids harbour the SUF pathway (Balk and Pilon, 2011). Mitochondria perform a prime role in FeS proteins biogenesis. First, the ISC machinery is responsible for the maturation of FeS proteins inside the organelle, but it also supports the generation of FeS proteins in the cytosol and nuclei. The ISC system provides a substrate, possibly a form of sulphur (Netz et al., 2007), that is exported from mitochondria to serve in the cytosolic FeS-cluster-assembly (CIA) machinery (Lill et al., 2006; Roy et al., 2003). The ISC export machinery includes the ABC transporter Atm1, sulphydryl oxidase Erv1 and glutathione.

All the above-mentioned biogenesis systems share a common paradigm: the assembly of an FeS cluster, which occurs on a scaffold protein and is followed by the transfer and integration of the cluster into the polypeptide chain of the target apoprotein. Each of these steps involves the participation of several proteins and cofactors that perform partial biosynthetic reactions. The following chapter summarises the current knowledge regarding the components of these processes, though not all of the partial reactions have been identified. Figure 2 illustrates the general steps of the biosynthesis of an FeS protein, and Table 1 provides a list of the corresponding proteins and cofactors in the various machineries.

Basic requirements for the formation of an FeS cluster are inorganic iron and sulphide, yet cells have to prevent an unregulated release of these potentially toxic components. A cysteine desulfurase releases the required sulfur from cysteine that is converted to alanine. The sulfur (S^0) is transiently bound to the cysteine desulfurase via a persulfide group at a conserved cysteine residue and is subsequently transferred to a scaffold protein either directly or via a helper protein (SufE). In bacteria, cysteine desulfurase is named NifS, IscS or SufS, depending on the particular machinery (reviewed in Hidese et al., 2011). The mitochondrial homologue of IscS (Nfs1) requires the additional protein Isd11 that promotes the formation of the persulfide (Pandey et al., 2011).

Similarly, to guarantee accurate iron delivery to scaffold proteins, specific iron donors are

needed. This function in the ISC system is most likely performed by the iron-binding protein frataxin. Frataxin is highly conserved in prokaryotes and eukaryotes, and loss of its function in humans leads to the neurodegenerative disorder Friedreich's ataxia (Busi and Gomez-Casati, 2012). Structural studies have demonstrated that frataxin is a component of a multiprotein complex together with the cysteine desulfurase and the scaffold protein (IscU or Isu1 and Isu2 in yeast) (Prischi et al., 2010).

The assembly of the FeS cluster further depends on electrons that are needed for the reduction of sulfur released from cysteine (S^0) to sulphide present in the FeS cluster (S^{2-}). Ferredoxin fulfils this function in the ISC machinery, whereas in the NIF system, the ferredoxin-like domain of the scaffold NifU is probably sufficient for the electron transfer (Mühlenhoff et al., 2003). The complex of the reductase Tah18 and Dre2 is part of an electron transfer chain that functions in the CIA machinery (Netz et al., 2010).

The so-called scaffold proteins serve as a platform for the *de novo* synthesis of an FeS cluster, a process that is still not fully understood. The new cluster is transiently bound to the conserved cysteine residues of the scaffold; hence, it can be transferred to the target apoprotein. There are a number of scaffolds present in different machineries and organisms (Table 1). The principal molecular scaffolds are the IscU (Isu) proteins, which are among the most conserved proteins in evolution (Johnson et al., 2005). Members of the IscA family function as alternative scaffolds, yet may also act as iron donors (Krebs et al., 2001; Wang et al., 2010). In the eukaryotic CIA machinery, the function of scaffolds is performed by P-loop NTPases Cfd1 and Nbp35 (Hausmann et al., 2005).

The final step in the biogenesis is the transfer of the transient FeS cluster from the scaffold to the protein, which is converted from the apo form to the holo form. Specific chaperone systems that are involved in this process have three roles: to induce dissociation of the preassembled FeS cluster from the scaffold, to guarantee its accurate and specific transfer to the target apoprotein, and to assist the correct integration of the FeS cluster into the proper acceptor site. However, thus far, these three partial reactions have not been separated experimentally. In eukaryotes, the transfer is facilitated by the mitochondrial chaperone Hsp70, which cooperates with two accessory proteins. The first protein is the co-chaperone of the DnaJ type (Jac1) that enables the interaction of HSP70 with IscU (Dutkiewicz et al., 2004), and the second protein is Mge1, which facilitates ADP/ATP exchange on HSP70 (Lill and Mühlenhoff, 2006). In the cytosolic CIA machinery, Nar1 and Cia1 are involved in cluster transfer (Balk et al., 2004, 2005). An auxiliary, non-essential role

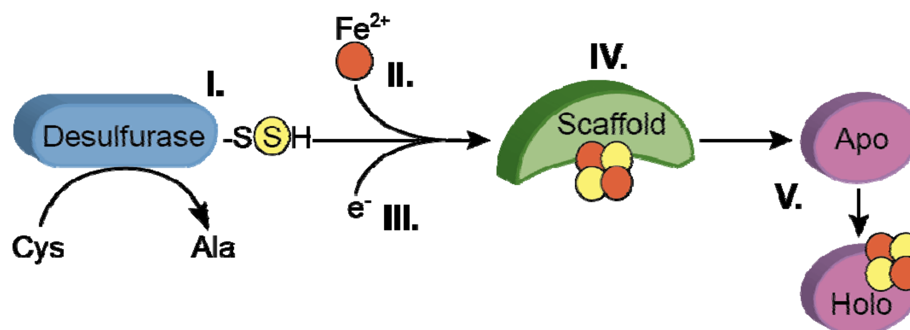

Figure 2. The generalised main steps of FeS protein biosynthesis. The list of corresponding proteins that function in the various biosynthesis machineries is provided in Table 1.

		Bacterial			Eukaryotic	
	Function	**NIF**	**SUF**	**ISC**	**Mitochondrial ISC**	**Cytosolic CIA**
I.	Cysteine desulfurase	NifS	SufS	IscS	IscS (Nfs1)	-
	Accessory protein	-	SufE	-	Isd11	
II.	Iron donor	-	-	CyaY	Frataxin (Yfh1)	-
III.	Electron transfer	NifU middle domain	-	Ferredoxin	Ferredoxin (Yah1) Ferredoxin reductase (Arh1)	Dre2, Tah18
IV.	U-type scaffold	NifU N-terminal domain	SufU	IscU	IscU (Isu1, Isu2)	-
	A-type scaffold	IscA	SufA	IscA, ErpA	IscA, (Isa1p, Isa2p) Iba57 *for aconitase*	-
	NFU-type scaffold	NifU C-terminal domain	-	Nfu	Nfu	-
	Scaffold	-	$SufBC_2D$	-	-	-
	P-loop NTPase scaffold	-	-	-	Ind1 *for complex I*	Cfd1, Nbp35
V.	Transfer proteins	-	$SufBC_2D$, SufA	HscA, HscB	Hsp70 (Ssq1), Jac1, Mge1, Grx5	Nar1, Cia1

Table 1. Components of various machineries for FeS proteins biosynthesis. Yeast homologues are shown in brackets. The numbering in the first column corresponds to the steps of biosynthesis, as indicated in Figure 2. Specialiazed functions of Iba57 and Ind1 are italicized.

in the transfer was reported for the mitochondrial protein glutaredoxin Grx5 (Mühlenhoff et al., 2003).

In addition to the ISC components required for the synthesis of general FeS proteins, components with more specific roles in FeS assembly are present in mitochondria. Two homologues of IscA (Isa1 and Isa2) that interact with Iba57 are specifically required for the maturation of aconitase-type FeS proteins and for the activation of radical SAM enzymes (Mühlenhoff et al., 2007; Gelling et al., 2008). Another case of a specialised FeS assembly component is mitochondrial P-loop NTPase Ind1, which is crucial for the assembly of respiratory complex I (Bych et al., 2008; Sheftel et al., 2009).

The active site of [FeFe] hydrogenases, termed the H cluster, requires for its maturation a specific set of three proteins-HydE, HydF and HydG-which are referred to as the Hyd machinery. In recent years, great progress in elucidating the mechanisms of H-cluster biosynthesis has been accomplished. It is now generally accepted that the H cluster is built in stages: the [4Fe4S] cluster is first synthesised and inserted by the housekeeping machinery (ISC or SUF), and consecutive synthesis and insertion of the [2Fe] subcluster is accomplished by the Hyd machinery (Mulder et al., 2010). The reactions of H-cluster biosynthesis have to be tightly controlled because toxic CO and CN^-, as well as hydrolytically sensitive dithiolate, are produced. First, HydE delivers the dithiolate to the central component of the machinery, the scaffold protein HydF (McGlynn et al., 2008). The [2Fe2S] cluster of HydF is either used as a substrate or replaced by the action of HydE (Nicolet and Fontecilla-Camps, 2012). HydG subsequently donates CN^- and CO ligands that were produced by tyrosine cleavage (Driesener et al., 2010). These two processes probably require GTP hydrolysis that is facilitated by the N-terminal GTPase domain of HydF. The nascent [2Fe] subcluster is then transferred from HydF to the hydrogenase. After the transfer, HydF likely contains only a [4Fe4S] cluster, and an external yet unidentified protein may be required to re-assemble the [2Fe2S] cluster in HydF to start a new synthetic cycle (Figure 3).

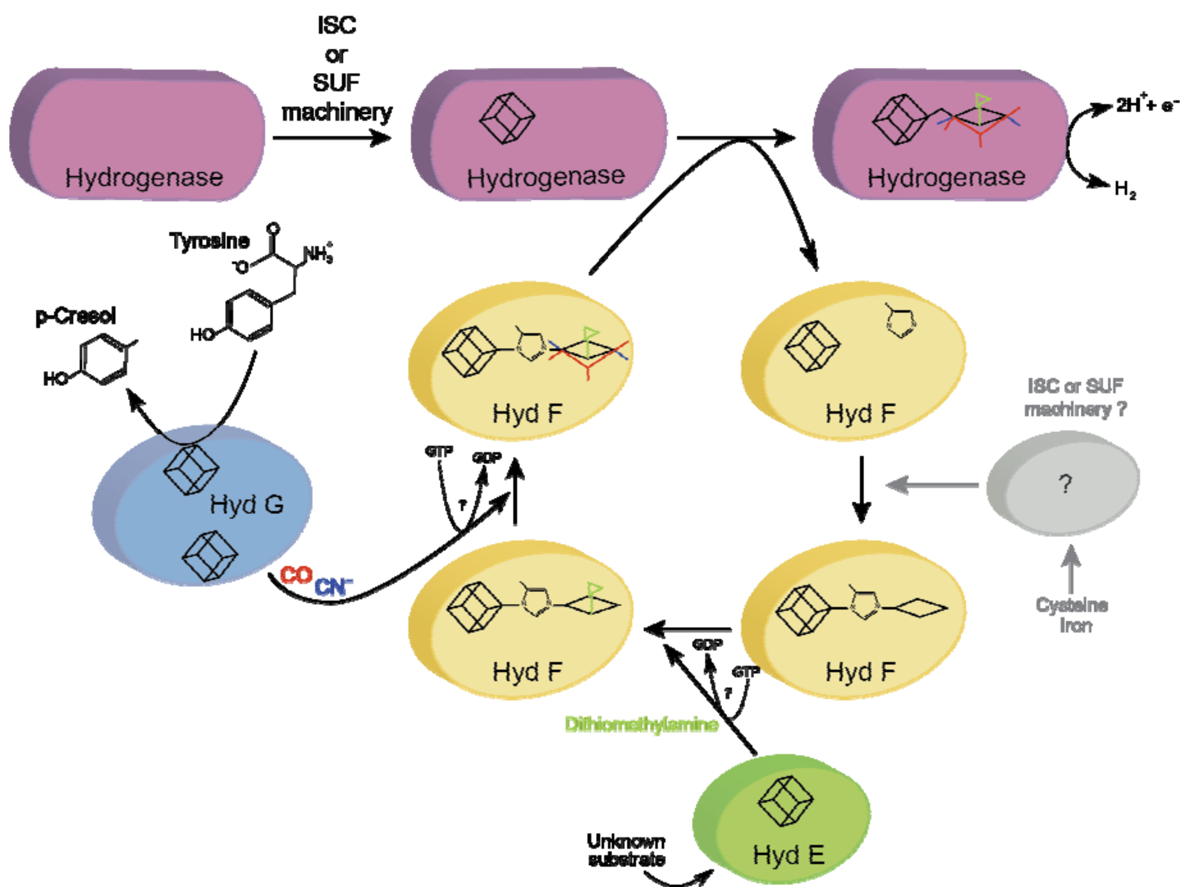

Figure 3. The assembly and insertion of the active site of [FeFe] hydrogenases. Presented according to Nicolet and Fontecilla-Camps (2012).

5. FeS proteins biogenesis in anaerobic parazitic protozoa

5.1. *Trichomonas vaginalis*

The identification of a cysteine desulfurase homologous to IscS in the genome of *T. vaginalis* indicated that the mitochondrial ISC machinery of higher eukaryotes might also be conserved in trichomonads (Tachezy et al., 2001). Indeed, a latter study demonstrated the hydrogenosomal localisation of IscS and the ability of isolated organelles to catalyse the assembly and insertion of an FeS cluster into apoferredoxin (Sutak et al., 2004) Subsequently, Dolezal et al. (2007) confirmed the hydrogenosomal localisation of a *T. vaginalis* frataxin homologue that is the probable iron donor. This study also revealed that hydrogenosomal frataxin is able to partially restore defects in haem and FeS-cluster synthesis in Δ*yfh1* mutants of *S. cerevisiae*. Another investigation demonstrated the efficient complementation of frataxin-deficient *Trypanosoma brucei* (Long et al., 2008). Thus far, *T. vaginalis* represents the only known organism with two genes encoding frataxin homologues (Carlton et al., 2007). Sequencing of the complete *T. vaginalis* genome revealed a number of other components of the mitochondrial-type ISC

machinery (Carlton et al., 2007), and their localisation within hydrogenosomes has been confirmed by proteomic analyses (Rada et al., 2011; Schneider et al., 2011). Interestingly, our studies demonstrated that virtually all members of the machinery are upregulated in iron-restricted conditions, suggesting their functional relationship and most likely a common regulatory mechanism for their gene expression (Horváthová et al., in press; Beltrán et al., in preparation) (Table 2).

Importantly, the gene encoding IscU stands out as the only single-copy gene, which renders it an excellent candidate for a gene-silencing experiment that would shed more light on the functionality of the trichomonads hydrogenosomal ISC system. However, multiple genes encoding alternative scaffolds such as Nfu and IscA are present in the hydrogenosome (Table 2).
Unlike mitochondria, in which two types of IscA homologues (Isa1 and Isa2) are found, only IscA-2 encoding genes were identified in hydrogenosomes. As mentioned above, Isa1, Isa2 and Iba57 are confined in mitochondria for the maturation of aconitase and the activation of SAM enzymes, namely biotin synthase and lipoic-acid synthase (Gelling et al., 2008). In hydrogenosomes, no aconitase is present; nevertheless, functional HydE, a homologue of biotin synthase, is required for hydrogenase maturation. We can therefore assume that IscA-2 may be involved in the activation of HydE.

The components that ensure transfer of the transient FeS cluster to the apoprotein in *Trichomonas* hydrogenosomes are as follows: chaperon HSP70, its co-chaperone J-protein (Jac1), and nucleotide exchange factor GrpE (Mge1). In Table 2, four homologues of HSP70 that were detected in hydrogenosomal proteome are listed; however, we cannot be certain which of them are actually included in the FeS cluster assembly.

Seven homologues of [2Fe2S] ferredoxin are present in *T. vaginalis* hydrogenosomes. Beltrán et al. (in preparation) found that ferredoxins 3, 6 and 7, unlike the rest of the homologues, are upregulated under iron-restricted conditions (albeit their upregulation did not reach the cut off limit); the same trend was also observed by Horváthová et al. (in press). Therefore, the participation of these three ferredoxins in FeS-cluster biosynthesis seems to be presumable, whereas the other ferredoxins are most likely required for electron transport associated with the energy metabolism of the hydrogenosome. This hypothesis, however, needs to be verified experimentally.

Mitochondrial P-loop NTPase Ind1 is required as a scaffold or transfer protein for the assembly of respiratory complex I (Bych et al., 2008; Sheftel et al., 2009). Four homologues of Ind1 were found in *Trichomonas* hydrogenosomes (Table 2), which correspond with the presence

of a reduced form of complex I within the organelle (Hrdy et al., 2004). Together with the absence of this protein in mitosome-harbouring organisms that are devoid of complex I, this finding highlights the specific function of Ind1 in the biogenesis of the respiratory complex (Jedelský et al., 2011).

No candidates for proteins of the ISC export machinery were identified in the *T. vaginalis* genome. However, genes coding for members of the cytosolic FeS-cluster assembly system (CIA), P-loop NTPases Cfd1 and Nbp35, hydrogenase homologue Nar1, and WD40 repeat domain protein (Cia1) are present in *T. vaginalis* (Tachezy and Smíd, 2008). Furthermore, a homologue of Tah18, which was demonstrated to deliver electrons for the early steps of CIA, was identified in *T. vaginalis*; however, its partner in the electron transfer chain (Dre2) is unknown. It remains to be investigated whether a functional cooperation between hydrogenosomal ISC and cytosolic CIA machineries occurs and, if so, which membrane components are involved.

The most specific FeS-cluster biosynthesis that takes place in *Trichomonas* hydrogenosomes is involved in the maturation of [FeFe] hydrogenase. As described above, this process is mediated by the specialised Hyd machinery. To date, the Hyd maturases have been characterised in only two eukaryotic species: in the hydrogenosomes of *T. vaginalis* and in the chloroplasts of the green alga *Chlamydomonas reinhardtii* (Posewitz et al., 2004; Putz et al., 2006). Recently, the Hyd machinery was found in the genomes of other hydrogenase-possessing eukaryotic protists such as *Naegleria gruberi*, *Acanthamoeba castellanii, Mastigamoeba balamuthi* and *Trimastix pyriformis* (Hug et al., 2010). Thus far, the complete set of *hyd* genes has been identified only in *Naegleria* (Fritz-Laylin et al., 2010). Genes encoding some components of the machinery were found in the other protists; however, because their genome sequences are not complete, the absence of the remaining *hyd* genes cannot be confidently assessed (Hug et al., 2010).

Function	Protein name	Gene ID	Transcriptome	Proteome
Cysteine desulfurase	IscS	TVAG_239660	+ ●	+
Accessory protein	Isd11	TVAG_242760	+	+ ●
		TVAG_215080	-	+
Scaffolds	IscU	TVAG_432650	-	+
	Nfu	TVAG_146780	+ ●	+ ●
		TVAG_044500	+	+ ●
		TVAG_008840	+ ●	+ ●
		TVAG_451860	+ ●	+ ●
	IscA-2	TVAG_055320	+	+ ●
		TVAG_456770	+	+ ●
		TVAG_361540	+	+ ●
P-loop ATPase	Ind1	TVAG_027170	+	+
		TVAG_076230	+	+
		TVAG_217870	+	+
		TVAG_277380	+	+ ●
Frataxin		TVAG_114560	+	+
		TVAG_182150	+ ●	-
Ferredoxin	Fdx6	TVAG_251200	+	+
	Fdx3	TVAG_213140	+	+
	Fdx7	TVAG_078730	+	+
Chaperone	Hsp70	TVAG_237140	+	+
		TVAG_340390	+	+
		TVAG_433130	+	+ ●
		TVAG_253630	+	+ ●
Accessory proteins	Jac1	TVAG_422630	-	-
		TVAG_032090	-	+
		TVAG_305730	+	+
		TVAG_019190	-	+
	Mge (GrpE)	TVAG_182340	+	+
		TVAG_297650	+	+
Hyd system	HydE	TVAG_027060	+	-
		TVAG_329200	-	+ ●
	HydF	TVAG_205390	+	+ ●
	HydG	TVAG_469020	+ ●	+ ●
		TVAG_257780	+	+

Table 2. Components of *T. vaginalis* hydrogenosomal ISC machinery.
\+ detected
\- not detected
● upregulated in iron restricted conditions
Presented according to Dolezal et al. (2007), Rada et al. (2011), Schneider et al. (2011), Horváthová et al. (in press), Beltrán et al. (in preparation)

5.2. *Giardia intestinalis*

The mitosomes of *G. intestinalis* are the sites of FeS-protein maturation, which is probably their only conserved mitochondrial function (Tovar et al., 2003; Tachezy and Smíd, 2008). Similar to mitochondria and hydrogenosomes, the mitosomal machinery is of the ISC type. The recent proteomic study of giardial mitosomes identified key components of the machinery: the cysteine desulfurase IscS; the scaffolds IscU, Nfu and IscA; and proteins acting late in FeS-protein biosynthesis, including the monothiol glutaredoxin 5, chaperone HSP70, its co-chaperone HscB (Jac1) and nucleotide exchange factor GrpE (Jedelský et al., 2011). However, compared with the hydrogenosomal or mitochondrial ISC assembly machinery, some of the components are unknown. A striking deviation is the absence of frataxin, given that it was detected in mitosomes of *E. cuniculi* (Goldberg et al., 2008); genes encoding frataxin are present in the genomes of *C. parvum* and a close relative of *Giardia*, *Spironucleus vortens*. It is possible that the role of the iron donor is played by IscA-2, as was described by Ding et al. (2004). The function of IscA-2 in the activation of radical SAM enzymes is unlikely because no Hyd proteins are present in *Giardia*.

Two important gaps in understanding the function of the giardial ISC machinery are the sources of the reducing equivalents and ATP. Only one component of an electron transport chain ([2Fe2S] ferredoxin) was identified within the mitosome, whereas no ferredoxin reductase has been identified thus far. Similarly, no candidate that could facilitate the import of ATP into the organelle has been revealed (Jedelský et al., 2011).

The fact that no other FeS proteins have been found in *Giardia* might suggest that the main role of the organelle is to export preassembled clusters (or other compounds) for the maturation of extramitosomal FeS proteins.

5.3. *Entamoeba histolytica*

Unlike *G. intestinalis*, the mitosomes of *E. histolytica* lack the ISC machinery and instead possess a non-redundant NIF system. Its components NifS and NifU were determined to be acquired by lateral gene transfer from ε-proteobacteria, which was the first demonstration of the NIF system in eukaryotes (Ali et al., 2004; van der Giezen et al., 2004). Moreover, the ability of the system to catalyse the general FeS-cluster assembly was shown (Ali et al., 2004). As no putative mitochondria-like targeting signals were revealed, it was suggested that NifS and NifU operate in the cytosol (van der Giezen et al., 2004). Therefore, the biological function(s) of amoebal mitosomes remained uncertain for many years. Maralikova et al. (2010) suggested the dual cellular distribution of NifS and NifU in both the cytosol and mitosomes, as evidenced by

immunoelectron microscopy. However, Mi-ichi et al. (2009) did not detect Nif proteins in the mitosomal proteome, and cytosolic distribution was also suggested by Dolezal et al. (2010). Interestingly, a free-living *Mastigamoeba balamuthi* also possesses the NIF system, suggesting that the replacement of the mitochondrial type-ISC assembly machinery by the bacterial type-NIF system occurred in a common ancestor of *Entamoeba* and *Mastigamoeba* (Gill et al., 2007)

Similarly to *Trichomonas* and *Giardia*, components of the cytosolic CIA machinery were identified in the genome, whereas no candidates for the export of FeS intermediates from the mitosome to the cytosol have been found (Loftus et al., 2005; Clark et al., 2007).

It is noteworthy that two other physiological functions, rubrerythrin-mediated oxygen detoxification and the sulfate-activation pathway, have been retained within entamoebal mitosomes, indicating the unusual divergence and mosaic nature of the organelle (Mi-ichi et al., 2009; Maralikova et al., 2010).

Components of the Hyd machinery are believed to be present in all organisms that possess the active [FeFe] hydrogenase, with the exception of *G. intestinalis* and *E. histolytica* (Hug et al., 2010). As mentioned above, *Giardia* and *Entamoeba* are capable of producing low levels of molecular hydrogen; however, they do not possess *hyd* genes. Given the structural complexity of the hydrogenase active site, it seems unlikely that it could be fully assembled without maturases. It is possible that the active site is assembled only partially, as was reported for the *C. reinhardtii* hydrogenase expressed in the absence of the corresponding maturases, into which the [2Fe] subcluster was not inserted (Mulder et al., 2010). This hypothesis is consistent with the low hydrogen production of *Giardia* and *Entamoeba*, as the site with only a [4Fe4S] component in the H cluster might still catalyse H_2 evolution, albeit at significantly lower levels than the properly assembled active site (Nicolet and Fontecilla-Camps, 2012). The other possibility is that the Hyd-encoding genes may be present, but they are so divergent that they are undetectable. Nonetheless, it would be very interesting to reveal the composition of the active site of the hydrogenases that are found in organisms lacking the corresponding maturases, such as *G. intestinalis* and *E. histolytica*.

6. The impact of iron on trichomonad gene expression

It is well documented that trichomonads require high levels of iron. Moreover, *T. vaginalis* has to cope with constantly changing iron concentrations during the menstrual cycle. It has been shown that addition of 50 – 100 μM Fe is required for optimal growth of *Tritrichomonas foetus in*

vitro (Tachezy et al., 1996), whereas bacteria require ~0.2 μM Fe. Gorrell (1985) demonstrated that the EPR signal for ferredoxin and the activity of PFOR and hydrogenase significantly increased if *T. vaginalis* was grown in a medium containing an iron concentration elevated to 200 μM. Given the importance of FeS proteins in the trichomonads' energy metabolism, it is not surprising that the availability of iron in the cell environment affects the metabolic activity of the hydrogenosome. However, Vanácová et al. (2001) demonstrated that iron is required for expression of both FeS and non-FeS proteins involved in *T. foetus* hydrogenosomal pyruvate metabolism. In trichomonads that grow under iron-limited conditions, hydrogenosomal metabolism is significantly decreased, and pyruvate is metabolised in the cytosol; the major end products are ethanol in *T. foetus* and lactate in *T. vaginalis*. Therefore, the ability to switch between hydrogenosomal and cytosolic energy metabolism is most likely important for the ability to quickly adapt to the changing availability of iron. Notably, similar changes in carbohydrate metabolism were observed in *T. vaginalis* that had impaired hydrogenosomal metabolism because of the induction of metronidazole resistance (Kulda et al., 1993). In contrast, components of the hydrogenosomal ISC assembly machinery exhibit substantial upregulation in iron-restricted conditions (Sutak et al., 2004; Dolezal et al., 2007; Horváthová et al., in press) (Table 2).

To establish and maintain the infection, *T. vaginalis* needs to adhere to mucosal epithelial cells (de Miguel et al., 2010). This process was reported to be upregulated by iron, which increases synthesis of various surface adhesins (Mundodi et al., 2006). Several hydrogenosomal enzymes have been claimed to have dual cellular localisation (so-called moonlighting proteins), with an alternative localisation to the cell surface where they can exhibit an adhesive function. This phenomenon was reported for enzymes that are upregulated in iron-rich conditions, such as ME, the α and β subunits of succinyl CoA synthetase and PFOR, which were designated as adhesins AP65, AP33, AP51 and AP120, respectively (Garcia et al., 2003; Alderete et al., 2004; Moreno-Brito et al., 2005; Meza-Cervantez et al., 2011). However, these data are controversial because the proposed surface proteins lack the detectable sequence features that are known to target and anchor proteins to the cell surface (Hirt et al., 2007).

Important roles in the host-parasite interactions are played by surface proteases (Klemba and Goldberg, 2002). Several studies have reported the upregulation of *Trichomonas* proteases under iron-restricted conditions, which may lead to increased cytotoxicity (Hernandez-Gutierrez et al., 2003; León-Sicairos et al., 2004; Alvarez-Sánchez et al., 2007; Kummer et al., 2008). However, in some cases, the opposite regulation was revealed (De Jesus et al., 2007), or contradictory data were published. For example, Solano-González et al. (2007) reported that that

iron had an insignificant effect on CP4 gene transcription but positively regulated the translation of CP4. In contrast, Kummer et al. (2008) isolated an extracellular protein fraction from *T. vaginalis* that they called CP30 and that contained CP2, CP3, CP4 and CPT, and these researchers demonstrated that trichomonads grown under -Fe conditions exhibited increased CP30 fraction protease activity. Additionally, iron positively regulates the expression of ecto-ATPases and ecto-phosphatases (De Jesus et al., 2006), and it increases trichomonad resistance to complement-mediated lysis (Alderete et al., 1995).

Two possible mechanisms of iron-dependent regulation have been suggested: (i) regulation at the transcriptional level, mediated by Myb-like transcription factors (Ong et al., 2006), and (ii) post-transcriptional regulation, mediated by an IRE/IRP-like system (Solano-González et al., 2007).

Myb-like transcription factors are exceptionally abundant in the *T. vaginalis* genome (Carlton et al., 2007). Three of these transcription factors (Myb1, Myb2 and Myb3) were demonstrated to regulate the temporal and iron-inducible transcription of malic enzyme by binding to two discrete Myb-protein recognition elements (MRE) in the 5'untranslated regions (5'UTR) of the malic-enzyme gene (Ong et al., 2006, 2007; Hsu et al., 2009). Recently, a novel core-promoter element (M3) that resembles MRE and a novel Myb-like protein (M3BP) that specifically recognises the M3 element were characterised, supporting the functionality of the Myb-mediated transcriptional regulation in *T. vaginalis* (Smith et al., 2011).

Post-transcriptional regulation, which is most likely mediated by an IRE/IRP-like system and is common in other eukaryotes (Wang and Pantopoulos, 2011), was reported for two cysteine proteases, TVCP4 and TVCP12 (Solano-González et al., 2007; Torres-Romero and Arroyo, 2009). However, a putative IRP-like protein in *T. vaginalis* that binds the IRE-like structures remains to be identified.

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AIMS OF THE THESIS

- To develop bioinformatic tool for automated search for putative N-terminal hydrogenosomal presequences in order to identify FeS proteins and components of FeS cluster assembly machinery that are targeted to the hydrogenosme of *T. vaginalis*.

- To characterize putative [FeFe] hydrogenase maturases from hydrogenosomes of *T. vaginalis*.

- To investigate iron-regulated genes of *T. vaginalis* using transcriptomic approach.

- To investigate iron-dependent changes in *T. vaginalis* hydrogenosomal proteome.

PUBLICATIONS

Putz, S., Dolezal, P., Gelius-Dietrich, G., **Boháčová, L.,** Tachezy, J., and Henze, K. (2006). **Fe-hydrogenase maturases in the hydrogenosomes of *Trichomonas vaginalis*.** Eukaryot.Cell *5*, 579–586.

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CONCLUSIONS

The hydrogenosomal energy metabolism of *Trichomonas vaginalis* relies on the activity of FeS proteins, such as PFOR, ferredoxin and hydrogenase. To obtain more complete information about hydrogenosomal pathways, with particular focus on FeS proteins, we have screened *in silico* *T. vaginalis* predicted gene products for putative hydrogenosomal N-terminal presequences. These presequences ensure targeting of nuclear-encoded hydrogenosomal proteins to the organelle and are proteolytically cleaved upon import (Bradley et al., 1997). A sequence-searching application "Hunter" was developed based on the amino acid composition of presequences of all the confirmed hydrogenosomal proteins that are known to date. This search predicted proteins with putative hydrogenosomal targeting signals, primarily proteins involved in energy metabolism, electron transport and ISC assembly machinery. The analysis facilitated gene annotations of the *T. vaginalis* genome, particularly helped to recognise multiple gene copies of hydrogenosomal genes, and substantially contributed to the reconstruction of a novel metabolic map of the *Trichomonas* hydrogenosome (Carlton et al., 2007). The application was applied in two other investigations of our group: (i) Smíd et al. (2008) examined the interactions of the hydrogenosomal processing peptidase of *T. vaginalis* and mitosomal processing peptidases of *G. intestinalis* with their substrates and used the application to search for the positively charger residues in the N-terminal presequences, and (ii) Beltrán et al. (in preparation) used the application for the processing of proteomic data to distinguish hydrogenosomal proteins from contamination.

The active site of [FeFe] hydrogenases, namely the H cluster, is a unique catalytic centre composed of a [4Fe4S] cluster bridged to a [2Fe] subcluster, which is coordinated by a dithiolate bridging ligand and carbon monoxide and cyanide ligands. It has been postulated that specialised maturases termed Hyd proteins are necessary to introduce the [2Fe] subcluster into the H cluster in bacteria (Meyer, 2007). We have identified three members of the Hyd machinery (HydE, HydF and HydG) in the hydrogenosome of trichomonads and have proposed their function in the maturation of the H cluster (Putz et al., 2006) HydE and HydG are radical SAM enzymes involved in the synthesis of ligands; HydF is proposed to function as a scaffold protein. *Trichomonas vaginalis* is, after *Chlamydomonas reinhardtii*, the second eukaryotic species in which Hyd proteins have been described. Our study also provided the first evidence of the monophyly of eukaryotic *hyd* genes, and we have proposed that the acquisition of [FeFe] hydrogenases together with Hyd maturases occurred once during eukaryotic evolution. These results were later confirmed by the work of Hug et al. (2010), who demonstrated that well-supported eukaryotic clades were recovered after including several newly discovered eukaryotic

genes coding for Hyd proteins.

The availability of iron was shown to have an important impact on the expression of several *T. vaginalis* genes encoding enzymes of energy metabolism and genes associated with the parasite's infectivity. To obtain a more comprehensive picture of the regulated genes and to understand the adaptations to various growth conditions, we explored changes in transcriptome between trichomonads growing in iron-rich versus iron-depleted media. Using microarray analysis and EST library sequencing, we have identified 308 and 336 iron-regulated genes, respectively. According to both methods, approximately half of the genes were upregulated in iron-rich conditions, and half were upregulated in iron-restricted conditions. The transcriptional differences revealed several important changes in the physiology of the pathogen. Under iron-rich conditions, cytosolic and hydrogenosomal carbohydrate metabolism as well as methionine catabolism were upregulated, whereas in iron-restricted conditions, a marked upregulation of the ISC-assembly machinery and a set of cysteine proteases was detected. One of the most striking observations from this investigation is the differential regulation of individual copies of the expanded gene groups, with some of the copies being regulated by iron, while expression of others was not iron-dependent. This finding possibly reflects the early events of functional diversification of individual gene copies after gene duplication (Horváthová et al., in press).

Proteomic analysis was focused specifically on *T. vaginalis* hydrogenosomes under iron limitation; hydrogenosomes from the cells cultivated under iron-rich conditions were used as a control. Our survey revealed 49 iron-regulated proteins, 24 of them where upregulated while 25 were downregulated in iron deficient conditions. Consistently with the previous work, components of the ISC pathway were upregulated in iron-restricted conditions, while proteins involved in the energy metabolism were downregulated. Interestingly, our observation that one or several of multiple protein homologues of hydrogenosomal proteins are differentially regulated, was confirmed.