Hydrogenosomes, membrane-bounded organelles that compartmentalize the terminal reactions of cellular energy metabolism, were first described in the parabasalid flagellate Tritrichomonas foetus as subcellular compartments that produce hydrogen and ATP (adenosine triphosphate). Since then, these organelles have been described in a number of different unicellular eukaryotes adapted to microaerobic or anoxic environments. Recent studies have led researchers to consider hydrogenosomes as variations of mitochondria adapted to anaerobic environments, a concept that is supported by the finding of rudimentary mitochondrial-remnant organelles in organisms previously considered devoid of energy-generating organelles (the Archezoa). The relationship of these energy-generating organelles to each other and to the mitochondrion has been examined by many researchers, and several theories have been put forward to explain their origins. In this article, we hope to correct some misconceptions concerning the relationships of the hydrogenosomes so far described, and to put forward an argument that supports the concept that hydrogenosomes evolved repeatedly, either from a protomitochondrion or from differentiated mitochondria.

Keywords: mitochondria, hydrogenosome, anaerobes, energy metabolism, microbiology

Hydrogenosomes are membrane-bounded organelles, approximately 1 to 2 micrometers in size, that compartmentalize the terminal reactions of cellular energy metabolism (figure 1). Hydrogenosomes were first described in the flagellate Tritrichomonas foetus in a seminal publication by Lindmark and Müller (1973) as subcellular compartments that produce hydrogen and ATP (adenosine triphosphate). Since then, these organelles (or variations of them) have been described in a number of different unicellular eukaryotes adapted to microaerobic or anoxic environments (Roger 1999). More recent studies have led researchers to consider these organelles as variations of mitochondria adapted to anaerobic environments (Biagini et al. 1997, Embley et al. 2003). This concept is supported by the finding of rudimentary mitochondrial-remnant organelles in organisms that were previously considered devoid of energy-generating organelles (the Archezoa; Cavalier-Smith 1993). The relationship of these energy-generating organelles to each other, regardless of whether they produce hydrogen or not, and to the mitochondrion has been examined by many researchers, and several theories have been put forward in an attempt to explain their origins (Martin and Müller 1998, Dyall et al. 2000, Hackstein et al. 2001, Martin et al. 2001). In this article, we hope to correct some misconceptions concerning the relationships of the various hydrogenosomes so far described, and to put forward an argument supporting the concept that hydrogenosomes evolved repeatedly, either from a protomitochondrion or from differentiated mitochondria. This view takes into account the structural and biochemical diversity of the hydrogenosomes observed among different taxa, and clearly demonstrates that these organelles should not be expected to be biochemically identical among the diverse organisms harboring them. Even the apparent common denominator—hydrogen production—can become marginal, as in the case of the symbiotic anaerobic chytrid fungi from the herbivore gastrointestinal tract (Boxma et al. 2004). The common denominator for all these organelles seems to be that they function as a compartment for the terminal reactions of an anaerobic energy metabolism without an electron transport chain. This compartmentalization may allow the generation of extra ATP, or better regulation of pathways that replenish (through anapleurotic reactions) or use (through catabolic reactions) certain key intermediates of the cellular energy metabolism, or both.

Mitochondrial origins of the hydrogenosome

Under anaerobic conditions, the mitochondrial electron transport chain cannot use oxygen as a terminal electron acceptor, and energy conservation by the generation of ATP cannot be accomplished. Yet certain mitochondria appear capable of using alternative environmental electron acceptors (e.g., nitrate), or metabolic (Krebs cycle) intermediates such as...
as fumarate, as endogenous electron acceptors that allow a rudimentary function of the electron transport chain even in the complete absence of oxygen (Tielens et al. 2002). In other eukaryotes, however, the adaptation to anoxic niches has reduced the mitochondria to inconspicuous cellular compartments, with a concomitant loss of the electron transport chain and its energy conservation capacities. Nevertheless, certain mitochondrial functions, such as the production of acetyl coenzyme A and keto acids for lipid and carbohydrate biosynthesis, respectively, are maintained even in the absence of a functioning electron transport chain. To date, it appears that there are no extant protozoa lacking mitochondria or hydrogenosomes, or the rudiments of these organelles (Martin and Müller 1998). The presence of vestigial mitochondrial remnant organelles in what were once considered amitochondriate organisms (archezoa) clearly suggests that the progenitor of all extant protozoa was a mitochondrion-containing cell and is an indication of the evolutionary success of this acquisition (figure 2). These structures are of varying complexity and appearance. Among the mitochondrial remnant organelles of the intestinal parasites, for example, the organelle associated with Entamoeba 60-kilodalton (kDa) heat shock protein (HSP60) has been termed “crypton,” as this subcellular structure was previously hidden and its function cryptic (figure 3). Likewise, the organelle containing Giardia 60-kDa chaperonin (CPN60, one of a family of specialized heat shock proteins), of putative mitochondrial origin, has been termed a “mitosome” (figure 4). These organelles function in the assembly of iron–sulfur clusters, which function as the redox-active components of hydrogenase, ferredoxin, and other molecules, but notably, none of these organelles is involved in classic redox-coupled energy conservation (Tovar et al. 1999, 2003, Vanacova et al. 2003). The presence of the anionic phospholipid cardiolipin, which is primarily localized in the inner membranes of mitochondria, has been demonstrated in Giardia using a specific nonyl acridine orange–cardiolipin fluorescence stain (figure 4c; David Lloyd, Cardiff University, Cardiff, Wales, United Kingdom, personal communication, 26 January 2005). That these specialized cardiolipin-containing membranes demonstrate functions in electron transport and in generating membrane potential (Lloyd et al. 2002) supports the growing evidence that Giardia intestinalis may not be primitive, but instead may be derived from an aerobic, mitochondria-containing flagellate.

Interestingly, the apicomplexan parasite Cryptosporidium parvum lacks both mitochondrial and apicoplast genomes (an apicoplast is a non-photosynthetic plastid-like organelle, present in other members of the Apicomplexa phylum). There are also no indications of any tRNA (transfer RNA) synthetases with mitochondrial or apicoplast targeting signals (Abrahamsen et al. 2004) in this species. Yet antibodies raised against the heat shock protein CPN60 do indicate the presence of remnant mitochondria (Riordan et al. 2003). The presence of mitochondrial remnant organelles in Giardia, in contrast, is inferred from the identification of iron–sulfur cluster synthesizing proteins IscS and IscU, and the presence of cardiolipin-containing membranes that are capable of reducing redox-sensitive dyes (Tovar et al. 1999, 2003, Lloyd et al. 2002, Vanacova et al. 2003; David Lloyd, Cardiff University, Cardiff, Wales, United Kingdom, personal communication, 26 January 2005). However, antibodies raised toward either HSP60 or hydrogenase failed to reveal a colocalization of these proteins with IscS and IscU at the subcellular level. The latest candidate organisms reported to have mitochondrial remnant organelles are the microsporidia (Trachipleistophora hominis and Nosema locustae). This conclusion is based on the identification of a mitochondrial-type 70-kDa heat shock protein (HSP70) in these organisms (Germot et al. 1996, Williams et al. 2002). Caution should be exercised.

Figure 1. Electron micrographs of Trichomonas foetus. (a) Seven hydrogenosomes (H) can be identified in the cytoplasm (A, axostyle; G, Golgi apparatus; N, nucleus). (b) At higher magnification, the double membrane that surrounds the hydrogenosomes can be identified (M, marginal plate). Scale bar: 1 micrometer. (c) Trichomonas vaginalis (viewed through light microscopy) after a BSTP reaction in the presence of hydrogen, which allows the cytochemical identification of hydrogenase activity (Zwart et al. 1988). The natural size of the cell is approximately 10 × 45 micrometers. Modified from Hackstein and colleagues (2001).
when interpreting this information, however, as an HSP60 gene has not been found in any of the microsporidia studied so far, and the iron–sulfur containing enzymes identified do not possess an N-terminal targeting signal typical of mitochondrial imported peptides. Moreover, there is no evidence that the HSP70-positive organelles possess a membrane potential, since they do not stain with MitoTracker Red (Williams et al. 2002).

The presence of an anaerobic mitochondrial-like organelle in Blastocystis hominis (Straminopiles) has been proposed mainly on the basis of redox-sensitive dyes (Nasirudeen and Tan 2004). However, the functional relevance of the Blastocystis organelle is unknown, because it has been reported that this structure lacks cytochromes and a functional tricarboxylic acid cycle (Nasirudeen and Tan 2004).

The amount of information available for hydrogenosomes is much greater than that for mitochondrial-like (and similar) organelles, and it is this information that allows creation of a coherent evolutionary scenario to relate the origins of all these organelles. Notably, there is no evidence that energy-conserving pathways have been retained in any of the non-hydrogenosomal mitochondrial remnant organelles, which were classified earlier as “type I anaerobes” (Martin and Müller 1998, Müller 1998, Tovar et al. 2003). The energy metabolism of these type I anaerobes relies on cytosolic fermentation pathways (Rosenthal et al. 1997, Müller 1998), and the significance of compartmentalized hydrogen production and redox activity in Giardia has to be confirmed (Lloyd et al. 2002). Most of the genes encoding cytosolic fermentation enzymes were present in the ancestral eukaryote, but some of these enzymes may have been acquired by lateral gene transfer from anaerobic eubacteria and archaea. Phylogenetic analysis of Giardia lamblia, Entamoeba histolytica, and the anaerobic chytrids supports the idea that a number of fermentation enzymes were acquired by lateral gene transfer, potentially from different sources (Rosenthal et al. 1997, Hackstein et al. 1999). However, lateral gene transfer from prokaryotes to eukaryotes other than by endosymbiosis is
highly controversial (Doolittle 1998), and even the detection of lateral gene transfer among prokaryotes might be flawed by the limitations of phylogenetic reconstruction (Koonin 2003).

Nevertheless, the available evidence strongly suggests that all extant eukaryotes (including the type I anaerobes discussed above) arose from a common ancestor that harbored a protomitochondrion-like endosymbiont (Martin and Müller 1998, Martin et al. 2001). This ancestral endosymbiont must have been a facultatively anaerobic eubacterium (Gabaldon and Huynen 2003), which in aerobic environments evolved into present-day mitochondria, retaining an electron transport chain that uses oxygen as the terminal electron acceptor.

Evolving in anaerobic niches, the universal endosymbiont lost its electron transport chain and its basic capacities for compartmentalized energy conservation, giving rise to type I anaerobes (Martin and Müller 1998). Alternatively, evolution in anaerobic niches could give rise to hydrogenosome-bearing “type II anaerobes” (see below), which have lost their electron transport chain but retained an energy-conserving and hydrogen-producing compartment (figure 5; Martin and Müller 1998, Martin et al. 2001). Although this scenario—also known as the hydrogen hypothesis—is likely to provide one possible explanation for the origin of the eukaryotic cell, the evolutionary history of hydrogenosomes appears to be much more complex, and reveals that evolution found several alternative solutions for the problem of adapting to anoxic niches.

**Hydrogenosomes: Organelles that can use protons as electron acceptors**

In certain anaerobic protists and some anaerobic chytrid fungi, the adaptation to anoxic niches led to the evolution of type II anaerobes, which are defined by the presence of hydrogenosomes or membrane-bounded organelles that compartmentalize the terminal reactions of the cellular energy metabolism (Martin and Müller 1998). In marked contrast, type I anaerobes did not retain functional structures and at best contained mitochondrial remnant organelles. Characteristically, hydrogenosomes import pyruvate (or malate), which is oxidatively decarboxylated to acetyl coenzyme A by the action of a pyruvate:ferredoxin oxidoreductase (figure 5). An acetate:succinate coenzyme A transferase and a succinate thiokinase mediate the formation of acetate and ATP (figure 5), as in similar reactions in the mitochondria of the kinetoplastidae and helminths (Tielens et al. 2002). The reduction equivalents that are formed during the decarboxylation of pyruvate are not used to fuel an electron transport chain, as in mitochondria; rather, they are removed from the cell by a hydrogenase, which reduces protons, resulting in the formation of molecular hydrogen (Müller 1993, Embley and Martin 1998). Alternatively, anaerobic chytrid fungi avoid the generation of reduction equivalents by using pyruvate:formate lyase instead of pyruvate:ferredoxin oxidoreductase for the nonoxidative splitting of pyruvate into acetate and formate, rendering hydrogen production a marginal metabolic route (figure 6; Akhmanova et al. 1999, Boxma et al. 2004). Notably, the hydrogenosomes of certain ciliates, such as *Nyctotherus ovalis* (figure 7), retained a much more mitochondrion-like type of hydrogenosomal metabolism (table 1).

Hydrogenosomes do not coexist with mitochondria, and they have not been detected in plants, in multicellular animals, or in eukaryotic microorganisms that face extended periods of aerobiosis during their life cycles. Given that hydrogenosomes are physiologically different and occur in phylogenetically unrelated taxa, which in general comprise both aerobic and anaerobic organisms, it is our contention that they have evolved several times and are not directly related to a common progenitor cell (Biagini et al. 1997, Martin and Müller 1998, Roger 1999, Hackstein et al. 2001, Embley et al. 2003).

**Hydrogenosomes of Trichomonas vaginalis**

The hydrogenosomes of the trichomonads (phylum Parabasalia) have been studied intensively for more than 30 years (Lindmark and Müller 1973, Müller 1993). Upon initial inspection, with the exception of a double membrane, these

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**Figure 4. Laser-scanning confocal micrograph of Giardia intestinalis stained with (a) the membrane potential dye rhodamine 123 and (b) 5-cyano-2,3-ditolyl tetrazolium chloride, a tetrazolium dye that is reduced to a red-fluorescent formazan. (c) Nonyl acridine orange–cardiolipin fluorescent stain demonstrating cardiolipin-containing organelles in G. intestinalis. Images (a) and (b) are reproduced with permission from Lloyd and colleagues (2002); (c) is kindly supplied by David Lloyd.**
organelles were considered both mor-
phologically and biochemically distinct
from mitochondria (figure 1; Benchimol et al. 1996). However, subsequent bio-
chemical and molecular studies have
changed this view. Trichomonad hydro-
genosomes possess mitochondrial-like
chaperonins; 10-kDa, 60-kDa, and 70-
kDa heat shock proteins (Bui et al. 1996,
Germot et al. 1996); 31-kDa hydrogeno-
somal membrane proteins (HMP31) of
the mitochondrial carrier family (Dyall et al. 2000); and circumstantial evidence (N-
terminal extensions) in favor of import
machinery like that in mitochondria
(Dyall et al. 2004a). Also, the presence of
an acetatesuccinate coenzyme A trans-
ferase (Tielens et al. 2002) that is believed
to be shared by these organelles and by
certain mitochondria might suggest a mi-
tochondrial ancestry for the hydrogeno-
somes of Trichomonas (table 1; Dyall et al.

However, trichomonad hydrogeno-
somes are clearly different from mito-
chondria, as they lack a genome,
ribosomes, cytochromes, an electron
transport chain, cardiolipin, and cristae
(Benchimol et al. 1996, Müller 1998,
Clemens and Johnson 2000, Voncken et al.
2002a). Recently identified 24- and 51-
kDa proteins homologous to mitochon-
drial nicotinamide adenine dinucleotide
(NAD) dehydrogenases are not part of a
mitochondrial electron transport chain.
Rather, they seem to fuel the hydrogenase
reactions (Dyall et al. 2004b, Hrdy et al. 2004).
Moreover, the import machinery of trichomonad hydrogenosomes seems
to exhibit peculiar characteristics that are not shared with mi-
tochondria (Dyall et al. 2000, 2004a). Like mitochondria,
trichomonad hydrogenosomes import pyruvate that results
from glycolysis, but they do not use a pyruvate dehydrogenase for
the catabolism of pyruvate, as mitochondria do. Instead,
these hydrogenosomes metabolize pyruvate through a
pyruvate:ferredoxin oxidoreductase and hydrogenase to
Acetate formation from acetyl coenzyme A is coupled to the
substrate-level phosphorylation of succinate via the enzyme
acetatesuccinate coenzyme A transferase; this route yields one
molecule of ATP per molecule of pyruvate consumed (figure
5). Additional ATP formation seems to be feasible through
the generation of a proton motive force (Humphreys et al.
1994). Although the generation of a proton motive force has
not yet been studied in detail, the generation of a proton gra-
dient by trichomonad hydrogenosomes has been demon-
strated (Humphreys et al. 1994). In addition, trichomonad
hydrogenosomes can serve as cellular Ca\(^{2+}\) (calcium ion)
stores (Humphreys et al. 1994, Benchimol et al. 1996).
Therefore, it might be concluded that trichomonad hydrogenosomes
are capable of generating a proton motive force and a proton
gradient, even if a mitochondria-like electron transport chain is absent (table 1).

Hydrogenosomes of anaerobic ciliates

Ciliates belong to the “crown group” of eukaryotes (Sogin 1991), and anaerobic species evolved in at least 8 of the 22 or-
ders of ciliates as classified by Corliss (1979). There is evidence
that anaerobic ciliates evolved secondarily from aerobic ances-
tors, since some higher ciliate taxa comprise both aerobic
and anaerobic species (Embley et al. 1995, 2003, Fenchel and
Finlay 1995, Hackstein et al. 2001). Bona fide hydrogenosomes
are present in 7 of the 22 orders, but the evidence that these
hydrogenosomes evolved from mitochondria is circumstan-
tial (Fenchel and Finlay 1995). Notably, Akhmanova and col-
leagues (1998a) have presented straightforward evidence of
the presence of a mitochondrial genome in the hydrogeno-

Figure 5. Metabolic scheme for a generalized type II anaerobe (e.g., Trichomonas
vaginalis; Müller 1993, 1998). Pyruvate is formed in the cytoplasm (C) by glycoly-
sis, imported into the hydrogenosome (H), and metabolized to acetate and carbon
dioxide (CO\(_2\)) under formation of molecular hydrogen (H\(_2\)). ATP (adenosine
triphosphate) is formed by substrate-level phosphorylation by the enzymes acetate
succinyl CoA transferase (2) and succinate thiokinase (3). ATP is exported by the
ADP/ATP carrier (5). The electrons resulting from the oxidative decarboxylation of
pyruvate are transferred to a ferredoxin by pyruvate:ferredoxin oxidoreductase
(1) and to protons by an iron hydrogenase (4). Recently, the presence of an NADH
(nicotinamide adenine dinucleotide, reduced form) dehydrogenase has been pos-
tulated, which must be able to transfer electrons from reduced NAD (nicotinamide
denine dinucleotide) to the hydrogenase (Dyall et al. 2004b, Hrdy et al. 2004).
some of N. ovalis, an anaerobic, heterotrichous ciliate that inhabits the intestinal tract of cockroaches (Akhmanova et al. 1998a, van Hoek et al. 2000). This genome, identified by immunocytochemistry, hosts ribosomal RNA (rRNA) genes that are abundantly expressed, and phylogenetic analysis reveals a clustering among the mitochondrial rRNA genes of aerobic ciliates (van Hoek et al. 2000, Hackstein et al. 2001). Since the phylogenies of the nuclear 18S rRNA genes of the ciliates are congruent with the ribosomal small subunit (SSU) rRNA genes of their mitochondria and hydrogenosomes (Akhmanova et al. 1998a, van Hoek et al. 2000, Hackstein et al. 2001), it is likely that the hydrogenosomes of N. ovalis evolved from the mitochondria of aerobic ciliates. Moreover, the hydrogenosomes of N. ovalis possess cristae and putative ribosomes and thus morphologically resemble mitochondria (figure 7; Akhmanova et al. 1998a). Therefore, it seems reasonable to assume that the hydrogenosomes of heterotrichous ciliates evolved from mitochondria that adapted to anaerobic environments. Notably, the hydrogenosomes of rumen ciliates, which are only distantly related to N. ovalis, seem to be quite different (table 1).

Akhmanova and colleagues (1998a) have shown that ciliate hydrogenosomes possess an iron hydrogenase that is encoded by a multisubunit gene located on a macronuclear minichromosome. This hydrogenase represents a novel type of iron hydrogenase that allows hydrogen formation to be coupled directly to the re-oxidation of reduced nicotinamide adenine dinucleotide (NADH). The iron hydrogenase is linked covalently with a protein, which possesses NAD and flavin mononucleotide (FMN) binding sites, and a ferredoxin-like module that makes it possible to transfer electrons to the catalytic site of the hydrogenase (Akhmanova et al. 1998a). The origin of the hydrogenase is of central importance not only for the hydrogen hypothesis but also for understanding the evolution of the various types of hydrogenosomes. All eukaryotic iron hydrogenases contain a highly conserved N-terminal iron–sulfur cluster, as well as most of the H-cluster residues (Balk et al. 2004). This observation includes the 63-kDa hydrogenase-like proteins termed NARF (nuclear prelamin A recognition factor), which have representatives in all aerobic eukaryotes studied so far and are considered to have evolved from a common ancestor. NARF-like proteins neither produce nor consume hydrogen (Balk et al. 2004), and appear to form a monophyletic cluster with eukaryotic iron hydrogenases, with the possible exception of the N. ovalis hydrogenase (Akhmanova et al. 1998a, Nixon et al. 2003). However, because of the high conservation of the hydrogenases and the sensitivity for species sampling, statistical support for either of these phylogenies is poor. Hydrogenases are also found in the eubacteria, representatives of which have been implicated in the origin of the mitochondrion. One division in particular, the purple phototrophic bacteria or proteobacteria, which form the largest and most physiologically diverse of all the eubacteria, are subdivided into

Figure 6. Energy metabolism of the anaerobic chytridiomycete fungus Piromyces sp. E2. The figure shows a scheme of the metabolic pathways involved in the production of major end products, which are representative for a bacterial-type mixed-acid fermentation. Numbers indicate the following enzymes: (1) hexokinase, glucose-6-phosphate isomerase, phosphofructokinase 1, aldolase and triose phosphate isomerase; (2) glyceraldehyde-3-phosphate dehydrogenase; (3) phosphoglycerate kinase, phosphoglycerate mutase, and enolase; (4) phosphoenolpyruvate carboxykinase; (5) malate dehydrogenase; (6) fumarase; (7) fumarate reductase; (8) pyruvate kinase; (9) lactate dehydrogenase; (10) cytosolic pyruvate formate-lyase; (11) alcohol dehydrogenase E; (12) pyruvate import into hydrogenosomes; (13) malic enzyme; (14) hydrogenase; (15) hydrogenosomal pyruvate formate-lyase; (16) acetate:succinate coenzyme A transferase; (17) succinyl coenzyme A synthetase; (18) ADP/ATP carrier. Analysis of the metabolic fluxes revealed that the formation of hydrogen via the malate route can become marginal under certain culture conditions (Boxma et al. 2004). Abbreviations: AccA, acetyl coenzyme A; ADHE, alcohol-aldehyde dehydrogenase; EtOH, ethanol; FUM, fumarate; G3P, glyceraldehyde-3-phosphate; MAL, malate; NAD, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced form; OXAC, oxaloacetate; PEP, phosphoenolpyruvate; PFL, pyruvate formate-lyase; PYR, pyruvate; SUCC, succinate. Reproduced with permission from Boxma and colleagues (2004).
Table 1. Properties of various hydrogenosomes and mitochondrial-remnant organelles.

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<th>Methanogenic endosymbiont</th>
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<td>No mAAC</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>Mastigomycotina</td>
<td>Spiellomyctales</td>
<td>Hydrogenosome</td>
<td>HSP60</td>
<td>[Fe]</td>
<td>PFL</td>
<td>No</td>
<td>ND</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Abbreviations: Cpr, chaperonin; [Fe], iron only; HMP, hydrogenosomal membrane protein; HSP, heat shock protein; kDa, kilodalton; mAAC, mitochondrial ADP/ATP carriers; NARF, nucleolar prelamin A recognition factor; ND, not determined; PDH, pyruvate dehydrogenase; PFL, pyruvate formate lyase; PFO, pyruvate ferredoxin oxidoreductase; PNOR, pyruvate NADH oxidoreductase.
the purple photosynthetic bacteria, comprising the alpha, beta, gamma, and delta proteobacteria. The delta proteobacteria comprise the sulfate and sulfate-reducing bacteria. Analysis of mitochondrial genomes indicates that the mitochondrion has a single endosymbiotic origin from an alpha proteobacterial-type progenitor. However, the case for an alpha proteobacterial origin of the hydrogenosome and its relatives is not as clear, because of the lack of organellar genomes in these subcellular compartments. Even the identification of the first alpha proteobacterial hydrogenase could not solve the phylogenetic puzzle. One of us (J. H. P. H.) recently showed that the genes encoding hydrogenases of rumen ciliates support a common eukaryotic origin of all eukaryotic hydrogenases and NARF-like proteins, with the exception of the hydrogenase of \( N. \ ovalis \), which appears to be a mosaic of proteins of delta proteobacterial and beta proteobacterial origins. In contrast, the iron hydrogenases of rumen ciliates and anaerobic chytrids clearly belong to the eukaryotic cluster. They are similar to iron hydrogenases from \( T. \ vaginalis \), \( Neocallimastix \), \( Piromyztes \), and \( Spironucleus \). Phylogenetic analysis of the hydrogenases of anaerobic chytrids clusters them with the extremely short hydrogenases of green algae, which function in plastidic electron transport (Akhmanova et al. 1998a, Voncken et al. 2002b, Nixon et al. 2003). However, the origin of these eukaryotic hydrogenases from a hypothetical hydrogenase-containing universal endosymbiont remains unclear; in particular, there is no convincing support for the assumption that all eukaryotic hydrogenases (including NARF-like proteins) evolved from an alpha proteobacterial ancestor.

**Hydrogenosomes of anaerobic chytrids: An alternative adaptation to anaerobic environments**

Anaerobic chytrids are important symbionts in the gastrointestinal tract of many herbivorous mammals. Their life cycle consists of a flagellated zoospore stage alternating with a vegetative phase when a multinucleated mycelium is formed.

The hyphae of the rhizomycelial system attach to plant-derived particles of the digesta and secrete a broad spectrum of fibrolytic enzymes, which in combination are very efficient in digesting plant polymers (Yarlett 1994). These organisms are highly adapted to intestinal environments; their optimal growth temperature coincides with the body temperature of their mammalian hosts, and they live and multiply under anoxic conditions during almost all of their life cycle (Yarlett 1994). The anaerobic chytrids evolved from mitochondria-bearing ancestors, as DNA sequence analysis reveals a clustering of both aerobic and anaerobic chytrids (Voncken et al. 2002a). Also, an analysis of biochemical and morphological traits consistently establishes a close relationship between chytrids and other fungi (Voncken et al. 2002a). Consequently, there is no doubt that the chytrids living in the gastrointestinal tract of herbivorous mammals have secondarily adopted an anaerobic way of life.

Anaerobic chytrids, such as \( Neocallimastix \) and \( Piromyztes \), possess hydrogenosomes instead of mitochondria (figure 8; Marvin-Sikkema et al. 1994, Yarlett 1994). However, these organelles are clearly different from the hydrogenosomes of the ciliate \( N. \ ovalis \) (figure 7), the amoeboflagellate \( Psalteriomonas \), the parabasalid \( T. \ vaginalis \) (figure 1; Coombs and Hackstein 1995, Hackstein et al. 2001). Like the hydrogenosomes of the amoeboflagellate \( P. \ lanterna \) and of the parabasalid \( T. \ vaginalis \), they lack a genome, but unlike \( T. \ vaginalis \) hydrogenosomes, the chytrid hydrogenosomes rely on malate, not pyruvate, for hydrogen formation (figure 6, table 1). The imported malate is oxidatively decarboxylated by a hydrogenosomal malic enzyme, and until recently, it had been postulated that the resulting pyruvate is oxidized further by pyruvate:ferridoxin oxidoreductase to acetyl coenzyme A. The reduced equivalents should then be transferred via

**Figure 7.** The hydrogenosome \((H)\) of \( Nyctotherus \) ovalis at higher magnification looks like a mitochondrion (glutaraldehyde–osmium tetroxide \([\text{OsO}_4]\) fixation). The inner and outer membrane, cristae-like invaginations of the inner membrane (arrowheads), and putative 70S ribosomes can be identified (arrows). Abbreviation: m, methanogenic endosymbionts. Reproduced with permission from Akhmanova and colleagues (1998a).

**Figure 8.** Electron micrograph of \( Neocallimastix \) hydrogenosomes. (a) Hydrogenosomes in a whole zoospore, where the organelles appear to be clustered around the flagella apparatus; (b) rhizoids, where the hydrogenosomes appear to be randomly distributed in small groups (they also occur in sporangia). The organelles are about 0.5–1.0 micrometer (\( \mu m \)) in diameter and have a finely granular appearance. Marker bars = 1 \( \mu m \). Reproduced with permission from Yarlett (1994).
ferredoxin to the hydrogenase, thus maintaining the redox balance (Marvin-Sikkema et al. 1994). However, Akhmanova and colleagues (1999) and Boxma and colleagues (2004) showed that the hydrogenosomes of anaerobic chytrids perform a bacterial-type mixed acid fermentation during which pyruvate is not oxidized but split by pyruvate formate-lyase into acetyl coenzyme A and formate (figure 6). Consequently, the formation of reduced equivalents is avoided, because the hydrogenosome excretes formate and acetate as end products of its energy metabolism. Moreover, the vast majority of the carbon flow through the hydrogenosome is mediated by pyruvate, which is imported from the cytosol and metabolized in the hydrogenosome without hydrogen formation (Boxma et al. 2004). Obviously, the hydrogenosomes of anaerobic chytrids followed a different strategy when adapting to anaerobic environments: avoiding the formation of reduced equivalents renders hydrogen production a rudimentary metabolic activity for these organelles. Akhmanova and colleagues (1999) and Boxma and colleagues (2004) also demonstrated that several enzymes of mitochondrial origin lacked N-terminal peptide extensions that could act as targeting signals for the mitochondrial membrane, and consequently these truncated proteins were retargeted to the cytoplasm and were no longer present in the hydrogenosome (Akhmanova et al. 1998b).

Functional and phylogenetic analysis of the ADP/ATP carriers from anaerobic chytrid hydrogenosomes clearly supports a fungal mitochondrial origin for these organelles (Voncken et al. 2002a, Tjaden et al. 2004). Given that chytrid hydrogenosomes lack a genome, the ADP/ATP carriers and HSP60 are the best available markers for tracing the evolutionary history of these organelles. Phylogenetic analysis of both genes unequivocally reveals a fungal mitochondrial ancestry (Voncken et al. 2002a), supporting the earlier finding that typical mitochondrial enzymes had been retargeted to the cytoplasm in the course of the evolution of the chytrid hydrogenosomes (Akhmanova et al. 1998b, Hackstein et al. 1999, 2001).

Chytrid hydrogenosomes are therefore clearly distinct from the hydrogenosomes of Trichomonas that formed the basis for the Martin–Müller hydrogen hypothesis for the evolution of the eukaryotic cell (table 1). Both the origin of the organelle (i.e., the universal endosymbiont in the case of Trichomonas and a differentiated fungal mitochondrion in the case of the anaerobic chytrids) and the evolutionary strategies to adapt to anoxic environments are different. Because the hydrogenosomes of Trichomonas also lack a genome, an analysis of the hydrogenosomal ADP/ATP carriers should provide clues for or against the hydrogen hypothesis. However, trichomonad hydrogenosomes do not host mitochondrial-type ADP/ATP carriers (Tjaden et al. 2004). Instead, they use a different member of the mitochondrial carrier family—HMP31 for ADP/ATP exchange—that is phylogenetically and biochemically distinct from the mitochondrial-type ADP/ATP carriers (Dyall et al. 2000, Tjaden et al. 2004). Phylogenetic analysis of these carrier proteins indicates that the trichomonad HMP31 carrier proteins branch earlier than the mitochondrial ADP/ATP carriers, in agreement with the predictions derived from the hydrogen hypothesis (table 1).

Repeated evolution of hydrogenosomes as adaptations to anaerobic environments

There is a lot of circumstantial evidence that hydrogenosomes might have evolved repeatedly, not only in the trichomonads, the ciliates, and the chytridiomycete fungi discussed above, but in other widely separated lines of eukaryotes. Hydrogenosomes have been identified in amoebo-bae (Monoplychis, Sawyeria) and in several flagellated eukaryotes, such as Trimastix, oxymonads, and trichomonads (Embley and Martin 1998, Roger 1999, Hackstein et al. 2001, Martin et al. 2001, Embley et al. 2003). However, the major arguments were based on the patchy distribution of hydrogenosome-bearing organisms in the Tree of Life (http://tolweb.org/tree/phylogeny.html). The Tree of Life is based on an 18S rRNA phylogeny, and it is widely accepted that this tree is biased and not suited to resolve certain evolutionary relationships (Embley et al. 2003). Notably, the monophyly of mitochondria could be established only on the basis of complete mitochondrial genomes (Gray et al. 1999). As hydrogenosomes—with one exception—lack an organelle genome, there is no straightforward approach to retrieving their phylogenetic relationships. Obviously, host phylogenies are of limited value in addressing the question of whether hydrogenosomes evolved repeatedly and, if so, from the same or different endosymbiotic ancestors. Therefore, it has to be stressed that there is also no straightforward evidence that all the hydrogenosomes evolved from mitochondria, or from an ancestor common to both mitochondria and hydrogenosomes (Gabaldon and Huynen 2003, Dyall et al. 2004a).
Because of the lack of hydrogenosomal genomes (van der Giezen et al. 1997, Clemens and Johnson 2000, Hackstein et al. 2001, Leon-Avila and Tovar 2004), a potential mitochondrial ancestry can be deduced only from the phylogenetic analysis of nuclear-encoded "organellar" genes (Embley et al. 2003, Dyall et al. 2004a). Such proteins are synthesized in the cytoplasm and subsequently imported into the hydrogenosome. This information will remain fragmentary until the proteomes of the various hydrogenosomes and the complete genomes of their hosts have been unraveled. Moreover, a phylogenetic analysis of the genes encoding these proteins might provide erroneous information, because many of the mitochondrial proteins have different ancestors.

Finally, proteins that are crucial for hydrogenosomal function (hydrogenases, pyruvate:ferredoxin oxidoreductases, or pyruvate formate lyases) do not belong to the normal repertoire of an aerobic eukaryotic cell or its organelles. These enzymes could be acquired from ancestral eukaryotic precursors, from eubacterial or archaeal endosymbionts, or (through lateral gene transfer) from the DNA of food bacteria (Doolittle 1998, Martin et al. 2001, Brown 2003). Currently, only the analysis of proteins, which fulfill a key role in both mitochondria and hydrogenosomes, can help to answer the questions regarding the origin of hydrogenosomes. Only in the heterotrichous ciliate N. ovalis has a hydrogenosomal genome been demonstrated (Akhmanova et al. 1998a), and phylogenetic analysis of its organelle SSU rRNA genes has revealed its mitochondrial ancestry (van Hoek et al. 2000, Hackstein et al. 2001), allowing a matching of the phylotypes of the various hydrogenosomes and the complete genomes of their hosts to be unraveled. Moreover, a phylogenetic analysis of the genes encoding these proteins might provide erroneous information, because many of the mitochondrial proteins have different ancestors.

Nuclear DNA sequences from Trichomonas and its relatives have suggested that hydrogenosomes and mitochondria share a common origin with each other and with alpha-proteobacteria (Martin and Müller 1998, Gray et al. 1999, Kurland and Andersson 2000, Embley et al. 2003). Such a proteobacterial ancestor, which formed a symbiotic association with an archaeal host, is believed to have evolved into mitochondria or hydrogenosomes by a differential loss of genes (Martin and Müller 1998). In both lines, this evolution involved the development of organelle transporters, which export the ATP generated inside the organelle to the benefit of the host (Voncken et al. 2002a). Recent studies indicate that the ADP/ATP translocators of trichomonad hydrogenosomes belong to the family of mitochondrial solute carriers, although they are clearly distinct from the ADP/ATP carriers of mitochondria (Tjaden et al. 2004). This finding argues for a distinct evolution of the trichomonad hydrogenosomes and of all mitochondria from the very beginning of eukaryogenesis, in agreement with the hydrogen hypothesis of Martin and Müller (1998).

In clear contrast, the functional and phylogenetic analysis of the ADP/ATP carriers of hydrogenosomes in the anaerobic chytrid Neocallimastix and Piromyces reveals that these carriers are of an unequivocally fungal mitochondrial origin. Also, the ADP/ATP carriers of the anaerobic ciliate N. ovalis are of ciliate mitochondrial origin, confirming the assumption that the hydrogenosomes of chytrids and ciliates evolved not only independently of each other but also later than the hydrogenosomes of Trichomonas and, notably, from differentiated mitochondria rather than from a pluripotent mitochondrion or premitochondrion (table 1).

Analysis of the fermentation patterns of the chytrids and the presence of certain enzymes (pyruvate formate lyase and alcohol dehydrogenase E), which are unique to these type II anaerobes and most likely acquired by lateral gene transfer, supports the uniqueness of chytrid hydrogenosomes (Boxma et al. 2004). Although the hydrogenases of the anaerobic chytrids and the trichomonads belong to the eukaryotic heritage, a phylogenetic analysis of the hydrogenase of N. ovalis argues for an acquisition by lateral gene transfer. The N. ovalis hydrogenase is a composite of a typical iron hydrogenase, a ferredoxin-like component (24-kDa subunit), and a binding protein for NADH and reduced FMN (51-kDa subunit). The hydrogenase and the accessory components are of different eubacterial origins (Akhmanova et al. 1998b). These modules allow the exploitation of organelle-derived NADH, which is likely to be generated by a mitochondrial-type pyruvate dehydrogenase. Thus, the available functional and phylogenetic evidence allows the conclusion that the hydrogenosomes of trichomonads, chytrids, and ciliates are substantially different, and that they evolved independently from each other. The hydrogenosomes of trichomonads evolved from a hydrogen-producing protomitochondrion-like ancestral organelle, the hydrogenosomes of chytrids from fungal mitochondria, and the hydrogenosomes of N. ovalis from the mitochondria of an aerobic ciliate retaining substantial features of a classical mitochondrion (figure 7). There is evidence that even the hydrogenosomes of ciliates are not the same, and it is merely the lack of information to date that has allowed only speculation about the biochemical and phylogenetic diversity of the other hydrogenosomes. In essence, hydrogenosomes are organelles that facilitate a compartmentalized energy metabolism in anoxic niches. They are fascinating products of the evolutionary tinkering of eukaryotic cells adapting to life without oxygen, from the beginnings of eukaryogenesis through the course of time. A detailed analysis of the relationship between mitochondria, hydrogenosomes, and mitochondrial remnants requires a deeper knowledge of the phylogenies and subcellular localization of HSP60 and the enzymes for iron–sulfur biosynthesis. Finally, the question of why only unicellular organisms evolved hydrogenosomes (or mitochondrial relics) might be answered tentatively by speculating that the loss of the electron transport chain observed in all hydrogenosomes studied so far will inevitably impair the apoptotic cascade, with the consequence that the morphogenesis and homeostasis of multicellular organisms will be hampered dramatically.

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