A double membrane surrounds the hydrogenosomes of the anaerobic fungus \textit{Neocallimastix frontalis}

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Abstract

The structure of hydrogenosomes of the anaerobic fungus \textit{Neocallimastix frontalis} was analyzed using routine preparations for transmission electron microscopy, freeze-fracture and immunocytochemistry. They appeared as round or elongated structures, always enveloped by two distinct, but tightly apposed membranes. Images of organelle division were very similar to those observed in trichomonad protozoa. These observations suggest that hydrogenosomes are homologous organelles in unrelated species weakening the hypothesis of a polyphyletic origin and supporting the evidence that fungal hydrogenosomes are probably derived from an endosymbiont relationship.

Keywords: Hydrogenosome; Filamentous fungus; \textit{Neocallimastix frontalis}; Freeze-fracture; Immunocytochemistry

1. Introduction

Hydrogenosomes are membrane-bounded organelles involved in the production of ATP and of molecular hydrogen [1]. They are found in several anaerobic species, such as protozoa of the Trichomonadida order [2,3], in rumen ciliates [4–6], some free-living ciliates [7] and anaerobic fungi [8,9]. They contain enzymes that participate in the metabolism of pyruvate formed during glycolysis [1]. The most extensive studies on this organelle were carried out in trichomonad species where it was demonstrated that it is surrounded by a double membrane, presenting an internal vesicle and calcium deposits [10]. Similarities between hydrogenosomes and mitochondria, such as the division process, calcium incorporation, internal membranes and the presence of mitochondrial/hydrogenosomal-type amino-terminal extensions in two sequenced proteins were demonstrated [10,11].

The ultrastructure of the fungal hydrogenosome is poorly described [1]. In the fungus \textit{Neocallimastix} sp. L2, Marvin-Sikkema et al. [9,12] found a single
membrane lining the hydrogenosome. They also found a relationship between hydrogenosomes and peroxisomes concerning the presence of the characteristic peroxisome targeting the SKL signal in some hydrogenosomal proteins. After these findings some authors claimed that the hydrogenosomes present different morphological characteristics which could reflect fundamental differences in the biological nature of hydrogenosomes of various organisms. A polyphyletic origin for this organelle was proposed [1,13]. However, recent molecular studies demonstrated that primary sequences of the hydrogenosomal proteins β-succinyl CoA synthetase [14] and malic enzyme [15] have targeting sequences that present some common features with mitochondrial targeting signals. So the evolutionary origin of hydrogenosomes should be clarified by a detailed examination of their structure in fungi.

In this paper we studied the fine structure of the hydrogenosomes of the fungus Neocallimastix frontalis using improved preparation methodology for transmission electron microscopy and freeze-fracture. The similarities with the trichomonad hydrogenosomes and mitochondria are also discussed.

2. Materials and methods

2.1. Fungal strain and culture conditions

The fungus Neocallimastix frontalis MCH3 isolated from sheep rumen (Laboratoire de Microbiologie CRZV, INRA, Theix, France) was grown anaerobically at 39°C for 3–4 days in culture flasks containing 100 ml of liquid synthetic medium [16] supplemented with 0.2% cellobiose.

2.2. Electron microscopy

Cells were fixed at room temperature in 2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.2). After fixation, the cells were washed in PBS (phosphate buffered saline) and post-fixed for 15 min in 1% OsO4 in 0.1 M cacodylate buffer plus 5 mM CaCl2 and 0.8% potassium ferricyanide [17]. Cells were dehydrated and embedded in Epon. Thin sections were stained and observed in a Zeiss 900 electron microscope.

2.3. Freeze-fracture

After routine fixation, increasing the concentration of glycerol in cacodylate buffer to a final concentration of 30% (v/v) led to cryopreservation. Specimens were rapidly frozen in the liquid phase of freon 22, cooled with liquid nitrogen and immediately transferred to liquid nitrogen. The frozen specimens were freeze-fractured at −115°C in a Balzers BAF 300 freeze-etching machine. The specimens were shadowed with platinum carbon at 2 × 10−6 Torr after fracturing. Replicas were recovered in distilled water, cleaned with sodium hypochlorite and examined in a Zeiss 900 electron microscope.

2.4. Cryo-ultramicrotomy and immunocytochemistry

After fixation (4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, overnight at 4°C) the samples were infused in a mixture of 25% (w/v) polyvinylpyrrolidone (PVP) and 2.3 M sucrose for 30 min, plunged into liquid nitrogen and transferred to a cryo-ultramicrotome (Ultracut Reichert). Cryosections were obtained at a temperature range of −80 to −100°C and collected on coated nickel grids. For immunolabeling the cryosections were washed in PBS/albunin 3%, quenched in 50 mM NH4Cl for 30 min, and subsequently incubated for 3 h in the presence of the antibody recognizing β-succinyl CoA synthetase. Antibody production was performed as previously described [14]. The cryosections were incubated in the presence of 10 nm gold-labeled goat anti-rabbit IgG antibodies and thinly embedded in a mixture of 9:1 (v/v) of 3% polyvinyl alcohol (PVA) and uranyl acetate [18].

3. Results and discussion

The rumen anaerobic fungus Neocallimastix frontalis presents a two-stage life cycle, the motile zoospore, and the zoosporangium fixed to the cell wall plant particles by filamentous rhizoids. We have examined the rhizoids, sporangia and zoospores by routine electron microscopy, immunocytochemistry and freeze-fracture. N. frontalis showed the presence of hydrogenosomes distributed randomly over the hyphae, zoospores and sporangia (Fig. 1). Spherical
Fig. 1. General view of *N. frontalis* in a routine thin section showing the overall distribution of hydrogenosomes in the rhizoid. The arrows point to hydrogenosomes in process of division. H, hydrogenosome; W, cell wall. Scale bar: 1 µm.

Fig. 2. *N. frontalis* zoospore fixed according to the glutaraldehyde osmium tetroxide-potassium ferricyanide procedure with 5 mM CaCl₂ added to this last step. Two closely apposed outer and inner unit membranes of the hydrogenosome (H) are seen (arrowheads). W, cell wall. Scale bar: 0.1 µm.

Fig. 3. Freeze-fracture image of the hydrogenosome in the rhizoid of *N. frontalis*. Differences between inner and outer hydrogenosomal membranes (star and asterisk) appeared concerning size and distribution of intramembranous particles. Scale bar: 0.16 µm.

Fig. 4. Immunocytochemical localization of β-succinyl CoA synthetase in the zoospore hydrogenosomes of *N. frontalis*. The gold particles are located in the hydrogenosomal matrix. Note the double membrane (arrowheads). Scale bar: 0.1 µm.

and elongated forms were frequently observed (Figs. 1 and 4). The hydrogenosome matrix was finely granular, with no core or nucleoid (Figs. 1, 2 and 6). Internal membranous profiles were occasionally seen (Figs. 5 and 6). A double membrane envelope was always present in all hydrogenosomes examined (Figs. 2 and 5). The two membranes were so tightly apposed that they could appear as a single membrane if the sample was not well fixed and/or a very thin section was not obtained. Immunocytochemical experiments also showed that the β-succinyl CoA synthetase was localized in the matrix of a double-membrane bounded organelle (Fig. 4). Immunocytochemistry has previously shown β-succinyl CoA synthetase in trichomonads to be located exclusively in the hydrogenosomal matrix [19], confirming that the organelles enveloped by two membranes are hydrogenosomes. There was no internemembrane space
(Figs. 2 and 4). Each membrane was 6.5 nm thick, while the plasma membrane in the same cell measured 7.2 nm (not shown). Our present observations showed that *N. frontalis* hydrogenosomes have two tightly apposed membranes in both zoospores and hyphal stages of the organism (Figs. 2 and 5). In Fig. 1b of the Marvin-Sikkema study [12], the authors pointed to a hydrogenosome, claiming the presence of a single membrane. However, a double membrane can be observed in these micrographs. We noticed that when good sample preservation was attained and very ultra-thin, gray colored sections were obtained, it was always possible to distinguish two membranes in the whole circumference of the hydrogenosome. The membranes are denser, thinner and more compact than the plasma membrane observed in the same cell. We have adapted Hepler’s method [17] where the post-fixation is carried out in an osmium tetroxide solution containing potassium ferri-cyanide and calcium for only 15 min, which improved the visualization and preservation of membranous structures. The double membrane in the hydrogenosomes of *N. frontalis* was also confirmed by freeze-fracture where at least two fracture faces of the outer membrane and one fracture face of the inner membrane could be identified (Fig. 3). Apparently no special domains, with an organized array of particles, existed in the hydrogenosomal membranes, although they presented different sizes and distribution of intramembranous particles. Yarlett et al. [8] presented isolated hydrogenosomes claiming regions of increased electron density and in some, projections giving a ‘dumb-bell’ appearance and an internal membrane. We did not find in hydrogenosomes of *N. frontalis* regions of increased electron density. Internal membranes were occasionally observed resembling the hydrogenosomes of the trichomonads [20,21]. On the other hand, it was proposed [9] that in hyphae the hydrogenosomes are micro-body-like, regularly shaped, round to oval while in zoospores they are elongated and have a peduncular extension. In our study we found hydrogenosomes round or in division where they are elongated or present larger size in all stages so far examined.

Routine preparations frequently showed hydrogenosomes in the process of division (Figs. 1, 5 and 6).
Some of them were elongated, containing a membranous septum (Fig. 1). Images suggesting the gradual invagination of the inner hydrogenosomal membrane characteristic of the partition process were observed (Fig. 5) while the outer hydrogenosomal membrane appeared intact. During a late step of the partition division process, the hydrogenosomes become larger and an invagination of the inner hydrogenosomal membrane divides the hydrogenosomal matrix into two compartments (Fig. 6). The N. frontalis hydrogenosomes followed a division process identical to that previously described for trichomonad hydrogenosomes [21]. Morphological evidence was presented showing that trichomonad hydrogenosomes, like mitochondria, divide by two distinct processes: segmentation and partition [20,21]. In the segmentation process, the hydrogenosome grows, becoming elongated with the appearance of a constriction in the central portion. In the partition process, the division begins by an invagination of the inner hydrogenosome membrane, forming a transverse septum, separating the organelle matrix into two compartments. We have frequently observed both these processes of hydrogenosome division in N. frontalis. Our morphological data supporting the hypothesis of a common evolutionary origin for mitochondria and trichomonad hydrogenosomes [21,22] could be extended to fungal hydrogenosomes.

Previous reports [9,12] presenting fungal hydrogenosomes with a single membrane supported the hypothesis of Cavalier-Smith [13] that in anaerobic fungi the hydrogenosomes were possibly derived from peroxisomes and did not, as in protozoa, evolve from mitochondria or from symbiotic bacteria. Furthermore, Marvin-Sikkema et al. [12] observed a cross-reaction of the anti-(C-terminal) SKL antiserum with Neocallimastix sp. L2 hydrogenosomes. On the other hand, van der Giezen et al. [23] proposed a second hypothesis based on the presence in fungal hydrogenosomes of enzymes leading to the production of hydrogen as pyruvate:ferredoxin:oxidoreductase, ferredoxin and hydrogenase which are usually encountered in prokaryotic anaerobes. Fungal hydrogenosomes could have evolved from the endosymbiotic uptake of a bacterium of a clostridial type. Our data presenting a double membrane surrounding the N. frontalis hydrogenosomes favor the hypothesis of a mitochondrial origin. Molecular support for this hypothesis has come recently from the cloning of N. frontalis genes encoding hydrogenosomal proteins such as β-succinyl CoA synthetase [14] and malic enzyme [15]. These proteins which are encoded by nuclear genes and targeted to the hydrogenosomes present presequences sharing common features with known mitochondrial targeting signals. The presence of hydrogenosomes in unrelated species such as protozoa and fungi raised the question of the appearance and evolution of this organelle. Since the hydrogenosomes of N. frontalis studied here did not present a single membrane as previously published but are surrounded by a double membrane, the hydrogenosomes of these unrelated organisms which have a similar morphological configuration may have had a similar origin.

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