Hydrogenosomes of *Metopus contortus* physiologically resemble mitochondria

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The anaerobic free-living ciliated protozoon *Metopus contortus* is a grazer in anoxic marine sediments. It does not possess mitochondria, but it does have specialized organelles termed hydrogenosomes which release hydrogen gas. The cationic lipophilic cyanine dye DiOC₂(3) is an indicator of transmembrane electrochemical potential. With the aid of confocal laser scanning microscopy (CLSM), the association of this dye with hydrogenosomes *in situ* was followed. Flow cytometric measurements showed that fluorescence of the membrane potential dye decreased in response to an elevated pH_i in the cell. CLSM also revealed localization of fluorescence of the calcium probe Fluo 3-AM, and of the transmembrane pH gradient probe BCECF-AM, within the lumen of the hydrogenosomes. In addition, hydrogenosomal inclusions were detected. X-ray microanalysis of these electron-dense granules revealed high levels of calcium, phosphate and magnesium. It is concluded that *M. contortus* hydrogenosomes are calcium stores, have a membrane potential, and an alkaline lumen. These physiological features resemble those of mitochondria in aerobic protozoa.

Keywords: *Metopus contortus*, hydrogenosomes, transmembrane electrochemical potential, transmembrane pH gradient, calcium phosphate inclusions

INTRODUCTION

Metopus contortus, a free-living ciliate, is a ubiquitous filter-feeder in anoxic marine sediments which plays a vital role in the phagotrophic chains of anaerobic communities (Fenchel & Finlay, 1990). It does not have mitochondria, but it does have specialized redox organelles, the hydrogenosomes, which release hydrogen gas (Finlay & Fenchel, 1989; Fenchel & Finlay, 1992). Hydrogenosomes are found in a phylogenetically broad range of organisms. In the parasitic flagellate *Trichomonas vaginalis*, the hydrogenosomes contain phosphate-bound and free calcium (Chapman *et al.*, 1985; Humphreys *et al.*, 1994) and have a transmembrane electrochemical potential ($\Delta\psi$) (Humphreys *et al.*, 1994), whilst hydrogenosomes from the rumen chytrid fungus *Neocallimastix* have a transmembrane pH gradient

(ΔpH) and a novel ATPase (Marvin-Sikkema *et al.*, 1994). The cattle parasite *Tritrichomonas foetus* has also been shown to possess calcium inclusions localized in the hydrogenosome (Benchimol *et al.*, 1982). Little is known of the function of *M. contortus* hydrogenosomes within the cell or of their role in relation to the endosymbiotic methanogenic bacteria which they harbour (Finlay & Fenchel, 1991; Embley *et al.*, 1992). The endosymbiotic methanogenic bacteria may convert the hydrogenosomally produced hydrogen, carbon dioxide and acetate into methane and water (Fenchel & Finlay, 1992, 1994). The apparent close juxtaposition of symbionts and hydrogenosomes (Finlay & Fenchel, 1991) is believed to aid the hydrogen transfer (Fenchel & Finlay, 1991, 1992). Removal of the endosymbiont, by the methanogen inhibitor bromoethanesulphonate (BES), leads to an approximately 30% reduction in growth rate and yield of the ciliate (Fenchel & Finlay, 1991). With the aid of confocal laser scanning microscopy (CLSM) and energy-dispersive X-ray microanalysis, this study has investigated *M. contortus* hydrogenosomes for the presence of calcium, a $\Delta\psi$ and a ΔpH . In addition, flow cytometric measurements revealed the effect of dissolved hydrogen on the membrane potential of the hydrogenosomes.

Abbreviations: BCECF-AM, 2',7'-bis(2-carboxyethyl)-5-carboxyfluorescein, acetomethyl ester; BES, bromoethanesulphonate; CLSM, confocal laser scanning microscopy; DiOC₂(3), dihexyloxycarbocyanine; Fluo 3-AM, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxy-9-xanthenyl)-phenoxy]-2-(2-amino-5-methylphenoxy)ethane-*N,N,N'*-tetraacetic acid, acetomethyl ester.

METHODS

Isolation and culture conditions. *M. contortus* was isolated by T. Fenchel (University of Copenhagen, Denmark) and B. J. Finlay from anaerobic marine sands in Denmark and cultured anaerobically in medium C75S in sealed glass serum vials with a nitrogen headspace at 20 °C (Finlay & Fenchel, 1989). Methanogen-free (asymbiotic) cells were obtained by the addition of 50 mM BES, a selective inhibitor of methanogenic bacteria (Smith, 1983; Fenchel & Finlay, 1991).

Electron microscopy. Cells were fixed in 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde (1:1 mixture) for 10 min, followed by transfer to 6% (v/v) glutaraldehyde (30 min), 2% (w/v) OsO₄ in 0.1 M sodium cacodylate buffer (45 min), and 0.5% (w/v) uranyl acetate (1 h). Dehydration in increasing concentrations of ethanol was followed by embedding in Spurr resin and sectioning (Finlay & Fenchel, 1989). Cells for electron probe X-ray microanalysis were single fixed in 4% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (30 min) followed by dehydration and embedding in LR White resin.

CLSM. Asymbiotic cells were stained with either cyanine DiOC₇(3) (final concentration 2 µg ml⁻¹), BCECF-AM (final concentration 2 µg ml⁻¹) or Fluo 3-AM (2 mM final concentration) for 15 min together with 2% (w/v) DABCO (1,4-diazabicyclo[2.2.2]octane), a free-radical scavenging 'anti-bleach' agent (Johnson *et al.*, 1982). Methyl cellulose (10%, w/v; pH 7) was added to reduce the motility of the cells. A Molecular Dynamics Sarastro 2000 Confocal Scanning Microscope was used. Fluorescence excitation was set at 488 nm and emission at 510 nm. Series of optical sections (0.5 µm thick) were taken through entire organisms at a spacing of 2 µm. Data were collected on a Silicon Graphics Indigo UNIX workstation running Molecular Dynamics 'Image Space' software.

Electron probe X-ray microanalysis. Energy-dispersive X-ray microanalysis of hydrogenosomal inclusions was carried out with a JEOL JEM-1210 transmission electron microscope equipped with a Link ATW Pentafet X-ray detector (138 eV resolution) and a Link 'ISIS' analyser (Oxford Instruments), with an accelerating voltage of 80 kV.

Flow cytometry. Asymbiotic cells were incubated with the cyanine dye DiOC₇(3) (2 µg ml⁻¹ final concentration) for 15 min in the presence of either hydrogen (0.81 mM dissolved concentration; Wilhelm *et al.*, 1977) or nitrogen (0.7 mM dissolved concentration; Wilhelm *et al.*, 1977). Flow cytometry was performed on a Skatron Argus 100 high-pressure mercury arc lamp based dual parameter flow cytometer. A fluorescein isothiocyanate filter block was used allowing excitation at 470–490 nm, band stop at 510 nm, and emission at 520–550 nm. Acquisition of signals from *M. contortus* (but not from food bacteria) was determined by gating with forward angle light scatter (an indicator of cell size). Typically, fluorescence signals from 800 to 1000 asymbiotic *M. contortus* cells were acquired for each experiment.

RESULTS

The electron micrograph of a transverse-sectioned *M. contortus* cell confirms the peripheral localization of the hydrogenosomes and methanogens (Fig. 1a), whilst the close proximity of hydrogenosomes and methanogens is clearly demonstrated in Fig. 1(b). Treatment with the specific methanogenesis inhibitor BES appears very effective at removing the endosymbiotic methanogenic

Fig. 1. Electron micrographs of *M. contortus*. (a) Transverse section of a whole cell showing the peripheral location of the hydrogenosomes (H) and methanogens (M); bar, 10 µm. (b) Methanogen/hydrogenosome complexes; bar, 2 µm. (c) BES-treated cells are cleared of the symbiotic methanogens, leaving only the hydrogenosomes; bar, 1 µm.

Fig. 2. (a–f) CLSM optical sections (0.5 μm thick at 2 μm increments) of a live BES-treated *M. contortus* cell stained with the cationic dye cyanine DiOC₇(3) (2 $\mu\text{g ml}^{-1}$), an indicator of $\Delta\psi$; bar, 5 μm .

Fig. 3. CLSM single optical section of a fixed (0.5%, v/v, formalin) *M. contortus* cell incubated with cyanine DiOC₇(3) (2 $\mu\text{g ml}^{-1}$). Bar, 5 μm .

bacteria without affecting the apparent location and morphology of the hydrogenosomes (Fig. 1c).

Specific association of the cationic lipophilic cyanine dye DiOC₇(3) to hydrogenosomes was apparent in live (Fig. 2) but not in dead (Fig. 3) protozoa, indicating the presence of a $\Delta\psi$ across the hydrogenosomal membranes. However, it was not clear to us whether the

Fig. 4. Flow cytometric measurements of live BES-treated *M. contortus* cells incubated with cyanine DiOC₇(3) (2 $\mu\text{g ml}^{-1}$) under a dissolved concentration of (a) 0.7 mM nitrogen and (b) 0.8 mM hydrogen.

association of the dye was to the inner or outer hydrogenosomal membrane. CLSM optical sections of the cell (Fig. 2a–f) revealed the accumulation of the dye in the cortical region where hydrogenosomes are found. No other membrane type in the cell appeared to have an affinity for the membrane potential dye.

Flow cytometric measurements of the fluorescence from the hydrogenosome-bound membrane potential dye under an atmosphere of nitrogen (0.7 mM dissolved concentration; Fig. 4a) was shown to decrease in response to an elevated atmosphere of hydrogen (0.8 mM dissolved concentration; Fig. 4b). A decrease in fluorescence from the membrane potential dye is directly correlated with a reduction in the $\Delta\psi$ across the hydrogenosome membranes.

Single fixed non-stained asymbiotic *M. contortus* cells were revealed to contain inclusions in the matrix (and

Fig. 5. Transmission electron micrograph of a single fixed non-stained asymbiotic *M. contortus* cell showing electron-dense inclusions in the hydrogenosomes (arrows). Bar, 0.5 μm .

Fig. 6. Qualitative energy-dispersive X-ray analytical spectrum of hydrogenosomal inclusions (see Fig. 5).

sometimes in between the inner and outer membranes, not shown) of hydrogenosomes (Fig. 5). Energy-dispersive X-ray microprobe spectra of these electron-dense granules revealed a high concentration of calcium and phosphate and some magnesium (Fig. 6). Background counts from the embedding resin were negligible but were nevertheless subtracted from the dot analysis of the hydrogenosomal inclusions. The titanium peak shown in the spectrum (Fig. 6) was as a result of electron scattering from the titanium grid which is used to support the samples. These results suggest that the hydrogenosomes contain calcium (and some magnesium) which has precipitated with phosphate to form the observed granules.

CLSM of live asymbiotic *M. contortus* cells loaded with the calcium-specific fluorophore Fluo 3-AM revealed the presence of calcium in the hydrogenosomes (Fig. 7). It should be noted that of the cells which were viewed, hydrogenosomal compartmentalization of the calcium

Fig. 7. CLSM optical single section of a BES-treated *M. contortus* cell stained with the calcium-specific probe Fluo 3-AM (2 mM). Bar, 5 μm .

probe was observed only in cells which appeared damaged (i.e. cells with little ciliary movement and vacuolated cytoplasm) by the handling procedure, or those which had been over-exposed to the confocal laser. This observation suggests that higher levels of calcium may enter the hydrogenosomes during periods of cell 'stress'.

CLSM also revealed compartmentalization of the ΔpH probe BCECF-AM. Optical sections of a live asymbiotic cell (Fig. 8) again revealed that the localization of the probe was in the periphery of the cells in the vicinity of the hydrogenosomes. BCECF-AM is a non-fluorescent molecule which becomes highly fluorescent within the cytoplasm where the AM group is cleaved by non-specific esterases, yielding a highly fluorescent molecule with a pK_a of 6.98. The emission intensity is pH-dependent with greater intensity at higher pH. The observed fluorescence indicates that the internal pH of hydrogenosomes is alkaline.

DISCUSSION

In this study, we have demonstrated that hydrogenosomes from the anaerobic free-living ciliate *M. contortus* possess calcium, a $\Delta\psi$ and a ΔpH (inside

Fig. 8. (a–d) CLSM optical sections of a live BES-treated *M. contortus* cell stained with the Δ pH-specific probe BCECF-AM ($2 \mu\text{g ml}^{-1}$). Bar, $10 \mu\text{m}$.

alkaline versus outside). Hydrogenosomes of trichomonads have been observed to have the same properties (Chapman *et al.*, 1985; Humphreys *et al.*, 1994; Yarlett *et al.*, 1987); however, there is no detectable F_0F_1 -type ATPase associated with these

organelles (Lloyd *et al.*, 1979; Turner & Luschbaugh, 1991), although it is possible that alternative energy-yielding ATPases are present. Significantly, *Neocallimastix* hydrogenosomes contain a novel ATPase (Marvin-Sikkema *et al.*, 1994) which is believed to

function as a proton-translocating ATPase, which in turn generates a pH gradient across the hydrogenosomal membrane. We have yet to determine whether these organelles in *M. contortus* have ATPase activity, but it is most likely that the ΔpH and $\Delta\psi$ contribute to a $\Delta\mu$ across the hydrogenosome membrane, enabling the import and export of metabolites and end-products. Of course, the possession of a $\Delta\psi$ is not unique to mitochondrial membranes, and it should be noted that proton gradients are present across other membrane types.

The presence of a ΔpH and $\Delta\psi$ in *M. contortus* hydrogenosomes may also be exploited to drive the accumulation of calcium into hydrogenosomes, as in the case of *T. foetus* and *T. vaginalis* (Benchimol *et al.*, 1982; Chapman *et al.*, 1985). Mitochondria are believed to function as temporary stores of excess cytosolic calcium when the plasma membrane of a cell is injured (Carafoli, 1987). They are able to accumulate calcium at high concentrations by precipitating with phosphate, forming hydroxyapatite deposits in the matrix (Carafoli, 1987). Our results suggest that calcium uptake in the hydrogenosomes of *M. contortus*, and of other anaerobic protozoa, may be as important a mechanism as it is in mitochondria of aerobic organisms.

In recent years, the origin of hydrogenosomes has been the subject of some debate. Based on biochemical evidence, it has been suggested that hydrogenosomes originate from symbiosis with a *Clostridium*-like anaerobic bacterium (Müller, 1980; Whatley, 1981). The alternative explanation is that hydrogenosomes are modified mitochondria which have functionally adjusted to their anaerobic environment by way of gaining and losing specific biochemical machinery (Cavalier-Smith, 1987; Finlay & Fenchel, 1989). The loss of mitochondrial characters such as cytochrome activity has been reported in the intestinal 'anaerobic' protozoan parasite *Blastocystis hominis* (Zierdt, 1986). Similarly, hydrogenosomes are also believed to be a functionally heterogeneous group of organelles. The free-living microaerophilic amoebamastigote *Psalteriomonas lanterna* possesses two populations of hydrogenosomes. Both types react with fluorescein-isothiocyanate-labelled hydrogenase antiserum, but only the 'mature' hydrogenosomes show hydrogenase activity (by reaction with BSPT [2-(2'-benzothiazolyl)-5-styryl-3-(4'-phthalhydrazidyl)tetrazolium chloride]; Coombs & Hackstein, 1995).

A mitochondrial origin of hydrogenosomes has been suggested by a recent molecular study of *T. vaginalis* cpn-60 chaperonin coding region (Horner *et al.*, 1996), which revealed a high sequence similarity to mitochondrial cpn-60 sequences. In addition, a cpn-60-like protein in *Tritrichomonas mobilensis* and *Tritrichomonas augusta* has been shown by Bozner (1996) to be localized in the hydrogenosome. A similar study by Bui *et al.* (1996) on the chaperonin proteins of *T. vaginalis* (cpn-10, cpn-60 and cpn-70) also demonstrated a homology with mitochondrial chaperonin proteins. However, Bui *et al.* (1996) reached a different conclusion to

this observation. They propose that in *T. vaginalis*, hydrogenosomes and mitochondria are derived from the same progenitor organelle and that exploitation of aerobic niches gave rise to mitochondria whereas exploitation of anaerobic niches gave rise to hydrogenosomes. In addition, hydrogenosomes from the rumen fungus *Neocallimastix frontalis* L2, which have been described to be morphologically different to protozoan hydrogenosomes by possessing only a single membrane, have been shown to possess mitochondrial-like targeting signals on the hydrogenosomal malic enzyme (van der Giezen *et al.*, 1997) and β -succinyl-CoA synthetase (Brondijk *et al.*, 1996).

Morphologically, the hydrogenosomes of *M. contortus* and of other free-living anaerobic ciliates have been shown to resemble mitochondria (Finlay & Fenchel, 1989). Embley *et al.* (1995) have suggested a multiple origin of hydrogenosomes in anaerobic ciliates within the radiation of aerobic ciliates and propose that a modification of pre-existing mitochondria better explains this distribution than the occurrence of multiple convergent endosymbiosis. It would appear that our results, which suggest a resemblance in the physiological role of hydrogenosomes and mitochondria, favour the idea that hydrogenosomes are modified mitochondria which have altered some of their biochemical features in order to cope more favourably with an anaerobic lifestyle. However, the broad phylogenetic distribution of hydrogenosomes and morphological differences (such as the presence of a single membrane in the hydrogenosomes of *Neocallimastix*) suggests that a polyphyletic origin of hydrogenosomes (Cavalier-Smith, 1987) cannot be dismissed.

The endosymbiotic methanogenic bacteria of *M. contortus* have previously been shown to increase the growth rate and yield of their host (Fenchel & Finlay, 1991). The flow cytometric data support the theory (Fenchel & Finlay, 1992; Finlay & Fenchel, 1992) that the endosymbionts closely associated with the hydrogenosomes act as 'hydrogen sinks', by reducing the pH_2 of the cell cytoplasm. In our study, the presence of an elevated pH_2 in the culture medium (and presumably in the cell cytoplasm) reduced the $\Delta\psi$ of the hydrogenosomes (Fig. 4). As a result, this may slow down the accumulation of substrates or the expulsion of products, which in turn could reduce the overall growth rate of the cell.

Further work will be concerned with isolating the hydrogenosomes, in order to further elucidate the bioenergetics of the hydrogenosome. If indeed hydrogenosomes of *M. contortus* are biochemically modified mitochondria, the elucidation of the mechanics for such a transition in function will pose challenging work. At present this work is hampered by the lack of an axenic medium for the culture of these free-living anaerobic ciliates.

ACKNOWLEDGEMENTS

We would like to thank M. Turner and K. J. Clarke for help with TEM sample preparation, R. M. Hindle for assistance

with ciliate cultures and J. Morgan for helpful discussions. This work was carried out during a NERC postgraduate studentship (G.A.B.).

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Received 16 August 1996; revised 20 December 1996; accepted 13 January 1997.