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The *Trichomonas vaginalis* hydrogenosome proteome is highly reduced relative to mitochondria, yet complex compared with mitosomes

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ABSTRACT

The human pathogen *Trichomonas vaginalis* lacks conventional mitochondria and instead contains divergent mitochondrial-related organelles. These double-membrane bound organelles, called hydrogenosomes, produce molecular hydrogen. Phylogenetic and biochemical analyses of hydrogenosomes indicate a common origin with mitochondria; however identification of hydrogenosomal proteins and studies on its metabolism have been limited. Here we provide a detailed proteomic analysis of the *T. vaginalis* hydrogenosome. The proteome of purified hydrogenosomes consists of 569 proteins, a number substantially lower than the 1,000–1,500 proteins reported for fungal and animal mitochondrial proteomes, yet considerably higher than proteins assigned to mitosomes. Pathways common to and distinct from both mitochondria and mitosomes were revealed by the hydrogenosome proteome. Proteins known to function in amino acid and energy metabolism, Fe–S cluster assembly, flavin-mediated catalysis, oxygen stress response, membrane translocation, chaperonin functions, proteolytic processing and ATP hydrolysis account for ~30% of the hydrogenosome proteome. Of the 569 proteins in the hydrogenosome proteome, many appear to be associated with the external surface of hydrogenosomes, including large numbers of GTPases and ribosomal proteins. Glycolytic proteins were also found to be associated with the hydrogenosome proteome, similar to that previously observed for mitochondrial proteomes. Approximately 18% of the hydrogenosomal proteome is composed of hypothetical proteins of unknown function, predictive of multiple activities and properties yet to be uncovered for these highly adapted organelles.

1. Introduction

Trichomonas vaginalis, a pathogenic protist, causes the most common non-viral sexually transmitted human infection worldwide, with ~170 million cases reported annually (WHO, 2001; Johnston and Mabey, 2008). This parasite belongs to a group of microaerophilic and anaerobic unicellular eukaryotes that lack conventional mitochondria and instead contain related specialised double-membrane organelles called hydrogenosomes (Lindmark et al., 1975; Shiflett and Johnson, 2010). These organelles, which are also found in specific fungi (chytrids) and ciliates (Boxma et al., 2004, 2005), are defined by the ability to produce molecular hydrogen. Hydrogenosomes are polyphylogenetic and have arisen independently in several eukaryotic lineages (Embley and Hirt, 1998).

Some eukaryotes lack either hydrogenosomes or mitochondria and instead contain highly reduced, double-membrane bound organelles called mitosomes (Mai et al., 1999; Tovar et al., 1999; Williams et al., 2002; Putignani et al., 2004; Regoes et al., 2005; Shiflett and Johnson, 2010). Studies demonstrating the presence of mitochondrial-type proteins in hydrogenosomes and mitosomes, together with similarities in the biogenesis of hydrogenosomes and mitochondria, support the hypothesis that these organelles evolved from a single α-proteobacterial endosymbiont (reviewed in Shiflett and Johnson, 2010). The relationship between hydrogenosomes, mitosomes and mitochondria has generated much debate and raises the question whether the acquisition of the endosymbiont that gave raise to mitochondria may have been present in the earliest eukaryotic cell (reviewed in Martin et al., 2001). Efforts to trace the origin of the *T. vaginalis* hydrogenosome have relied on phylogenetic analyses of nuclear-encoded hydrogenosomal proteins as there is no genome to allow analyses of organellar genes (Clemens and Johnson, 2000). Hypotheses put forth differ primarily in whether a single endosymbiotic event gave rise to both hydrogenosomes and mitochondria through divergent

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evolution or whether an additional second endosymbiont contributed to the formation of hydrogenosomes in *T. vaginalis* (Dyall et al., 2004a; Embley, 2006; Shiflett and Johnson, 2010). As the list of analysed proteins has grown, a consensus has emerged that hydrogenosomes, mitosomes and other mitochondrion-like organelles evolved from a single endosymbiont that also gave rise to mitochondria (reviewed in Shiflett and Johnson, 2010).

Until recent years, the only characterised function for *T. vaginalis* hydrogenosomes was carbohydrate metabolism, specifically in the conversion of pyruvate and malate to the end products of ATP, acetate, CO_2 and hydrogen (Muller, 1993). Hydrogenosomes are also the target and site of activation of the 5-nitroimidazole drugs used to treat trichomoniasis (Narcisi and Secor, 1996). Recently, enzymes responsible for iron–sulphur (Fe–S) cluster assembly typically found in mitochondria have been localised to the *T. vaginalis* hydrogenosome (Tachezy et al., 2001; Carlton et al., 2007; Dolezal et al., 2007). Similarly, mitosomes have also been shown to be the site of Fe–S biogenesis (Tachezy et al., 2001; Regoes et al., 2005; Goldberg et al., 2008). This supports the theory that the only required function for mitochondria is Fe–S biogenesis and may be why these organelles have been preserved throughout evolution (Lill and Kispal, 2000). In *Entamoeba histolytica,* the machinery for Fe–S cluster assembly has been reported in both the cytosol and the mitosome (Maralikova et al., 2010). This mitosome also houses proteins involved in a sulphate activation pathway, indicating an additional potential function (Mi-Ichi et al., 2009).

Both hydrogenosomes and mitochondria contain many more metabolic pathways than mitosomes (Shiflett and Johnson, 2010). Mitosomes appear to be extremely reduced in complexity and none have been demonstrated to generate ATP (Shiflett and Johnson, 2010). Hydrogenosomes can generate ATP by substrate level phosphorylation but not via oxidative phosphorylation as mitochondria do. Hydrogenosomes also lack a trichloroacetic acid (TCA) cycle, cytochromes and members of complexes I–IV, with the exception of NADH dehydrogenase 51 kDa (Ndh51) and 24 kDa (Ndh24) subunits (Dyall and Johnson, 2000; Dyall et al., 2004b; Hrdy et al., 2004). To mediate reduction of reactive oxygen species (ROS), *T. vaginalis* contains a bacterial-type thioredoxin reduction system within the hydrogenosome (Coombs et al., 2004; Putz et al., 2005). The organism, however, lacks peroxisomes and the glutathione reducing pathway normally present in mitochondria.

Being devoid of a genome, all *T. vaginalis* hydrogenosomal proteins are nuclearly encoded, synthesized in the cytosol and subsequently targeted and translocated into the organelle. Many hydrogenosomal matrix proteins contain conserved N-terminal presequences that are similar to sequences known to target proteins to the mitochondrial matrix (Bradley et al., 1997; Hausler et al., 1997). In the case of the ferredoxin protein, the presequence has been shown to be necessary for targeting and translocation of the protein into hydrogenosomes in vitro (Bradley et al., 1997). Homologues of several proteins known to be involved in protein import and the biogenesis of yeast mitochondria are present in the hydrogenosome. These include mitochondrial-like chaperones Hsp70, Hsp60 and Hsp10, a processing peptidase and putative members of the translocation machinery (Pam18 and Tim17/22/23 orthologs) (Bui et al., 1996; Dolezal et al., 2006; Brown et al., 2007; Carlton et al., 2007; Smid et al., 2008; Shiflett and Johnson, 2010).

The sequencing of the *T. vaginalis* genome allowed bioinformatic identification of putative hydrogenosomal proteins through screening for the conserved N-terminal presequence motif. We originally identified 138 putative hydrogenosomal presequences in the genome using this approach (Carlton et al., 2007). Subsequently, using a less strict consensus sequence, 222 putative hydrogenosomal precursor proteins were found (Smid et al., 2008). These data support the presence of additional metabolic pathways in the organelle but suffer from the weakness of the inability to detect proteins that contain divergent targeting signals or lack an N-terminal presequence and may mistakenly identify a non-hydrogenosomal protein (Mentel et al., 2008).

In this study, we conducted a proteomics analysis of the *T. vaginalis* hydrogenosome to gain a better understanding of the metabolic processes of this organelle. These studies allow the comparison of a hydrogenosome proteome with the mitochondrial proteomes of yeast, protistan and human mitochondria, and the mitosome proteomes of *E. histolytica* and *Giardia lamblia*, further defining similarities and differences between these organelles (Mi-Ichi et al., 2009; Jedelsky et al., 2011). Using multiple fractionation techniques, 569 proteins were identified in the *T. vaginalis* hydrogenosome, uncovering new members of known hydrogenosomal pathways and revealing new metabolic pathways present in this unique organelle.

2. Materials and methods

2.1. Parasite culture

Trichomonas vaginalis strain T1 were grown in Diamond's medium supplemented with 10% (v/v) horse serum and iron as described previously (Diamond, 1957). Transformed *T. vaginalis* T1 cultures were grown as described (Delgadillo et al., 1997).

2.2. Isolation of hydrogenosomes

Hydrogenosomes of *T. vaginalis* strain T1 were purified by collecting cells grown as described in Section 2.1 by centrifugation. All procedures were done at 4 °C. Cell pellets were washed twice in SMD (0.25 M sucrose, 0.01 M morpholine propane sulphonic acid (MOPS) pH 8.0, 0.01 M DTT) and resuspended in SMDI (SMD plus 25 μg/ml *N-p*-tosyl-L-lysine chloromethylketone (TLCK) and 10 μg/ml leupeptin). Cells were lysed in a Stansted cell disruptor with 30 psi front pressure and 12 psi back pressure. Unbroken cells were pelleted by centrifugation at 1,000*g* for 10 min and the supernatant was then centrifuged at 5,000*g* to pellet the organelles. This pellet was resuspended in 45% Percoll in SMDI and subjected to centrifugation at 68,000*g* at 4 °C for 1 h. The fraction containing hydrogenosomes (Bradley et al., 1997) was collected from the resulting Percoll gradient, washed twice with 10 vol. of SMDI and reisolated by centrifugation at 7,500*g* for 10 min. Hydrogenosomes were resuspended in freezing buffer (0.25 M sucrose, 0.01 M morpholine propane sulphonic acid pH 8.0, 0.5% BSA, 8% glycerol) and stored at −80 °C.

2.3. Fractionation of hydrogenosomal proteins by sodium carbonate or sodium hydroxide extraction

Isolated hydrogenosomes were alkaline extracted at 1 mg/ml in 0.1 M Na_2CO_3, pH 11.5 or in 0.5 M NaOH, for 30 min at 4 °C. The insoluble pellet and soluble supernatant were subsequently obtained by centrifugation at 100,000*g* for 30 min. The proteins found in the supernatant were TCA-precipitated and resuspended in sample buffer. The proteins from the insoluble pellet were resuspended in 0.1 M Na_2CO_3 and subjected to a second centrifugation step at 100,000*g* for 20 min. The insoluble pellet from the second centrifugation step was resuspended in SDS–PAGE sample buffer. Approximately 100 μg of total protein from the soluble fraction and approximately 10 μg of total protein from the membrane fraction were resolved on 15% SDS–PAGE gels and stained with 0.04% Coomassie Brilliant Blue G-250/3.5% perchloric acid. Proteins from both the soluble and insoluble membrane fractions from the sodium carbonate extraction and only the insoluble membrane

fraction from the sodium hydroxide extraction were excised and prepared for mass spectrometry analysis as described in Section 2.6.

2.4. Fractionation of hydrogenosomal proteins by zinc chelating column chromatography

Isolated hydrogenosomes (0.5 mg/ml) were solubilised in 1% Triton X-100, 0.3 M NaCl, 20 mM Tris pH 7.5, with a protease inhibitor cocktail (Sigma) for 30 min and insoluble material removed by centrifugation at 100,000*g* at 4 °C for 20 min. The solubilised material was fractionated with the Amersham Chelating Sepharose Fast Flow column, which had been charged with Zn^{2+} and equilibrated with the solubilisation buffer. Fractions from the flow-through and the subsequent washes were collected. Wash steps included the solubilisation buffer supplemented with the following: 1 M NaCl for the first wash, 50 mM NaCl for the second wash, 20 mM imidazole/300 mM NaCl for the third wash, 250 mM imidazole/300 mM NaCl for the fourth wash and 50 mM EDTA/1 M NaCl to strip the remaining proteins from the column. The collected fractions were precipitated using TCA and resuspended in SDS–PAGE sample buffer. Samples from the third and fourth washes were resolved by 8–16% Tris SDS–PAGE (Criterion, USA) and stained with 0.04% Coomassie Brilliant Blue G-250/3.5% perchloric acid. Protein bands were excised and prepared for mass spectrometry analysis as described in Section 2.6.

2.5. Fractionation of hydrogenosomal proteins using sodium carbonate extraction and sucrose gradient

Isolated hydrogenosomes (1.3 mg/ml) were alkaline extracted in 0.1 M Na_2CO_3, pH 11.5, for 30 min at 4 °C. Then 2.4 M sucrose/0.1 M Na_2CO_3 was added to adjust the final concentration to 1.5 M sucrose/0.1 M Na_2CO_3 in a final volume of 4 ml. A sucrose gradient was established by overlaying the extracted protein solution with 3.75 ml of 1.4 M sucrose/0.1 M Na_2CO_3 and then 3 ml of 0.25 M sucrose/0.1 M Na_2CO_3. The gradient was subjected to centrifugation for 4 h in a SW41 rotor (Beckman, USA) at 274,000*g* at 4 °C. Aliquots from the interface of the top and middle layer were precipitated using TCA, resuspended and resolved on 8–16% SDS PAGE gels, then analysed by western blot, Coomassie blue stain or silver stain. Interface proteins were excised and prepared for analysis by mass spectrometry. Western blot analysis was also used to compare the protein collected from the interface layer with proteins from the middle and bottom layers of the sucrose gradient. Protein bands were excised and prepared for mass spectrometry analysis as described in Section 2.6.

2.6. Protein gel extraction in preparation for LC–MS/MS analysis

Following gel electrophoresis, gel slices containing the target protein(s) were excised from the gel and incubated for 10 min with 0.1 M NH_4HCO_3 followed by a 1:1 solution of NH_4HCO_3 and acetonitrile. These two wash steps were repeated three times in succession and then gel slices were dried in a Speedvac. The gel slices were then incubated in 10 mM DTT in 0.1 M NH_4HCO_3 for 1 h at 60 °C, followed by incubation in 50 mM iodoacetimide in 0.1 M NH_4HCO_3 for 45 min at 45 °C. The gel slices were then washed as described above and dried in a Speedvac. Gel slices were then incubated with 83 ng/μl of sequencing-grade trypsin (Promega, USA) in 0.1 M NH_4HCO_3 and incubated for 45 min at 4 °C. NH_4HCO_3 (0.1 M) was added to the gel slices, which were then incubated overnight at 37 °C. Water was added and the supernatant was subsequently removed. A solution of 50% acetonitrile with 1% trifluoroacetic acid (TFA) was then added to the gel slices and incubated for 10 min. The supernatant was removed and added to the pool of peptides. The last step was repeated three to five times. The peptides were then dried in a Speedvac and analysed by LC–MS/MS.

2.6.1. Protein identification and analysis of protein sequences

Protein identification was accomplished by reversed-phase high performance LC–MS/MS using a hybrid quadrupole time-of-flight (QTOF) mass spectrometer. A QSTAR Pulsar XL QTOF mass spectrometer (Sciex/Applied Biosystems, Toronto, ON, Canada) was equipped with a nanoelectrospray (nanoESI) interface (Protana, Odense, Denmark) and a Dionex/LC Packings (Sunnyvale, CA, USA) nano-HPLC system. The nano-HPLC system was equipped with a homemade precolumn (150 μm × 5 mm) and analytical column (75 μm × 150 mm) packed with Jupiter Proteo C12 resin (particle size 4 μm; Phenomenex, USA). The dried peptides were resuspended in 1% (v/v) formic acid (FA) solution; 6 μL of sample solution was loaded onto the precolumn for each LC–MS/MS run. The precolumn was washed with the loading solvent (0.1% FA) before the sample was injected onto the LC column. The eluants used for the LC were 0.1% FA (solvent A) and 95% acetonitrile containing 0.1% FA (solvent B). The flow rate was 200 nL/min and the following gradient was used: 3% B to 6% B in 6 s, 6% B to 24% B in 18 min, 24% B to 36% B in 6 min, 36% B to 80% B in 2 min, and maintained at 80% B for 7.9 min. The column was finally equilibrated with 3% B for 15 min before the next run. Electrospray ionisation was performed using a 30 μm (inner diameter) nano-bore stainless steel online emitter and a voltage set at 1900 V.

Peptides identified by LC–MS/MS were analysed using the MASCOT sequence database searching program (Matrix Science, London, UK) against the *T. vaginalis* peptide sequence database (www.trichdb.org v1.0) (Perkins et al., 1999). Derived from the size of the *T. vaginalis* genome and the protein query database, all searches were performed allowing for phosphorylated Ser, Thr and Tyr, oxidised Met, carbamidomethylated Cys and N-terminal pyro-Gly, using a mass tolerance of 0.3 Da. A MASCOT score of 42 corresponds to a *P* value <0.05; thus, peptides with scores <42 were excluded from the dataset. This dataset was further analysed by the Basic Local Alignment Search Tool (BLAST) using the BLOSUM62 scoring matrix (Altschul et al., 1990). Individual examination of the program output included analysis of sequences with the use of programs available from the Swiss Institute of Bioinformatics (SIB) (ftp://ftp.expasy.org/databases/uniprot) and ClustalX or MUSCLE alignment programs, identification of motif or domains, and identification of any predicted transmembrane domains using the TMpred or HMMTOP prediction algorithm. In addition, the KEGG Orthology-Based Annotation System (KOBAS) program was used to compare the sequences with protein orthologs found in other organisms and predict putative associated metabolic pathways. Putative hydrogenosomal targeting sequences were also identified with a perl script regular expression corresponding to the consensus sequences ML(S/T/A) X(1..15)R (N/F/E/XF), MSLX (1..15)R(N/F/XF) or MLR(S/N)F.

To compare the hydrogenosomal proteome with mitochondrial protein sequences from *Homo sapiens* and *Saccharomyces cerevisiae,* the mitochondrial proteomes were obtained from the MitoMiner database (http://mitominer.mrc-mbu.cam.ac.uk) (Smith and Robinson, 2009). The *Trypanosoma brucei* mitochondrial proteome (Panigrahi et al., 2009) was obtained from the TriTrypDB (http://tritrypdb.org/tritrypdb/), and the mitochondrial *Tetrahymena thermophila* protein sequences (Smith et al., 2007) were obtained from GenBank.

2.7. Immunofluorescent microscopy

Trichomonas vaginalis T1 transfectants were fixed in 3.5% formaldehyde/0.2% Triton X-100/PBS solution. Cells were preincubated in blocking buffer (PBS/3% BSA) prior to incubation with mouse

anti-HA monoclonal antibody (Covance) and/or rabbit anti-Hsp70 polyclonal antibody diluted 1:5,000 (v/v) in blocking buffer. Secondary Alexa Fluor-488 anti-mouse and Alexa Fluor-594 anti-rabbit antibodies (Invitrogen), diluted 1:5,000 (v/v) were added for 1 h. Cells were mounted in Prolong Gold antifade mounting medium (Invitrogen). Images were taken on a Zeiss Axioscope 2 microscope and analysed using Axiovision LE software (Zeiss).

3. Results and discussion

3.1. Identification of hydrogenosomal proteins by mass spectrometry

We determined the proteome of the *T. vaginalis* hydrogensome to better define its metabolic pathways and to allow comparison with the proteomes of mitochondria and mitosomes. Hydrogenosomes were purified using gradient density centrifugation (Bradley et al., 1997). We showed that this method yields highly purified hydrogenosomes as judged by electron microscopy and the absence of activities of cytosolic enzymes (Bradley et al., 1997). Hydrogenosomal proteins were extracted using a combination of sodium hydroxide, sodium carbonate extractions, sucrose gradients and zinc chromatography. Multiple fractionation strategies were employed to optimise inclusion of proteins in the proteome. Proteins were then subjected to trypsin digestion and the resulting peptides were identified by mass spectrometry. The complete proteome dataset was found to contain 569 proteins (Supplementary Tables S1–S3). Present in the proteome were 66 previously characterised hydrogenosomal proteins and their paralogs, confirming both their localisation and our methodology (Table 1). Of the 569 proteins identified, 175 proteins can be placed in pathways previously associated with the hydrogenosome such as energy metabolism, Fe–S cluster assembly, oxygen stress response and amino acid metabolism, better defining the organelle's metabolic capacity (Fig. 1, Supplementary Table S1). Many of these proteins are also candidates for playing a role in biogenesis or protein and solute membrane translocation. In addition, we identified 101 hypothetical proteins which are mostly unique to the *T. vaginalis* genome (Fig. 1, Supplementary Table S1). Our proteome also contains 137 small GTPases and related proteins as well as 123 proteins that are likely associated with the external surface of the organelle (Fig. 1, Supplementary Table S2). Thirty-three proteins were classified as contaminants as they are homologues of proteins known to be localised in the endoplasmic reticulum, nucleus or vesicles in other organisms (Fig. 1, Supplementary Table S3). Definitive classification as a contaminant, however, awaits future experiments to directly test whether these proteins localise to hydrogenosomes. To independently confirm the hydrogenosomal localisation of proteins assigned to the protein, 13 randomly selected proteins were epitope-tagged and expressed in *T. vaginalis* transfectants and localised by immunofluorescent microscopy using an antibody against hydrogenosomal Hsp70 as the organelle marker. Twelve of the 13 proteins were seen to co-localise with Hsp70, consistent with the low level of assigned contaminants (data not shown). Nevertheless, based on the association of hydrogenosome with other subcellular structures and the difficulty of obtaining purity of any subcellular fraction, our preparations are likely to contain contaminating, non-hydrogenosomal proteins.

The peptides used to identity these proteins had MASCOT scores ranging from 42-1,843 in a skewed bell curve distribution pattern (Fig. 2A). The majority of the proteins (66%) correspond to MASCOT scores ranging from 50–200 and approximately 63% of the proteins were found to have $\geqslant 3$ matched peptides (Fig. 2A and B). The proteome consists of 468 (82%) unique proteins and 101 (18%) proteins that correspond to protein families with multiple sequences containing such high identity at the amino acid level that the expressed genes could not be distinguished by the derived peptides (Supplementary Table S4). In comparison, most mitochondrial proteomes have been shown to contain ~1,000 proteins (Sickmann et al., 2003; Reinders et al., 2006; Smith et al., 2007), whereas the recently published *G. lamblia* mitosomal proteome contains 139 proteins (Jedelsky et al., 2011), and the *E. histolytica* mitosomal proteome contains only 95 proteins (Mi-Ichi et al., 2009).

The number of proteins (101/569; ~18%) annotated as hypothetical in the hydrogenosome proteome (Supplementary Table S1) is comparable to that observed for mitochondrial proteomes. For example, 25% of the 750 proteins found in the *S. cerevisiae* mitochondrial proteome do not have a known function (Sickmann et al., 2003). This is also true for mitosomes; approximately 28% of the *G. lamblia* mitosome proteome consists of hypothetical proteins (Jedelsky et al., 2011), however ~67% of the *E. histolytica* mitosome proteome is hypothetical (Mi-Ichi et al., 2009). Twenty-six percent of the hypothetical proteins in the hydrogenosomal proteome are predicted to have at least one transmembrane domain (Supplementary Table S5) using the prediction algorithms TMHMM 2.0 (Krogh et al., 2001), HMMTOP 2.0 (Tusnady and

Table 1
Known *Trichomonas vaginalis* hydrogenosomal proteins and their representation in the proteome.

Protein	# Paralogs (Proteome)	# Paralogs (Genome)
Energy metabolism		
Hydrogenosome malic enzyme	7	15
Hydrogenase	5	14
Ferredoxin	3	7
Pyruvate:ferredoxin oxidoreductase (PFO)	6	7
Adenylate kinase	1	8
Acetate:succinate CoA-transferases	3	4
Succinate thiokinase (α subunit)	3	3
Succinate thiokinase (β subunit)	3	3
NADH dehydrogenase (24 kD subunit)	1	1
NADH dehydrogenase (51 kD subunit)	1	2
Oxygen scavenging system		
Thioredoxin (Trx)	1	32
Thioredoxin peroxidase (TrxP), Type 1	1	3
Thioredoxin peroxidase (TrxP) Type 2	1[a]	7
Rubrerythrin	1	6
Iron superoxide dismutase (SOD)	2	7
Amino acid metabolism		
H-protein	2	2
L-protein	1	1
Serine hydroxymethyltransferase	1	1
Fe–S cluster assembly/hydrogenase maturation		
IscS	1	2
IscU	1	1
IscA	2	3
HydG	1	2
Chaperones		
Hydrogenosomal Hsp10	4	4
Hydrogenosomal Hsp60	3	3
Hydrogenosomal Hsp70	3	3
Peptidases		
Hydrogenosome processing peptidase (α subunit)	1	1
Hydrogenosome processing peptidase (β subunit)	1	1
Membrane proteins		
Hydrogenosome membrane protein (Hmp35)	3	3
Hydrogenosome membrane protein (Hmp31 MCF)	3	3

[a] Multiple protein sequences that cannot differentiate between individual genes based on spectra.

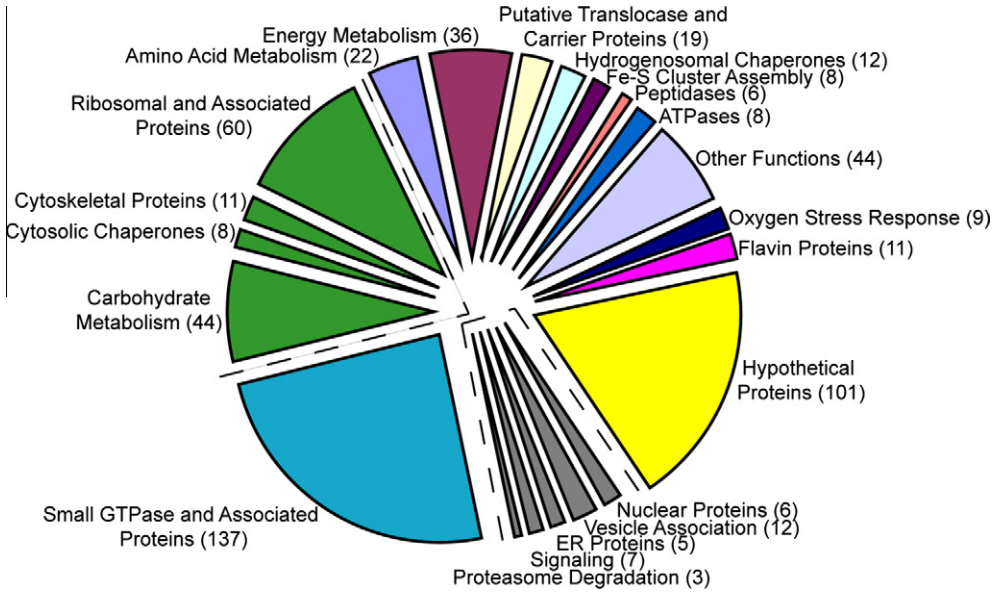

Fig. 1. Functional distribution of the 569 proteins identified in the *Trichomonas vaginalis* hydrogenosome proteome. Proteins involved in amino acid or energy metabolism, membrane functions, oxygen stress response and other functions are depicted on the top right in multiple colours. Hypothetical proteins are indicated in yellow and small GTPase and associated proteins are shown in blue. Proteins predicted to be associated with the external surface of the organelle are depicted in green and contaminants are indicated in grey. Numbers of proteins in each category are listed in parentheses.

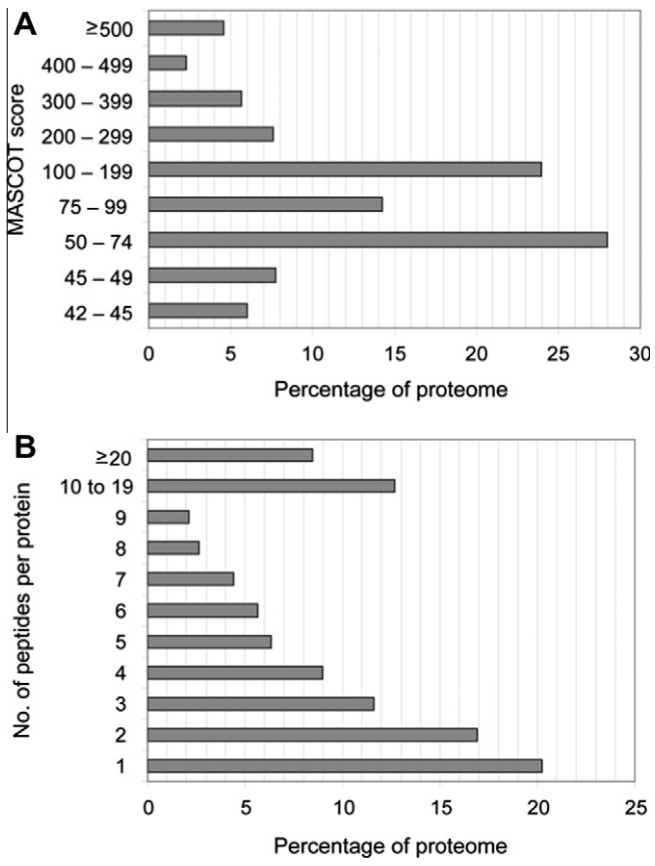

Fig. 2. Statistical analysis of the *Trichomonas vaginalis* hydrogenosome proteome. (A) Distribution of MASCOT scores. MASCOT scores ranged from 42 to 1,843. The top MASCOT score is reported for those proteins identified in multiple fractions. MASCOT Scores >42 correspond to a *P* value <0.05 in this dataset. (B) Number of peptides per protein. Peptides per protein ranged from 1 to 128.

Simon, 2001) or SPOCTOPUS (Viklund et al., 2008). Further investigation of these hypothetical proteins may reveal proteins that function as additional members of the protein translocase machinery, carriers or proteins involved in biogenesis and homeostasis. These hypothetical proteins likely also represent novel functions associated with each type of organelle.

3.2. The highly redundant nature of the hydrogenosomal proteome

The *T. vaginalis* genome is highly redundant, containing many multi-copy gene families (Carlton et al., 2007). This is reflected in the hydrogenosomal proteome where multiple paralogs of protein families are present (Table 1, Supplementary Table S4). For example, the genome contains 15 genes encoding malic enzymes and seven of these distinct proteins are found in the proteome; similarly six of seven predicted pyruvate ferredoxin oxidoreductases (PFOs) are present and three of seven ferredoxin proteins are also found in the organelle (Table 1). Although it is not known whether the three ferredoxin paralogs are functionally redundant, their presence could explain the lack of an observable phenotype when a single ferredoxin gene was deleted by homologous recombination (Land et al., 2004). Multiple genes encoding four types of hydrogenases, as well as two hydrogenase-related proteins, are present in the genome (Carlton et al., 2007). The proteome contains four hydrogenase proteins, two type I and two type II, differing in the number of Fe–S domains, together with a previously documented but uncharacterised type IV hydrogenase containing a hydrogenase domain and a C-terminal electron-supplying reductase region (Supplementary Table S1). Determining whether the different hydrogenases present in the hydrogenosome have overlapping or distinct catalytic activities awaits further analyses.

3.3. Comparative analysis of the proteome and expressed sequence tag (EST) databases

The observation that in some cases every member of a hydrogenosomal protein family is found in the proteome while other families lack the full complement of proteins (Table 1, Supplementary Table S4) may be the result of regulated expression of multigene families under different conditions. To address this possibility, we compared the expression profile of proteins found in the

hydrogenosome proteome with a *T. vaginalis* EST database generated from cells grown under the same normal growth conditions used to prepare organelles for proteomic analyses (http://tvx-press.cgu. edu.tw/). Thirteen protein families comprised of 3–10 paralogs that had at least one paralog absent from the proteome were compared. From this dataset of 71 proteins, 34 are present in the proteome and 29 of these are expressed under normal growth conditions (Fig. 3). Of the remaining 37 proteins absent from the proteome, 31 are only expressed under specialised conditions or lack ESTs under all conditions tested. This observation, while based on a small dataset, raises the possibility that *T. vaginalis* has used gene expansion and differential gene expression as a way of adapting quickly to changing environmental pressures.

3.4. Functional categorisation of the proteome

The proteins composing the hydrogenosome proteome were analysed using BLAST to assign putative functions based on sequence similarity to previously identified proteins in the NCBI database (Supplementary Tables S1–S3; Altschul et al., 1990). Proteins were further examined using KOBAS to search the KEGG database and build putative metabolic pathways (Kanehisa et al., 2008). These analyses led to the discovery of several new pathways and putative functions for the trichomonad hydrogenosome, as well as providing additional information on pathways previously assigned to the organelle.

As shown in Fig. 1, ~1/3 of the proteome is involved in metabolic processes including sugar (44 proteins), amino acid (22 proteins) and energy metabolism (36 proteins), protein membrane translocation (14 proteins), chaperonin functions (12 proteins), Fe–S cluster assembly (eight proteins), proteolytic processing (peptidases) (six proteins), ATP hydrolysis (eight proteins), oxygen stress response (nine proteins), flavin-mediated catalysis (11 proteins) and other known functions. Although a limited role in amino acid metabolism has been previously implicated for the hydrogenosome, these data greatly expand the role of this organelle in this process (Mukherjee et al., 2006a,b). The identification of additional proteins with activities previously described in *T. vaginalis* hydrogenosomes, such as Fe-S cluster assembly or maturation, peptidases and flavin proteins, further broaden our understanding of metabolic activities compartmentalised within this organelle.

The majority of identified proteins in our proteome were not previously assigned to the organelle, however 66 characterised hydrogenosomal proteins and their paralogs were found (Table 1).

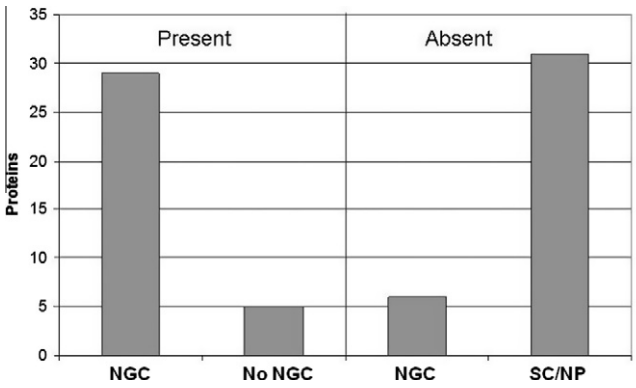

Fig. 3. Comparison of expressed sequence tags (ESTs) for the presence or absence of the corresponding protein in the *Trichomonas vaginalis* hydrogenosome proteome. Seventy-one proteins comprising 13 gene families were compared. Twenty-nine of the 34 proteins represented in ESTs from cells grown under the conditions used to isolate hydrogenosomes for proteomic analysis (NGC) are present in the proteome, whereas 31 of the 37 proteins not represented in the proteome are either expressed under specialised conditions (SC) or not presence (NP) in EST databases.

As discussed in Section 3.1, a substantial number of hypothetical proteins (101) were found, pointing to the presence of multiple activities and properties yet to be revealed (Fig. 1, Supplementary Table S1). Further studies will be necessary to determine what percentage of these hypothetical proteins are localised to the hydrogenosome; nevertheless their high frequency is predictive of as-yet uncovered hydrogenosomal activities. We classified 123 proteins as organelle-associated proteins that are not predicted to compartmentalise within the organelles, but instead associate with the external surface of hydrogenosomes (Supplementary Table S2, Fig. 1). These include proteins involved in carbohydrate metabolism (glycolysis, gluconeogenesis and the pentose phosphate pathway, 44 proteins), cytosolic chaperones (eight proteins), cytoskeletal proteins (11 proteins) and ribosomal proteins (60 proteins). Additionally, an exceptionally large number of GTPase proteins (137 proteins) were found, consistent with the massive expansion of this protein family in the *T. vaginalis* genome (Supplementary Table S1, Fig. 1; Lal et al., 2005; Carlton et al., 2007). It is probable that the GTPases are primarily components of cytoskeletal structures that have been shown to associate with the external surface of the hydrogenosome (Benchimol, 2009), however some may function within the organelle. Due to this ambiguity we did not classify the GTPases as contaminants; although many are likely to be associated with the outer surface of the organelle. Confirmatory localisation using immunomicroscopy of parasites transfected with tagged candidate hydrogenosomal proteins will be required to definitively classify them as hydrogenosomal.

3.5. Metabolic pathways of the hydrogenosome

The *T. vaginalis* hydrogenosome has previously been shown to be a site of ATP production, Fe–S cluster biosynthesis, and oxygen stress response (Muller, 1993; Tachezy et al., 2001; Coombs et al., 2004; Sutak et al., 2004; Putz et al., 2005). Molecular hydrogen, acetate and CO_2 are by-products of ATP production via substrate level phosphorylation. Proteins known to be involved in energy metabolism include malic enzyme, PFO, [2Fe–2S] ferredoxin (Fdx), succinate thiokinase (SCS), adenylate kinase (AK), [Fe] hydrogenase (Hyd), Ndh51 and 24 kDa Ndh24 subunits and acetyl:succinate CoA-tranferase (ASCT) (Johnson et al., 1990; Lahti et al., 1992; Lahti et al., 1994; Hrdy and Muller, 1995b; Bui and Johnson, 1996; Horner et al., 2000; Dyall et al., 2004b; Hrdy et al., 2004; van Grinsven et al., 2008). All of these proteins were represented in the proteome, in several instances by multiple paralogs (Table 1, Supplementary Tables S1–S4).

3.5.1. Fe–S cluster pathway

Multiple proteins involved in energy metabolism contain Fe–S clusters and are dependent on enzymes required for Fe–S biosynthesis for their maturation (Lill, 2009). Similar to mitochondria and mitosomes, enzymes involved in the Iron–Sulphur Cluster (ISC) machinery are localised to the hydrogenosome (Tachezy et al., 2001; Tovar et al., 2003; Regoes et al., 2005; Dolezal et al., 2007). Twelve genes encoding proteins similar to components of the ISC assembly or protein maturation pathways are present in the *T. vaginalis* genome (Carlton et al., 2007). We identified eight of these proteins in the proteome: IscU, IscA, IscS, Fdx, Hsc20 (HscB), GrpE (Mge1), HydF and HydG (Fig. 4A, Supplementary Table S1). Three proteins of the ISC pathway missing from the proteome are frataxin, HydE and an Isd11-like protein that contains a putative N-terminal presequence (Richards and van der Giezen, 2006). Of these, frataxin and HydE have been localised in the *T. vaginalis* hydrogenosome (Dolezal et al., 2007; Putz et al., 2006). Two additional proteins found in mitochondria that compose the Fe–S export machinery, Atm1 and Erv1, are absent from

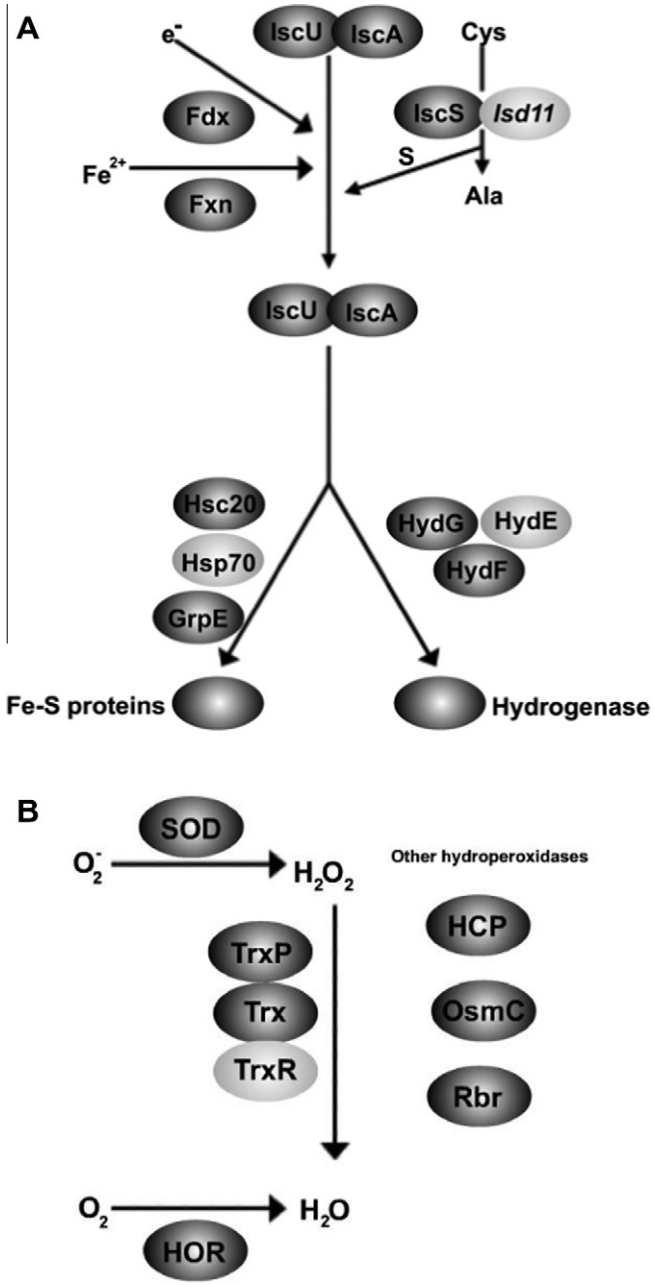

Fig. 4. Proteins from known pathways localised to the *Trichomonas vaginalis* hydrogensome. (A) Iron–Sulfur (Fe–S) cluster assembly pathway. Proteins involved in Fe–S cluster assembly were identified in the hydrogenosome proteome (dark grey); some proteins not present in the proteome have been identified in the genome (light grey). (B) Proteins involved in oxygen stress response were also identified in the hydrogensome proteome (dark grey). Only one protein, TrxR (light grey), known to be involved in this pathway, was not found in the proteome.

the proteome and genes encoding these proteins are also not readily identifiable in the genome (Lill and Muhlenhoff, 2008).

The mitosome of *G. lamblia* contains only eight proteins known to be involved in ICS transfer and assembly: IscS, IscU, Nfu, IscA, Glutaredoxin5, Hsp70, HscB, and GrpE (Jedelsky et al., 2011). During reductive evolution, *E. histolytica* has lost the ISC machinery entirely and instead gained the Nif system, likely through a lateral gene transfer from a ε-proteobacterium (Ali et al., 2004; van der Giezen et al., 2004). This system has been described in only one other eukaryotic organism, *Mastigamoeba balamuthi*, where it is predicted to be cytosolic (Gill et al., 2007; Maralikova et al., 2010). In *E. histolytica* the Nif machinery has been demonstrated to possess a dual localisation in both the cytosol and mitosome (Maralikova et al., 2010).

3.5.2. Oxygen stress response

Inhibition of specific oxygen sensitive hydrogenosomal proteins, such as PFO and [Fe]-hyd, can block energy metabolism in the organelle (Hrdy and Muller, 1995a; Page-Sharp et al., 1996). Iron-dependant superoxide dismutase (SOD) proteins that convert reactive oxygen superoxide species (O_2^-) to hydrogen peroxide (H_2O_2) have been characterised but not previously localised in *T. vaginalis* (Viscogliosi et al., 1998). SOD activity has been reported in both the cytosol and the hydrogenosomes of the related parasite *Tritrichomonas foetus* (Lindmark and Muller, 1974). Proteins encoded by two of the seven genomic copies of SOD were identified in the *T. vaginalis* hydrogenosome proteome (Fig. 4B, Table 1). Mitochondria typically utilise a glutathione system and catalase to remove H_2O_2, proteins that are not present in trichomonads (Muller, 1993; Coombs et al., 2004). Instead proteins of the thioredoxin pathway (Trx, TrxP, TrxR) that convert H_2O_2 into H_2O have been localised to hydrogenosomes, as has peroxidase rubrerythrin (Rbr) (Coombs et al., 2004; Putz et al., 2005). Three of these four proteins, Trx, TrxP and Rbr, were also identified in our proteomic data (Fig. 4B, Supplementary Table S1). In addition a protein with weak similarity to the OsmC/Ohr hydroperoxidase protein of bacteria was present, which may represent another pathway for the metabolism of H_2O_2 (Fig. 4B, Supplementaryl Table S1; Rehse et al., 2004). A protein that is up-regulated in bacteria by H_2O_2, the hybrid cluster protein (HCP) is also present in the proteome (Fig. 4B, Supplementaryl Table S1; Almeida et al., 2006).

To confirm the localisation of this protein in hydrogenosomes, a C-terminally HA-tagged HCP protein was expressed in *T. vaginalis*. Immunostaining of the transformants showed that HCP-HA colocalised with the hydrogenosomal marker protein Hsp70 (Fig. 5B). Another protein that plays a role in oxygen stress response that is also present in the hydrogenosome proteome is the recently identified flavo-diiron protein called the hydrogenosome oxygen reductase (HOR) (Fig. 4B). This protein has been shown to metabolize another ROS, O_2 (Smutna et al., 2009). Several of the proteins involved in these pathways appear to be the result of lateral gene transfer based on phylogenetic analyses, including HCP, Trx, TrxP, TrxR, Rbr and quite possibly OsmC (Coombs et al., 2004; Rehse et al., 2004; Putz et al., 2005; Andersson et al., 2006).

3.6. Amino acid metabolism pathways identified in the proteome

The first evidence that hydrogenosomes, like mitochondria, may be involved in amino acid metabolism came when the H and L proteins of the Glycine Cleavage System (GCS), and the serine hydroxymethyltransferase protein (SHMT) were identified and characterised as hydrogenosomal (Mukherjee et al., 2006a, b). However the apparent absence of other proteins required in these pathways for glycine and serine metabolism left the role of these proteins undefined. Our proteomic data now show that in addition to these proteins, 10 other protein families involved in amino acid metabolism are present in the hydrogenosome (Fig. 5A, Supplementary Table S1).

Three of these proteins, phosphoserine aminotransferase (PSAT), methionine-γ-lyase (MGL) and cysteine synthase (CS), have been shown to be involved in the conversion of phosphohydroxypyruvate and homocysteine to cysteine in *T. vaginalis* (Westrop et al., 2006). CS localises to mitochondria and plastids in *Arabidopsis thaliana* (Hesse et al., 1999) and PSAT is found in mitochondria in spinach nodules (Hoa le et al., 2004). However, in *T. vaginalis* localisation of the pathway was predicted to be cytosolic based on the lack of classic presequences on these proteins. To further address whether this pathway exists in hydrogenosomes, C-terminally

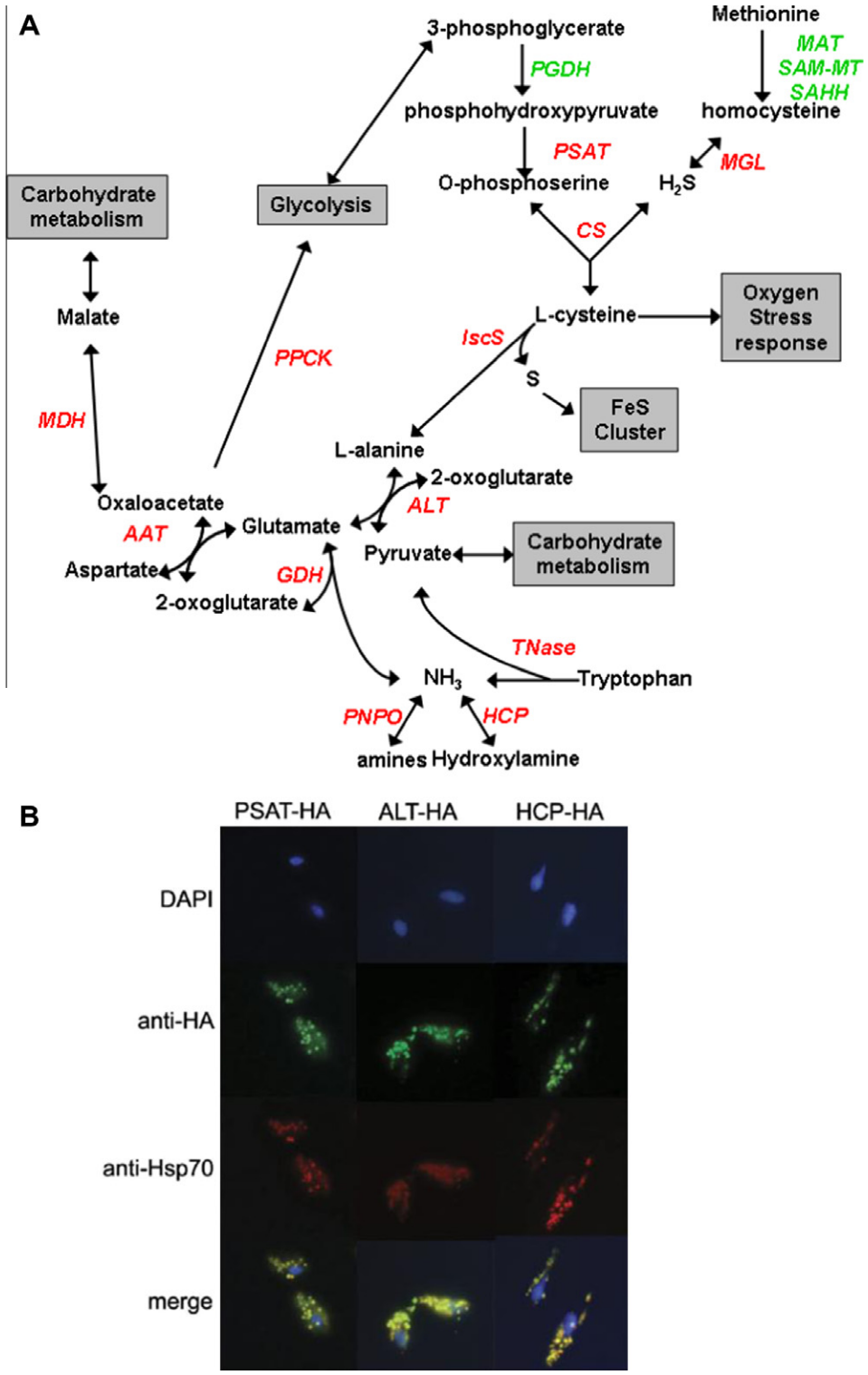

Fig. 5. Proteins involved in amino acid metabolism pathways identified in the *Trichomonas vaginalis* hydrogenosome proteome. (A) Proteins involved in amino acid metabolism pathways found in the hydrogenosome proteome are labelled red and proteins found in the genome are labelled green. Grey boxes list intersecting pathways. PGDH, phosphoglycerate dehydrogenase; PSAT. phosphoserine aminotransferase; CS, cysteine synthase; MGL, methionine-g-lyase; MAT, SAM-MT, SAHH, methionine adenosyltransferase, S-adenosyl-methioinine-dependent methyltransferase, S-adenosylhomocysteine hydrolase, respectively; IscS, iron sulphur cluster C protein; ALT, alanine aminotransferase; Tnase, tryptophanase; HCP, hybrid cluster protein; PNPO, pyridoxamine 5′-phophate oxidase; GDH, glutamate dehydrogenase; AAT, aspartate aminotransferase; MDH, malate dehydrogenase; PPCK, phosphoenolpyruvate carboxykinase. (B) Subcellular localisation of PSAT, ALT and HCP was confirmed using immunofluorescence. *Trichomonas vaginalis* cells were transformed with C-terminally HA-tagged PSAT, ALT or HCP and analysed as described in Section 2.7 (green). The hydrogenosome was stained with anti-Hsp70 (red). Merged images show co-localisation of PSAT-HA, ALT-HA and HCP-HA with Hsp70 in the hydrogenosome. Nuclei are stained with DAPI.

HA-tagged PSAT (PSAT-HA) was expressed in *T. vaginalis*. The resulting immunostaining showed that PSAT-HA colocalised with hydrogenosomal Hsp70, confirming its localisation in the organelle (Fig. 5B).

The cysteine generated by this pathway (Fig. 5A) is a substrate for IscS to generate alanine and molecular sulphur used in ISC formation (Tachezy et al., 2001; Sutak et al., 2004). Alanine can then be converted to glutamate by the four distinct alanine aminotransferases (ALT) present in the proteome. To confirm the presence of this enzyme in the organelle, a C-terminally HA-tagged protein (ALT-HA) was shown to co-localise with Hsp70 in transfected *T. vaginalis* (Fig. 5B). These data indicate that enzymes that provide a source of cysteine for the hydrogenosomal Fe–S biogenesis pathway are also localised in the organelle.

The *T. vaginalis* CS has been shown to utilise O-phosphoserine as well as the more common substrate, O-acetylserine, in the synthesis of cysteine in vitro (Westrop et al., 2006). However the genes required for the synthesis of O-acetylserine are absent from the *T. vaginalis* genome. The use of O-phosphoserine by the *T. vaginalis* CS is surprising as this was previously observed exclusively in mycobacteria and Archeae (Westrop et al., 2006; Agren et al., 2008). Additionally, CS has only been identified in a few mitochondria, whereas MGL is absent and PSAT has only been previously documented in one organism's mitochondrion (Hoa le et al., 2004).

Two additional enzymes involved in amino acid metabolism, aspartate aminotransferase (AAT) and glutamate dehydrogenase (GDH), that catalyse the reversible metabolism of aspartate, glutamate, oxoglutarate and oxaloacetate, were also present in the hydrogenosomal proteome (Fig. 5A, Supplementary Table S1). GDH would also allow for the conversion of NH_3, which is a by-product of a number of enzymes identified in the hydrogenosome proteome.

Compared with hydrogenosomes, human mitochondria harbour a larger number of amino acid metabolic pathways: 11 amino acids can be synthesized in human mitochondria and 17 can be metabolized (Guda et al., 2007). Very little, however, is known about amino acid metabolism in mitochondria of other organisms. In an effort to gain a better understanding of similarities and differences between *T. vaginalis* hydrogenosomes and mitochondria we compared our proteome and the mitochondrial proteomes of *H. sapiens*, *S. cerevisiae*, *T. brucei* and *T. thermophila* using KOBAS (Mao et al., 2005; Wu et al., 2006). We identified metabolic pathways for 16 amino acids in *H. sapiens*, 12 in *S. cerevisiae*, eight in *T. brucei*, nine in *T. thermophila* and six in *T. vaginalis* (Supplementary Table S6).

Comparison of the KOBAS results showed that the Ala, Asp, Glu pathway and the Gly, Ser pathway are conserved in the hydrogenosome and the majority of the mitochondria proteomes (Supplementary Table S6). Additionally, the mitochondrial proteomes of *H. sapiens* and *S. cerevisiae* reveal the presence of enzymes involved in cysteine metabolism, a feature shared with the hydrogenosome, albeit using different enzymes (Supplementary Table S6). In contrast, pathways that are present in the mitochondrial proteomes but absent from the hydrogenosome include the Val, Ile and Leu biosynthesis and degradation pathway; proline metabolism is also present in all of the mitochondrial proteomes but not the hydrogenosomal proteome (Supplementary Table S6). However, it is unlikely that *T. vaginalis* has lost these pathways as enzymes such as branched chain aminotransferase, known to play a major role in Val, Ile and Leu degradation, is present in the *T. vaginalis* genome. The differences in conservation of the Val, Ile, Leu and Pro pathways in the analysed mitochondrial proteomes but not the *T. vaginalis* hydrogenosomes suggests that these pathways play an essential role within mitochondria.

Conservation of the Ala, Asp, Glu, Gly and Ser pathways in both hydrogenosomes and mitochondria emphasises key similarities between the organelles, as does the presence of all or the majority of proteins involved in the GCS pathway (H-, L-, T-, P-proteins and SHMT proteins) in the hydrogenosome and mitochondrial proteomes. None of the current mitosome proteomes appear to contain amino acid metabolism pathways (Mi-Ichi et al., 2009; Jedelsky et al., 2011). A more in-depth analysis of the evolution of these pathways should provide a better understanding of the similarities and differences between these organelles.

3.7. Identification of hydrogenosomal membrane proteins

Eighty-nine proteins in the proteome were identified as potential membrane proteins based on TMHMM (Krogh et al., 2001), HMMTOP (Tusnady and Simon, 2001) and SPOCTOPUS (Viklund et al., 2008) analyses (Supplementary Table S7). Membrane proteins are difficult to identify by proteomic analyses due to hydrophobicity and solubility properties, therefore this subset of proteins is often under-represented (Santoni et al., 2000) and it is likely that many hydrogenosomal membrane proteins were not detected. The membrane proteins we have identified include both predicted translocases and carrier family proteins, as well as several hypothetical proteins (Supplementaryl Table S7).

3.7.1. Translocases

Only a few proteins found in the two membranes that surround the hydrogenosome have been characterised. Lacking a genome, all proteins involved in the metabolic pathways housed in the hydrogenosome must be imported. Proteins must traverse the outer membrane, the intermembrane space (IMS) and the inner membrane, and complexes containing multiple proteins have evolved to serve in these roles. As biochemical analyses of the translocation machinery of the *T. vaginalis* hydrogenosome are limited, we and others have adopted bioinformatics approaches to identify putative mitochondrial-like translocase proteins in the *T. vaginalis* genome (Table 2).

Saccharomyces cerevisiae mitochondrial proteins are initially recognised and transported across or into the outer membrane by the Translocase of the Outer Membrane (TOM) complex (Neupert and Herrmann, 2007; reviewed in Lithgow and Schneider, 2010). The import of outer membrane β-barrel proteins also requires an additional complex called the Sorting and Assembly Machinery (SAM) (Neupert and Herrmann, 2007; Lithgow and Schneider, 2010). Of the seven proteins that form the *S. cerevisiae* mitochondrial TOM complex, four related proteins similar to one of these were present in the hydrogenosome proteome (Table 2, Supplementary Table S1). These proteins share the porin-3 domain with Tom40 based on Pfam domain predictions (http://pfam.sanger.ac.uk). Caution is required in interpreting this, however, as the Tom40 protein is a member of the Pfam PF01459 porin-3 superfamily. The presence of this domain in a membrane protein does not necessarily imply that it is involved in protein import, as these domains are also found in mitochondrial voltage-dependent anion channels (VDAC). Functional data will be necessary to demonstrate its identity. A single porin-3-domain containing protein was identified in both mitosome proteomes (Mi-Ichi et al., 2009; Jedelsky et al., 2011). In *G. lamblia* it is postulated to act as both a general import pore for proteins as well as serving in ion exchange (Jedelsky et al., 2011). In trypanosome mitochondria, a single porin-3 protein is also present, but has been demonstrated functionally to be a VDAC (Pusnik et al., 2009).

In addition to the porin-3 domain containing proteins, the hydrogenosome proteome contains a protein that was previously identified as Sam50 by Hidden Markov Modeling (Table 2, Supplementary Table S1; Dolezal et al., 2006). No Sam50 was identified in the *G. lamblia* or *E. histolytica* mitosome proteomes (Mi-Ichi et al., 2009; Jedelsky et al., 2011), however a Sam50-like gene is present in the *E. histolytica* genome (Loftus et al., 2005) and the protein it encodes has been localised to the mitosome in *E. histolytica* via

Table 2
Comparative analyses of mitochondrial translocase proteins found in *Saccharomyces cerevisiae* and *Tetrahymena thermophilia* mitochondria, the *Trichomonas vaginalis* hydrogenosome and mitosomes of *Giardia lamblia* or *Entamoeba histolytica*.

Translocase complex member	*S. cerevisiae* mitochondria	*T. thermophilia* mitochondria	*T. vaginalis* hydrogenosome	*G. lamblia* & *E. histolytica* mitosome
Outer membrane proteins				
Tom70	X			
Tom40	X	X	X[a]	X[a]
Tom22	X	X		
Tom20	X			
Tom7	X	X		
Tom6	X			
Tom5	X			
Sam50	X	X	X	X[b]
Inner membrane proteins				
Tim50	X	X		
Tim44	X	X	X	
Tim21	X			
Tim17/22/23	X	X	X	
Motor complex proteins				
Pam18	X	X	X	X
Pam16	X	X	X	X

X indicates the presence of a homologue.
[a] These proteins have a porin-3 domain found in both Tom40 and VDAC. They have not been shown to function as a Tom40 biochemically.
[b] Not present in the *G. lamblia* and *E. histolytica* proteomes; however, this protein localises to the *E. histolytica* mitosome.

immunofluoresence (Dolezal et al., 2010). Unlike that observed for the *T. vaginalis* hydrogenosome and the *E. histolytica* mitosome, it appears that the *G. lamblia* mitosome lacks a Sam50 as the gene is absent from its genome (http://giardiadb.org/giardiadb/).

The presence of proteins with weak similarity to Tom40 raises the possibility that a very divergent TOM complex exists; however, screening for additional members of the TOM complex failed to identify any candidates (Table 2). The absence of TOM complex members suggests that the translocase machinery in the hydrogenosome may be distinct from that found in mitochondria or that similarity is insufficient for identification by bioinformatics. Biochemical analyses will therefore be critical in defining these complexes. We have previously described an abundant hydrogenosomal integral membrane protein of approximately 35 kDa, Hmp35 (TVAG_104250), found in a stable ~300 kDa complex, and with characteristics of a translocation pore (Dyall et al., 2003). This protein and two similar proteins, TVAG_031860 and TVAG_216170, were identified in the hydrogenosome proteome (Supplementary Table S1). The Hmp35 complex may function as an outer membrane protein translocase, acting in addition to or in place of Tom40 in these organelles.

In mitochondria, proteins are transferred from the TOM complex and across the IMS by soluble chaperone complexes (small translocase of the inner membrane (TIM) proteins), which include the Tim9–Tim10 and Tim8–Tim13 complexes (Neupert and Herrmann, 2007; Lithgow and Schneider, 2010). No small Tim proteins similar to those found in mitochondria IMS were found in the hydrogenosome proteome, nor have any been identified in the mitosome proteomes. The absence of these proteins in the hydrogenosome may reflect the paucity of inner membrane proteins found in hydrogenosomes, large sequence divergence in these small Tims, or they may simply be low abundance in hydrogenosomes. Their absence in mitosomes likely reflects the extreme reduction that has occurred in these organelles.

Two TIM complexes, the Tim23 complex and the Tim22 complex, exist in yeast mitochondria (Neupert and Herrmann, 2007; Lithgow and Schneider, 2010). The Tim23 complex mediates the translocation or membrane insertion of preproteins containing an N-terminal presequence (Neupert and Herrmann, 2007; Lithgow and Schneider, 2010). Matrix preprotein translocation is further assisted by the presequence translocase-associated motor (PAM) complex, which is localised on the matrix side of the Tim23 complex (Neupert and Herrmann, 2007; Lithgow and Schneider, 2010). The Tim22 complex mediates the insertion of inner membrane proteins containing internal signal sequences (Neupert and Herrmann, 2007; Lithgow and Schneider, 2010). Tim22, Tim17 and Tim23 are evolutionarily related and are grouped into the Tim17/22/23 protein family (Pfam PF02466).

Several putative homologues of the TIM and PAM complexes were identified in the hydrogenosomal proteome (Table 2, Supplementary Tables S1 and S5). Three putative homologues of the Tim 17/22/23 family are present in the *T. vaginalis* genome and all three were found in the hydrogenosome proteome, together with a Tim44 homologue and Pam16 (Dolezal et al., 2006). Together with the previously identified Pam18 like protein (Dolezal et al., 2005), many of the members of the Tim23 inner membrane complex and PAM complex have been identified via sequence similarity in *T. vaginalis*, although the similarity is very low (Dolezal et al., 2006). Again, further investigation will determine whether these putative hydrogenosomal translocase proteins are functionally equivalent to their mitochondrial counterparts, including whether they form separate complexes with different roles in substrate import.

This is in sharp contrast to *G. lamblia* mitosomes, which contain no Tim17/22/23 homologues (Table 2; Jedelsky et al., 2011). These organelles do however contain conserved Pam16 and Pam 18 proteins, as well as Hsp70, indicating that the motor complex is well-conserved evolutionarily across phyla and organelle types, despite considerable diversification (Table 2; Jedelsky et al., 2011). The mitosome of *E. histoytica* has only Hsp70 and entirely lacks any proteins with homology to Tim17/22/23 or Pam 16/18 (Table 2; Mi-Ichi et al., 2009).

3.7.2. Carrier family proteins

Another abundant membrane protein, Hmp31 (TVAG_237680), which is phylogenetically related to the mitochondrial carrier protein family (MCF), and two Hmp31 paralogs were present in the proteome (Table 1; Dyall et al., 2000). BLAST analyses indicate that these proteins, TVAG_051820 and TVAG_262210, share 49% and 41% sequence identity at the amino acid level and 69% and 62% sequence similarity, respectively, with Hmp31, although their function has not been assessed. Three additional MCF proteins were also found. This protein family is responsible for transporting a variety of molecules, including ADP/ATP, glutamate, thiamine

pyrophosphate and succinate/fumurate, across the mitochondrial inner membrane (Kunji, 2004). Compared with the MCFs in *Arabidopsis thaliana* (45) and yeast (35), there are relatively few MCFs in *T. vaginalis*, most likely due to the fewer metabolites produced by a reduced number of metabolic pathways in the hydrogenosome. Further investigation will be required to determine whether these proteins can function as MCFs, as possible aspartate/glutamate or oxodicarboxylate carriers or as a malate-aspartate shuttle system.

Both the *G. lamblia* and *E. histolytica* mitosomes contain transporters. *Giardia lamblia* contains seven; three members of the major facilitator superfamily and four ABC transporter family proteins (Jedelsky et al., 2011). The *E. histolytica* mitosome is further reduced, containing only four distinct transporter proteins; an ADP/ATP transporter, a sodium/sulphate symporter, an ABC transporter and a phosphate transporter (Chan et al., 2005; Mi-Ichi et al., 2009; Dolezal et al., 2010).

3.8. Characterisation of the hydrogenosomal presequence

Many hydrogenosomal matrix proteins contain cleavable N-terminal presequences thought to be necessary for targeting proteins to the organelle (Johnson et al., 1990, 1993; Lahti et al., 1992, 1994; Lange et al., 1994; Hrdy and Muller, 1995a; Bui and Johnson, 1996; Bradley et al., 1997; Mentel et al., 2008). Analyses of a limited subset of presequences revealed that they typically contain hydroxylated amino acids (Ser and Thr) and Leu residues, and a conservation of an Arg residue at the -2 or -3 position relative to the cleavage site; these features are also common in mitochondrial presequences, although hydrogenosomal presequences are typically much shorter than those of mitochondrial targeted proteins (Dyall and Johnson, 2000). Several mitosome-targeted proteins also contain presequences; these are even shorter than those found on hydrogenosome-bound proteins but also contain hydrophobic amino acids (Burri and Keeling, 2007).

A search of the *T. vaginalis* genome for genes encoding putative hydrogenosomal matrix proteins identified 138 proteins with a predicted presequence, 53 of which encode hypothetical proteins (Carlton et al., 2007). Putative sequence motifs compiled from these data identified the motifs M(L/I)(S/T/A/C/G/Q/K/N) or M(S/T)(L/I) at the N-terminus and R(N/F/XF/S/A/I/E/G/Q/Y/XY) within the first 20 amino acid residues of the protein. These motifs were used to search the hydrogenosome proteome. Ninety-five of the 569 proteins (81 known and 14 hypothetical) had matching N-terminal sequences (Supplementary Table S8). These data indicate that either the majority of hydrogenosomal proteins do not possess a presequence or that the parameters used to define the presequence are too stringent. Proteins have been demonstrated to be targeted to the *T. vaginalis* hydrogenosome in the absence of an N-terminal presequence and only a few putative presequences have been experimentally validated as targeting signals, leaving the role of a presequence unclear (Mentel et al., 2008). It is notable that ~1/3 of mitochondrial matrix proteins lack an identifiable targeting signal and many mitosome targeting signals appear to be dispensable (Gakh et al., 2002; Burri and Keeling, 2007). An explanation for the absence of presequences is the presence of yet-to-be-defined internal signal sequences for matrix-targeted proteins. The combination of bioinformatics and proteomics will be helpful in teasing out the requirements of protein translocation, possibly redefining the presequence motif and identifying putative internal targeting sequences.

A comparison of the predicted presequences of paralogous proteins in the proteome revealed that while some contain the predicted Arg at the −2 or −3 position, others have a Lys residue at this position. This exchange was found in IscS, Hsc20, flavodoxin protein (Wrba-like), MRP protein and in ALT paralogs (Supplementary Table S8). A presequence search substituting a Lys for Arg in the motif above identified 18 additional proteins with putative presequences. The binding pocket of the β subunit of the hydrogenosomal processing peptidase (HPP) which binds presequence substrates is large enough to accommodate varied presequences and should permit a Lys residue to be exchanged for an Arg residue (Brown et al., 2007; Smid et al., 2008). Experimental data testing targeting and cleavage of such a presequence will be necessary to determine whether a Lys at the −2 or −3 position is permitted.

To determine an average length and amino acid composition of predicted hydrogenosomal presequences and the residues immediately downstream of the cleavage site we performed a LOGOs analysis on the first 25 amino acids of these proteins containing a predicted presequence with either Arg or Lys at the −2 or −3 position (Supplementary Fig. S1A) (http://weblogo.berkeley.edu/logo.cgi). The average length of the putative hydrogenosomal presequence was found to be 8–10 amino acids, with 84% of the presequences being ⩽14 residues in length (Supplementary Fig. S1A). Hydroxylated residues, specifically Ser, and hydrophobic residues such as Ala, Phe, Ile and Leu were common in the first seven residues. The majority of the N-terminal presequences also started with MLS, which is strikingly similar to the consensus N-terminus of targeting signals in *T. thermophila* mitochondrial matrix protein presequences (MLSK) (Smith et al., 2007). The aligned predicted cleavage sites reveal that Phe and Asn are preferred at the −1 position relative to the cleavage site and hydrophobic and hydroxylated residues are preferred at positions +1 and +2 (Supplementary Fig. S1B).

3.9. Identification of externally associated proteins

The purified hydrogenosome fractions used to determine the proteome were not subjected to mild protease treatment in order to remove proteins associated with the outer surface of the organelle, thus allowing for the identification of hydrogenosome-associated proteins. Approximately 22% of the proteome (124 proteins) are likely to be externally associated based on the assigned localisation and properties of these proteins in other organisms (Supplementary Table S2). This group includes proteins that share sequence similarity with glycolytic enzymes, ribosomal proteins, cytosolic chaperones and cytoskeletal proteins (Fig. 1). Similar externally associated proteins are also found in mitochondrial proteomes (Giege et al., 2003; MacKenzie and Payne, 2004; Ohlmeier et al., 2004; Anesti and Scorrano, 2006; Brandina et al., 2006; Graham et al., 2007). Moreover, the presence of cytoskeletal proteins in the hydrogenosomal proteome is expected as hydrogenosomes can be seen closely associated with components of the cytoskeleton in electron micrographs (Benchimol, 2009).

Although the presence of glycolytic enzymes may have been expected, the presence of the entire glycolytic pathway in this proteome is unprecedented (Supplementary Table S2). Glycolytic enzymes were reported in multiple mitochondrial proteomes including those of *A. thaliana* (Heazlewood et al., 2004), *S. cerevisiae* (Reinders et al., 2006), human heart (Taylor et al., 2003) and *T. thermophila* (Smith et al., 2007), although typically only a few members of the pathway were found. In contrast, four of the eight glycolytic enzymes in the hydrogenosomal proteome are found in multiple isoforms (Pi-dependent fructose 6-P 1-phosphotransferase, pyruvate dikinase, phosphofructokinase and pyruvate kinase) (Supplementary Table S2). *Trichomonas vaginalis* genes encoding both pyrophosphate and ATP-dependent glycolytic enzymes have been previously documented (Slamovits and Keeling, 2006) and the presence of enzymes of both types in the proteome indicate their co-localisation within the cell. The presence of glycolytic proteins in mitochondrial proteomes was originally thought to be contamination, however recent studies have shown that glycolytic proteins form complexes on the surface of these

organelles (Giege et al., 2003; Graham et al., 2007). Our data indicate that this may also be the case for the hydrogenosome.

Ribosome and ribosome-associated proteins were found to constitute 11% of the proteome (Fig. 1, Supplementary Table S2). The association of ribosomal proteins with the hydrogenosome has not been previously noted, however mammalian mitochondria are known to associate with cytosolic ribosome populations (MacKenzie and Payne, 2004). It is notable that the *T. vaginalis* genome contains unusually large ribosomal protein gene families, the expression of which may contribute to the large number of ribosomal proteins associated with hydrogenosomes (Lal et al., 2005).

Another group of proteins that appear to associate with the hydrogenosome are small GTPase proteins (Fig. 1, Supplementary Table S1). GTPase and associated proteins represent 24% of the hydrogenosomal proteome, which is a significantly higher proportion than the 6% found in the yeast mitochondrial proteome (Sickmann et al., 2003). In mitochondria GTPases and associated proteins are known to be involved in fusion, fission, motility and maintenance of inner membrane architecture (Rube and van der Bliek, 2004; McBride et al., 2006). For example, the mitochondrial Rab protein, Ypt11p, has been shown to be involved in mitochondrial retention and inheritance and dynamin related proteins (DRPs) are involved in fission (Frederick et al., 2004; Rube and van der Bliek, 2004; Boldogh and Pon, 2007). It is possible that these hydrogenosomal proteins carry out similar functions. However, with the sheer number of GTPases identified it is also likely that they have other unknown roles or are merely organelle-associated and do not function within the hydrogenosome.

3.10. Conclusions

The work described here has greatly added to our knowledge of the hydrogenosome of *T. vaginalis*. Previously thought to play a role in only a few metabolic pathways, these analyses indicate the *T. vaginalis* hydrogenosome to be a far more complex organelle. In-depth phylogenetic analyses of pathways identified in the proteome should allow for a better understanding of the evolution of this organelle. Determining whether these proteins and pathways also exist in the hydrogenosomes of fungi and ciliates will provide a better understanding of the evolution of these divergent organelles. Currently, proteomes for the mitosomes of both *G. lamblia* and *E. histolytica* are available (Mi-Ichi et al., 2009; Jedelsky et al., 2011). These proteomes indicate that the mitosome family of mitochondrial-related organelles are substantially more reduced than the hydrogenosome. Further studies made possible by comparison of organellar proteomes will serve to broaden our knowledge of the various functions and biogenetic properties that both unify and differentiate eukaryotic organelles.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2011.10.001.

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