Fermentation enzymes of *Giardia intestinalis*, pyruvate:ferredoxin oxidoreductase and hydrogenase, do not localize to its mitosomes

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It is becoming increasingly clear that the so-called remnant organelles of microaerophilic unicellular eukaryotes, hydrogenosomes and mitosomes, are significantly reduced versions of mitochondria. They normally lack most of the classic mitochondrial attributes, such as an electron transport chain and a genome. While hydrogenosomes generate energy by substrate-level phosphorylation along a hydrogen-producing fermentation pathway, involving iron–sulfur-cluster-containing enzymes pyruvate:ferredoxin oxidoreductase (PFO) and hydrogenase, whether mitosomes participate in ATP synthesis is currently unknown. Both enzymes were recently described in the mitosome-bearing diplomonad *Giardia intestinalis*, also shown to produce molecular hydrogen. As published data show that giardial PFO is a membrane-associated enzyme, it could be suspected that PFO and hydrogenase operate in the mitosome, in which case the latter would by definition be a hydrogenosome. Using antibodies against recombinant enzymes of *G. intestinalis*, it was shown by Western blot analysis of subcellular fractions and by confocal immunofluorescence microscopy of whole cells that neither PFO nor hydrogenase localize to the mitosome, but are mostly found in the cytosol. The giardial mitosome is known to play a role in iron–sulfur cluster assembly and to contain chaperones Cpn60 and mtHsp70, which assist, in particular, in protein import. In mitochondria, transmembrane potential is essential for this complex process. Using MitoTracker Red and organelle-specific antibodies, transmembrane potential could be detected in the *Trichomonas vaginalis* hydrogenosome, but not in the *G. intestinalis* mitosome. These results provide further evidence that the *Giardia* mitosome is one of the most highly reduced mitochondrial homologues.

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INTRODUCTION

As described in the literature, the main function of mitochondria is aerobic respiration. Indeed, the ATP yield of aerobic respiration approaches 34 moles per mole of glucose utilized (Saraste, 1999), while a ubiquitous glycolysis (Embden–Meyerhof–Parnas pathway) produces only 2 moles (Dandekar *et al.*, 1999). Along with aerobic mitochondria, which use molecular oxygen as a terminal electron acceptor, versatile mitochondria exist, which also use alternative electron acceptors such as fumarate and oxidized nitrogen compounds (Tielens & Van Hellemond, 1998; Takaya *et al.*, 1999). Mitochondria are also involved in various metabolic processes, such as iron–sulfur cluster

assembly, which is an essential mitochondrial function in yeast (Lill & Mühlenhoff, 2008).

Mitochondria are convincingly argued to have originated once in evolution from within the alpha subdivision of Proteobacteria (Gray *et al.*, 1998). Numerous phylogenetic data point to the order *Rickettsiales* of obligate endosymbionts as a taxonomic source of organelles (Emelyanov, 2001, 2003a, b; Fitzpatrick *et al.*, 2006; Williams *et al.*, 2007). Many unicellular eukaryotes, either parasitic or inhabiting niches with little oxygen, were once thought to lack mitochondria. For a long time, these species were classified into the kingdom *Archaezoa*, a paraphyletic group of organisms whose lack of mitochondria was deemed to be a primitive condition (Cavalier-Smith, 1987). Some of them, such as the Archamoeba *Entamoeba histolytica* and many ciliates, nevertheless branch on phylogenetic trees with eukaryotes, bearing typical mitochondria, thus suggesting that they have a secondarily amitochondriate nature (Embley, 2006; Embley & Martin, 2006). Among the most enigmatic *Archaezoa* are parabasalids, such as

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Abbreviations: DIC, differential interference contrast; ER, endoplasmic reticulum; FCCP, 4-(trifluoromethoxy)-phenylhydrazon; IFA, immunofluorescence analysis; PFO, pyruvate:ferredoxin oxidoreductase.

Trichomonas vaginalis, and diplomonads, exemplified by *Giardia intestinalis*, whose phylogenetic position on eukaryotic trees has not been defined unequivocally (Arisue *et al.*, 2005; Keeling *et al.*, 2005; Hampl *et al.*, 2009). Nowadays, a wealth of molecular data has overturned the Archezoa concept in that all the known extant Archezoa, including the two above and some other enigmatic protists, have been shown to contain mitochondrion-derived organelles (Tovar *et al.*, 2003; Embley & Martin 2006; Hampl *et al.*, 2008). First, it was found that *T. vaginalis* and *G. intestinalis* possess components of the ISC system of iron–sulfur cluster assembly (Tachezy *et al.*, 2001), which is characteristic of mitochondria (Lill & Mühlenhoff, 2008). Phylogenetic analysis of cysteine desulfurase (IscS), which is an essential component of the ISC, affiliated monophyletic eukaryotes, including these protists, with the order *Rickettsiales* to the exclusion of free-living alphaproteobacteria, suggesting that diplomonad ancestors possessed bona fide mitochondria (Emelyanov, 2003a). At the same time, a tiny remnant organelle, called a mitosome, was revealed in *G. intestinalis*, based on localization of the components of the ISC system and their co-localization with bacterial-type chaperones (Tovar *et al.*, 2003; Regoes *et al.*, 2005). The presence and phyletic affiliation of the ISC system with mitochondria as well as its hydrogenosomal localization were also shown for *T. vaginalis* (Sutak *et al.*, 2004), which was already known to contain a hydrogenosome, an organelle producing molecular hydrogen via the fermentation pathway and generating ATP (Müller, 1993). Second, hydrogenosomes, mitosomes and classic yeast mitochondria were shown to possess a conserved protein import pathway (Bradley *et al.*, 1997; Dolezal *et al.*, 2005; Regoes *et al.*, 2005).

The reduced organelles of mitochondrial origin described above lack the electron transport chain and a genome (Embley & Martin, 2006). Their energy metabolism relies exclusively on glycolysis and fermentation (Müller, 2003). In hydrogenosomes, pyruvate undergoes oxidative decarboxylation by pyruvate:ferredoxin oxidoreductase (PFO) yielding acetyl-CoA and reduced ferredoxin. Iron-only hydrogenase reoxidizes the latter, coupled to reduction of protons (Müller, 1993, 2003). As in some mitochondria (Van Hellemond *et al.*, 1998), ATP is generated via the concerted action of two enzymes. Acetyl:succinate CoA transferase converts acetyl-CoA to succinyl-CoA. Typically a mitochondrial enzyme, succinyl-CoA synthetase, also known as succinate thiokinase (a component of the Krebs cycle), hydrolyses succinyl-CoA coupled with ATP synthesis (Müller, 1993; van Grinsven *et al.*, 2008). Energy metabolism of hydrogenosomal eukaryotes like *Trichomonas* was called type II metabolism (Müller, 2003). In *Giardia*, which possesses type I metabolism (Müller, 2003), acetyl-CoA and reduced ferredoxin are also produced through the action of PFO, but reduced ferredoxin has long been thought to reoxidize via a different route (Townson *et al.*, 1996). This sort of energy production was ascribed to the cytoplasm in agreement with the first data on the cytosolic

localization of PFO in *Giardia* (Lindmark, 1980). It should be noted that neither mitosome nor hydrogen gas production by *G. intestinalis* was known at that time. Remarkably (and of unknown significance), unlike in organisms with hydrogenosomes, acetyl-CoA is directly converted to acetate by the action of ADP-forming acetyl-CoA synthetase coupled with ATP synthesis in mitosome-bearing protists (Sánchez *et al.*, 2000; Hjort *et al.*, 2010).

Subsequently, *G. intestinalis* PFO was reported to be a membrane-associated enzyme (Ellis *et al.*, 1993; Townson *et al.*, 1996). Subsequently, *Giardia* was shown to generate molecular hydrogen. The amount of hydrogen produced is one-tenth of that of *T. vaginalis* per cell (Lloyd *et al.*, 2002a), i.e. in view of cell size difference (Müller, 2003) these protists may produce comparable amounts of hydrogen per unit of cell volume. Notably, unlike *Trichomonas*, *G. intestinalis* does not possess hydrogenase maturation enzymes, which are normally essential for hydrogenase activity (Pütz *et al.*, 2006). Two genes encoding putative iron-only hydrogenases were also found in a *G. intestinalis* genome (Morrison *et al.*, 2007). Taken together, these data raise the question of whether PFO and hydrogenase localize to the giardial mitosome, i.e. whether it is a form of hydrogenosome. To address this issue, we cloned and overexpressed *Giardia* PFO and hydrogenase in *Escherichia coli*, and raised polyclonal (monospecific) antibodies against purified recombinant proteins. These were used in co-localization experiments and Western blot analysis of subcellular fractions. As organellar markers, antibodies against giardial IscS and Cpn60, which are known to localize to the mitosome (Tovar *et al.*, 2003; Dolezal *et al.*, 2005; Regoes *et al.*, 2005), were used. Using MitoTracker Red and organelle-specific antibodies, we also searched for transmembrane potential in the *Giardia* mitosome. A similar search for hydrogenosomal potential was also done for *T. vaginalis* to be compared with *G. intestinalis*, given that published data did not identify the organelle itself using hydrogenosome-specific molecular markers (Humphreys *et al.*, 1998).

METHODS

Growth of *G. intestinalis* and *T. vaginalis*. *G. intestinalis* strain WB trophozoites were propagated in TYI-S-33 medium, which contained 0.05 % bovine bile, antibiotics (0.1 mg penicillin ml⁻¹ and streptomycin) and 10 % (v/v) heat-inactivated bovine serum (Tachezy *et al.*, 2001). For immunofluorescence analysis (IFA), the cells were grown in either 2 ml vials or 7 ml plastic tubes to form a subconfluent layer (48 h at 37 °C). For preparative growth, a 2 ml plastic vial with a confluent layer was chilled in an ice bath for 20 min and the material (2 × 10⁶ cells ml⁻¹) was transferred to a 50 ml sterile flask containing complete TYI-S-33 medium. After 48 h of growth, when the confluent layer was formed, the flask was chilled as above and its contents (2–3 × 10⁶ cells ml⁻¹) were transferred to two triple flasks (Nunc, approximately 0.75 l each). A confluent layer of *Giardia* cells was typically reached after 48–60 h incubation at 37 °C. After cooling, the cells (1.5–2 × 10⁶ cells ml⁻¹) were pelleted at 900 g for 8 min at 4 °C followed by washing twice with 10 min centrifugations with HSDP buffer composed of 10 mM HEPES-KOH pH 7.2, 0.25 M sucrose,

4 mM DTT, 0.1 mM PMSF, 10 µg leupeptin ml⁻¹ and 50 µg *N*-tosyl-L-lysine chloromethyl ketone ml⁻¹. Finally, the cells were suspended in 6 ml HSDP and snap-frozen in liquid nitrogen as 3 × 2 ml aliquots. *T. vaginalis* (strain G3) cells were grown in modified Diamond's medium at 37 °C (Hirt *et al.*, 2003) in 7 ml plastic tubes, till cell density reached 5 × 10⁵ c.f.u. ml⁻¹.

Generation of antisera to *G. intestinalis* proteins. *G. intestinalis* PFO (GenBank accession no. L27221) and Fe-only hydrogenase (AF242293) genes were amplified by PCR from genomic DNA using designed primers (sequences available from the authors on request) and cloned into expression vector pET-16b (Novagen). The constructs were confirmed by sequencing and transformed into *E. coli* BL21 cells. Full-length PFO and hydrogenase were expressed as recombinant His-tagged proteins, and the resulting inclusion bodies were partially purified by using BugBuster reagent (Novagen). The proteins were then purified by a preparative PAGE. Hydrogenase (0.25 mg) and PFO (1.5 mg) were used for the commercial production of rat and rabbit polyclonal antisera (Eurogentec), respectively. Cloning and production of monospecific antibody against *Giardia* Cpn60 were performed in a similar way.

Measurement of organellar transmembrane potential. Subconfluent cultures of *G. intestinalis* in three 2 ml sterile vials were treated (protected from light) as follows. 4-(Trifluoromethoxy)-phenylhydrazine (FCCP, Sigma-Aldrich) was added from 80 mM stock solution in acetone to one tube at a final concentration of 100 µM, followed by 1 h incubation at 37 °C. MitoTracker Red was added from 40 µM stock solution in DMSO to each tube at a final concentration of 0.5–1.5 µM, and incubation was continued for 1 h. Tubes were chilled in an ice bath for 20 min, centrifuged at 4 °C for 4 min at 900 g, and washed once with ice-cold PBS with recentrifugation. All subsequent treatments were carried out at room temperature. Each sample was suspended in 0.5 ml PBS, and 0.5 ml 6% formaldehyde in PBS was added to each tube. Fixation was continued at room temperature for 30–40 min followed by washing with PBS once with centrifugation for 4 min at 900 g. The cells were incubated for 5 min in 0.5 ml PBS containing 0.1 M glycine, and permeabilized for 20 min in 0.5 ml PBS containing 2% BSA and 0.2% Triton X-100. After washing with PBS with centrifugation as above, the cells were suspended in 0.2 ml PBS and attached to poly-L-lysine-coated slides of eight-well glass slides (Nunc) for 1 h. The cells were then blocked by using 2% BSA for 2.5 h (Regoes *et al.*, 2005; Stefanic *et al.*, 2006). Rabbit anti-IscS (1:400) or anti-Cpn60 (1:100), and anti-rabbit secondary antibody conjugated to Alexa-fluor 488 (Molecular Probes) were diluted in PBS containing 2% BSA and 0.2% Triton X-100. In control experiments with non-organellar markers, rabbit anti-PFO (1:150) or rat anti-hydrogenase (1:200) was used. Incubation with each antibody proceeded for 1 h, with three washes between incubations with PBS containing 0.5% BSA and 0.05% Triton X-100 (Stefanic *et al.*, 2006). Finally, the samples were washed with PBS, mounted with DAPI-containing anti-fade mounting reagent (Vectashield), and observed under a laser scanning confocal microscope (Leica, TCS SP2 UV) fitted with a ×63 objective (Plan Apo, 1.32 nA). Images were collected using LCS V2.61 software (Leica Microsystems) and processed with Adobe Photoshop CS2. After treatment with FCCP and MitoTracker Red (0.5 µM final concentration) or MitoTracker Red alone (as above but in 7 ml plastic tubes), *T. vaginalis* cells were pelleted at 350 g for 5 min at 4 °C, washed once with potassium PBS (PPBS, 10 mM potassium phosphate pH 7.2 and 0.15 M NaCl), and fixed for 15 min at room temperature in the same buffer containing 3% formaldehyde. After two washes with PPBS with centrifugations as above, the cells were permeabilized for 10 min at room temperature in PPBS with 0.2% Triton X-100. The cells were washed once with PPBS, suspended in the same buffer and attached for 1 h to glass slides, as for *Giardia*. The samples were treated with 0.1 M glycine, and blocked in 5% BSA for 0.5 h. Primary and secondary antibodies were diluted in

PPBS containing 2% BSA. Rabbit antibody against hydrogenosomal malic enzyme and monoclonal antibody against non-organellar marker α -tubulin (Delgado-Viscogliosi *et al.*, 1996) were diluted 1:500 and 1:1000, respectively. *T. vaginalis* cells were washed three times with PPBS after 1 h incubations with antibodies (Hirt *et al.*, 2003). The samples were mounted for IFA as above.

Immunolocalization of PFO and hydrogenase in *G. intestinalis*.

Trophozoites of *G. intestinalis* were grown in 7 ml plastic tubes and prepared for confocal microscopy essentially as described above. Incubation with primary antibody (rabbit anti-IscS or anti-Cpn60) was followed by incubation with anti-rabbit secondary antibody conjugated to Alexa-fluor 594 (Molecular Probes), and subsequent treatment with a second (rat anti-hydrogenase) primary antibody was followed by incubation with anti-rat secondary antibody conjugated to Alexa-fluor 488 (Molecular Probes). As anti-PFO antibody was raised in rabbits, we first purified anti-IscS immunoglobulins using the Melon IgG spin purification kit (Pierce) and then biotinylated these with the EZ-link Sulfo NHS-LC biotinylation kit (Pierce) to enable detection via the streptavidin conjugate (streptavidin–Alexa 488, Invitrogen).

Purification of *G. intestinalis* mitochondria. All steps were done at 4 °C unless otherwise stated. Cell suspension (2 ml) from liquid nitrogen (see above) was thawed, and 5 ml HSDP buffer was added, followed by ultrasound treatment for 15 s in an ice bath. The completeness of cell breakage was monitored by light microscopy. The suspension was centrifuged at 3000 g for 20 min to remove nuclei and cell debris. An aliquot for electrophoresis and Western blot analysis was saved as the S3 fraction. Post-nuclear supernatant (7 ml) was centrifuged at 5600 g for 0.5 h. The supernatant was ultracentrifuged at 50 000 g for 1.5 h. The resulting pellet was resuspended in 7 ml HSDP and resedimented at 100 000 g for 1 h. The resulting pellet was resuspended in 0.7 ml HSDP, and an aliquot was saved as P50. The rest was layered on a 10–35% (w/v) linear Nycodenz (Axis-Shield) density gradient, prepared with HSDP in an Ultra-Clear centrifuge tube, and ultracentrifuged at 155 000 g for 2 h. Material of the second band from the top (approx. 16% Nycodenz) was diluted seven times with HSDP and pelleted at 175 000 g for 1 h. The supernatant obtained above after the centrifugation at 50 000 g (S50) was centrifuged in parallel, and the supernatant was saved as S175. Pelleted material of fraction 2 of the linear Nycodenz gradient was resuspended in 0.2 ml HSDP, loaded onto a discontinuous 15, 17.5 and 20% (w/v) Nycodenz gradient, and ultracentrifuged at 199 000 g for 1.5 h. Material from the 15/17.5% interphase was diluted seven times with HSDP, pelleted at 150 000 g for 1 h, loaded onto a second discontinuous Nycodenz gradient (13.5, 16, 18.5%) and centrifuged again at 150 000 g for 1 h. Material from the 13.5/16% interphase was diluted seven times with HSDP, pelleted at 150 000 g for 1 h, resuspended in 0.1 ml HSDP, divided into four aliquots of 25 µl, quickly frozen in liquid nitrogen, and stored at –80 °C as the mitochondrial fraction. All fractions were analysed by Western blot using antibodies against mitochondrial markers IscS and Cpn60. Contamination of mitochondrial material with endoplasmic reticulum (ER) was checked by using monoclonal antibody against the protein disulfide isomerase-2 (PDI-2) (Stefanic *et al.*, 2006).

Fractionation of giardial cells by differential centrifugation. All procedures were done essentially as described above. S3 supernatant was centrifuged in an 80 Ti rotor at 5600 g for 0.5 h, and the pellet (P6) washed with 7 ml HSDP with recentrifugation, while the supernatant (S6) was centrifuged for 1 h at 50 000 g. Subsequent ultracentrifugations at 100 000 and 190 000 g were carried out for 1 h, and each pellet was washed with recentrifugation to remove trapped material. To keep the correct supernatant/pellet ratio, 10 µl of each supernatant (7 ml) and 1 µl of each resuspended pellet (0.7 ml) were taken for SDS-PAGE followed by immunoblotting. Monoclonal antibodies against PDI-2 (membrane-associated ER enzyme) and PDI-1 (enzyme of ER lumen) served as ER markers (Stefanic *et al.*, 2006).

SDS-PAGE and Western blotting. Proteins of whole cells, centrifugation pellets and supernatants, and partially purified mitochondria were separated by SDS-PAGE, as described by Laemmli (1970). They were transferred from gels onto nitrocellulose membranes using a semi-dry transfer unit, followed by staining with Ponceau S (Sigma, diluted five times with water). Membranes were processed following standard protocols. They were destained, blocked with 5 % skimmed milk (Merck), and incubated with the anti-IscS (1 : 6000), rabbit anti-Cpn60 (1 : 200), rabbit anti-PFO (1 : 1000), rat anti-hydrogenase (1 : 300), mouse anti-PDI-2 (1 : 400 000) or anti-PDI-1 (1 : 200 000) antisera, followed by secondary anti-rabbit, anti-rat or anti-mouse antibodies conjugated to peroxidase (Sigma). The blots were developed using enhanced chemiluminescence (Amersham Biosciences).

RESULTS

Non-mitosomal localization of *G. intestinalis* fermentation enzymes

Using laser scanning confocal immunofluorescence microscopy, co-localization of hydrogenase with IscS

(Fig. 1a) and Cpn60 (not shown) was not found. Similarly, PFO did not co-localize with IscS (Fig. 1b), and PFO and hydrogenase did not co-localize with each other (Fig. 1c). Both PFO and hydrogenase are seen as rather scarce cytosolic enzymes, which may be a reason for the absence of co-localization signal. Notably, both enzymes are also partially present in very small aggregates, which may represent some unknown organelles. At any rate, neither enzyme localized to clearly visible mitochondria (Fig. 1a, b).

In immunoblot analysis, both enzymes were detected in whole cells and high-speed supernatants of the cell lysate, but not in the 50 000 g pellet and Nycodenz density gradient fractions, which are highly enriched in the mitochondria (Fig. 2a, b). When additional subcellular fractions were prepared using a higher centrifugation speed, PFO and (to a much lesser extent) hydrogenase were shown to partition between the 190 000 g pellet and supernatant; however, neither was found in the 100 000 g

Fig. 1. Cellular localization of *G. intestinalis* Fe-only hydrogenase (a) and PFO (b), and their co-localization (c) *in situ* using fluorescence confocal microscopy. (a) The polyclonal antiserum against mitochondrial marker cysteine desulfurase IscS (red) labels discrete structures reminiscent of *Giardia* mitochondria within the cytosol (Tovar *et al.*, 2003; Dolezal *et al.*, 2005; Regoes *et al.*, 2005), while the rat polyclonal antiserum raised against hydrogenase (green) shows diffuse and patchy distribution. No co-localization is observed. (b) Biotinylated antibody (1 : 50) to IscS (green) does not co-localize with PFO (red). (c) PFO (red) and hydrogenase (green) do not co-localize with each other. The diplomonad's nuclei are stained with DAPI (blue). Differential interference contrast (DIC) images of the same fields are also shown.

(or less) pellet (Fig. 3). This observation may reconcile earlier data, showing that PFO localizes to the cytosol in one case (Lindmark, 1980) or that 70 % of PFO associates with membrane fractions in other studies (Ellis *et al.*, 1993; Townson *et al.*, 1996). It is not clear from which cellular membrane the structures sedimentable at 190 000 *g* are derived, but it is unlikely to be the ER, given that the 50 000 *g* pellet contains marker proteins for ER but no signal for PFO and hydrogenase. Accordingly, P190 enriched in PFO contains only trace amounts of ER marker PDI-2 (Fig. 3). This partial membrane localization of fermentation enzymes may be consistent with the observation of small aggregates in IFA (see above). PFO and hydrogenase may, however, be contained in different structures, given that P190 contains only trace amounts of hydrogenase but 50 % PFO. This fact may explain the absence of co-localization of PFO and hydrogenase. In contrast with fermentation enzymes, mitochondrial markers IscS (Fig. 2c) and Cpn60 (Figs 2c and 3) were detected in the 50 000 *g* pellet and partially purified mitosome.

Taken together, these data strongly suggest that the mitosome of *G. intestinalis* is not a compartment of H₂-evolving fermentation, which seems to proceed mostly in the cytoplasm instead.

If present, the mitochondrial transmembrane potential of *Giardia* is below the detection limit

While PFO and hydrogenase are non-mitosomal enzymes, the giardial mitosome still needs to import at least the components of the ISC system and chaperones mtHsp70 and Cpn60 (Dolezal *et al.*, 2005; Regoes *et al.*, 2005). Many preproteins imported by classic mitochondria contain amino-terminal cleavable presequence, mitochondrion-targeting sequence, with basic residues at the extreme N terminus (Mokranjac & Neupert, 2008). However, it is not very clear whether positive charge is required for electrophoretic transport across the polarized inner membrane or for binding to a specific receptor, or both.

A transmembrane potential is known to be a prerequisite for import of most mitochondrial proteins in yeast (Mokranjac & Neupert, 2008).

We undertook a search for organellar potential by confocal fluorescence microscopy using MitoTracker Red and organelle-specific antibodies. MitoTracker Red, also called CMXRos (Invitrogen), was chosen because it was shown to be a more reliable tracer of potential in classic mitochondria compared with many other fluorescent dyes and to be fixed better than, for example, rhodamine 123 (Pendergrass *et al.*, 2004). As high transmembrane potential is not expected in mitochondrion-derived organelles, control experiments involved FCCP to depress any putative potential, to be compared with samples without protonophore treatment. For the same comparison purpose, other controls included MitoTracker and antibodies against non-organellar proteins. The data presented show that the hydrogenosome of *T. vaginalis* does possess transmembrane potential (Fig. 4a). Even after pretreatment of *Trichomonas* culture with FCCP, some co-localization of malic enzyme (hydrogenosomal marker) with MitoTracker Red may be seen (Fig. 4b). As expected, no co-localization was observed when using antibody against the cytosolic marker tubulin (not shown).

Using antibodies against mitochondrial markers IscS (Fig. 5) and Cpn60 (not shown), no co-localization of these antibodies with MitoTracker was shown, regardless of whether *Giardia* cells were pretreated with protonophore (Fig. 5b) or not (Fig. 5a). A similar pattern was observed when using antibodies against non-mitosomal enzymes PFO (Fig. 5c) or hydrogenase (not shown). An inference here is that even such a sensitive method as fluorescence microscopy failed to reveal transmembrane potential in the giardial mitosome. Even if some potential exists, it is below the detection limit of the technique used. As giardial plasma membrane, like plasma membrane of other eukaryotic cells, contains transmembrane potential (Biagini *et al.*, 2000), MitoTracker Red is evenly distributed in the cytosol. Its absence in the cytoplasm of *T. vaginalis*

Fig. 2. Western blot analysis of fractionated *G. intestinalis* cells. Giardial proteins were separated by 10 % SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were incubated with antisera to (a) hydrogenase, (b) PFO and (c) IscS and Cpn60. *G. intestinalis* whole cells (C), the pellet after centrifugation of a cleared lysate at 50 000 *g* (P), the supernatant after ultracentrifugation of 50 000 *g* supernatant at 175 000 *g* (S) and the mitosome-rich fraction after purification via three consecutive Nycodenz density gradients (M) are shown. Molecular masses (in kDa) of protein markers are indicated.

Fig. 3. Differential centrifugation of cleared giardial lysates. Immunoblot of the centrifugation pellets and supernatants with various antibodies. Names of the detected proteins with antibodies are given to the right. P, Pellet; S, supernatant. Numbers indicate $10^{-3} \times g$.

(Fig. 4) may be due to the relatively high MitoTracker Red concentration in the hydrogenosome, so that cytosolic content represents an actually invisible background. As fluorescent dye does not penetrate the giardial mitosome, unlike the hydrogenosome, only the cytosolic background is seen (Fig. 5).

DISCUSSION

The *G. intestinalis* remnant organelle is not a hydrogenosome

In light of the fact that *G. intestinalis* produces molecular hydrogen (Lloyd *et al.*, 2002a) and PFO was reported to be a membrane-associated enzyme (Ellis *et al.*, 1993; Townson *et al.*, 1996), we readily suggested that its mitochondrion-derived organelle is nothing but a hydrogenosome. However, this appears not to be the case: the mitosome does not contain fermentation enzymes.

Thus, while both the *G. intestinalis* mitosome and *Trichomonas* hydrogenosome possess the same mitochondrion-derived iron-sulfur cluster assembly system (Tachezy *et al.*, 2001), they differ in the localization of the iron-sulfur-cluster-containing fermentation enzymes PFO and hydrogenase. It is indeed puzzling why H_2 -evolving fermentation proceeds in hydrogenosomes, including those of *Trichomonas* (Müller, 2003), but not in giardial mitosomes.

As PFO, generating acetyl-CoA, does not localize in the mitosome, and ATP is generated through the action of ADP-forming acetyl-CoA synthetase (Sánchez *et al.*, 2000), it is highly unlikely that the giardial mitosome produces ATP. In that case, the latter enzyme would have to be localized in the mitosome, and the mitosome would, in turn, have to import acetyl-CoA from the cytoplasm. How giardial mitosomes make or take up ATP, which is obviously required for mtHsp70 and Cpn60 functions, remains to be answered, as *Giardia* appears to lack an ATP/ADP carrier of any type (Hjort *et al.*, 2010). In contrast,

Fig. 4. Detection of *T. vaginalis* hydrogenosome transmembrane potential by using confocal microscopy. (a) Cells pretreated with MitoTracker Red (0.5 μM) were bound to a coverslip and incubated with rabbit polyclonal antibody to malic enzyme (organellar marker) followed by incubation with anti-rabbit antibody conjugated to Alexa-fluor 488. (b) Before treatment with MitoTracker Red the cells were incubated for 1 h in the presence of 0.1 mM FCCP. For other details see the legend to Fig. 1.

Fig. 5. IFA measurement of transmembrane potential in the *G. intestinalis* mitosome. (a) Anti-IscS serum (green) was used to detect mitosomes within the cells prestained with MitoTracker Red. (b) Before addition of MitoTracker Red, the cells were incubated with FCCP (0.1 mM). (c) Along with MitoTracker Red, anti-PFO serum (green) was used as a non-mitosomal marker. For other details see the legend to Fig. 1.

Entamoeba histolytica, another free-living microaerophilic protist, possesses an ATP/ADP carrier of mitochondrial type (Chan *et al.*, 2005), and the PFO and hydrogenase of this organism were suggested not to function in the mitosome (Aguilera *et al.*, 2008).

Thus far, the only known function of the *G. intestinalis* mitosome remains to be an involvement in iron–sulfur cluster assembly by means of a mitochondrion-derived ISC system (Hjort *et al.*, 2010). Archamoebae (*Entamoeba histolytica* and *Mastigamoeba balamuthi*) are known to possess an NIF system of iron–sulfur cluster assembly instead of an organelle-associated ISC system, which was suggested to have been laterally transferred from epsilonproteobacteria and localizes in both the cytosol and the mitosome (Gill *et al.*, 2007; Maralikova *et al.*, 2010). This raises the question of why *Giardia* retained mitochondrial-like ISC (or Archamoebae acquired the NIF system to replace ISC), especially given that its important iron–sulfur-containing enzymes, PFO and

hydrogenase, do not function in the mitosome, as is also suggested for *Entamoeba* (Aguilera *et al.*, 2008). The simplest explanation is that *G. intestinalis* or even diplomonads as a whole had no opportunity for a non-orthologous replacement of ISC machinery with the much simpler (Maralikova *et al.*, 2010) NIF system. Another possibility is that, unlike amoebae, *G. intestinalis* could have been unable to direct components of the NIF system into the mitosome, that is of course if iron–sulfur cluster assembly is needed in the giardial mitosome, as it probably takes place in the *Entamoeba* mitosome containing the iron–sulfur protein rubrerythrin (Maralikova *et al.*, 2010).

As anaerobic energy metabolism appears to be quite ubiquitous among eukaryotes (Embley & Martin, 2006) and supposedly relates to eukaryogenesis itself (Martin & Müller, 1998; Emelyanov, 2003c, 2007; Embley & Martin, 2006), the evolutionary origins of eukaryotic PFO and hydrogenase deserve much attention. Phylogenetic data

based on PFO and iron-only hydrogenase sequences have been published on numerous occasions. While phylogenetic analysis of PFO typically shows a monophyly of eukaryotes with the bacterial donor taxon currently being uncertain, hydrogenase has an apparently polyphyletic distribution (Horner *et al.*, 1999; Embley *et al.*, 2003; Embley, 2006; Hampl *et al.*, 2008; Hug *et al.*, 2010). The hypotheses thought to be most relevant for the origin of anaerobic energy metabolism are based on syntrophy-driven endosymbiosis (Martin & Müller, 1998). The most parsimonious and popular scenario, the so-called hydrogen hypothesis, speculates that H₂-evolving fermentation, like glycolysis, another anaerobic process, has been acquired by an archaeal host from an alphaproteobacterial endosymbiont which has given rise to mitochondria (Martin & Müller, 1998). However, fermentation (e.g. Hug *et al.*, 2010) and glycolysis (Emelyanov, 2003c, 2007) enzymes do not affiliate eukaryotes with alphaproteobacteria, unlike aerobic respiration (Emelyanov, 2003b). Furthermore, the hydrogen hypothesis suffers from many other drawbacks (Emelyanov, 2007). A more complex scenario of eukaryogenesis, called fusion theory, invokes two consecutive endosymbioses at the origin of eukaryotes (Gupta, 1998; Emelyanov, 2003c). In these models, the mitochondrial origin has been preceded by an endosymbiosis of a member of the archaea in a fermenting eubacterium, which could be a donor of PFO and hydrogenase. It should be noted, however, that phylogenetic data did not identify any particular eubacterial group that could be the donor taxon of fermentation enzymes (see references above), while analysis of some glycolytic enzymes and valyl-tRNA synthetase pointed to beta- and gammaproteobacteria as possible eubacterial partners of the first endosymbiotic event (Emelyanov, 2003c, 2007).

In contrast with the *Trichomonas* hydrogenosome, the *G. intestinalis* mitosome is not a charged organelle

Mitochondrion-targeting sequences of classic organelles are known to contain positively charged amino acid residues at the extreme N terminus (Mokranjac & Neupert, 2008). Recent examination of *Giardia* mitosomal and *Trichomonas* hydrogenosomal preproteins showed that: (i) most mitosomal precursors lack cleavable presequences, and when present, they do not contain terminal basic residues and (ii) some hydrogenosomal precursors do contain positively charged amino acid residues at the N terminus (Smíd *et al.*, 2008). These observations raised the question of whether remnant organelles of these organisms possess transmembrane potential. Earlier data suggested that the hydrogenosome of *T. vaginalis* is a charged organelle. Rhodamine 123 and cationic fluorescent dyes have been used to address the question (Humphreys *et al.*, 1998), but an organelle as such has not been identified *in situ* independently using hydrogenosome-specific markers. It was also shown that *in vitro* import of proteins into the *T. vaginalis* hydrogenosome is blocked by the protonophore *m*-chlorophenylhydrazine,

although at a very high (0.6 mM) concentration (Bradley *et al.*, 1997). Rhodamine 123 was shown to stain some structures within the giardial cytosol, but these are too large in size and too scarce in number to be mitosomes, which was unknown at that time (Lloyd *et al.*, 2002b).

In the present work, a co-localization approach was used, i.e. the organelle was identified independently of organelle-penetrating dye (here MitoTracker Red) using antibodies specific to organellar proteins. While transmembrane potential could easily be detected in the *Trichomonas* hydrogenosome, it could not be revealed in the *G. intestinalis* mitosome under similar conditions. It is notable that this observation correlates with the absence of positively charged amino acid residues in presequences of mitosome-targeted proteins (Smíd *et al.*, 2008). It is not clear why MitoTracker Red did not reveal the structures in the giardial cytoplasm that were observed by Lloyd *et al.* (2002b) using rhodamine 123 (see above), and it is also not known what these large structures are. It should be pointed out that, in our work, MitoTracker Red showed punctate distribution in some cases (Fig. 5). It is unclear whether this is an artefact of the fixation process or staining of some new tiny organelles, e.g. those described recently in the *G. intestinalis* cytoplasm as tubulovesicular structures (Abodeely *et al.*, 2009).

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