Giardia intestinalis, a eukaryote without hydrogenosomes, produces hydrogen

David Lloyd, James R. Ralphs and Janine C. Harris

Author for correspondence: David Lloyd. Tel: +44 29 2087 4772. Fax: +44 29 2087 4305. e-mail: LloydD@cardiff.ac.uk

The microaerophilic flagellated protist Giardia intestinalis, the commonest protozoal agent of intestinal infections worldwide, is of uncertain phylogeny, but is usually regarded as the earliest branching of the eukaryotic clades. Under strictly anaerobic conditions, a mass spectrometric investigation of gas production indicated a low level of generation of dihydrogen (2 nmol min⁻¹ per 10⁷ organisms), about 10-fold lower than that in Trichomonas vaginalis under similar conditions. Hydrogen evolution was O₂ sensitive, and inhibited by 100 µM metronidazole. Fluorescent labelling of G. intestinalis cells using monoclonal antibodies to typical hydrogenosomal enzymes from T. vaginalis (malate enzyme, and succinyl-CoA synthetase α and β subunits), and to the large-granule fraction (hydrogenosome-enriched, also from T. vaginalis) gave no discrete localization of epitopes. Cell-free extracts prepared under anaerobic conditions showed the presence of a CO-sensitive hydrogenase activity. This first report of hydrogen production in a eukaryote with no recognizable hydrogenosomes raises further questions about the early branching status of G. intestinalis; the physiological characterization of its hydrogenase, and its recently elucidated gene sequence, will aid further phylogenetic investigations.

Keywords: early branching eukaryotes, diplomonads, membrane inlet mass spectrometry, hydrogenase

INTRODUCTION

The ability of eukaryotes to produce dihydrogen is somewhat limited, despite the widespread occurrence of genes encoding hydrogenase (Horner et al. 2000). Dihydrogen is produced by some green algae in the light under anaerobic conditions, e.g. in Chlamydomonas reinhardtii (Happe et al., 1994), and in Scenedesmus obliquus (Schnackenberg et al., 1993). Other lower eukaryotes that generate hydrogen gas include the microaerophilic protozoa (Yarlett et al., 1982) and chytrid fungi, e.g. Neocallimastix patriciarum (Yarlett et al., 1986, 1987; Rees et al., 1998) isolated from the rumen. Like the microaerophilic pathogen Trichomonas vaginalis (Paget & Lloyd 1990), and the cattle parasite Tritrichomonas foetus (Lloyd et al., 1983), these protists all possess hydrogenosomes, organelles specialized to produce dihydrogen from pyruvate (Lindmark & Müller, 1973). Similar organelles have been shown in the free-living ciliates Plagiopyla frontata and Metopus contortus (Fenchel & Finlay, 1992; Biagini et al., 1997a).

Hydrogenosomes have inner and outer membranes (Finlay & Fenchel, 1989), generate a transmembrane electrochemical potential (Humphreys et al., 1994, 1998) and have intra-organellar Ca²⁺-containing granules (Benchimol et al., 1982; Chapman et al., 1985; Biagini et al., 1997b); these are all characteristics shared by mitochondria (Lloyd, 1974). It is now generally believed that hydrogenosomal convergence in disparate descendant phyla (Lloyd et al., 1983) represents the emergence of secondarily derived mitochondria (Biagini et al., 1997c). Evolutionary selection pressures that favoured the necessary modifications (loss of respiratory chains that use cytochromes, and the proton-translocating systems that generate ATP by oxidative phosphorylation) were life under anoxic conditions (Fenchel & Finlay, 1995) or under conditions of low O₂ in microaerobic refuges (e.g. in sediments or in parasitic or symbiotic niches; Lloyd & Williams, 1993).

One of the lower eukaryotic groups adapted to these conditions, the diplomonads, includes Giardia intestinalis, an important human pathogen that causes an estimated 2–8 × 10⁹ infections each year. Although this organism can be life-threatening in the immunocompro-
mised, the very young or the very old (Adam, 1991), it is of uncertain phylogeny. It has been commonly assumed that this ‘anaerobic amitochondriate’ protist is ‘ancient and primitive’ on account of its many bacterial characteristics (pyrophosphate-biased energy metabolism, arginine dihydrolase pathway of energy production, etc.; Brown et al., 1998), and on the basis of RNA trees, that it represents an extant survivor typical of the earliest branch of the eukaryotes (Sogin, 1991; Leipe et al., 1993; Madigan et al., 2000). However, this assumption has recently been questioned (Embly & Hirt, 1998), as genes for several typically mitochondrial proteins have been shown to be present in G. intestinalis (Solty & Gupta, 1994; Horner et al., 1996; Roger et al., 1998) and in T. vaginalis. Although open to various interpretations, this startling discovery might place the diplo-
monad as a recent derivative of an aerobic lineage. Furthermore, electron-transport activities (Ellis et al., 1993), and transmembrane electrochemical potential generation are associated with distinctive sites in this organism (Lloyd et al., 2002). These observations may be interpreted as suggesting that G. intestinalis is not an early branching eukaryote, but having been derived from an antecedent with mitochondria, should be allocated to a crown taxon (Lake, 1994). Here we show, despite earlier claims to the contrary, that G. intestinalis possesses hydrogenase and produces dihydrogen, even if it does not possess identifiable hydrogenosomes.

**METHODS**

**Organisms and cultures.** Giardia intestinalis Portland-1 strain ATCC 30888, was a gift of Michael R. Edwards, University of New South Wales, Sydney, Australia. Cultures were main-
tained in liquid N₂ after cryopreservation in the presence of 10% (v/v) dimethyl sulphoxide. Tryptophoites were cultured axenically and anaerobically in screw-capped Nunclon tubes (Life Technologies) at 37°C axenically and anaerobically in screw-capped Nunclon tubes

**Harvesting.** After chilling for 20 min in an ice bucket, tubes were shaken gently to dislodge adherent organisms. Cell numbers were counted and then centrifugation was at 1000 g (3000 r.p.m.) for 4 min at room temperature in a bench centrifuge (MSE Minor). After washing once with phosphate-buffered saline (pH 7.4, PBS) and recentrifugation, organisms were finally resuspended in PBS or 0.31 M mannitol solution (both at pH 7.4) and kept at 4°C.

**Mass spectrometry.** Dissolved gases were measured using a Hal series quadrupole mass spectrometer (Hiden Analytical) fitted with an inlet probe covered by a gas-permeable polymer membrane (1.56 mm outside diameter, 0.5 mm internal di-
diameter, 1 cm length) sealed into a 1 cm length of quartz-glass tube. The inlet orifice was 100 μm diameter, and the membrane used was either silicone rubber (permeable to low-molecular

**Preparation of cell-free extracts.** Packaged organisms were resuspended in 5 vols 0.31 M mannitol, then mixed with an equal volume of 0.1 mm diameter acid- and distilled-water washed glass beads in screw-cap sealed 2 ml Nunclon tubes under argon. They were shaken at 4000 Hz for 20 s in a Braun MSK cell disintegrator. Beads and unbroken cells were removed by centrifugation at 1000 g for 2 min. Assays were performed using the supernatant.

**Immunolocalization.** Following the method of Brugerolle et al. (2000), washed cell suspensions were fixed in PBS, pH 7.4, containing 3.5% formaldehyde for 20 min at room temper-
ate. The cells were then permeabilized in PBS containing 0.5% Triton X-100 for 10 min. After treatment with 0.1 M glycine in PBS for 15 min, the cells were resuspended in 200 μl of the primary antibody (undiluted mAb supernatant) and incubated for 2 h at room temperature. The antibodies used (kindly provided by Dr Guy Brugerole, Clermont-Ferrand) were anti-malic enzyme (15 D7), anti-x-subunit-succinyl-CoA synthetase (IC8), anti-beta-subunit-succinyl-CoA synthetase (SH6) and anti-granule (XIV D2). After this period the cells were washed three times in PBS (3 x 30 min) and then incubated for 1 h with anti-mouse Ig, IgG/M antibody conjugated with fluorescein isothiocyanate. After final washes in PBS (3 x 30 min), the cells were mounted in 1:1 (v/v) glycerol/PBS containing 10 mg 1,4-diazabicyclo-(2,2,2)-octane (DABCO) ml⁻¹. Cells were viewed using a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope. Control specimens were treated with the secondary antibody only.

**Confocal laser scanning microscopy.** Cells prepared for immunofluorescence were incubated with 0.31 M mannitol instead of PBS and viewed using a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope. Specimens were scanned using a 25 mW argon laser with appropriate excitation and emission filters for fluorescein (488/510 nm). Specimens were examined using oil-immersion objectives, × 60 (50 μm confocal aperture) and × 100 (100 μm confocal aperture). Series sections through samples were taken at 1 μm intervals (512 × 512 pixels; ~ 0.5 μm thick), and three-dimen-
sional constructs were prepared using Molecular Dynamics Volume Workbench running in Silicon Graphics UNIX workstation.

**Materials.** Gases were from Air Products; traces of O₂ were removed from research-grade N₂, using an ‘oxytrap’ column.
(Alltech Associates). Tryptone was purchased from Becton-Dickinson, and trypticase from BioMérieux. Fetal calf serum was supplied by GibcoBRL, and Nunclon screw-capped culture tubes and cryopreservation vials were supplied through Life Technologies. All other chemicals were supplied by Sigma-Aldrich-Fluka.

RESULTS

Fig. 1 shows the responses of a washed intact cell suspension of *G. intestinalis* to successive cycles of anoxia and aerobiosis after addition of 20 mM glucose, as monitored by membrane inlet mass spectrometry using a silicone rubber interface. After equilibration to the anaerobic state, dissolved CO$_2$ and ethanol (a major fermentation product under anoxic conditions), attained steady states. Switching the mobile gas phase to trace O$_2$ (using a gas mixture containing 0.4 kPa O$_2$ in N$_2$) gave accelerated fermentation, even though the level of dissolved O$_2$ was less than the detection limit of the mass spectrometer (0.1 µM O$_2$); this was indicated by elevated ethanol production and an increased CO$_2$ concentration. The response time of the system is longer for ethanol than for fixed gases. Monitoring at $m/z = 2$ (for H$_2$) indicated an increase, but calibrations in parallel with those at $m/z = 31$ confirmed that this signal is derived by the fragmentation of ethanol in the vacuum system (Fig. 2a). The fact that the $m/z = 2$ trend analysis exactly follows that at $m/z = 31$ confirms that this does not arise from dihydrogen; in this experimental configuration (open to gas flow) the latter behaves as do all “fixed gases”, i.e. reach steady states and do not accumulate as does a low-molecular-mass volatile (e.g. ethanol).

In order to avoid the interference of ethanol at $m/z = 2$ while measuring H$_2$, a chemical trap for ethanol (alcohol dehydrogenase plus semicarbazide) was included in the cell suspension, but this did not obviate the problem, as the mass spectrometer was able to compete with the enzyme for ethanol and still gave signals at both mass numbers.

A solution to this problem involved using a more selective gas-permeable membrane. Of several polymers (polyethylene, polypropylene, Teflon and polyethylene terephthalate), the last (Mylar) proved most successful with respect to restricted ethanol permeability. Fig. 2(b) shows that even at very high concentrations, ethanol gives no responses, but H$_2$ can penetrate rapidly to give a mass spectrometric response with a short half-time. Fig. 3 shows an experiment on glucose utilization by whole cells of *G. intestinalis* in PBS using this Mylar inlet system to monitor gases continuously in a closed vessel. Initially, the organisms consumed dissolved O$_2$, and this became undetectable by 8 min. Only after this, when almost complete anoxia was attained, did the signal at $m/z = 2$ increase as H$_2$ was produced and accumulated continuously. Injection of air-saturated PBS (final [O$_2$] = 8 µM) gave an almost immediate cessation of H$_2$ generation. Resumption of H$_2$ evolution only occurred when added O$_2$ had been depleted again. Finally, addition of 100 µM metronidazole gave complete arrest of hydrogenogenesis.

Experiments with cell-free extracts

Fig. 4 shows the absorbance changes at 600 nm during incubation of cell-free extract with methyl viologen in closed cuvettes under an atmosphere of either N$_2$ or H$_2$ in a buffer containing 40 mM dithiothreitol. No dye reduction occurred in the presence of heat-inactivated cell-free extract. The presence of hydrogenase in the active extract was indicated by dye reduction under H$_2$ at a rate proportional to the volume of extract added. No reaction occurred under a gas phase of N$_2$ or when air was admitted to the reaction mixture during the hydrogenase reaction. CO (3 µM) inhibited the reaction by 20% when H$_2$ was present at 820 µM; 16 µM CO gave 70% inhibition.

ImmunocytoLOGY

Incubation of fixed *G. intestinalis* with fluorescent-labelled monoclonal antibodies to hydrogenosomes isolated from *T. vaginalis* or to typical hydrogenosomal enzymes (also purified from *T. vaginalis*) as described by Bruggerole et al. (2000) gave no discrete localization as revealed by examination using confocal laser scanning microscopy. The fluorescent hydrogenosome labels (fluorescein $\lambda_{exc} = 488$ nm, $\lambda_{emiss} = 510$ nm) gave positive results for the characteristic distribution of these organelles and for the hydrogenosomal location of malate enzyme and succinyl-CoA synthetase in *T. vaginalis*, but in *G. intestinalis* only faint yellow-green fluorescence was observed throughout the organisms (not shown). Thus no discrete localization of typical hydrogenosomal epitopes was found in the diplomonad.
DISCUSSION

The lack of mitochondria and peroxisomes, and the possession of only a rudimentary Golgi body in the trophozoite may not necessarily point to a ‘primitive’ status for *G. intestinalis*, but rather to the secondary loss of completely functional organelles during evolution as a parasite (Lloyd et al., 1983; Embley & Hirt, 1998).

Our observation that this organism produces dihydrogen, despite its apparent lack of a discrete structural entity that can be recognized as a hydrogenosome, even by the criterion of immunological cross-reaction with proteins isolated from the hydrogenosomes of another...
microaerophilic lower eukaryote, *T. vaginalis* (Brugerolle *et al.*, 2000), provides a further reason for a new emphasis on its uncertain taxonomic position. Dihydrogen production rates (2 nmol min⁻¹ per 10⁷ organisms) were about 10-fold lower than in *T. vaginalis* (Ellis *et al.*, 1992).

The hydrogenase responsible for the dihydrogenase generation in *G. intestinalis* shows characteristics typical of an (iron-only) [Fe] hydrogenase. Thus its activity, as measured by H₂-dependent reduction of methyl viologen, is extremely O₂ sensitive and is also inhibited by low concentrations of CO. Other classes of hydrogenase include those containing Ni, or Ni and Se as well as acid-labile sulphur; these hydrogenases are relatively CO insensitive (Cammack *et al.*, 1985).

Other microaerophilic protists containing hydrogenosome-localized hydrogenase include the parasitic species, the trichomonads (Lindmark & Müller, 1973), six different species of the symbiotic rumen protists examined (see Yarlett *et al.*, 1981, 1983a, b, 1984; Hillman *et al.*, 1985; Ellis *et al.*, 1991a, b, c), chytrid fungi (Yarlett *et al.*, 1986, 1987; Rees *et al.*, 1998) and the highly anaerobic free-living sediment-dwelling ciliate *Metopus contortus* (Biagini, 1997a). Clearly, these organisms are widely separated phylogenetically (Lloyd *et al.*, 1983). Molecular analysis, as well as likelihood ratio tests and parametric bootstrapping, suggest that the [Fe] hydrogenases in microaerophilic lower eukaryotes are not monophyletic, even though those from organisms not bearing hydrogenosomes, *Entamoeba histolytica* and *Spironucleus barkhanus*, together with a newly described third hydrogenase from *T. vaginalis*, might possibly be (Horner *et al.*, 2000).

The complete sequence of the *G. intestinalis* hydrogenase gene (http://www.ncbi.nlm.nih.gov:80/, GenBank accession no. AF242293), available only after the experiments described here were completed (J. E. Nixon, J. Field, J. Samuelson & M. L. Sogin, unpublished), places *Giardia* close to *E. histolytica* and *S. barkhanus*. The *Spirotrichomonas* sequence is also complete (Horner *et al.*, 2000), and neither that gene nor the one for *Giardia* hydrogenase shows any obvious N-terminus motif that would target it to an organelle. This observation correlates with our unsuccessful attempts to locate hydrogenosomes in *G. intestinalis* reported here.

Monoclonal antibodies for hydrogenosomal malic enzyme, α and β-subunits of succinyl-CoA synthetase, and granules were incubated with *T. vaginalis* (positive control) and *G. intestinalis* to see if this organism contained any of the hydrogenosomal enzymes. All of the *T. vaginalis* tests showed discrete localizations within the cells and no fluorescence when the cells were incubated with fluorescently labelled secondary antibody only (negative control). All of the localizations in the trichomonad were discrete, and in organelles of the same dimensions and distributions. However, incubation of *Giardia* with the same antibodies did not result in any discrete localization within the cells. This suggests that *Giardia* does not contain any of the hydrogenosomal proteins screened for by this assay; however, this does not necessarily indicate that these are not present. Monoclonal antibodies are highly specific, and even a change of one amino acid of an enzyme can result in a different epitope; then no binding of the antibody would occur.

Extensive mechanistic research on hydrogenases indicates that evolutionary relationships can be elucidated. This first report of an hydrogenase in *G. intestinalis* and the progress of the *Giardia* genome project (Adam, 2000) paves the way for molecular analyses (Horner *et al.*, 2000) that may help phylogenetic studies on *G. intestinalis* as has been the case for *E. histolytica* (Rosenthal *et al.*, 1997). These studies may also have important implications for hypotheses regarding the origin of eukaryotes (Gupta & Golding, 1996; Lopez-Garcia & Moreira, 1999).

Further work is in progress to determine the specific location of the hydrogenase in *G. intestinalis*, especially with regard to its membrane association. Possible functions include intracellular redox balancing by this ‘output hydrogenase’. Previous metabolic analysis of fermentation pathways reveals tight control of apportionment of electron flux between pathways divergent from the pyruvate pool to alanine, or ethanol or acetate + CO₂ (Paget *et al.*, 1990, 1993) most likely via the redox balance of the NADH/NAD⁺ couple (Paget *et al.*, 1993). Intracellular O₂ concentration is presumably sensed in order to provide the homeodynamic modulation at this important branch point (Lloyd *et al.*, 2002). The O₂ sensitivity of hydrogenase reported here strongly suggests that dihydrogen serves as an alternative product, the generation of which can serve as a process for the dissipation of excess reducing power, only necessary under highly anaerobic conditions. Clearly, control of intracellular redox state is paramount in an environment that has fluctuating redox and O₂ levels. Other possible functions may also come to light if the hydrogenase turns out to be a reversible transmembrane enzyme with a dual hydrogen-uptake/output role, also capable of the generation of a proton-translocating transmembrane electrochemical potential.

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**REFERENCES**


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