Hydrogenosomes

*eukaryotic adaptations
to anaerobic environments*

Frank Voncken

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Chapter 1

Introduction

Introduction

There are many ways to survive in anaerobic environments

Life on earth evolved under anaerobic conditions until photosynthesis provided the basis for the evolution of aerobic organisms. But until now, a wealth of anoxic and microaerobic environments persisted that provided niches for the evolution of the most divergent and complex microbial communities. In the gastro-intestinal tracts of men and animals, for example, extraordinary complex and numerous anaerobic communities are found (Savage 1977; Breznak 1982; Miller and Wolin 1986; Cruden and Markovetz 1987; Breznak and Brune 1994; Brune 1998). Also freshwater and deep ocean sediments, continental aquifers, and porous rocks host bewildering anaerobic microbiota (Ghoirse 1997). Such anaerobic communities exhibit remarkable species diversity, but prokaryotic organisms and unicellular eukaryotes clearly predominate (Fenchel and Finlay 1995).

Organisms that can persist in such anaerobic communities are highly adapted to life in the absence of oxygen. Prokaryotes, for example, evolved a wide variety of anaerobic respiration processes: instead of oxygen, they can use alternative, environmental electron acceptors such as nitrate, sulphate, carbonate, iron or protons. Alternatively, and sometimes additionally, many prokaryotes invented a broad spectrum of fermentation pathways in order to maintain their oxidation-reduction balance (Schlegel 1992; Brock and Madigan 1991). Eukaryotes, on the other hand, have to rely nearly exclusively on glycolysis for their survival under anaerobic conditions. With a few exceptions that will be discussed below, anaerobic eukaryotes did not evolve anaerobic "respiration" processes. Evolution in anaerobic niches clearly favoured those eukaryotes that succeeded to maintain their redox-balance by fermentation. For example, anaerobic eukaryotes degrade glucose to ethanol, lactate or other partially reduced compounds. However, the glycolytic Embden-Meyerhoff pathway yields only two mol of ATP per mol glucose that is metabolised to pyruvate, and reduced cofactors such as NADH are wasted in the various fermentation products with no or only a rather limited energy yield. In the presence of oxygen, on the other hand, the mitochondrial electron transport chain of (aerobic) eukaryotes allows the oxidation of the reduced cofactors that are generated in the Embden-Meyerhoff pathway and the TCA cycle with a gain of (calculated) 38 mol ATP from the degradation of 1 mol glucose. The anaerobic metabolism of eukaryotic cells is therefore characterised by a permanently low energy status.

Under anaerobic conditions, the mitochondrial electron transport chain cannot be used for the generation of ATP, and the main function of the mitochondria, energy conservation, cannot be fulfilled. Therefore, it is not surprising that a number of anaerobic eukaryotes lack mitochondria. The absence of mitochondria, however, can be interpreted in different ways - either as a primitive or as a derived character of the anaerobic eukaryotic cell. For many years, anaerobic eukaryotes without mitochondria (or hydrogenosomes) have been interpreted as "primitive" organisms; therefore, they were called "archaezoa". These organisms were supposed to be relics of primitive eukaryotes that evolved before the advent of mitochondria, since the evolution of the

eukaryotic cell occurred under anoxic conditions (Cavalier-Smith 1983; Margulis 1993; Fenchel and Finlay 1995; Martin 1996; Martin and Müller 1998; Müller 1998; Embley and Hirt 1998). However, the assumption that the extent amitochondriate eukaryotes are "primitive" is no longer favoured. Currently, amitochondriate parasites such as *Giardia*, *Entamoeba*, and different microsporidia are regarded as highly derived eukaryotes. Persuasive evidence has been presented that they possess mitochondrial chaperons and tRNA synthetases that seem to be relics of an ancestral mitochondrion (see for example Clark and Roger 1995; Roger et al. 1998; Hashimoto et al. 1998; Embley and Hirt 1998). Since all these (nuclear-encoded) mitochondrial proteins are located in the cytoplasm of the amitochondriate anaerobes, their existence is most easily explained by the secondary loss of mitochondria. Thus, it is likely that - at least in certain amitochondriate anaerobic protists - the adaptation to permanent or phasic anaerobiosis resulted in a loss of mitochondria (*type I anaerobes*, c.f. Martin and Müller 1998)

However, adaptation to anaerobiosis does not necessarily imply a loss of mitochondria: the yeast *Saccharomyces cerevisiae* can grow under aerobic and anaerobic conditions. Cultivation under anaerobic conditions does not allow the generation of ATP by mitochondria. ATP synthesis relies completely on the fermentation of glucose to

Figure 1. Scheme of the glucose catabolic pathway in yeast. Big box represents the cytosol, small box inside of it represents the mitochondrion. PEP, phosphoenolpyruvate; ADH, alcohol dehydrogenase; PDH, pyruvate dehydrogenase; OAA, oxaloacetate; NDH, NADH-dehydrogenase; SDH, succinate dehydrogenase; UQ, ubiquinone; II-IV, mitochondrial complex II, III and IV.

ethanol. In the presence of oxygen, however, the fermentation pathway is inhibited and the yeast's metabolism is switched to aerobic respiration with its much higher energy yield ("Pasteur-effect"). Interestingly, the mitochondrial electron transport chain became modified in *S. cerevisiae*, since its mitochondria lack complex I of the electron transport chain (MIPS/MITOP database). Instead, yeast possesses two genes encoding NADH-dehydrogenases (NDH1 and NDH2). These dehydrogenases allow reoxidation of NADH in the mitochondria and the cytoplasm - albeit without the extrusion of protons (Fig. 1).

Adaptive modifications of mitochondrial metabolism and the compartmentalisation of glycolytic pathways

Similar to yeast, also multicellular organisms such as parasitic helminths, freshwater snails, mussels, lugworms, and other marine invertebrates are exposed to more or less extended periods of anaerobiosis during their development. These animals envisaged substantial changes of their mitochondrial metabolism in adaptation for anoxic or microaerobic environments (Tielens 1994; van Hellemond and Tielens 1994; Tielens and van Hellemond 1998a,b). Most of these organisms evolved a peculiar variant of anaerobic respiration, "malate dismutation", where endogenous fumarate is reduced to succinate by the enzyme fumarate reductase. In these organisms, fumarate serves as an electron sink. This process requires adaptations of the mitochondrial electron transport chain, i.e. a rhodoquinone as an electron-carrier (van Hellemond et al. 1995), but it allows the use of mitochondrial complex I for the generation of a proton gradient also under anoxic conditions (Fig. 2). In a *parallel* reaction, another malate molecule is metabolised to acetyl-CoA via pyruvate. With the aid of an acetate:succinate CoA-transferase and a succinate thiokinase, substrate-level phosphorylation allows the generation of ATP and the regeneration of succinate. One might conclude that this adaptation allows the mitochondrial compartment to be maintained for the generation of a proton-motive force (PMF) that can be used for the generation of additional ATP.

In unicellular kinetoplastidae such as *Leishmania* and *Trypanosoma* species, the glycolytic metabolism is compartmentalised in organelles unique to kinetoplastidae, the so-called glycosomes (Clayton and Michels 1996; Opperdoes et al. 1998). These peroxisome-like organelles produce G-3-P, 3PGA, and/or malate. These intermediates of the glycolytic degradation are metabolised further in the cytoplasm or in the mitochondria. This happens in many different ways, since the mitochondrial metabolism of different kinetoplastidae can vary substantially between species, developmental stages, and the availability of oxygen (Tielens and van Hellemond 1998a; van Hellemond et al. 1998). The common denominator of these adaptations seems to be the maintenance of the mitochondrial compartment for the generation of energy.

Hydrogenosomes: organelles that use H^+ as electron acceptor

In certain anaerobic protists and some anaerobic chytridiomycete fungi, however, the adaptation to anoxic niches led to the evolution of "hydrogenosomes" ("*type II anaerobes*", Martin and Müller 1998; Fig. 3 (Brul and Stumm 1994)). These hydrogenosomes are membrane-bound organelles with a size of approximately 1-2 micrometer that compartmentalise certain reactions of cellular energy metabolism.

Figure 2. Scheme of the glucose catabolic pathway in helminths. Big box represents the cytosol, small box inside of it represents the mitochondrion. PEP, phosphoenolpyruvate; OAA, oxaloacetate; PEPCK, phosphoenolpyruvate carboxykinase; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; PDH, pyruvate dehydrogenase; AST, acetate: succinate CoA transferase; STK, succinate thiokinase; RQ, rholoquinon; FR, ferredoxin; I-II, mitochondrial complex I and II.

Characteristically, hydrogenosomes import pyruvate (or malate) that is oxidatively decarboxylated to acetyl-CoA by the action of a pyruvate : ferredoxin oxidoreductase (PFO, see Fig. 4). An acetate:succinate CoA-transferase and a succinate thiokinase mediate the formation of acetate and ATP, similar to the situation in the mitochondria of the kinetoplastidae and helminths (van Hellemond et al. 1998; Tielens and van Hellemond 1998 a,b) . The reduction equivalents that are formed during the decarboxylation of pyruvate are used by a hydrogenase to reduce protons under the formation of molecular hydrogen (Müller 1993; Embley and Martin 1998).

Hydrogenosomes do not co-exist with mitochondria, and they have not been detected in organisms that face extended periods of aerobiosis during their life cycles. But since there is no doubt that hydrogenosomes were "invented" several times in evolution in different phylogenetically disparate lineages of eukaryotes (Fig. 3; Biagini et al. 1997a; Embley et al. 1997; Martin and Müller 1998), the question arose whether all hydrogenosomes are the same (Müller 1993; Coombs and Hackstein 1995; Embley et al. 1997).

Figure 3. An unrooted phylogenetic tree based on 16S-like rRNA sequence information (Sogin 1991). The various groups of microorganisms in which species with hydrogenosomes occur are indicated with an asterisk (*). The groups marked with a hash (#) are unicellular eukaryotes ('protists'). Within the ciliates, members of different taxa contain hydrogenosomes (Brul and Stumm 1994).

Hydrogenosomes of anaerobic ciliates: highly evolved mitochondria that produce hydrogen

Ciliates belong to the "crown group" of eukaryotes (Fig. 3; Sogin 1991, Schlegel 1994; Hirt et al. 1998), and in three to four different taxa, anaerobic ciliates with hydrogenosomes are found. There is broad agreement that anaerobic ciliates evolved secondarily from aerobic, mitochondria-bearing ancestors; some higher ciliate taxa even comprise both aerobic and anaerobic species (Fenchel and Finlay 1995; Embley et al. 1997). Electron microscopic studies revealed that the hydrogenosomes of anaerobic ciliates resemble mitochondria, and, therefore, it had been postulated that the hydrogenosomes of ciliates evolved from mitochondria (Fenchel and Finlay 1991a; Gijzen et al. 1991; Müller 1993). Recently, Akhmanova et al. (see Appendix) presented evidence for the presence of a "mitochondrial" genome in the hydrogenosomes of *Nyctotherus ovalis*, an anaerobic, heterotrichous ciliate that inhabits the intestinal tract of

Figure 4. Scheme of the glucose catabolic pathway in anaerobic ciliates. Big box represents the cytosol, small box inside of it represents the hydrogenosome. Question marks indicate enzymes, the existence of which is uncertain. PEP, phosphoenolpyruvate; OAA, oxaloacetate; AST, acetate : succinate CoA transferase; STK, succinate thiokinase; I-IV, mitochondrial complex I, II, III and IV.

cockroaches (Akhmanova et al. 1998a; van Hoek et al. 1998, 1999). This genome hosts rRNA genes that are abundantly expressed, and phylogenetic analysis reveals a clustering among the mitochondrial rRNA genes of aerobic ciliates. Since also the phylogenies of nuclear rRNA genes of the ciliates are congruent with those of their mitochondria and hydrogenosomes (Akhmanova et al. 1998a; van Hoek et al. 1998, 2000), an evolution of the ciliate's hydrogenosomes from the mitochondria of aerobic ciliates is likely. Moreover, the hydrogenosomes of *Nyctotherus ovalis* also look like mitochondria - e.g. they possess cristae and ribosomes (see Appendix: Akhmanova et al. 1998a). Therefore, it seems reasonable to assume that the hydrogenosomes of heterotrichous ciliates evolved from mitochondria that adapted to anaerobic environments. One crucial step was the expression of a hydrogenase.

Akhmanova et al. (see Appendix) have shown that the ciliate's hydrogenosomes possess a [Fe]-hydrogenase that is encoded by a macronuclear mini-chromosome and transcribed into a single mRNA. This hydrogenase represents a novel type of an [Fe]-hydrogenase that might be the most parsimonious solution to the problem of making a functional, hydrogen-producing enzyme complex, since H₂-formation seems to be coupled directly to the reoxidation of NADH. DNA sequence analysis has shown that

this has been achieved by combining a [Fe]-hydrogenase with the NAD and FMN binding sites of a mitochondrial complex I-like protein that might be able to transfer electrons to the catalytic site of the hydrogenase (see fig 4, Akhmanova et al 1998a). One might conclude that this enzyme complex not only guarantees the redox balance of the hydrogenosome it seems to be able to facilitate the generation of a proton gradient. Biagini et al have shown that the pH of the lumen of the hydrogenosomes of certain rumen ciliates and the free-living heterotrichous ciliate *Metopus contortus* is alkaline and capable of accumulating Ca^{2+} (Biagini et al 1997).

Thus, there is suggestive evidence that the hydrogenosomes of ciliates evolved from the mitochondria of their aerobic ancestors. Moreover, it is likely that they are still capable of generating a PMF. Unfortunately, the inability to culture these ciliates axenically has precluded more detailed molecular and physiological analysis of the consequences of this adaptation to anaerobiosis until now.

Hydrogenosomes of Trichomonas vaginalis

The hydrogenosomes of the trichomonads (*Parabasalina*) have been studied for nearly 30 years (c f Muller 1993). It has been shown that they are bound by a double membrane like mitochondria, but in contrast to mitochondria and the above-mentioned hydrogenosome of *Nyctotherus ovalis* they lack a genome, ribosomes and cristae (Benchimol et al 1982, 1996a/b). All aerobic mitochondrial functions are lacking in the hydrogenosomes of *Trichomonas* (Muller 1993, Martin and Muller 1998). However, the presence of a set of mitochondrial-like chaperons (HSP 10, 60, and 70, c f Bui et al 1996, Germot et al 1996, Hashimoto et al 1998), and evidence in favour of a mitochondrial like import machinery (Bradley et al 1997, Haussler et al 1997, Dyall et al 2000) suggest a mitochondrial ancestry of the hydrogenosomes of *Trichomonas* (Muller 1997, Sogin 1997, Palmer 1997, Dyall and Johnson 2000, Rotte et al 2000).

Also the presence of an acetate succinate CoA-transferase (van Hellemond et al 1998) that is shared between mitochondria and these hydrogenosomes can be interpreted in favour of a mitochondrial ancestry of hydrogenosomes. However, hydrogenosomes of *Trichomonas* are clearly different from mitochondria since they lack cytochromes, an electron transport chain, and cardiolipin (Muller 1988, 1993, 1998). Like mitochondria, they import pyruvate that results from glycolysis - but they do not use a pyruvate dehydrogenase for catabolism. Rather, the hydrogenosomes metabolise pyruvate through a pyruvate ferredoxin oxidoreductase (PFO) and hydrogenase to acetate, carbon dioxide and hydrogen (Fig 5, Muller 1993, 1998). Acetate formation is coupled to substrate level phosphorylation of succinate that yields 1 ATP per mol of pyruvate consumed. Additional ATP formation seems to be feasible by the generation of a PMF (Humphreys et al 1994). Although the generation of a PMF has not yet been studied in detail, it could be shown that the hydrogenosomes of Trichomonads generate a proton gradient. In addition, these hydrogenosomes can serve as cellular Ca^{2+} -stores (Lloyd et al 1979, Yarlett et al 1987, Chapman et al 1985, Benchimol et al 1982, 1996a/b). Therefore, it might be concluded that the hydrogenase of *Trichomonas* is capable of generating a PMF in the hydrogenosomes - even if a mitochondrial-like or prokaryotic electron transport chain is absent.

Figure 5. Scheme of the glucose catabolic pathway in *Trichomonas vaginalis*. Big box represents the cytosol, small box inside of it represents the hydrogenosome. Question marks indicate enzymes and transporters, the existence of which is uncertain. PEP, phosphoenolpyruvate; OAA, oxaloacetate; PEPCK, phosphoenolpyruvate carboxykinase; AST, acetate : succinate CoA transferase; ADH, alcohol dehydrogenase; STK, succinate thiokinase; PFO, pyruvate : ferredoxin oxidoreductase; FD ox/red, ferredoxin oxidised/reduced; I-IV, mitochondrial complex I, II, III and IV?

Hydrogenosomes of anaerobic chytrids: another way to make a hydrogenosome

Anaerobic chytrids are important symbionts in the gastro-intestinal tract of many herbivorous mammals (see Trinci et al. 1994 and references therein). These organisms are highly adapted to intestinal environments: their optimal growth temperature coincides with the narrow temperature range set by the body temperature of their mammalian hosts, and during their whole life cycle, they thrive under anoxic conditions. Only the poorly characterised resting stages become exposed to oxygen and must possess a certain resistance to oxygen (for review see Orpin 1994). The life cycle of all anaerobic fungi studied to date consists of an alternation between motile, flagellated zoospore stages and a vegetative phase where a complex, multi-nucleated rhizomycelial system is formed. The hyphae of the rhizomycelial system attach to plant-derived particles of the digesta, and, by the production and excretion of a broad spectrum of (cellulolytic and other) enzymes, the intestinal chytrids contribute significantly to the digestion of plant polymers (Teunissen et al. 1991; Orpin 1994; Yarlett 1994; Dijkerman 1997).

These highly specialised anaerobic chytrids evolved from mitochondria-bearing, aerobic ancestors, since phylogenetic analysis of their rRNA genes has shown unequivocally that yeasts and fungi belong to a "crown group" of eukaryotic micro-organisms that includes both aerobic and anaerobic chytrids (Sogin 1991; Knoll 1992; Beakes 1998). DNA sequence analysis reveals a clustering of both aerobic and anaerobic chytrids (Dore and Stahl, 1991; Li and Heath, 1992; Bowman et al., 1992). Also an analysis of biochemical (Ragan and Chapman 1978) and morphological traits (Li et al., 1993) consistently establishes a close relationship between chytrids and other fungi. Moreover, for the aerobic chytrids, a congruency between nuclear ribosomal RNA- and mitochondrial protein-based trees has been demonstrated (Paquin et al. 1995). Consequently, there is no doubt that the chytrids that live in the gastro-intestinal tract of herbivorous mammals have secondarily adopted an anaerobic life style (Paquin et al. 1996).

In contrast to their aerobic relatives, anaerobic chytrids such as *Neocallimastix* and *Piromyces* lack mitochondria. Instead, they possess hydrogenosomes (Yarlett et al. 1986; Müller, 1993). However, these organelles seem to be different from the hydrogenosomes of the ciliate *Nyctotherus ovalis*, the amoebflagellate *Psalteriomonas lanterna* and the parabasilid *Trichomonas vaginalis* (Coombs and Hackstein 1995; Hackstein et al. 1997; 1998). Like the hydrogenosomes of the amoebflagellate *Psalteriomonas lanterna* and of the parabasilid *Trichomonas vaginalis* they lack a genome, but notably, the chytrids hydrogenosomes import malate and not pyruvate (Fig. 6). The imported malate is oxidatively decarboxylated by a hydrogenosomal malic enzyme, and it has been postulated that the resulting pyruvate is oxidised further by pyruvate : ferredoxin oxidoreductase (PFO) to acetyl-CoA. The reduction equivalents should be taken up by a ferredoxin and transferred to a hydrogenase that is believed to maintain the redox balance (Marvin-Sikkema et al. 1992, 1993a, 1994)

Earlier investigators also claimed that the hydrogenosomes of anaerobic chytrids are bound by a single membrane (Yarlett et al. 1986; Marvin-Sikkema et al. 1992, 1993a/b). Moreover, it has been postulated that peroxisomal targeting signals sort hydrogenosomal proteins to their subcellular destination (Marvin-Sikkema et al. 1993). Recently, however, van der Giezen and collaborators challenged the hypothesis that the hydrogenosomes of chytrids resemble peroxisomes. They provided evidence for putative N-terminal targeting signals on hydrogenosomal proteins that can direct these proteins to the mitochondria of transgenic yeasts (Brondijk et al. 1996; van der Giezen et al. 1997, 1998). This observation stimulated a re-investigation of the ultrastructure of chytrid hydrogenosomes that were now interpreted in favour of mitochondrial traits. Especially the presence of internal membrane structures seemed to argue for the presence of double membranes surrounding the chytrid hydrogenosomes (Benchimol. et al. 1997; van der Giezen et al. 1997). In addition, evidence has been presented that the lumen of the hydrogenosomes of chytrids is alkaline, and it is likely that both differences in the electric charge and proton gradients exist between the hydrogenosomes and the cytoplasm (Biagini et al. 1997c). However, notwithstanding the continuing discussion about the validity and meaning of these findings, the studies of Marvin-Sikkema (1994, Thesis) and van der Giezen (1998, Thesis) have revealed a substantial number of

Figure 6. Scheme of the glucose catabolic pathway in the anaerobic chytrid *Neocallimastix* sp. L2, based on the studies described by Marvin-Sikkema et al. (1993a, 1994). Big box represents the cytosol, small box inside of it represents the hydrogenosome. PEP, phosphoenolpyruvate; OAA, oxaloacetate; PEPCK, phosphoenolpyruvate carboxykinase; malate DH, malate dehydrogenase; PFL, pyruvate formate-lyase; STK, succinate thiokinase; PFO, pyruvate : ferredoxin oxidoreductase; FD ox/red, ferredoxin oxidised/reduced; AST, acetate : succinate CoA transferase; I-IV, mitochondrial complex I, II, III and IV?

differences between hydrogenosomes of chytrids and those of *Trichomonas*.

In conclusion, the examples for the central anaerobic energy metabolism of eukaryotes that have been discussed here suggest that there are many ways to adapt to anaerobic environments. These adaptations range from the loss (or absence) of mitochondria to the evolution of the various types of hydrogenosomes. Amitochondriate eukaryotes without hydrogenosomes ("*type I anaerobes*", Müller and Martin 1998; Müller 1998) use fermentation pathways to conserve energy and to maintain the redox balance, whereas eukaryotes with hydrogenosomes ("*type II anaerobes*"; Müller and Martin 1998) gain additional ATP from the compartmentalisation of their energy metabolism. The fumarate reduction in the mitochondria of helminths allows the extrusion of protons by a part of the electron transport chain also under anaerobic conditions. The activity of a hydrogenase in the hydrogenosomes of anaerobic ciliates,

trichomonads and chytrids facilitates the use of protons as electron acceptors and their removal from the matrix of the organelle. The common denominator of both adaptations is the maintenance of a compartment that allows the generation of a proton gradient.

All the "adaptations" to anaerobic environments described here are the irreversible result of evolution. Since there is evidence that the various hydrogenosomes are different, it is necessary to discuss the evolution of hydrogenosomes in more detail.

The evolution of hydrogenosomes: paradigms and problems

There is no doubt that hydrogenosomes evolved repeatedly in widely separated lines of eukaryotes (Fig. 3; Müller 1993, 1997; Brul and Stumm 1994; Embley et al. 1997, Biagini et al. 1997; Palmer 1997; Sogin 1997; Embley and Martin 1998; Embley et al. 1995; Hirt et al. 1998). Even within the ciliate clade, hydrogenosomes evolved three to four times (Embley et al. 1995). And, as discussed earlier, the hydrogenosomes of the various anaerobes are structurally and functionally different. Their evolution from mitochondria (or phylogenetically related endosymbiotic α -proteobacteria) is clearly favoured. Sogin (1997) and Martin and Müller (1998) argued that hydrogenosomes and mitochondria share a common ancestor among the α -proteobacteria. Such a proteobacterial ancestor must have been a facultative anaerobe that became engaged in symbiosis with an archaeal host. In aerobic environments, mitochondria evolved, whereas under anaerobic conditions, evolution favoured the loss of respiratory functions of the endosymbiont thereby promoting the evolution of hydrogenosomes (Martin and Müller 1998). In their hypothesis, Martin and Müller (1998) theorise solutions for a number of odd problems in the evolution of the eukaryotic cell. Their hypothesis offers a solution to the obvious dilemma that is created by a potential common origin of mitochondria and hydrogenosomes. The evolution from a common α -proteobacteria ancestor into mitochondria that strictly depend on oxygen on the one hand, and into hydrogenosomes that are irreversibly damaged by the exposure to oxygen, appears strange under the "classical" endosymbiont hypothesis. However, the hydrogen hypothesis offers a persuasive solution for this dilemma. In short, the hydrogen hypothesis assumes that the eukaryotic cell evolved from the syntrophic association of a heterotrophic facultative anaerobic (α -proteo) bacterium (= symbiont) and a strictly anaerobic autotrophic archaeobacterium (= host). The eubacterial symbiont was supposed to depend on reduced carbon sources, whereas the archaeal host should thrive on (geological) hydrogen and carbon dioxide that could be either derived from a geological source or from the symbionts metabolic wastes. In the absence of a geological source, the host immediately becomes strictly dependent on the symbiont. Subsequently, this syntrophic association can evolve into a mitochondrion-bearing eukaryote in aerobic and into a hydrogenosome-bearing protist in anaerobic environments. Martin and Müller (1998) suggested that this could be achieved by losses of either the "aerobic enzymes" or losses of enzymes (genes) of amitochondriate (cytoplasmic) energy metabolism from the symbiont on the way to become an organelle. A third variant, i.e. loss of the symbiont/organelle, should lead to the "Type I" amitochondriate anaerobes. RNA phylogenies indicated that this many times occurred, and in different eukaryotic lineages (Martin and Müller 1998). Therefore, both the total loss of the symbiont/organelle and the loss of

enzymes for aerobic metabolism seems to be a general phenomenon that follows the same rule every time.

The most straightforward approach to prove a mitochondrial ancestry of all hydrogenosomes would be a phylogenetic analysis of a potential hydrogenosomal genome. Until now, only in the heterotrichous ciliate *Nyctotherus ovalis*, evidence for the presence of a hydrogenosomal genome could be presented (Akhmanova et al. 1998a; see Appendix). The phylogenetic analysis of the SSU of the organellar ribosomal repeat revealed its mitochondrial ancestry (van Hoek et al. 2000). Moreover, since also the ultrastructure of these hydrogenosomes reveals classical mitochondrial traits such as double membranes, cristae, and ribosomes, it is likely that the hydrogenosomes of ciliates are highly specialised mitochondria. One has to conclude that they acquired or expressed a hydrogenase in order to cope with the consequences of an impaired function of the electron transport chain under anaerobic conditions.

However, the hydrogenosomes of the parabasalid *Trichomonas vaginalis*, the amoeboflagellate *Psalteriomonas lanterna* and the chytrids *Piromyces* and *Neocallimastix* lack a genome (Müller 1993, van der Giezen 1998; Hackstein et al. 1997, 1998; Embley et al. 1997; c.f. Margulis 1993). Therefore, a straightforward approach to unravel the evolutionary ancestry of these hydrogenosomes is impossible. The lack of a genome and, consequently, also organellar protein synthesising machinery implies that nuclear genes must encode all of the approximately 300 hydrogenosomal proteins (Sogin 1997). All hydrogenosomal proteins must be synthesised in the cytoplasm, targeted to the hydrogenosome, and imported into the organelle (Lahti and Johnson 1991). A phylogenetic analysis of these genes might allow a reconstruction of the descent of each of these proteins, but it cannot be excluded that these proteins have different ancestries (Andersson and Kurland 1998; Martin 1999). At least hydrogenase and PFO - enzymes that are crucial for the function of most of the hydrogenosomes - do not belong to the normal repertoire of an aerobic eukaryotic cell or its organelles. These enzymes can be derived from ancestral organellar (for example, mitochondrial) proteins, from eubacterial or archaeal endosymbionts, or acquired by lateral gene transfer from the DNA of food bacteria (Cavalier-Smith 1987; Müller 1993; Doolittle 1998).

However, the knowledge about ancient eubacterial and archaeal genomes, and the potential transfer of genes from endosymbionts or food bacteria to the eukaryotic nucleus is rather limited. Therefore, the results of a phylogenetic analysis of hydrogenosomal proteins might be rather puzzling and not sufficient to allow unequivocal conclusions about the ancestry of the organelle (Martin and Herrmann 1998; Martin et al. 1998; Doolittle 1998). Lastly, also an analysis of hydrogenosomal targeting signals and the hydrogenosomal import machinery might not allow a simple explanation. There is evidence for the presence of both potential C-terminal (peroxisomal: e.g. the tri-peptide SKL, Marvin-Sikkema et al. 1993b) and of N-terminal (mitochondrial-like) extensions that might function as targeting signals for hydrogenosomal proteins of chytrids (Brondijk et al. 1996; van der Giezen et al. 1997, 1998).

A rationale to unravel a potential polyphyly of hydrogenosomes

Chytrid hydrogenosomes seem to be especially suited to address the problem of a potential polyphyletic origin of hydrogenosomes since their ultrastructure and physiology differs in several aspects from the prototypical hydrogenosomes of *Trichomonas*. Therefore, in this thesis, a number of genes encoding hydrogenosomal proteins of anaerobic chytrids will be analysed and compared to their homologues in aerobic eukaryotes, free-living anaerobic eubacteria and hydrogenosome-bearing anaerobic eukaryotes in order to gain insights into the nature of functional and evolutionary relationships between hydrogenosomes and mitochondria. Moreover, the subcellular localisation of genuine mitochondrial proteins will be studied in the chytrids, as well as the presence or absence of potential targeting signals. Also, special attention will be paid to the ultrastructure of the hydrogenosomes of chytrids - both of isolated hydrogenosomes obtained by cell fractionation and of the hydrogenosomes present in the zoospores of the chytrids.

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Chapter 2

Cytosolic enzymes with a mitochondrial ancestry from the anaerobic chytrid *Piromyces* sp. E2.

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Summary

The anaerobic chytrid *Piromyces* sp. E2 lacks mitochondria, but contains hydrogen-producing organelles, the hydrogenosomes. We are interested in how the adaptation to anaerobiosis influenced enzyme compartmentalisation in this organism. Random sequencing of a cDNA library from *Piromyces* sp. E2 resulted in the isolation of cDNAs, encoding malate dehydrogenase, aconitase and acetohydroxyacid reductoisomerase. Phylogenetic analysis of the deduced amino acid sequences revealed that they are closely related to their mitochondrial homologues from aerobic eukaryotes. However, the deduced sequences lack N-terminal extensions, which in the corresponding mitochondrial enzymes from aerobic eukaryotes function as mitochondrial leader sequences. Subcellular fractionation and enzyme assays confirmed that the corresponding enzymes are located in the cytosol. Since anaerobic chytrids evolved from aerobic, mitochondria-bearing ancestors, we suggest that - in the course of the adaptation from an aerobic to an anaerobic life style - mitochondrial enzymes were retargeted to the cytosol with the concomitant loss of their N-terminal leader sequences.

Introduction

Anaerobic chytrids are important symbionts in the gastro-intestinal tract of many herbivorous mammals (see Trinci et al. 1994 and references therein). They are highly adapted to these intestinal environments and, during their whole life cycle, they thrive under anoxic conditions (for review see Orpin 1994). The life cycle of all anaerobic fungi studied to date consists of an alternation between motile, flagellated zoospore stages, and a vegetative phase where a complex rhizomycelial system is formed. The hyphae of the rhizomycelial system attach to plant-derived particles of the digesta, and, by the production and excretion of a broad spectrum of (cellulolytic and other) enzymes, the intestinal chytrids contribute significantly to the digestion of plant polymers (Teunissen et al. 1991; Orpin 1994).

These highly specialised anaerobic chytrids evolved from mitochondria-bearing aerobic ancestors, since phylogenetic analysis of their rRNA genes has shown unequivocally that yeasts and fungi belong to a "crown group" of eukaryotic microorganisms that includes the aerobic and anaerobic chytrids (Sogin 1991; Knoll 1992). DNA sequence analysis reveals a clustering of both aerobic and anaerobic chytrids (Dore and Stahl 1991; Li and Heath 1992; Bowman et al. 1992). Also an analysis of biochemical (Ragan and Chapman 1978) and morphological traits (Li et al. 1993) consistently establishes a close relationship between chytrids and other fungi. Moreover, for the aerobic chytrids, a congruency between nuclear ribosomal RNA- and mitochondrial protein-based trees has been demonstrated (Paquin et al. 1995). And lastly, the complete nucleotide sequence from the mitochondrial genome of *Allomyces macrogynus*, an aerobic chytrid, has been determined (Paquin and Lang 1996). This genome exhibits all traits characteristic of an eufungal mitochondrial genome, albeit an ancestral one. Consequently, there is no doubt that the chytrids that live in the gastro-

intestinal tract of herbivorous mammals have secondarily adopted an anaerobic life style.

In contrast to their aerobic relatives, anaerobic chytrids such as *Neocallimastix* and *Piromyces* lack mitochondria. Instead, they possess membrane-bound organelles, called hydrogenosomes (Yarlett et al. 1986; Müller 1993). These organelles are involved in anaerobic energy metabolism. Malate is imported into the organelles where it undergoes oxidative decarboxylation to pyruvate. The reduction equivalents formed in this process are excreted as molecular hydrogen via a H_2 : ferredoxin oxidoreductase (Marvin-Sikkema et al. 1993b). Since evidence has accumulated that the hydrogenosomes of the parabasalid *Trichomonas* and mitochondria share a common ancestor (see reviews by Müller 1997; Sogin 1997; Embley et al. 1997; Biagini et al. 1997), it is possible that the hydrogenosomes of anaerobic chytrids also evolved from the mitochondria of their aerobic ancestors. Recently, Martin and Müller (1998) discussed the evolution of aerobic and anaerobic eukaryotes in more detail. They postulated that the evolution of a hydrogenosome from a mitochondrion (or its progenitor) would entail the loss of the respiratory pathway, and they emphasised that losses likely occurred several times in evolution in different lineages of eukaryotes (Martin and Müller 1998).

The lack of a genome in the hydrogenosomes of the chytrids *Piromyces* and *Neocallimastix* makes it impossible to reconstruct their ancestry directly (Müller 1993; van der Giezen et al. 1997b; c.f. Margulis 1993; Hackstein et al. 1997, 1998; Embley et al. 1997). Only the phylogeny of the genes that encode hydrogenosomal proteins, the type of hydrogenosomal targeting signals, and the hydrogenosomal import machinery can provide indirect evidence for the evolutionary history of the hydrogenosome (Sogin 1997). The molecular basis of the changes that accompany the transition from aerobic mitochondrial to anaerobic hydrogenosomal energy metabolism are still poorly understood. A crucial event in the adaptation to anoxic environments and the evolution of hydrogenosomes is the expression or acquisition of a hydrogenase and a pyruvate: ferredoxin oxidoreductase. However, the questions why these enzymes become compartmentalised whereas others are targeted to a different subcellular compartment, and why these changes eventually causes the loss of the hydrogenosomal genome, have not been answered yet. Similarly, potential changes in enzyme compartmentalisation and losses of enzyme activities that might be required to cope with the new environment on the one hand, and to avoid an interference with the metabolic routes that evolve in response to the novel evolutionary constraints on the other hand, are far from being understood. Moreover, as Martin and Müller (1998) have pointed out, also a total loss of mitochondria can be envisaged under conditions of adaptation to anaerobic environments.

Regardless whether the mitochondrion became modified or lost during the adaptation to anoxic environments, the repression or factual loss of the tricarboxylic acid (TCA) cycle in anaerobic chytrids (aerobic chytrids do possess a complete TCA cycle, see Ragan and Chapman 1978) and of enzymes involved in the oxidative phosphorylation must have serious consequences for chytrid cellular metabolism, since mitochondria, besides their function in the energy-metabolism, provide intermediates for biosynthesis of cellular components. Also the synthesis of branched-chain amino acids that is known to be located in the mitochondria of aerobic fungi might be affected by the modification

or the loss of mitochondria (Ryan and Kohlhaw 1974). Moreover, in mammals and apicomplexan parasites, mitochondria are involved in purine and pyrimidine biosynthesis (Gutteridge et al 1979, Prapunwattana et al 1988, Krungkrai et al 1991). Therefore, a loss (or repression) of these functions potentially could result in auxotrophy of the organisms.

However, it has been shown that anaerobic chytrids are still capable of growth on monosaccharides and ammonia as the sole carbon and nitrogen sources (Teunissen et al 1991, Dijkerman et al 1997). Therefore, anaerobic chytrids must be able to synthesise all amino acids and nucleotides, including those that normally require functional mitochondria for their biosynthesis in the absence of functional mitochondria. This suggests that at least some of the mitochondrial functions have been retained in the amitochondriate chytrids - in the absence of functional mitochondria.

To address these questions, we have performed random sequencing of *Piromyces* sp E2 cDNAs in order to identify genes with a mitochondrial ancestry. Here, we describe the cDNAs encoding TCA cycle enzymes malate dehydrogenase (MDH) and aconitase, and a cDNA that encodes acetohydroxyacid reductoisomerase, an enzyme involved in the biosynthesis of isoleucine and valine. We show that these enzymes are of mitochondrial origin, lack mitochondrial targeting signals, and are active in the cytosol of *Piromyces* sp E2.

Experimental procedures

Organism and growth conditions

Piromyces sp E2 (ATCC 76762) was cultured in medium M2, supplemented with 0.5% fructose as a carbon source (Teunissen et al 1991). Biomass for the preparation of DNA, RNA, or homogenates for subcellular fractionation and enzyme assays was harvested after 40-48 hours of growth at 39°C.

RNA and DNA isolation

RNA was prepared by the guanidinium-chloride method (Chirgwin et al 1979). For preparation of poly(A)⁺ RNA, a mRNA purification kit (Pharmacia) was used according to the manufacturer's protocol. *Piromyces* sp E2 genomic DNA was prepared as described by Brownlee (1994).

Construction and random sequencing of the Piromyces sp E2 cDNA library

The *Piromyces* sp E2 cDNA library in the vector lambda ZAPII was constructed with the help of the ZAP-cDNA synthesis kit (Stratagene). As starting material, 7.5 µg of poly(A)⁺ RNA, isolated from *Piromyces* sp E2 grown on 0.5% fructose, was used. The titer of the primary library was 2×10^6 plaque-forming units per ml. An aliquot of this library was converted to pBluescript SK⁻ clones by mass excision with the ExAssist helper phage (Stratagene). Clones were picked at random and sequenced with the M13 reverse primer to determine the sequence of the 5' part of the cDNAs. The sequences of interesting cDNA clones were completed by generating shorter subclones in pUC18 and

by using internal sequencing primers. Sequencing was performed with the ABI PRISM Model 310 automatic sequencer, using a dRhodamine terminator cycle sequencing ready reaction DNA sequencing kit (Perkin-Elmer Applied Biosystems).

Southern and Northern blotting

Piromyces sp. E2 genomic DNA was digested by several restriction enzymes (see Fig. 4) and separated on 0.7% agarose gels (10 µg DNA per lane). Total RNA was separated on 1.2% agarose-formaldehyde gels (15 µg RNA per lane). Gels were blotted to Hybond N+ membranes (Amersham). Different cDNAs, used as probes (see Fig. 4 and 5), were labelled by PCR with α -[³²P]dATP. Hybridisation was performed in 0.5 M sodium phosphate buffer, pH 7.0, 7% SDS, 1% bovine serum albumin, 1mM EDTA at 60°C. Filters were washed stringently, with 50 mM sodium phosphate buffer, 0.5% SDS at 60°C.

Sequence analysis

Sequences were analysed with the help of the GCG Sequence Analysis package (Devereux et al. 1984). Phylogenetic trees were constructed with the aid of the programs in PHYLIP V3.5c (Felsenstein 1993).

Enzyme assays

Subcellular fractionation of *Piromyces* sp. E2 was performed essentially as described by Marvin-Sikkema et al. (1993b), but the homogenisation medium was substituted for 20 mM HEPES pH 7.4, 250 mM sucrose, and 2 mM dithiothreitol. Hexokinase was assayed as described by Bergmeyer et al. (1983). Malic enzyme activity was determined with the method of Kremer et al. (1989). Hydrogenase activity was measured as described by Marvin-Sikkema et al. (1993b). MDH was assayed according to Stams et al. (1984). Aconitase was assayed as described by Reeves et al. (1971). The specificity of the aconitase enzyme assay was supported by the complete inhibition of the aconitase activity by 10 mM fluorocitrate (see Table 1). Acetohydroxyacid reductoisomerase activity was measured as described by Magee and Hereford (1969), using α -acetolactate as substrate. α -Acetolactate was prepared by hydrolysis of ethyl-2-acetoxy-2-methylacetoacetate (Aldrich) with NaOH (Park et al. 1995). In order to permeabilise the membrane-bound hydrogenosomes, Triton X-100 was added to a final concentration of 0.2% (v/v). All enzyme assays were performed at 39°C.

Proteinase K protection assay

Isolated hydrogenosomes (0.4 mg protein) were incubated anaerobically (N₂ gas phase) in 0.5 ml homogenisation medium with or without 50 µg proteinase K. The hydrogenosomes were permeabilised by the addition of Triton X-100 (2% v/v). After 30 minutes incubation on ice, the proteolytic digestion was terminated by the addition of 20 mM phenylmethylsulfonylfluoride (PMSF). Aconitase and malic enzyme activities were assayed as described above, but now in the presence of 20 mM PMSF and Triton X-100 (2% v/v).

Results

cDNAs encoding malate dehydrogenase, aconitase and acetohydroxyacid reductoisomerase

In order to identify highly expressed genes from the anaerobic chytrid *Piromyces* sp. E2, a cDNA library was constructed. Randomly selected cDNA clones were sequenced. Clones with a high similarity to malate dehydrogenase (clone pa49), aconitase (clone pa14) and acetohydroxyacid reductoisomerase encoding genes (clones pa1 and pa62) were identified. These cDNA clones were analysed in detail.

The first cDNA clone (pa49) contained one open reading frame (ORF), encoding a protein of 316 amino acids with high similarity to malate dehydrogenases (MDHs). MDH-encoding genes can be grouped into a “mitochondrial” and a “cytosolic” lineage (Joh et al. 1987; Cendrin et al. 1993; Ocheretina and Scheibe 1997). The “mitochondrial” lineage includes the mitochondrial MDHs from animals, plants, and fungi, and the glyoxysomal MDHs from plants. Cytosolic MDHs from plants and animals and the chloroplast MDH constitute the “cytosolic” lineage. Comparison of the deduced MDH sequence with MDHs from both lineages revealed that it displayed 61-67% identity to mitochondrial MDH sequences. When compared to the MDHs from the “cytosolic” lineage, it displayed only 25-27% identity. Phylogenetic analysis of the mitochondrial MDH sequences, grouped the *Piromyces* sp. E2 MDH sequence in one clade together with the yeast MDHs (see Fig. 1A). It should be noted that in yeast, also the cytosolic and peroxisomal MDHs are derived from mitochondrial MDHs (Ocheretina and Scheibe 1997). Comparison of the *Piromyces* sp. E2 MDH sequence with the yeast MDHs revealed that the chytrid MDH is more closely related to the yeast mitochondrial MDH (62% identity) than to the yeast peroxisomal and cytosolic MDHs (56% and 55% identity, respectively).

The second cDNA clone (pa14) contained one ORF, encoding a protein of 755 amino acids with high similarity to aconitases. Similar to MDH, also the aconitase-encoding genes belong to two different lineages. The first lineage represents the mammalian cytosolic aconitases, the plant mitochondrial and cytosolic aconitases, and some eubacterial aconitases (Peyret et al. 1995; Zhou and Ragan 1995; Gruer et al. 1997). The mitochondrial aconitases from mammals, red algae and fungi constitute the other lineage. Pairwise comparison revealed that the chytrid aconitase showed the highest similarity (66-70% identity) with the aconitases from the mitochondria of mammals, algae and fungi. Also phylogenetic analysis showed clustering of the chytrid aconitase within the mitochondrial clade (see Fig. 1B). The exact branching order within this group could not be determined, because of the small number of available aconitase sequences and their high degree of identity.

The cDNA clones pa1 and pa62 encoded an ORF with high similarity to acetohydroxyacid reductoisomerases. One of these clones (pa1) was completely sequenced. The ORF encoded a protein of 352 amino acids. Pairwise comparison has shown that the deduced chytrid sequence displayed the highest similarity (57-64% identity) with the mitochondrial acetohydroxyacid reductoisomerases from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Neurospora crassa*. Also

A. malate dehydrogenase

B. aconitase

C. acetohydroxyacid reductoisomerase

Figure 1. Phylogenetic relations between malate dehydrogenases (A), aconitases (B) and acetohydroxyacid reductoisomerases (C). Phylogenetic trees were obtained using the neighbor-joining method (A and C) or the maximum likelihood method (B). Bootstrap values of 70% or more are indicated. For the aconitase tree (B), bootstrap values obtained for the indicated clades using the neighbor-joining method are shown in parentheses. The clades including fungal sequences are shaded with gray. Cellular localisation of the gene products is indicated: Cyt., cytosol; Chl., chloroplast; Mit., mitochondrion; Glyox., glyoxysome; Per., peroxisome. Uncertain localisation is indicated with a question mark. For the accession numbers of the source sequences, see legend to Fig. 2. Additional sequences, not included in Fig. 2 were: *S. scrofa* Mit., *Sus scrofa* mitochondrial MDH (P00346/Q95308); *C. reinhardtii* Mit., *Chlamydomonas reinhardtii* mitochondrial MDH (Q42686).

phylogenetic analysis revealed clustering of the *Piromyces* sp E2 acetohydroxyacid reductoisomerase sequence with the mitochondrial homologues from aerobic fungi (see Fig 1C)

Malate dehydrogenase aconitase and acetohydroxyacid reductoisomerase of Piromyces sp E2 lack N-terminal leader sequences

Many nuclear-encoded mitochondrial matrix proteins are sorted to their subcellular compartment by a N-terminal leader sequence. This leader sequence is enriched in positively charged and hydroxylated amino acids, and cleaved off upon import into the mitochondrial matrix (Hendrick et al 1989, von Heijne et al 1989). Also mitochondrial malate dehydrogenases, aconitases and acetohydroxyacid reductoisomerases are sorted to the mitochondrial matrix with the aid of N-terminal leader sequences (see Fig 2).

Analysis of the deduced MDH, aconitase and acetohydroxyacid reductoisomerase sequences of *Piromyces* sp E2 has shown that they lack N-terminal extensions. Their coding sequences start around the processing site of the mitochondrial N-terminal leader sequences of the mitochondrial isoforms from aerobic eukaryotes (see Fig 2). It is very unlikely that translation of the analysed cDNAs starts upstream of the first ATG codon, since the upstream sequences of all three cDNAs contain in-frame stop codons and are highly AT-rich, as characteristic for untranslated regions of anaerobic chytrids (see Fig 3, Reymond et al 1992, Durand et al 1995, Fanutti et al 1995). Apparently, the N-terminal extension, which in mitochondrial orthologues functions as a targeting signal, is absent from the genes described here.

Figure 2. Multiple alignments of the N- and C-terminal sequences of malate dehydrogenases (A), aconitases (B) and acetohydroxyacid reductoisomerases (C). Amino acids identical in more than 60% of the sequences are shaded with gray. Gaps are indicated by dashes. N-terminal transit peptides experimentally shown to be removed from the corresponding mature proteins are underlined. The PTS1 peroxisomal targeting signal of the yeast peroxisomal MDH is shown in bold. Since the C-terminal domains of different acetohydroxyacid reductoisomerase proteins are poorly conserved, only the C-termini of the fungal sequences are shown in C. The source sequences and data bank accession numbers are listed below. Malate dehydrogenases: pirE2 - *Piromyces* sp E2, this study, yeast per - *S. cerevisiae*, peroxisomal (P32419), yeast cyt - *S. cerevisiae*, cytosolic (P22133), yeast mit - *S. cerevisiae*, mitochondrial (P17505), rat mit - *Rattus norvegicus*, mitochondrial (P04636), mouse mit - *Mus musculus*, mitochondrial (P08249), citvu mit - *Citrullus vulgaris*, mitochondrial (P17783), eucgu mit - *Eucalyptus gunnii*, mitochondrial (P46487), citvu glyox - *Citrullus vulgaris*, glyoxysomal (P19446), cucs glyox - *Cucumis sativus*, glyoxysomal (P46488), ecoli - *Escherichia coli* (P06994). Aconitases: pirE2 aconitase - *Piromyces* sp E2, this study, yeast - *Saccharomyces cerevisiae* (P19414), yeast AcoX - *S. cerevisiae* (P39533), grave mit - *Gracilaria verrucosa*, mitochondrial (P49609), bov mit - *Bos taurus*, mitochondrial (P20004), pig mit - *Sus scrofa*, mitochondrial (P16276), caeel - *Caenorhabditis elegans* (P34455). Acetohydroxyacid reductoisomerases: pirE2 - *Piromyces* sp E2, this study, schpo mit - *Schizosaccharomyces pombe* (P78827), yeast mit - *S. cerevisiae* (P06168), neucr mit - *Neurospora crassa* (P38674), arath chl - *Arabidopsis thaliana* chloroplast (Q05758), spiol chl - *Spinacia oleracea*, chloroplast (Q01292), ecoli - *Escherichia coli* (P05793), bacsu - *Bacillus subtilis* (P37253).

A. MDH N-terminus

pirE2 Cyt. -----MVKVAVLGAAGGIGQPLSLLLKSH-FOV 27
 yeast Per. -----MVKVAILGASSGVGQPLSLLLKLS-FYV 27
 yeast Cyt. -----MPHSVTPSIEQDSLKTAILGAGGGIGQSLLLAQLOQY 40
 yeast Mit. -----MLSRVAKRAFSSSTVANPYKVTVLGAGGGIGQPLSLLLKLN-HKV 43
 rat Mit. -----MLSALARPVGAALRRSFSTSAONNAKVAVLGAGGGIGQPLSLLLKNS-PLV 50
 mouse Mit. -----MLSALARPAGAALRRSFSTSAONNAKVAVLGAGGGIGQPLSLLLKNS-PLV 50
 citvu Mit. -----MKASILLRSVRSAVSRSSSNRLLSRSFATESVPERKVAVLGAGGGIGQPLALLMKLN-PLV 60
 eucgu Mit. -----MRASMLRLIRSRSSSAAPRPHLLRRAYGSESVPERKVAVLGAGGGIGQPLALLMKLN-PLV 60
 citvu glyox. MQPIPDVNQRIARISAHLPKPKSQMEESSALRRANCRAGKGGAPGPKVAILGAGGGIGQPLAMMKMN-PLV 70
 cucs glyox. MQPIPDVNQRIARISAHLPKPKSQMEESSALRRANCRAGKGGAPGPKVAILGAGGGIGQPLAMMKMN-PLV 70
 ecoli -----MKVAVLGAAGGIGQALALLNTQLSESG 27

MDH C-terminus

pirE2 Cyt. GLYKACVEQLKANTAKGVNVNQA---- 316
 yeast Per. QLVNTAVKELRNKITEKGKSFILDS**SKL**- 342
 yeast Cyt. QMLPICVSQHKKNIDKLEFVASRSASS 376
 yeast Mit. EMLQCKKETLKKNIEKGVNVASK---- 334
 rat Mit. KMIAEAIPELKASTIKKGEDFVKNMK---- 338
 mouse Mit. KMIAEAIPELKASTIKKGEDFVKNMK---- 338
 citvu Mit. EGGLEKLPPELKASTIEKGIQAFANAN---- 347
 eucgu Mit. QGLEILPELKASTIEKGIKFAQ---- 347
 citvu glyox. IGLEKAKKELAGSTIEKGVSHRS---- 356
 cucs glyox. IGLEKAKKELAGSTIEKGVSHRS---- 356
 ecoli NALEGLMDTLKKDIALGEEFVNK---- 312

B. aconitase N-terminus

pirE2 Cyt. -----MATKIAMAFDQNNFIQ--YEKMAENIKIVRELRNE 34
 yeast -----MLSARSAIKRPVIRGLATVSNLTRDSKVNQNLLEDHDSFIN--YKQNVETLDEVKRLNE 57
 yeast AcoX -----MLSSANRFYIKRHLATHANMFPVSKNFQTKVPPYAKLLTNLDKIKQITNN 51
 grave Mit. -----MIAMDRIARIPARWTSRAFRVSAARQTPMSPLAHNELEPVYAAIDRLNLTVEKRLNE 60
 bov. Mit. -----MAPYSLLVSRLOKALGARQYHVASVLCQRAKVAMSHFEPNEYIR--YDLLEKNINIVVEKRLNE 61
 pig Mit. -----MAPYSLLVTRLOKALGVQYHVASVLCQRAKVAMSHFEPNEYIR--YDLLEKNINIVVEKRLNE 61
 caeel MRYHFLFGSLRNHLFSFGVGYCYREKLFNCCKLSFRPSKVAISKFPKSYLP--YEKLSQTVKIKDKRLKE 69

aconitase C-terminus

pirE2 Cyt. ILLNHTLNQIEWFKAGSALNLLAAAKKN- 755
 yeast AVLTETENDEQIEWFKYGSALNKKIKADEKK-- 778
 yeast AcoX IKAKHTMSKQIDFFFKAGSALNINIGNIRNE- 789
 grave Mit. ILLNHTFNDEQIEWFKAGSALNKKIKIDLGT-- 779
 bov. Mit. ILLNHTFNDEQIEWFKAGSALNRMKELQK-- 780
 pig Mit. ILLNHTFNDEQIEWFKAGSALNRMKELQK-- 781
 caeel VTLNHTFNDEQIEWFKAGSALNRMKEVFAKSK 788

C. acetoxyhydroxyacid reductoisomerase N-terminus

pirE2 Cyt. -----MSFRNSSRMAMKALRTMGSRRLATRSMSVMART - 33
 schpo Mit. -----MLRTQAARLICNSRVITAKRTFALAT 26
 neur Mit. -----MAARNCTKALRPLARQLATPAVQRRRTFVAAS 32
 arath Chl. -----MAAATSSIAPLSLSCPSPPSSSSKTLWSSKARTLALPNIGFLSSSSSKSLRSLTAVAGNGATGSSLAAR 67
 spiol Chl. MAATAATFTSLSSSSSTSAASAKALKQSPKPSALNLGFLGSSSTIKACRSLKAARVLPFGANGGGSALSAQMV 73
 ecoli ----- -
 bacsu ----- -
 pirE2 Cyt. -----MVKVINFGVDETVYERADFPQEKINEIFKD-DVFVVIGYCTOGRNCSRNLRD 52
 schpo Mit. IAAPRMRAWPRMTAPLMQTRGMVMDPAGTKENVWERSDWPREKLVDFYFN-DTLAILGSGSGHGGGLNARD 105
 yeast Mit. RAAAYSRAAPARFVKPMITTRLGLQINFGTVTVYERADWPREKLLDYFN-DTFALIGYSGSGYGGGLNARD 98
 neur Mit. AVRASVAVKAVAPARQVQVRGVKTMDFAGHKKEVHERADWPAEKLIDYFN-DTLALIGYSGSGHGGGLNARD 104
 arath Chl. MVSSSAVKAPVSLDFETSVFKKKEKVSALGYEYIVRGGRLDFKHLDPAFKGIQIGVIGWGSQGPAAQNLRD 140
 spiol Chl. SAPSINTPSATTFDFDSSVFKKEKVTLSGHDEYIVRGGRLDFKHLDPAFKGIQIGVIGWGSQGPAAQNLRD 146
 ecoli -----ANYFNTNLNRQQLAQLGKCRFMGRDFADAGSYLGQKKVIVGCGAAGLNLGLNMD 57
 bacsu -----MVKVYNGDIKENVLAKGTVAVIGYSGSGHAHALNKE 38

acetoxyhydroxyacid reductoisomerase C-terminus

pirE2 Cyt. EELKELRESQMWQTAVTVRSRLRPENOKVEKN 352
 schpo Mit. ELEEIRNLEIWKAGEVGRSLRPENHKKH-- 404
 yeast Mit. ELDITRNMEIWKVGEVKRLRPENQ----- 395
 neur Mit. EIDEIRNLEIWRAGK--RSLRPENOK----- 400

A. malate dehydrogenase

M V K V A V L G A A

AATATAATTATAAATCAAA**ATGGTTAAGGTCGCTGTTCTTGGTGCTGC** 48

B. aconitase

ATATAATCAAAGGATCTTATTTATTTAATATTTTATATATTTTTTTTC 48

M A T K V A M S A F

AAAAGTAATAGTAAGAAAA**ATGGCTACCAAAGTTGCTATGTCCGCTTT** 96

C. acetohydroxyacid reductoisomerase

M V K V I N F G G V

AATATACATTAAATCAAA**ATGGTTAAGGTTATTAACTTTGGTGGTGT**

Figure 3. 5' sequences of cDNAs encoding MDH (A), aconitase (B) and acetohydroxyacid reductoisomerase (C) from *Piromyces* sp. E2. The putative coding regions are shown in bold, with the encoded amino acids indicated above the nucleotide sequence. Stop codons present upstream of the translation start codon and in the same translation frame with it are underlined. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers Y16748, Y16747 and Y16743.

It has been reported previously that also the peroxisomal MDH from yeast lacks a N-terminal extension, although it probably has a mitochondrial ancestry (see Fig. 1; Ocheretina and Scheibe 1997). Instead, it contains a conserved carboxy-terminal peroxisomal targeting signal - the tripeptide SKL - which is responsible for the peroxisomal sorting of this isoform (McAlister-Henn et al. 1995). Sequence analysis of the deduced carboxy-terminal ends from the chytrid MDH, aconitase and acetohydroxyacid reductoisomerase encoding genes did not provide evidence for the presence of putative peroxisomal targeting signals.

Finally, for the yeast mitochondrial MDH it has been shown that the N-terminal leader sequence is not the only determinant for mitochondrial import. Deletion of the N-terminal extension still allows mitochondrial import of this MDH isoform (Thompson and McAlister-Henn 1989). This import relies on three positively charged residues near the N-terminus of the mature protein (Lys-25, Arg-30 and Lys-38). Their alteration together with a simultaneous removal of the N-terminal presequence abolishes mitochondrial import of this MDH protein (Small and McAlister-Henn 1997). In the deduced *Piromyces* sp. E2 MDH sequence, the corresponding positions are occupied by uncharged amino acids, suggesting that also this cryptic mitochondrial targeting signal is absent.

Localisation of the MDH, aconitase, and acetohydroxyacid reductoisomerase activities

Absence of the N-terminal leader sequences from the deduced MDH, aconitase and acetohydroxyacid reductoisomerase sequences suggests that their gene products are localised in the cytosol. To test this hypothesis, the activities of these enzymes were

assayed in the cytosolic and hydrogenosomal fractions from *Piromyces* sp E2 homogenates

The quality of the subcellular fractionation was controlled by measuring the activities of cytosolic and hydrogenosomal marker enzymes. As expected, the cytosolic marker enzyme hexokinase was exclusively present in the cytosolic fraction of *Piromyces* sp E2 (Table 1). The activities of the hydrogenosomal marker enzymes hydrogenase and malic enzyme could be detected in both the cytosolic and hydrogenosomal fractions (Table 1). However, they were significantly enriched in the hydrogenosomal fraction of *Piromyces* sp E2, which indicated that the majority of the isolated hydrogenosomes was intact (Marvin-Sikkema et al 1993b). Addition of Triton X-100, a detergent permeabilizing the hydrogenosomal membrane, resulted in a considerable increase of the measured hydrogenosomal enzyme activities (Table 1).

MDH and acetoxyhydroxyacid reductoisomerase activity were found exclusively in the cytosolic fraction of *Piromyces* sp E2 (see Table 1). Aconitase activity was found in both the cytosolic and hydrogenosomal fractions. However, a proteinase K protection assay revealed that no aconitase activity is present inside the hydrogenosomes (Table 2). After incubation of isolated hydrogenosomes with proteinase K, no aconitase activity could be detected, regardless whether the detergent Triton X-100 was added or not. These data indicated that the aconitase activity measured in the hydrogenosomal fraction (Table 1) results from adhesion of cytosolic aconitase to the outside of the isolated hydrogenosomes. In the control experiment, the hydrogenosomal malic enzyme (van der Giezen et al 1997a) could only be digested by proteinase K in the presence of the detergent Triton X-100 (Table 2).

Triton X-100	Cytosol	Hydrogenosomes	
	+	-	+
Hydrogenase	1.90	11.49	172.45
Malic enzyme (NADP)	0.19	4.42	20.60
Hexokinase	0.83	ND	ND
Malate dehydrogenase (NAD)	0.24	ND	ND
Acetoxyhydroxyacid reductoisomerase (NADP)	0.01	ND	ND
Aconitase	0.17	0.09	0.08
Aconitase + fluorocitrate	ND	ND	ND

Table 1 Specific enzyme activities ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein) in different subcellular fractions of *Piromyces* sp E2. ND, not detectable within the limits of the assay sensitivity ($1 \text{ nmol min}^{-1} \text{mg}^{-1}$ protein).

	Malic enzyme			Aconitase		
Proteinase K	–	+	+	–	+	+
Triton X-100	–	–	+	–	–	+
Specific Activity ($\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein)	3.79	3.99	0.23	0.10	ND	ND

Table 2. Proteinase K protection assays for hydrogenosomal enzymes of *Piromyces* sp. E2. ND, not detectable within the limits of the assay sensitivity ($1 \text{ nmol min}^{-1} \text{mg}^{-1}$ protein).

Thus, the three enzymes analysed here, i.e. MDH, aconitase, and acetohydroxyacid reductoisomerase, are exclusively located in the cytosol of *Piromyces* sp. E2.

Gene copy number and expression of MDH, aconitase, and acetohydroxyacid reductoisomerase at the mRNA level

The copy number of the MDH, aconitase, and acetohydroxyacid reductoisomerase encoding genes was determined by Southern blotting (Fig. 4). Hybridisation with the MDH probe revealed the presence of one gene copy with a high similarity to the probe (Fig. 4A). For aconitase similar results were found (Fig. 4B). The acetohydroxyacid reductoisomerase encoding gene is apparently present in several copies (Fig. 4C). It is unclear whether more than one of these gene copies is expressed. The two

Figure 4. Southern blots with genomic DNA from *Piromyces* sp. E2, probed with cDNAs encoding MDH (A), aconitase (B), and acetohydroxyacid reductoisomerase (C). DNA was digested with the following restriction enzymes: B - Bam HI, Bg - Bgl II, C - Cla I, E - EcoRI, S - Sst I, K - Kpn I, X - Xba I. Presence of several bands in (B) in the lanes with genomic DNA, digested with Bgl II and EcoRI, is due to the presence of internal Bgl II and EcoRI restriction sites in the aconitase cDNA.

acetoxyacid reductoisomerase cDNAs, isolated in our random screening, were identical in the overlapping region (400 bp) and are most likely derived from the same gene. Northern blotting revealed the presence of single transcripts for MDH, aconitase and acetoxyacid reductoisomerase (see Fig. 5). The estimated lengths of these transcripts were in good agreement with the lengths of the corresponding cDNAs.

Figure 5. Northern blots with total RNA from *Piromyces* sp. E2, probed with cDNAs encoding MDH (A), aconitase (B), and acetoxyacid reductoisomerase (C). In A, B, and C, the same blot was used.

Discussion

In this study, we have identified cDNA clones that encode MDH, aconitase, and acetoxyacid reductoisomerase from anaerobic chytrid *Piromyces* sp. E2. Phylogenetic analysis of the deduced protein sequences (Fig. 1) demonstrated that these enzymes are closely related to the corresponding mitochondrial isoforms from aerobic eukaryotes. DNA sequence analysis has revealed the absence of N-terminal extensions, which in the mitochondrial homologues function as a targeting signal (Fig. 2). The absence of such N-terminal leader sequences correlates with the cytosolic localisation of these enzymes in *Piromyces* sp. E2 (Table 1, 2), and one has to conclude that certain mitochondrial enzymes of the aerobic ancestor became localised in the cytoplasm as a consequence of the adaptation of chytrids to anaerobic environments.

Anaerobic chytrids lack mitochondria and several key enzymes of the TCA cycle (O'Fallon et al. 1991; Mountfort 1994; Yarlett et al. 1986; Yarlett 1994). However, they must still rely on the various TCA cycle intermediates and enzymes that are necessary for the biosynthesis of compounds that permit their growth on simple sugars and ammonia as sole source of carbon and nitrogen (Teunissen et al. 1991; Dijkerman et al. 1997). It has been shown previously that some important enzymes involved in biosynthetic reactions, such as phosphoenolpyruvate carboxykinase, are localised in the cytosol of anaerobic chytrids (Marvin-Sikkema et al. 1993b; Mountfort 1994; Yarlett 1994). Also a part of the TCA cycle - working in the reverse (reductive) direction from oxaloacetate, to malate, fumarate and finally succinate - is located in the cytosol (Marvin-Sikkema et al. 1993b). This metabolic pathway plays an important role in the synthesis of intermediates which are essential for several anabolic reactions. The MDH

is also playing a major role in main cellular carbon flow by converting oxaloacetate to malate, the sole substrate of hydrogenosomal metabolism (Marvin-Sikkema et al. 1993b; Marvin-Sikkema et al. 1994).

Since anaerobic chytrids can grow on glucose and ammonium as the sole carbon and nitrogen source (Dijkerman et al. 1997), they must contain a full set of enzymes necessary for the biosynthesis of all amino acids, purines and pyrimidines. In glucose-grown yeast, the disruption of the gene encoding the mitochondrial aconitase results in glutamate auxotrophy - in addition to respiratory defects (see McAlister-Henn and Small 1997 and references therein). Therefore, it is likely that in *Piromyces* sp. E2, after the adaptation to anaerobiosis and the "loss" of functional mitochondria and a complete TCA cycle, a cytosolic aconitase and a cytosolic isocitrate dehydrogenase (results not shown), must maintain the cellular pool of α -ketoglutarate. This intermediate is used to synthesise glutamate and other amino acids. Acetohydroxyacid reductoisomerase is crucial for the biosynthesis of branched amino acids (see Petersen and Holmberg 1986 and references therein). Thus, several biochemical pathways necessary for amino acid synthesis are localised in the cytosol in anaerobic chytrids.

We have shown here that MDH, aconitase, and acetohydroxyacid reductoisomerase - enzymes normally localised in the mitochondria of aerobic fungi - are expressed and active in the cytosol of the *Piromyces* sp. E2. Therefore, we have to conclude that many, if not all of the "mitochondrial" biosynthetic reactions are localised in the cytosol of this anaerobic chytrid. These aspects, and the fact that a part of the chytrid's carbohydrate metabolism is running "backward", might be of some relevance for the hypothesis of Martin and Müller (1998) for the evolution of the eukaryotic cell. They have postulated that the presence of biosynthetic and catabolic reactions in the same subcellular compartment might result in futile cycling. Consequently, one might conclude that the cytoplasmic localisation of MDH and aconitase is a necessary consequence of the evolution of a compartmentalised hydrogenosomal metabolism that uses malate and retains a ("mitochondrial") succinyl-thiokinase (Brondijk et al. 1996).

The adaptation to anoxic environments apparently involved the retargeting of several "mitochondrial" enzymes to the cytosol. There are several possible scenarios for the adaptation of the chytrids to anaerobiosis. The first scenario assumes a conversion of mitochondria into hydrogenosomes as postulated by Martin and Müller (1998), the second scenario, would postulate a loss of mitochondria, but, beyond the hypothesis of Martin and Müller, it would implicate the evolution of a hydrogenosome from a compartment different from the mitochondrion. In the first model the subcellular compartment and the mitochondrial import machinery are maintained. This should allow import of all mitochondrial proteins, also after a loss or repression of respiratory functions. Evidence in favour of this scenario is provided by the genes encoding hydrogenosomal malic enzyme and the β subunit of succinyl-CoA synthetase (Brondijk et al. 1996; van der Giezen et al. 1997a, 1998).

The second scenario assumes that the mitochondria have been lost during the transition from aerobiosis to anaerobiosis leading to "type I" anaerobes according to the hypothesis of Martin and Müller (1998). In this scenario, however, hydrogenosomes of chytrids must have evolved *de novo*, perhaps from microbody-like organelles as

postulated by Marvin-Sikkema et al. (1993a). This scenario is supported by the data on the hydrogenosomal adenylate kinase of *Neocallimastix* and *Piromyces*. This hydrogenosomal enzyme has a mitochondrial ancestry. It lacks a N-terminal extension, but contains a carboxy-terminal sequence, the tripeptide SKL, which is known as a peroxisomal targeting signal. Expression of the hydrogenosomal adenylate kinase in the heterologous host *Hansenula polymorpha* revealed that this tripeptide is functional in sorting this enzyme to peroxisomes (see Chapter 6, Voncken et al. unpublished).

As shown in this paper, also the chytrid MDH, aconitase, and acetohydroxyacid reductoisomerase have a mitochondrial ancestry. However, they lack mitochondrial targeting information and are located in the cytosol. This phenomenon might fit into both scenarios. Under the assumption, that mitochondria evolved into hydrogenosomes - while maintaining a mitochondrial import machinery - one can speculate that the presence of MDH, aconitase, and acetohydroxyacid reductoisomerase interfered with the evolving hydrogenosomal metabolism. Consequently, the exclusion of these enzymes from the hydrogenosomes might have required the removal of all targeting information.

If the mitochondria were completely lost in anaerobic chytrids, hundreds of nuclear genes encoding mitochondrial proteins would have remained functional. In the absence of a suitable subcellular compartment, the encoded proteins would stay in the cytosol - unless their synthesis is down-regulated. Also the N-terminal leader peptides, that normally are removed by specific mitochondrial peptidases, would be retained in the mature protein. The presence of such N-terminal extensions could potentially interfere with protein folding and enzyme activity (Danpure 1997). Thus, also the second assumption can provide arguments for the absence of mitochondrial leader sequences from the chytrid MDH, aconitase, and acetohydroxyacid reductoisomerase.

It is evident that more information about the subcellular localisation of "mitochondrial" enzymes and the nature of the chytrid hydrogenosomal import system(s) will be required to discriminate between the two scenarios described above. The origin of chytrid hydrogenosome remains elusive, since we are far from understanding the meaning of the data available so far. However, regardless whether the mitochondrial compartment has been lost, or alternatively, became transformed into a hydrogenosome, the adaptation to anoxic environments by the chytrid ancestors must have involved a loss of the mitochondrial genome, a loss of mitochondrial functions, the re-compartmentalisation of mitochondrial enzymes, and, perhaps, also the acquisition of novel enzymes. The data presented here show that such an adaptation required dramatic changes in subcellular sorting and import.

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Chapter 3

A hydrogenosome with pyruvate formate-lyase: anaerobic chytrid fungi use an alternative route for pyruvate catabolism

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Summary

The chytrid fungi *Piromyces* sp E2 and *Neocallimastix* sp L2 are obligatory amitochondriate anaerobes which possess hydrogenosomes. Hydrogenosomes are highly specialised organelles engaged in anaerobic carbon metabolism: they generate molecular hydrogen and ATP. Here we show for the first time that chytrid hydrogenosomes use pyruvate formate-lyase (PFL), and not pyruvate ferredoxin oxidoreductase (PFO) for pyruvate catabolism, unlike all other hydrogenosomes studied to date. Chytrid PFLs are encoded by a multigene family and are abundantly expressed in *Piromyces* sp E2 and *Neocallimastix* sp L2. Western blotting after cellular fractionation, proteinase K protection assays and determinations of enzyme activities reveal that PFL is present in the hydrogenosomes of *Piromyces* sp E2. The main route of the hydrogenosomal carbon metabolism involves PFL: the formation of equimolar amounts of formate and acetate by isolated hydrogenosomes excludes a significant contribution by PFO. Our data support the assumption that chytrid hydrogenosomes are unique and argue for a polyphyletic origin of these organelles.

Introduction

Hydrogenosomes are membrane-bound organelles found in a wide variety of unicellular anaerobic eukaryotes. These protists belong to phylogenetically rather unrelated groups, and it is likely that their hydrogenosomes evolved several times in different, phylogenetically disparate lineages (Embley et al 1995, Martin and Muller, 1998). Hydrogenosomes metabolise pyruvate or malate to hydrogen, acetate and CO₂, and they are characterised by their key enzymes hydrogenase and pyruvate ferredoxin oxidoreductase (PFO, Yarlett et al 1986, O'Fallon et al 1991, Marvin-Sikkema et al 1993a, Muller 1993, Trinci et al 1994). However, our knowledge about hydrogenosomes is mainly based on decade-lasting studies of the hydrogenosomes of *Trichomonas vaginalis* and its relatives, and it cannot be excluded that the hydrogenosomes of the various protists have deviating properties (Muller 1993, Coombs and Hackstein 1995).

There is evidence that the hydrogenosomes of anaerobic chytrids are different: their ultrastructure exhibits a number of traits that discriminate them clearly from the hydrogenosomes of *Trichomonas* (Yarlett et al 1986, Marvin-Sikkema et al 1993a/b, Benchimol et al 1996a/b). Moreover, it has been claimed that - in contrast to *Trichomonas* - the hydrogenosomes of chytrids rely on malate rather than pyruvate for their metabolism (Marvin-Sikkema et al 1994). Also the evidence for PFO activity in the hydrogenosomes of chytrids is poor. Notwithstanding that hydrogenosomes of chytrids possess a highly active hydrogenase, only low levels of PFO activity were measured in the hydrogenosomal fraction of these cells by Marvin-Sikkema et al (1993a). Measurements of fermentation products of isolated hydrogenosomes seemed to support the assumption that PFO is a key enzyme in the hydrogenosomes of the chytrid *Neocallimastix* - similar to the situation in the hydrogenosomes of the parabasalid

Trichomonas. However, formation of CO₂ by isolated hydrogenosomes could not be detected, although the formation of 2 moles of CO₂ per mole of malate utilised had been postulated (Marvin-Sikkema et al. 1994). Moreover, also the formation of substantial amounts of formate by the axenic cultures of the chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2 (Marvin-Sikkema et al. 1992; Voncken, unpublished results) was in conflict with the metabolic scheme that had been postulated (Marvin-Sikkema et al. 1993a, 1994). Because of these puzzling results, only an alternative, molecular genetic approach in combination with biochemical techniques promised a chance to unravel the elusive metabolism of chytrid hydrogenosomes. Since intensive attempts to isolate chytrid genes encoding PFO with the aid of PCR techniques have failed, we constructed a cDNA library using mRNA from rapidly growing, hydrogen producing cultures of the anaerobic chytrid *Piromyces* sp. E2. We anticipated that genes encoding hydrogenosomal enzymes must be highly expressed under conditions where maximum hydrogen production is observed (Teunissen et al. 1991). DNA sequencing of 90 randomly chosen clones of this cDNA bank failed to identify any PFO gene, but 6 out of the 90 clones exhibited a substantial homology to prokaryotic pyruvate formate-lyase (PFL), another pyruvate-converting enzyme. Here we show that mRNAs encoding putative PFLs are abundantly expressed, and that PFL activity can be detected in the hydrogenosomes of *Piromyces* sp. E2. We show that also the fermentation patterns of isolated hydrogenosomes support the assumption that PFL and not PFO is one of the key enzymes of the hydrogenosomal metabolism of anaerobic chytrids. We will discuss a potential eubacterial origin of the PFL of anaerobic chytrid fungi and its bearing for a potential polyphyletic origin of the hydrogenosomes.

Experimental procedures

Organisms and growth conditions

The axenic cultures of the chytrids *Piromyces* sp. E2 and *Neocallimastix* sp. L2 were grown anaerobically (N₂/CO₂ gas-phase) in medium M2, supplemented with 0.5% fructose (Teunissen et al. 1991; Marvin-Sikkema et al. 1990). Biomass was harvested after 40-48 hours of growth at 39°C.

Random screening of the cDNA library and sequence analysis

The *Piromyces* sp. E2 cDNA library in the vector λ ZAPII was constructed as described earlier (Akhmanova et al. 1998a). cDNA clones were picked at random and sequenced with the M13 reverse primer to determine the sequence of the 5' part of these cDNAs. The sequences of interesting cDNAs were completed by generating shorter subclones in pUC18 and by using internal sequencing primers. To obtain 5' terminal cDNA sequences, RACE-PCR was performed with *Piromyces* sp. E2 cDNA ligated into the λ ZAPII vector. The used RACE-PCR primers are: the universal M13 reverse primer; PFLrev1, 5'-GTCTTGGTAACTAAGGTACGAC-3'; PFLrev2, 5'-TA(AG)TCACCAA TAATACGACCACG-3'; PFLrev3, 5'-TCAAGGTAACCT GGCTTGTG-3'; PFLrev4, 5'-AGAACCATCA CCTTCGTATG-3' (see Fig. 1). The resulting RACE-PCR products

were subcloned into pCR2.1 (Invitrogen) and sequenced. Sequencing was performed with the ABI PRISM Model 310 automatic sequencer using a dRhodamine terminator cycle sequencing ready reaction DNA sequencing kit (Perkin-Elmer Applied Biosystems). Sequences were analyzed with the GCG Sequence Analysis package (Devereux et al 1984). Phylogenetic analysis was performed with the programs PHYLIP V3.5c (Felsenstein 1993) and PROTML (Adachi and Hasegawa 1992). Database accession numbers are *Clostridium pasteurianum* - Q46267 (Weidner and Sawers 1996), *Chlamydomonas reinhardtii* - P37836 (Dumont et al 1993), *E. coli* PFLB - P09373 (Rodel et al 1988), *E. coli* PFL3 - P42632 (Blattner et al 1997, Hesslinger et al 1998), *E. coli* PFLD - P32674 (Blattner et al 1993, Reizer et al 1995), *Haemophilus influenzae* - P43753 (Fleischmann et al 1995), *Streptococcus mutans* - Q59934 (Yamamoto et al 1996), *Lactococcus lactis* - AJ000326 (Arnau et al 1997).

Southern and Northern blotting

Genomic DNA was prepared from *Pyromyces* sp. E2 biomass according to the protocol of Brownlee (1994). DNA digested with different restriction enzymes was separated on 0.7% agarose gels. Total RNA was prepared by the guanidinium chloride method (Chirgwin et al 1979). For preparation of poly(A)⁺ RNA, a mRNA purification kit (Pharmacia) was used. RNA was separated on 1.2% agarose-formaldehyde gels. Gels were blotted to Hybond N⁺ membrane (Amersham). DNA probes were labelled by PCR with α -[³²P]dATP. To make probes from the pPFLa12 and pPFLh115 cDNA clones, the universal M13 forward and reverse primers were used for PCR. To generate probes, specific for the 'long' or 'short' type PFL, PCR was performed with the M13 reverse primer and the primer PFLrev4 using the clones pL1 ('long' type PFL) or pS2 ('short' type PFL) as a template. Hybridisation was performed in 0.5 M sodium phosphate buffer pH 7.0, 7% SDS, 1% BSA, 1 mM EDTA, at 60°C. Filters were washed stringently with 50 mM sodium phosphate buffer pH 7.0 and 0.5% SDS at 60°C.

Subcellular fractionation and Western blotting

Hydrogenosomal and cytosolic fractions were prepared from *Pyromyces* sp. E2 biomass essentially as described by Marvin-Sikkema et al (1993a), with the following modification: the potassium phosphate (KPi) buffer in the homogenisation medium was substituted for 20 mM K-HEPES buffer, pH 7.4. The hydrogenosomal fraction was subfractionated by carbonate extraction as described by Elgersma et al (1996). Proteins were separated on 7.5% polyacrylamide gels with 0.1% SDS and blotted to polyvinylidenedifluoride (PVDF) membrane by semi-dry transfer (Biorad) according to the manufacturers protocol. Rabbit antiserum raised against PFL protein of *Escherichia coli* was kindly provided by Dr. D. Kessler. It was used in a dilution of 1:250. Detection was performed using goat anti-rabbit antibodies conjugated to peroxidase (Boehringer Mannheim).

Proteinase K protection assay

Isolated hydrogenosomes (about 0.8 mg protein) were incubated anaerobically (N₂ gas-phase) in 1 ml digestion buffer containing 20 mM K-HEPES pH 7.4, 250 mM sucrose,

2 mM DTT, with proteinase K (0.0, 0.2 and 20.0 mg per ml) and detergent (0.1% Triton X-100 and 0.1% deoxycholate) in all possible combinations. After 30 minutes incubation on ice, the proteinase K digestion was stopped by the addition of 1 ml 15% (w/v) trichloroacetic acid. Protein was recovered by centrifugation.

Enzyme assays

To measure PFL activity, 0.5 ml of the cytosolic or hydrogenosomal fraction (0.5 mg protein) was added to 4.5 ml of assay mix, containing: 20 mM K-HEPES pH 7.5, 2 mM KP_i, 100 mM succinate, 5 mM MgADP, 2 mM DTT, 1.3 mM ferriammoniumsulfate, 0.12 mM CoA, and 30 mM pyruvate. The incubation was stopped after 60 min by heating (5 minutes, 100°C). Formation of formate was determined with formate-dehydrogenase (Boehringer Mannheim). Samples, heated (5 minutes, 100°C) before addition to the assay mix, were used as controls to correct for internal formate pools. Hypophosphite, a specific inhibitor of PFL activity (Plaga et al. 1988), was added to a final concentration of 10 mM. For enzyme assays with the hydrogenosomal fraction, Triton X-100 was added to a final concentration of 0.2% (v/v) in order to permeabilise the organelles. All enzyme assays were performed anaerobically (N₂ gas-phase) at 39°C.

Analysis of hydrogenosomal fermentation products

Isolated hydrogenosomes were resuspended in 20 mM K-HEPES buffer pH 7.4, supplemented with 250 mM sucrose and 2 mM DTT. From this suspension 0.2 ml (about 0.55 mg protein) was added to 2 ml of assay mixture, containing: 20 mM K-HEPES buffer pH 7.4, 250 mM sucrose, 2 mM DTT, 2 mM KP_i buffer, 100 mM succinate, 5 mM MgADP, and 5 mM L-malate. Incubations were performed anaerobically (N₂ gas-phase) at 39°C in 15 ml vials. Liquid samples were analysed for malate, pyruvate, acetate, and formate, with the help of Test Combinations purchased from Boehringer Mannheim. Gas samples were analysed by gas chromatography for the determination of the hydrogen concentration (Teunissen et al. 1991).

Results

*Isolation of PFL-encoding cDNAs from the *Piromyces* sp. E2 cDNA library*

Ninety clones, randomly chosen from a *Piromyces* sp. E2 cDNA library that has been described earlier (Akhmanova et al. 1998a), were partially sequenced. Among these cDNAs, six clones (named pPFLh104, pPFLh112, pPFLh114, pPFLh115, pPFLh140 and pPFLa12, see Fig.1) displayed a high degree of similarity to pyruvate formate-lyase (PFL)-encoding genes from different eubacteria. The longest of these cDNAs (pPFLh115) was sequenced completely. It contained an open reading frame (ORF) of 2040 bp which could be aligned over the whole length with a *pfl* gene of *Clostridium pasteurianum* (Weidner and Sawers 1996). However, the absence of an AT-rich 5' untranslated region (UTR) that is characteristic for genes of anaerobic chytrids (Durand et al. 1995; Fanutti et al. 1995; unpublished observations of the authors), and the lack of a translation start codon indicated that the pPFLh115 cDNA was incomplete.

Figure 1. PFL-encoding cDNA clones from *Piromyces* sp. E2. The scheme of the composite cDNA sequence PFL-L1 (assembled from clones pL1 and pPFLh115) is shown at the top, with the black box indicating the open reading frame. cDNA clones, identified by random screening (designated pPFL) or isolated by 5' RACE (the rest of the clones) are shown by thick lines. Short arrows indicate the position of the PFL-specific primers PFLrev1 to PFLrev4 (marked 1 to 4) used in this study. Bent arrows indicate the positions of the first ATG codon, and the asterisks indicate the position of the STOP codon. Groups of clones, identical in the overlapping regions are marked with accolades

In order to obtain the 5' part of this cDNA we performed a 5' rapid amplification of cDNA end-PCR (RACE-PCR) with the primers PFLrev1 or PFLrev3 (see Fig.1). With the primer PFLrev1, three clones (pL1, pL2 and pL3) were isolated. DNA sequence analysis of these clones revealed an exact match with the clone pPFLh115 over a contiguous stretch of 811 bp. Consequently, the clones pL1, pL2, pL3 and pPFLh115 must be derived from the same gene. Also one of the clones (pL4) that were obtained with the primer PFLrev3, must be derived from this *pfl* gene. However, the clones pL5, pL6, pL7 (also obtained with the primer PFLrev3, see Fig. 1) differed in several positions and must be derived from different *pfl* genes.

A complete gene encoding a putative PFL ('PFL-L1') was reconstructed from the clones pPFLh115 and pL1. The gene contained an ORF encoding a protein of 805 amino acids with a predicted molecular mass of 89 kDa. At the amino acid level, the predicted protein exhibited a substantial similarity with the PFL of *Clostridium pasteurianum* (77% similarity and 61% identity). The amino acids which are involved in catalysis, e.g. the neighbouring cysteines in the middle part of the protein (Knappe and Sawers, 1990)

and the glycine residue near the C-terminus (Wagner *et al.*, 1992; see Fig. 2B), are conserved. Also the C-terminus of PFL-L1 exhibits a high degree of similarity to the C-terminus of the *Clostridium pasteurianum* PFL (Fig. 2B). The N-termini, on the other hand, are rather divergent.

Alignment of PFL-L1 with the deduced *Clostridium pasteurianum* PFL sequence revealed that the chytrid sequence possesses a N-terminal extension of 61 amino acids (Fig. 2). This N-terminal extension is enriched in positively charged and hydroxylated amino acids. Since such an amino acid composition is similar to that of mitochondrial transit peptides (Hendrick *et al.* 1989; von Heijne *et al.* 1989), the N-terminal extension of PFL-L1 might represent a hydrogenosomal targeting signal (c.f. Brondijk *et al.* 1996; van der Giezen *et al.* 1997, 1998).

A. PFL N-termini

Piromyces pL1	MESLALSNNVS-VLANTVSVNAVAATKVAGVRMAKPSRALHTPAMKTT-LKTSKK--VPAMQAKTYATQAPC	67
Piromyces pL5	MESLTLTQVNNIAIAKSVSVNAVAATKVAGVRISKPSRAIHTPMTTSLKTSKSSFPISQTKTYATQAPC	71
Piromyces pL7	MESLTLTQVNNIAIAKSVSVNAVAATKVAGVRISKPSRAMHTTTPMTTSLKTSK---VQAMQAKTYATQAPC	68
Piromyces pS1	-----	-
Piromyces pS2	-----	-
C. pasteurianum	-----MFKQWE	6
H. influenzae	-----SELNEMOKLAWA	12
E.coli PFLB	-----SELNEKLATAWE	12
E.coli PFL3	-----MKVDLDTSDKLYADAWL	17
S. mutans	-----MATVKTNNTDVFKEAW	16
L. lactis	-----MKTEVTE-NIFEQAWD	15
Piromyces pL1	ITNDAAAKSEIDVEGWIKKHYTPYEGDGSEFL-AGPTEKTKKLFKAEEYLAKERANGGLYDVPDHPSTSI	136
Piromyces pL5	ITSDAAAKSEIDVEGWIKKHYTPYEGDGSEFL-AGPTEKTKKLFKAEEYLAKERANGGLYDVPDHPSTSI	140
Piromyces pL7	ITNDAAAKSEIDVEGWIKKHYTPYEGDGSEFL-SGPTEKTKKLFKAEEYLAKERANGGLYDVPDHPSTSI	137
Piromyces pS1	-----MQVIDVAKNIKENYTPYEGDASTFLVTDASAKTKDVNNKCELRABEIKTNGCLDVKNTISTV	63
Piromyces pS2	-----MQVIDVAKNIKENYTPYEGDASTFLVTDASAKTKDVNNKCELRABEIKTNGCLDVKNTISTV	63
C. pasteurianum	GFQDGEWTDNNVRDFTQKNYKEITGDKSEFL-KGPTEKTKKLVMDKAVSLILE-LKKGLLDVDTETISGI	74
H. influenzae	GFAGGDWQENNVNRDFTQKNYTPYEGDSEFL-AGPTEATTKLWESMEGIRIENHAPLVDVDTHTPSTSI	81
E.coli PFLB	GFTKGDWQENNVNRDFTQKNYTPYEGDSEFL-AGATEATTTLMDKVMGKVLNTHAPVDDTAVASTI	81
E.coli PFL3	GFKGTDWKNEINVRDFTQHNYTPYEGDSEFL-AEATPATTELWEKVMGIRIENATHAPVDDTNIATSI	86
S. mutans	GFKGTDWKDRASISRFVQDNYPYEGDSEFL-AGPTERSLHIKKVVEET---KAHYETREFPMDT-RITSI	82
L. lactis	GFKGTNWRDKASVTRFVQENYKPYEGDSEFL-AGPTERTLKVKKILIEDT---KNHYEEVGFPPDTRVTSI	82

B. PFL C-termini

Piromyces PFL-L1	LLDGYETRG-----AHHLNVNLKRETLSDAMAHPEPNYLNLTIRVSGYAVNFVKLTPOQQKEVIA	747
C. reinhardtii	ILDGYFANG-----GHHNVNLNRLMDAVEHPEKYPNLTIRVSGYAVHFARLTREQQLEVIA	188
C. pasteurianum	IMGGYFGGG-----AHHLNVNLNRETLDAMNNPDKYPYLTIRVSGYAVNFNRLSKDHQKEVIS	733
H. influenzae	LMDDYTHHEATVEG---GCHLNVNLNREMLDAMENPDKYPOLTIRVSGYAVRFNSLTKEQQQDVI	762
E.coli PFLB	LLDGYTHHEASTEG---GCHLNVNMNREMLDAMENPEKYPOLTIRVSGYAVRFNSLTKEQQQDVI	722
E.coli PFL3	LLDGYTHHEADVEG---GCHLNVNMNREMLDIEHPEKYPNLTIRVSGYACASTH-----	744
S. mutans	ILDGYTEGG-----GCHLNVNMDLKDQVDYDKIMNGE---DVIIVISGVCYNTKYLTPEQKTELQ	755
L. lactis	ILDGYTFGALINGTEFAGCHLNVNMDLKDQVDYDKIMRGE---DVIIVISGVCYNTKYLTPEQKQELTE	767
Piromyces PFL-L1	RTFHEKM-----	754
C. reinhardtii	RTFHDTM-----	195
C. pasteurianum	RTFHEKL-----	740
H. influenzae	RTFTESM-----	769
E.coli PFLB	RTFTQSM-----	759
E.coli PFL3	-----	-
S. mutans	RVEHEVLSDMDAATDLVNNK	775
L. lactis	RVEHEVLNDDDEVNHSNI	787

Figure 2. Multiple alignments of the deduced N-terminal (A) and C-terminal (B) sequences of different putative PFL enzymes. Amino acids identical in all sequences are in bold and shaded with dark grey. Amino acids, similar in more than 70% of the sequences are shaded with light grey. For database accession numbers see: Experimental procedures p. 46. Glycine residue, involved in catalysis (Wagner *et al.* 1992) is indicated by an arrow.

The high similarity between PFL-L1 of *Piromyces* sp. E2 and the PFL-encoding genes of the various eubacteria, including *Escherichia coli*, *Clostridium*, *Streptococcus* and *Lactococcus* (Rödel et al. 1988; Weidner and Sawers 1996; Yamamoto et al. 1996; Arnau et al. 1997) suggests a prokaryotic rather than an eukaryotic origin. A pairwise comparison of the C-terminal parts of different PFL enzymes strongly supports this interpretation. Therefore, it is not surprising that phylogenetic analyses using neighbour-joining, parsimony or maximum likelihood methods, revealed that the two eukaryotic sequences known to date (*Piromyces* sp. E2 and *Chlamydomonas reinhardtii*) do not form a separate clade (Fig.3).

Figure 3. Phylogenetic analysis of the C-terminal parts of the deduced PFL sequences by using the maximum likelihood method. C-terminal parts of the PFL sequences, corresponding to the sequence fragment available for *Chlamydomonas reinhardtii* (starting from amino acid residue 612 of *Piromyces* sp. E2 PFL-L1) were aligned with the program Pileup and the alignment was refined by visual inspection. The best tree obtained by an exhaustive search using PROTML program is shown. A tree with a similar topology was obtained with the neighbour-joining method. The numbers indicate the bootstrap values obtained for the same clades by using the neighbour-joining method.

Figure 4. Multiple alignments of the 5'-terminal (A) and 3'-terminal (B) nucleotide sequences of different putative PFL encoding cDNA fragments from *Piromyces* sp. E2. Coding sequences are shown in bold. The numbering corresponds to the nucleotide positions in the composite cDNA sequence PFL-L1. Nucleotides identical in all sequences are shaded with dark grey. Additionally, nucleotides conserved in more than 60% of the sequences are shaded with light grey in Fig. 4A. In Fig. 4B, the encoded amino acids (derived from the clone pPFLh115) are shown above the nucleotide alignment. The amino acids encoded by clones pPFLa12, pPFLh112 and pPFLh140 which are different from those encoded by pPFLh115 are also indicated. The start codon (ATG) is marked by a bent arrow, whereas the stop codon is marked with an asterisk. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers Y16739 (composite cDNA PFL-L1); Y16738 (clone pS2); Y16740 (clone pPFLh104); Y16741 (clone pL7); Y16742 (clone pPFLh114); Y16744 (clone pS1); Y16745 (clone pPFLh112); Y16746 (clone pPFLa12); Y16749 (clone pPFLh140); Y16750 (clone pL3).

A. 5' regions from different *Piromyces* sp. E2 PFL cDNA clones

pL1 -----ATAAATAAGAATTACTTATTGTAAAAACAAAAAATCTTAATATACCAATAATATTATTGTGAAA
pL5 TAAATACCTTAAGGAATTTAAATAATCTTTATATACCAAAAAAAGCTGTGAATTATTATTTTAAACAAACGCAATATATCCCTTTT
pL7
pS1
pS2 -----

pL1 -ATTTATATTTTTTTTTTACAAAAACAAAATAAATGGAAAGCTTAGCTTTATCCAATGTCAGT---GTTCTTGCTAACACTGTTCTGTT 86
pL5 ATAAAAATTAATTAATATATATTAATAAAAAAATGGAAAGCTTAACTTTAACTCAAGTTAATAATGCTATTGCCAAGTCAGTTTCAGTT
pL7 TTTATTATAAAAAAAGCTTTTTTAAAAACAAAATGGAAAGCTTAACTTTAACTCAAGTTAACAACGCTATTGCCAAGTCAGTTTCAGTT
pS1
pS2 -----

pL1 AAGCGTGTGCTGCCACCAAGGTCGTGGTGCAGAAATGGCCAAGCATCCGCGCGCTTCACACCCAGCTATGACACTACT---CT 173
pL5 AATGCTGTGCTGCTACTAAAGTTGCTGGTGTAGAAATCAGCAAGCGCATCCGCTGCATCCACACTTACGAATCTACGACTAGTCTT
pL7 AATGCTGTGCTGCTAAAGCTTGTGGTGTAGAAATCAGCAAGCATCCGCTGCATCCACACTTACGAATCTACGACTAGTCTT
pS1 -----TAACTACTTAAAAATATTAGAAATTAATTTTGGCTATTTTTAAATAT
pS2 -----ATAATACTACTTAAAAATATTAGAAATTAATTTTGGCTATTTTTAAATAT

pL1 AAGACTTCTAAGAAG-----GTCCAGCTATGCCAGCTAGCTCTCGGCACTCAAGCCACAGCATCACCAACGATGCTGCTGCCAAG 257
pL5 AAGACTTCTAAGAAGCTTCTCCATCTTCCAAACCAACCTTCGCTCACTCAAGCCACAGCATCACGACTAGTATGCGCGCTGCTAG
pL7 AAGACTCTAAG-----GTTCAAGCTATGCCAGCTAGCTCTCGGCACTCAAGCCACAGCATCACGACTATGATGCTGCTGCTAG
pS1 TTTTATATATTTTAAATTTAACATATCAAAATAATGTAATTTTAAATATTAAGTTATTTTATATATTATTATATATAATAAAGATG
pS2 TTTTATATATTTTAAATTTAACATATCAAAATAATGTAATTTTAAATATTAAGTTATTTTATATATTATTATATATAATAAAGATG

pL1 AGTGAATCTATGTTGAAGGTTGGATTAGAACCACTACACCCGATACGAAGGTGATGCTCTTCCCTT---GTGTGCCAATGAAGA 344
pL5 AGTGAATCTATGTTGAAGGTTGGATTAGAACCACTACACCCGATACGAAGGTGATGCTCTTCCCTT---GTGTGCCAATGAAGA
pL7 AGTGAATCTATGTTGAAGGTTGGATTAGAACCACTACACCCGATACGAAGGTGATGCTCTTCCCTT---GTGTGCCAATGAAGA
pS1 CAAATTTATTAAGCTTCCCAAGTGGCAAGCAAACTACACCCGATACGAAGGTGATGCTCTTCCCTTGTATCGAAGTTTGAAGTAA
pS2 CAAATTTATTAAGCTTCCCAAGTGGCAAGCAAACTACACCCGATACGAAGGTGATGCTCTTCCCTTGTATCGAAGTTTGAAGTAA

B. 3' regions from different *Piromyces* sp. E2 PFL cDNA clones

h140 V L H I R
h115 D A M A H P E N Y P N L T I R V S G Y A V N F V K L T P
pPFLh115 CACGCGATAGGCCCCAGAGAAATATCCAAACCTTACTATTGCTGTCTCTTCTACCTCTCAAGCTTGTGAAGTAAGTCGA 2402
pPFLh112 GATTTATGCTGCTTACGAGAAATATCCAAACCTTACTATTGCTGTCTCTTCTACCTCTCAAGCTTGTGAAGTAAGTCGA
pPFLh114 GATTTATGCTGCTTACGAGAAATATCCAAACCTTACTATTGCTGTCTCTTCTCTTCTACCTCTCAAGCTTGTGAAGTAAGTCGA
pPFLh140 GATTTATGCTGCTTACGAGAAATATCCAAACCTTACTATTGCTGTCTCTTCTCTTCTACCTCTCAAGCTTGTGAAGTAAGTCGA
pPFLh104 GATTTATGCTGCTTACGAGAAATATCCAAACCTTACTATTGCTGTCTTCTCTTCTACCTCTCAAGCTTGTGAAGTAAGTCGA
pPFLa12 CACGCGATAGGCCCCAGAGAAATATCCAAACCTTACTATTGCTGTCTTCTCTCTACCTCTCAAGCTTGTGAAGTAAGTCGA

h140 D E K T L
h112,a12 A
h115 Q Q Q K E V I A R T F H E K M *
pPFLh115 CAACAAACAAGAGAAATATGGCCCTACCTTCCACCAAGACGTAATATTGCGCTTCAAAAAGTAATCTTTTGAACCCACAAGT 2486
pPFLh112 GCTCAACAAGAAGAGATATTTGGCTCTACCTTCCACCAAGACGTAATATTGAATGAATAATATTTAATATATCTAGGAATAATTTTAA
pPFLh114 GATTTATGCTGCTTACGAGAAATATCCAAACCTTACTATTGCTGTCTCTTCTCTTCTACCTCTCAAGCTTGTGAAGTAAGTCGA
pPFLh140 GACCAACAAGAAGAGATATTTGGCTTACGCTTCCACCAAGCTCTTAAAGAGATTTAAAGGATATAAAATTTGTTTTCACATGA
pPFLh104 CAACCAACAAGAAGATATTTGGCTCTACCTTCCACCAAGACGTAATTTTGGAGAAATTTGAAAAAATAAAAAAATAAAC--
pPFLa12 GCTCAACAAGAAGATATTTGGCTCTACCTTCCACCAAGACGTAATATTTT-----

pPFLh115 TTTTATCCACAAAAGTGCTGAAAAATCAGTGACCAATTTTTTAAATCTTTTGAAGTGGTAAAAATTTTTATATATAAAC 2570
pPFLh112 AGAAGAAATACCTTACCTCTTTTATTTATTTTATTTTGGGTGCAAGAGGATAATAATATATAGTATCTACGAAAAAT
pPFLh114 TAAAAATATATATAAACAATAAAATGAAATGAAATATTAATTTAAAAATATTTAAATTACTAATCTTAATATATGCTAA
pPFLh140 AAAATATATCAATTTTTAATAATTAATAAAATTTTATGAAAAATATATCATAAATAATTAGGCATCTCCAATCAACCAACA
pPFLh104
pPFLa12

pPFLh115 CATCCCTCCCCAAAAATATATAAATAGTAAAAAGATTTTGTTTTATATTTAAAAAGTAAAAATTTTTAAATATTAATTTTAT 2654
pPFLh112 AAAATAAAAATATAAATAAATAAATAAAGAAATATACAAATATTAATATAGCAACAACAACAACAAAAATAATAATATAACA
pPFLh114 AATAATTTAGCTTTTACAATAAGAAAAAAATATTTATTTATATAAATAACACATAGTAAATAAAAAATAATCATATAAAAA
pPFLh140 CTATATAAATTTTATATAATAATAATATATAAAAAATTTAATTTTTTAATAATACAAAAAATAAATAATAATATATTTAT
pPFLh104
pPFLa12

pPFLh115 ATATAAAATAGTAAAAAAATTAATGCAAAATATAAAAAAGTAAATAAATAAATAAATAAATAAATAAATATATCAAA 2733
pPFLh112 AATAATACAAAAATAAATAATC-----
pPFLh114 ATACTATCAATAACT-----
pPFLh140 TAGTTTTAT-----
pPFLh104
pPFLa12

PFL proteins of Piromyces sp. E2 are encoded by a multigene family

All six cDNA clones obtained by random sequencing of the cDNA library (i.e. pPFLh104, pPFLh112, pPFLh114, pPFLh115, pPFLh140 and pPFLa12, see Fig.1) were different (Fig. 4B). Also the clones pL5, pL6 and pL7 (see Fig.1), representing the 5' part of PFL cDNAs, differed at multiple positions from the PFL-L1 sequence (Fig. 4A). The majority of the sequence differences were observed in the non-coding 5' and 3' UTRs (Fig. 4). In the coding regions, many synonymous, but also a few non-synonymous substitutions were present (Fig. 2 and 4). Our data strongly suggest that the genome of *Piromyces* sp. E2 possesses multiple, non-identical genes that encode several putative PFL isoforms. This assumption has been confirmed by Southern blotting of genomic DNA from *Piromyces* sp. E2 (Fig. 5). Since the PFL probe which was hybridised to the blot did not contain restriction sites for the enzymes that had been used for the digestion of the genomic DNA, the multiple bands observed on the blot argue for the presence of multiple copies of *pfl* genes. It is unlikely that restriction sites in introns contributed significantly to the complex pattern of restriction fragments, since introns in the genome of *Piromyces* sp. E2 are rare and small (F. Voncken, H. Harhangi, unpublished observations),

Because the *pfl-11* gene of *Piromyces* sp. E2 encodes a N-terminal extension that might function as a hydrogenosomal import- and targeting signal, the question arose whether all *pfl* genes encode such a N-terminal extension or whether some of the *pfl* genes lack such a N-terminus. The latter ones might encode a cytoplasmic PFL that has been postulated by Marvin-Sikkema et al. (1993a). Therefore, we performed a 5'RACE-PCR with the primer PFLrev2 (see Fig.1) in order to identify genes with and without a N-terminal extension. In addition to clones identical to pL1 (possessing the N-terminal extension), two shorter clones (pS1 and pS2) were identified which lacked the first 74

Figure 5. Southern blot of genomic DNA from *Piromyces* sp. E2. 10 µg of genomic DNA digested with Bam HI (B), Cla I (C), Eco RI (E), Kpn I (K) and Xba I (X) was loaded per lane. cDNA clone pPFLa12, containing the 3' terminal part of the PFL encoding gene, was used as probe. This probe does not contain restriction sites for the enzymes used for genomic DNA digestion.

N-terminal amino acids encoded by pL1 (Fig. 2). Moreover, they differed from each other by four nucleotide substitutions (one of them non-synonymous) (Fig. 2 and 4A). Comparison of the pS1 clone as a representative of the 'short' type PFL with the pL1 clone (a representative of the 'long' type PFL) revealed a 58.2% identity at the protein level.

Thus, it has to be concluded that a multigene family, encoding two types of PFL (e.g. 'short' and 'long' type PFL), is present in the genome of *Piromyces* sp. E2. Here, we have identified two clones coding for the 'short' type PFLs, which differed from each other by one amino acid substitution, and three clones encoding the 'long' type PFLs, which differed from each other by several amino acid substitutions and insertions/deletions (see Fig. 2).

Expression of pfl genes at the mRNA level

The transcription of the *pfl* genes of *Piromyces* sp. E2 was investigated by Northern blotting (Fig. 6). Two transcripts of 2800 and 3000 nucleotides, respectively, were observed on the Northern blot when the clone pPFLh115 was used as a probe (Fig. 6A). In order to investigate whether the two transcripts correspond to the 'long' and 'short' type of PFL, the probes derived from the 5' terminal parts of the clones pL1 ('long' type) and pS2 ('short' type) were hybridised to the same Northern blot lanes. The 'long' type probe hybridised to both transcripts, obviously due to a length polymorphism at the 3' end (c.f. Fig. 4B). The 'short' type probe hybridised only to the 2800 nt long mRNA (Fig. 6B, C). Consequently, it must be concluded that both, the 'long' and the 'short' types of *pfl* genes are abundantly expressed.

Also on Northern blots with RNA from a related anaerobic chytrid species, *Neocallimastix* sp. L2, two different transcripts could be readily detected using a *Piromyces* sp. E2 PFL probe (Fig. 6D). This suggests that abundant expression of *pfl* genes is a general feature of anaerobic chytrids.

Figure 6. Analysis of PFL expression by Northern blotting. **A.** Northern blot with 10 μ g of total RNA from *Piromyces* sp. E2, probed with the pPFLh115 clone. **B.** The same lane as in A, probed with the 5' part of pL1 clone ('long' type specific probe). **C.** The same lane as in A, probed with the 5' part of pS2 clone ('short' type specific probe). **D.** Northern blot with 1 μ g of poly(A)⁺ RNA from *Neocallimastix* sp. L2, probed with the pPFLh115 clone.

Figure 7. Western blot analysis of the subcellular distribution of PFL. Western blotting of different subcellular fractions from *Piromyces* sp. E2 (A) and *Neocallimastix* sp. L2 (B). Cyt, cytosolic fraction; Hdg, hydrogenosomal fractions (Hdg) are indicated as follows: Ma, hydrogenosomal matrix; PM, peripheral membrane-bound; and IM, integral membrane protein fraction. The antiserum used was directed against PFLB protein from *E. coli*.

Expression of PFL at the protein level

The subcellular localisation of PFL in *Piromyces* sp. E2 was investigated by cellular fractionation followed by Western blotting with an antiserum raised against pyruvate formate-lyase (PFLB) from *E. coli* (Conradt et al. 1984; Rödel et al. 1988). In both the hydrogenosomal and the cytosolic fraction of a homogenate of the mycelium, a doublet protein band with a molecular mass of approximately 80-82 kDa, was recognised by the antiserum (Fig. 7A). Such a doublet is characteristic for bacterial PFLs. It is the consequence of a specific fragmentation of the activated (free radical-bearing) form of PFL in the presence of oxygen (Wagner et al. 1992). The observed molecular mass of the hydrogenosomal PFL of *Piromyces* sp. E2 is 7 kDa lower than the predicted molecular mass of the 'long' type PFL from *Piromyces* sp. E2 (89 kDa). This difference might be the consequence of a proteolytic processing of the 'long' type PFL upon import into the hydrogenosomes, since a removal of the putative N-terminal import signal from this isoform could account precisely for the observed difference. However, since we cannot exclude that the 'short' type ('cytoplasmic') PFLs could also account for a cross-reacting protein with a molecular mass of ~81 kDa (if the C-terminal part of the 'short' PFLs does not contain significant deletions or insertions) a confirmation of the hydrogenosomal localisation is required. Also in *Neocallimastix* sp. L2 a cross-reacting protein of the same size could be detected by Western blotting in both the cytosolic and the hydrogenosomal fractions (Fig. 7B). Also in this chytrid species part of the hydrogenosomal PFL appears to be membrane-bound.

A proteinase K protection assay with isolated hydrogenosomes revealed that, in the absence of Triton X-100 (when the hydrogenosomal membranes are intact), PFL was protected against digestion by proteinase K (Fig. 8). Addition of Triton X-100 to the hydrogenosomal fraction resulted in a complete digestion of the cross-reacting protein (Fig. 8). Consequently, PFL proteins must be located inside the hydrogenosome.

Figure 8. Proteinase K protection assay with isolated hydrogenosomes. Hydrogenosomes were incubated in the absence (-) or presence of 0.2 (+) or 20 (++) $\mu\text{g/ml}$ proteinase K and in the presence (+) or absence (-) of detergent (0.1% Triton X-100 and 0.1% deoxycholate). The Western blot was incubated with antiserum against PFL from *E. coli*.

PFL activities in the hydrogenosomal and cytosolic fractions

Cellular fractionation and Western blotting revealed the presence of PFL in both the hydrogenosomes and the cytoplasm of *Piromyces* sp. E2 and *Neocallimastix* sp. L2. This subcellular localisation has been confirmed by enzymatic measurements of PFL activity (Table 1). In the hydrogenosomes, PFL activity exceeded the activity in the cytoplasm by a factor of two. The specificity of the PFL-assay has been tested: it was possible to inhibit the reaction completely by hypophosphite, a structural analogue of formate that acts as a suicide substrate (Plaga et al. 1988).

Incubation of the hydrogenosomal fraction with pyruvate or malate, respectively, confirmed that the hydrogenosomes used malate, but not pyruvate for hydrogen formation. However, in contrast to the observations of Marvin-Sikkema, our measurements revealed that approximately 1 mole of hydrogen was formed per 1 mole of malate utilised (Fig. 9, see also Marvin-Sikkema et al. 1994). Since acetate and formate were produced in equimolar amounts, too, a significant PFO activity in the hydrogenosomes could be excluded. However, only 0.7 mole of acetate and formate were formed per 1 mole of malate consumed. Since a concomitant accumulation of pyruvate (~0.3 mole per 1 mole of consumed malate) has been observed (Fig. 9), the low product levels can be explained by the assumption that the PFL activity is rate-limiting in isolated hydrogenosomes. Consequently, all data strongly support the assumption that PFL and not PFO is one of the key enzymes of the hydrogenosomal metabolism of chytrids.

	Cytosol	Hydrogenosomes
PFL	0.111	0.235
PFL + hypophosphite	ND	ND

Table 1. PFL activities ($\mu\text{mol mg}^{-1}$ protein) in the cytosolic and hydrogenosomal fractions of *Piromyces* sp. E2. PFL activity was measured by formate production after 60 minutes incubation at 39°C. Hypophosphite, a specific inhibitor of PFL activity, was added to a final concentration of 10 mM. ND, not detectable.

Figure 9. Consumption of malate and production of hydrogen, acetate, formate and pyruvate by isolated *Piromyces* sp. E2 hydrogenosomes. Suspensions of isolated hydrogenosomes (aliquots containing approximately 0.55 mg protein) were incubated anaerobically in the assay mixture (see Experimental Procedures) at 39°C. Liquid samples (excreted products plus hydrogenosomal contents) were analysed for malate, pyruvate, acetate, and formate. Gas samples were analysed by gas chromatography for the determination of the hydrogen concentration. The following symbols are used: ■ - pyruvate; ▲ - acetate; ○ - formate; x - hydrogen; ◆ - malate.

Discussion

In this study we have demonstrated for the first time that hydrogenosomes of anaerobic chytrids exhibit PFL activity - in contrast to all other hydrogenosomes that have been studied to date (Müller 1993; Embley et al. 1995; Biagini et al. 1998). The presence of PFL in the hydrogenosomes of *Piromyces* sp. E2 and *Neocallimastix* sp. L2 has clearly been demonstrated by subcellular fractionation, Western blotting (Fig. 7), proteinase K protection assay (Fig. 8), measurements of enzyme activities (Table 1) and the fermentation patterns (Fig. 9). Northern and Western blotting have revealed that PFL genes are highly expressed in both species. We have shown that the *pfl* genes of *Piromyces* sp. E2 constitute a multigene family which encodes 'long' and 'short' type PFLs. Both types are abundantly expressed, and it is likely that it is the 'long' type that encodes the hydrogenosomal PFL. It remains to be shown that the N-terminal extension

of 61 amino acids that is encoded by the 'long' PFL genes, represents a hydrogenosomal targeting signal since the evidence that the extension might be cleaved off after import into the hydrogenosome is circumstantial (Fig. 7). However, it has been shown that similar - albeit considerably shorter - N-terminal leader sequences have been described for other hydrogenosomal enzymes such as malic enzyme and succinyl-CoA synthetase (Brondijk et al. 1996; van der Giezen et al. 1997). These N-terminal extensions are cleaved upon import into the hydrogenosomes (van der Giezen et al. 1997, 1998).

The fermentation pattern measured after incubation of isolated hydrogenosomes with malate as a substrate is in agreement with the assumption that the main carbon flow in the hydrogenosomes of chytrids occurs via PFL (Fig. 9). The formation of equimolar amounts of formate and acetate definitely excludes that a significant PFO activity is present in chytrid hydrogenosomes (Fig. 9). Thus, all available evidence consistently shows that PFL - and not PFO - is one of the key enzymes in the hydrogenosomes of anaerobic chytrids. This discriminates the chytrid hydrogenosomes clearly from the hydrogenosomes of the parabasalids *Trichomonas vaginalis* and *Tritrichomonas foetus* (Steinbüchel and Müller 1986; Müller 1993) and the ciliate *Dasytricha ruminantium* (Yarlett et al. 1982) - the only other organisms, where the hydrogenosomal metabolism has been studied in more detail. Since there is no simple answer to the question *why* hydrogenosomes of chytrids use a PFL instead of a PFO, one might speculate whether the descent and the evolutionary history of the anaerobic chytrids can provide arguments to understand the reasons for this important difference.

These anaerobic chytridiomycete fungi are unrelated to trichomonads and ciliates: phylogenetic analysis of their rRNA genes, biochemical and morphological evidence has shown unequivocally that they belong to the 'crown group' of eukaryotic micro-organisms that secondarily adapted to an anoxic environment (Ragan and Chapman 1978; Sogin 1991; Dore and Stahl 1991; Knoll 1992; Li and Heath 1992; Bowman et al. 1992; Li et al. 1993). Evidence has been presented that this adaptation involved a re-targeting of mitochondrial enzymes such as the tricarboxylic acid cycle enzymes malate dehydrogenase and aconitase to the cytoplasm (Akhmanova et al. 1998a; Fig. 10). Since also the acetohydroxyacid reductoisomerase is located in the cytoplasm one might even speculate whether anaerobic chytrids lost their mitochondria during their adaptation to anaerobic environments.

Phylogenetic analysis strongly suggests that *Piromyces* sp. E2 acquired PFL from a prokaryotic source, perhaps from a *Clostridium*-like eubacterium (Fig. 3). Such a PFL enables the chytrid to avoid the generation of reduction equivalents during the metabolism of pyruvate (Fig. 10). However, the formation (and excretion) of formate and acetate does only allow the generation of one ATP by substrate-level phosphorylation. Compartmentalisation of the pyruvate catabolism, i.e. the evolution of hydrogenosomes, might allow the generation of extra ATPs by the generation of a proton motive force.

The presence of PFL instead of PFO in the hydrogenosomes of anaerobic chytrids has at least one obvious consequence: these hydrogenosomes cannot rely on pyruvate for hydrogen formation (c.f. Marvin-Sikkema et al. 1994; K. Hosea, unpublished results). The formation of molecular hydrogen requires reduction equivalents that, in the

hydrogenosomes of chytrids, seem to be provided by a hydrogenosomal malic enzyme that catalyses the oxidative decarboxylation of malate to pyruvate (Fig 10) This implies, too, that the NAD(P)H that is generated by the hydrogenosomal malic enzyme must be reoxidised by the hydrogenase - either directly or indirectly For anaerobic chytrids, we do not know yet how NAD(P)H is reoxidised, but recently we were able to provide evidence for a new type of 'iron-only' hydrogenase in anaerobic ciliates that might be capable of binding and reoxidising NAD(P)H directly (Akhmanova et al 1998b)

These observations might lead to the conclusion that the presence of PFL in a hydrogenosome is less favourable than a hydrogenosomal PFO, since a hydrogenosomal PFL requires an import machinery for malate and a hydrogenosomal malic enzyme (Fig 10) Naturally, we cannot exclude that the presence of PFL in anaerobic chytrids is purely accidental and is due to the particular evolutionary history of the anaerobic chytrids However, the potential secondary loss of mitochondria (Akhmanova et al 1998a, see Chapter 2) and the presence of PFL in both the cytoplasm and the hydrogenosomes of anaerobic chytrids might support a hypothetical scenario for the adaptation of the ancestral, mitochondriate chytrids to anaerobic environments

Our data suggest that the chytrid PFL has been acquired by lateral gene transfer (Fig 3) However, lateral gene transfer seems to be a rare event in eukaryotes (Syvanen 1994), and the assumption of independent acquisitions of either two different PFLs or one PFL and one PFO appear unlikely The generation of hydrogenosomal *pfl* genes by duplication of the genes encoding the cytoplasmic isoforms seems to be the most straightforward way to evolve a hydrogenosomal PFL, since gene duplication is an important evolutionary process in eukaryotes, and more frequent than lateral gene transfer, or the transfer of organellar genes to the nucleus (Ohno 1970, Mewes et al 1997, Martin and Herrmann 1998) Notably, the presence of multiple copies of PFL genes provides direct evidence for gene duplications in the chytrids (Fig 2,4,6) Therefore, a scenario that assumes (1) an acquisition of a cytoplasmic PFL by lateral gene transfer, (2) gene duplication events that create the multigene family of PFLs, and (3) compartmentalisation of a subfamily of PFLs in the hydrogenosomes might provide an explanation why PFL and not PFO is present in the hydrogenosomes of anaerobic

Figure 10. Scheme of the glucose catabolic pathway in anaerobic chytrids (*Piromyces* sp E2 and *Neocallimastix* sp L2), based on the studies, described by Marvin-Sikkema et al (1993a, 1994), Akhmanova et al (1998a) and present study Big box represents the cytosol, small box inside of it represents the hydrogenosome Question marks indicate enzymes and transporters, the existence of which is uncertain Abbreviations PEP, phosphoenolpyruvate, OAA, oxaloacetate, PEPCK, phosphoenolpyruvate carboxykinase, malate DH^M, malate dehydrogenase (of mitochondrial origin, also for aconitase superscript M indicates mitochondrial origin), succinate DH, succinate dehydrogenase, IDH^M, isocitrate dehydrogenase (origin uncertain), PK, pyruvate kinase, LDH, lactate dehydrogenase, PFL, pyruvate formate-lyase, ACDH, acetaldehyde dehydrogenase, ADH, alcohol dehydrogenase, AST, acetate succinate CoA transferase, STK, succinate thiokinase, AAC, ATP/ADP carrier, (I), putative mitochondrial complex I-homologous hydrogenase subunits, which would allow direct NAD(P)H oxidation by the hydrogenase (Akhmanova et al 1998b, see Appendix)

chytrids. Consequently, the evolution of PFL possessing hydrogenosomes by gene duplication and compartmentalisation might be a possible consequence of the adaptation of chytrids to anoxic environments - after the loss of their ancestral mitochondria and an acquisition of PFL by lateral gene transfer.

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Chapter 4

Multiple origins of hydrogenosomes: A mitochondrial-type ADP/ATP carrier in the hydrogenosomes of the anaerobic chytrid *Neocallimastix* sp. L2

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Summary

A gene encoding a mitochondrial-type ADP/ATP carrier (AAC) has been isolated from the amitochondriate, anaerobic chytridiomycete fungus *Neocallimastix spec L2*. Phylogenetic analysis has confirmed that this adenine nucleotide transporter belongs to the family of mitochondrial AACs. Expression of the cDNA in *E. coli* confers the ability to the bacterial host to incorporate preferentially ADP, but also ATP at significant rates. Biochemical and ultrastructural studies revealed that the ADP/ATP carrier is an integral component of the hydrogenosomal membranes. Notwithstanding the unusual structure of the hydrogenosomes of *Neocallimastix spec L2*, all data consistently argue for the presence of a functional mitochondrial-type AAC in the hydrogenosomal membranes, and, consequently, a mitochondrial origin of these organelles. Hydrogenosome-containing anaerobic ciliates possess similar, but clearly distinct mitochondrial-type AACs. Since the putative hydrogenosomal nucleotide carrier (Hmp31) of the anaerobic flagellate *Trichomonas vaginalis* is paralogous to the mitochondrial-type AACs of both, ciliate and chytrid hydrogenosomes, a multiple, independent origin of hydrogenosomes is likely.

Introduction

Eukaryotic cells are characterised by a high level of compartmentalisation. Notably, at least two of these subcellular compartments, i.e. mitochondria and plastids, are of endosymbiotic origin. They arose from prokaryotic ancestors in a complex evolutionary process that involved symbiotic association, transfer of genes from the endosymbiont to the genome of the host, loss of redundant or “useless” genes (Herrmann 1997, Martin and Herrmann 1998), the evolution or acquisition, respectively, of genes encoding proteins with “novel” properties (Andersson and Kurland 1999, Kurland and Andersson 2000, Karlberg et al 2000), and the retargeting of the nuclear-encoded proteins to the organelle (Schatz 1996, Neupert 1997). The evolutionary logic and driving force behind this compartmentalisation is still subject to discussion. The “textbook-version” of the endosymbiont theory assumes that eukaryotic compartmentalisation – and especially the evolution of mitochondria – was favoured by the enormous increase in energy yield resulting from the acquisition of an electron transport chain that allowed oxidative phosphorylation (OXPHOS, Gray et al 1999, Saraste 1999).

However, there are many anaerobic eukaryotes, which lack mitochondria and a compartmentalised energy metabolism. Since evidence has been provided that these organisms descended from mitochondriate ancestors (Roger 1999, Muller and Martin 1999), the above-mentioned “textbook”-explanation for the evolution of eukaryotic compartmentalisation seems unlikely. Furthermore, quite a number of unicellular anaerobes such as, for example, *Trichomonas vaginalis*, *Psalteriomonas lanterna*, *Nyctotherus ovalis*, *Piromyces* sp. and *Neocallimastix* sp., harbour “hydrogenosomes” instead of mitochondria (Muller 1993, Fenchel and Finlay 1995). Hydrogenosomes, like mitochondria, compartmentalise terminal reactions of the eukaryotic energy metabolism.

They generate hydrogen, carbon dioxide and ATP, supposedly by substrate level phosphorylation, but they lack an electron-transport chain (Müller 1993, 1998). Consequently, the energy-gain by hydrogenosomes is much lower than that of mitochondria, notwithstanding their presumed mitochondrial ancestry (Embley et al. 1997; Plümper et al. 1998; Hackstein et al. 1999; Rotte et al. 2000). The mere existence of such anaerobes challenges the codified endosymbiont hypothesis and questions our rationales for the evolution of cellular compartmentalisation (c.f. Andersson and Kurland 1999; Kurland and Andersson 2000).

Several hypotheses have been developed in order to cope with the secondary loss of mitochondria and the apparent non-existence of primitive eukaryotes ("archaezoa" sensu Cavalier-Smith 1993) that never hosted a mitochondrion-like symbiont or organelle in the course of their evolution (Roger 1999). The most important hypotheses are the hydrogen-hypothesis (Martin and Müller 1998), the syntrophy hypothesis (Moreira and Lopez-Garcia 1998; Lopez-Garcia and Moreira 1999), and the "ox-tox" hypothesis (Vellai et al 1998; Andersson and Kurland 1999). All these hypotheses assume the acquisition of an endosymbiont – as a rule after passing through a syntrophic stage. However, not only the symbiont and the host, also the conditions under which mitochondria and hydrogenosomes must have evolved are different in these hypotheses (for discussion, see Andersson and Kurland 1999; Kurland and Andersson 2000).

The analysis of mitochondrial and hydrogenosomal adenine nucleotide transporters should allow spotting of the evolution of both the mitochondrial and the hydrogenosomal compartments of eukaryotic cells. These organellar carrier proteins are intimately tangled with the process of eukaryogenesis, since the function of ATP-generating organelles such as hydrogenosomes and mitochondria, depends on transporter proteins in the organellar membrane that facilitate the vectorial exchange of adenine nucleotides between the matrix of the organelle and the cytoplasm of the eukaryotic cell. It is known for many years that mitochondria possess ADP/ATP carriers (AAC's), abundant proteins of the inner mitochondrial membrane that facilitate the import of ADP and the export of ATP (Aquila et al. 1987; Klingenberg 1989, 1992; Palmieri 1994). However, there is only circumstantial evidence for the existence of a "mitochondrial-type" nucleotide transporters in the hydrogenosomes of the anaerobic chytrid *Neocallimastix* sp. L2 (Marvin-Sikkema et al. 1994) and a putative nucleotide carrier (Hmp31) in the hydrogenosomes of the parabasal flagellate *Trichomonas* (Dyall et al. 2000). The evolutionary origin of these adenine nucleotide carriers is elusive, since the expression of such a protein should be highly detrimental for any ancestral prokaryotic organism: ATP – exporting transporters of mitochondria and, supposedly of hydrogenosomes as well, must represent an evolutionary novelty that is one of the prerequisites for the evolution of eukaryotic compartmentalisation (Andersson and Kurland 1999; Winkler and Neuhaus 1999; Kurland and Andersson 2000). Their evolution has been crucial for a successful eukaryogenesis, since ATP exporting proteins are an absolute requirement for the transformation of an autonomous symbiotic prokaryote into a non-autonomous, energy-generating compartment of the evolving eukaryotic cell. Therefore, the study of these proteins will allow to answer the question as to whether the eukaryotic AACs evolved independently in early diverging evolutionary lines that eventually gave rise to

both mitochondria and hydrogenosomes, or only once in the mitochondrial lineage that gave rise to both specialized mitochondria and a number of different hydrogenosomes as well.

Here we describe a gene from the anaerobic chytridiomycete fungus *Neocallimastix* sp. L2 that encodes a protein with a high level of sequence identity with the mitochondrial AACs of yeasts, fungi, animals and plants. Phylogenetic analysis of the gene encoding this hydrogenosomal adenine nucleotide carrier reveals that it clearly belongs to the mitochondrial-type AACs - like the AACs of the hydrogenosomes of anaerobic ciliates. By functional expression in *Escherichia coli*, we demonstrate that the hydrogenosomal protein functions as a "mitochondrial-type" AAC. Both, functional and phylogenetic evidence favour the assumption of multiple origins of hydrogenosomes. The implications for the evolution of the eukaryotic cell are discussed.

Experimental procedures

Organisms and growth conditions

Neocallimastix sp. L2 was cultured axenically under anoxic conditions in M2 medium, supplemented with 20 mM fructose, at 39°C (Teunissen et al 1991; Marvin-Sikkema et al. 1992). The aerobic ciliate *Tetrahymena thermophila* was cultured axenically in a proteose pepton, yeast extract, glucose, FeSO₄ EDTA medium. *Euplotes minuta* was grown in artificial seawater and fed with *E. coli*. The anaerobic heterotrichous ciliates of the *Nyctotherus ovalis* cluster were isolated by electromigration from the hindgut of cockroaches as described earlier (van Hoek et al. 1999).

Isolation of genomic DNA and messenger RNA

Genomic DNA of the chytrids was prepared according to the protocol of Brownlee (1994). Ciliate DNAs were isolated using a phenol/chloroform protocol. Total RNA from *Neocallimastix* sp. L2 was prepared by the guanidinium-chloride method (Sambrook et al. 1989). Poly(A)⁺ RNA was isolated with the Pharmacia[™] QuickPrep mRNA Purification Kit.

Isolation of the AAC-encoding cDNA

Adaptor-ligated cDNA was prepared from poly (A)⁺ RNA using the Clontech[™] Marathon cDNA Isolation Kit. Oligonucleotide primers based on highly conserved amino-acid regions of ADP/ATP translocators, and DNA polymerase chain reaction (PCR) were used to amplify DNA from adapter-ligated cDNA. A full-length cDNA was isolated by the Rapid Amplification of cDNA End (RACE) procedure as described in the protocol of the Clontech[™] Marathon cDNA Isolation Kit. The AAC-genes of the various ciliates were identified by PCR with degenerated primers. Sequencing was performed with the ABI PRISM Model 310 automatic sequencer, using a Rhodamine terminator cycle sequencing ready reaction DNA sequencing kit (Perkin-Elmer Applied Biosystems).

Northern and Southern blotting

Poly (A)⁺ RNA (5 µg) of *Neocallimastix* sp. L2 was size-fractionated on a 1% agarose-formaldehyde gel. Genomic DNA (10 µgram) from *Neocallimastix* sp. L2 and *Piromyces* sp. E2, was digested with restriction enzymes (see legend fig. 2) and separated on 0.7% agarose gels. The gels were blotted to Hybond N+ membranes (Amersham). The *hdgaac* probe (the full-length cDNA clone) was labelled with α -[³²P] dATP using PCR. Hybridisation was performed in 0.5 M sodium phosphate buffer pH 7.0, 7% SDS, 1% BSA, and 1 mM EDTA at 60°C. The membrane was washed stringently with 50 mM sodium phosphate buffer pH 7.0 and 0.5% SDS, at 60°C.

Sequence analysis

A sequence alignment was created with t_coffee (Notredame et al. 2000) from a representative set of sequences of the AAC family. Phylogenetic trees were subsequently constructed using Neighbor Joining (Saitou and Nei 1987) and Quartet Puzzling (Strimmer and von Haeseler 1996). Only positions that were present in all sequences were included. For Quartet Puzzling the JTT model of Amino Acid substitution was used (Jones et al. 1992), with four Gamma distributed rate categories.

The sequences and their Genbank identifiers or, if available, Swissprot names, are shown below (from top to bottom): ***, ***, g3220183, g7018577, AF340168, ADT_CHLRE, ADT1_WHEAT, ADT_NEUCR, ADT_SCHPO, ADT1_YEAST, ADT_KLULA, ADT2_YEAST, ADT3_YEAST, ***, ***, ***, ***, ***, AF343580, ADT_CHLKE, g7292557, ADT1_HUMAN, ADT2_HUMAN, ADT3_HUMAN, g3885438, g6321533, g6746567, YHG2_YEAST, GDC_HUMAN, FLX1_YEAST, g6325268, BT1_MAIZE, TXTP_YEAST, TXTP_HUMAN, g6912334, M2OM_HUMAN, MPCP_YEAST, MPCP_HUMAN, MCAT_HUMAN, g5565862.

A phylogeny of HSP60 sequences was derived using the same methods as for the AAC tree. The genbank identifiers are: *Piromyces* ***, *N. crassa* GI:7800840, *S. pombe* GI:1346314, *C. albicans* GI:3552009, *S. cerevisiae* GI:123579, *H. sapiens* GI:190127, *Arabidopsis* GI:116229, *D. discoideum* GI:1621639, *T. gondii* GI:5052052, *P. yoelii* GI:3885995, *Leishmania* M. GI:3023477, *E. gracilis* GI:2493645, *T. brucei* GI:249365, *E. histolytica* GI:2564749, *T. vaginalis* GI:1755053, *C. ruminantium* GI:1345759, *N. gonorrhoeae* GI:2119968, *Synechocystis* GI:2506274.

Subcellular fractionation

Hydrogenosomal and cytosolic fractions were obtained by differential centrifugation, using the protocol of Marvin-Sikkema et al. (1993b) with minor modifications. For the isolation of hydrogenosomal membranes, the hydrogenosomal fraction was resuspended in 100 mM Tris-HCL buffer pH 7.5. The hydrogenosomes were lysed by repeated freezing and thawing, and subsequently centrifuged (233,000 x g for 60 minutes, at 4°C). The pellet, consisting of the hydrogenosomal membranes, was extracted twice with 1 M KCl or 0.1 M sodium-carbonate (pH 11.5), for 60 minutes on ice. Subsequent centrifugation (233,000 x g for 60 minutes, at 4°C) resulted in a supernatant containing membrane-associated proteins, and a pellet that contained integral membrane proteins. All extraction steps were performed at 4°C, and in the presence of the Complete[™]

protease inhibitor mix (Boehringer Mannheim). Equal portions of all fractions were separated on 12.5% polyacrylamide gels containing 0.1% SDS (SDS-PAGE) and blotted to a PVDF membrane by semi-dry transfer (Biorad), using the manufacturers protocol. A polyclonal antiserum, directed against the mitochondrial ADP/ATP carrier (AAC) of the yeast *Saccharomyces cerevisiae*, was used to identify the putative hydrogenosomal AAC using goat-anti-rabbit antibodies conjugated to peroxidase (Boehringer Mannheim).

Proteinase K protection

For the proteinase K protection experiments, isolated hydrogenosomes (about 0.4 mg protein) were incubated anaerobically in a medium consisting of 0.5 ml 20 mM HEPES pH 7.4, 250 mM sucrose, and 2 mM DTT. Proteinase K (50 µg per 0.5 ml assay volume) or detergent (0.1% triton X-100 and 0.1% deoxycholate) was added when indicated (see Fig. 4c). After 30 minutes incubation on ice, the proteinase K treatment was terminated by the addition of 0.5 ml 15% (w/v) TCA. Aliquots of each incubation were separated on a 12.5% SDS-polyacrylamide gel, blotted to a PVDF membrane, and probed with the AAC antiserum.

N-terminal amino-acid sequencing

For the N-terminal amino-acid sequencing, the cross-reacting protein band was cut out from the PVDF membrane, and subjected to microsequencing (Applied Biosystems, Model 477A).

Cardiolipin analysis

Cardiolipins were analysed in a total lipid extract, which was prepared according to the methods described by Bligh and Dyer (1959). Negative ion mass spectrometry was performed on a Sciex API 365 triple quadrupole mass spectrometer equipped with an electrospray ion source. The presence or absence of cardiolipin ions in the mass spectra was studied by analysis of the fragments produced by Collision Induced Dissociation of the putative cardiolipin ions.

Electron microscopy, immuno-gold labeling and 3 dimensional reconstruction

Electron microscopy and immunocytochemistry with the *S. cerevisiae* anti-AAC was performed as described by Marvin-Sikkema et al. (1993a). For the osmotic treatment, hydrogenosomes were isolated as described above, and resuspended in (anoxic) isolation buffer (20 mM HEPES pH 7.4, 2 mM DTT) containing 0.0, 0.1, 0.25, 0.5, or 1.0 M of sucrose, and incubated for 30 minutes on ice. The hydrogenosomes were pre-fixed by the addition of glutaraldehyde (0.1% final concentration). After 15 minutes, the hydrogenosomes were collected by centrifugation and post-fixed in 2.5% glutaraldehyde for 2 hours on ice.

For a 3-D reconstruction of the hydrogenosomes, fixed and epon-embedded zoospores of *Neocallimastix* sp. L2 were subjected to serial sectioning. Sixteen (serial) electron micrographs were mounted on a digitizer tablet. The relevant contours were traced manually and stored in a database using the 3D-reconstruction software TDR-3Dbase (Verbeek et al. 1995). For each section, two reference points were included and

used to realign the contours to a consistent 3D stack of contours. The contour model was used to generate a voxel model of cells containing the hydrogenosomes (Verbeek 1999 and references therein). TDR-3Dbase, was developed for the MS-Windows (9x, NT) user interface. Here, a Summagraphics Bitpad Two was used for the input of the contours (Verbeek 1999). Visualisation of the volume model was accomplished with a SUN UltraSPARC 10 workstation.

Results

Molecular characterisation of a putative hydrogenosomal ADP/ATP carrier

Using PCR and the rapid amplification of cDNA ends (RACE) procedure, we isolated a 1051 bp cDNA ("*hdgaac*") from *Neocallimastix* sp. L2. DNA sequence analysis revealed the presence of a complete open reading frame of 924 bp (see EMBL database accession no. AF340168). The deduced amino acid sequence (hdgAAC) exhibits high similarity (42%-59% identity, 57%-77% similarity) with the sequences of well-characterised mitochondrial ADP/ATP carriers from the various aerobic eukaryotes. The sequence predicts a protein with an apparent molecular weight of 32 kDa, which is well within the expected size range of the AAC protein family (Palmieri 1994). The putative protein exhibits the characteristic tri-repeat structure that is observed in all AACs analysed so far (Saraste and Walker 1982; Aquila et al. 1987; Klingenberg 1989, 1992; Palmieri 1994). The clusters of charged residues are conserved. Moreover, adjacent to the hydrophobic region V, the motif RRRMMM can be identified that is conserved among all mitochondrial ADP/ATP translocators (Fig. 1).

Southern blotting argues for the presence of only one gene with high similarity to the *hdgaac* probe (Fig. 2a). Also, Northern blotting does not provide evidence for the expression of more than one *hdgaac* gene: hybridisation of poly (A)⁺ RNA with the *hdgaac* probe revealed the presence of a single transcript (Fig. 2b). Its length (1750 nucleotides) exceeds the length of the identified open reading frame (924 bp). This length difference is due to the presence of AT-rich 5' and 3' non-translated regions, which are characteristic for all *Neocallimastix* and *Piromyces* cDNAs analysed so far (Reymond et al. 1992; Durand et al. 1995; Fanutti et al. 1995; Akhmanova et al. 1998b, 1999).

Figure 1. Alignment of the deduced amino-acid (AA) sequence hdgAAC from *Neocallimastix* sp. L2 with putative hydrogenosomal AACs from anaerobic ciliates, HMP 31 of *Trichomonas* and representative mitochondrial AACs from aerobic eukaryotes. The three-domain structure that is characteristic for mitochondrial AACs is indicated by the letters A, B, and C. The six hydrophobic, membrane-spanning regions are boxed (I – VI). The RRRMMM motif is printed bold face. (It is not shown for the ciliate sequences because this motif has been used for the PCR primer). Gaps are indicated by dashes. The 15 N-terminal amino acids of the *Neocallimastix* AAC that have been confirmed by protein sequencing are boxed. The (cleaved) presequence of HMP 31 of *T. vaginalis* has been underlined.

Figure 2. Southern- and Northern-blots of the *hdgAAC* of *Neocallimastix* sp. L2. (A) Genomic DNA (10 µg) of *Neocallimastix* sp. L2 was digested with the restriction enzymes *Cla*I (C), *Eco*RI (E), and *Xba*I (X). Probing with *hdgaac*, labelled with [α - 32 P]dATP, does not provide evidence for more than one genomic copy of *hdgaac*. (B) Northern blot analysis of 5 µg of poly(A)⁺ RNA reveals the presence of a single transcript.

Phylogenetic analysis

Phylogenetic analysis shows that the deduced *hdgAAC* sequence of *Neocallimastix* sp. L2 clusters with the mitochondrial AACs of yeasts, fungi, and plants (Fig. 3). The branching order within the cluster cannot be resolved completely, but it is obvious that the *hdgAAC* sequence from *Neocallimastix* sp. L2 shares a common ancestry with the mitochondrial AACs of yeasts and fungi. High bootstrap values support a monophyly of all mitochondrial AACs, including the hydrogenosomal AAC from *Neocallimastix* sp. L2. Also, the putative hydrogenosomal AACs identified in a number of anaerobic ciliates belong to this cluster. Notably, not only the mitochondrial AACs, but also the (putative) hydrogenosomal AACs of *Neocallimastix* sp. L2 and the various *Nyctotherus* species are clearly distinct from the hydrogenosomal protein Hmp31of *Trichomonas* (Fig. 3; c.f. Dyall et al. 2000).

Figure 3. Phylogenetic tree of representatives of the mitochondrial carrier family. Phylogenetic trees were constructed using the Neighbor Joining (Saitou and Nei 1987) and Quartet Puzzling algorithms (Strimmer and von Haeseler 1996). Only positions that were present in all sequences were included in the calculations. For Quartet Puzzling the JTT (Jones et al. 1992) model of amino acid substitution was used, with four Gamma distributed rate categories. The displayed tree is a Neighbor Joining tree based on sequence identities. Only Neighbor Joining bootstrap values (left value) and Quartet Puzzling reliability values (right value) larger than 49 out of 100 are indicated. At the right, experimentally determined substrate specificities of the translocators, or other relevant features are indicated (the latter in italics). In contrast to the single (hydrogenosomal) AAC of *Neocallimastix*, several AAC isoforms seem to exist in anaerobic ciliates (*). *Nyctotherus* PA, *N. ovalis* from *Periplaneta americana* var. Bayer; *Nyctotherus* PA_B, *N. ovalis* from *Blaberus fuscus* var. Düsseldorf (van Hoek et al. 1998). For the swissprot names or genbank identifiers of the sequences in the tree: see Experimental procedures.

Substrate	Rate of transport (nmol mg ⁻¹ protein h ⁻¹)		
	E.coli-pET16b (control, 50 μ M)	E.coli-pIH6 (50 μ M)	E.coli-pIH6 (500 μ M)
α -[³² P]ATP	0.06 $\pm$ 0.01	0.84 $\pm$ 0.08	3.46 $\pm$ 0.45
α -[³² P]ADP	0.07 $\pm$ 0.01	3.96 $\pm$ 0.48	12.11 $\pm$ 0.81

Table 1. Uptake of radioactive labeled compounds into *E. coli*. Radioactive labeled compounds were present at a final concentration of 50 μ M for the control cells (*E.coli*-pET16b) and at a final concentration of 50 μ M or 500 μ M for the *E. coli*-cells (pIH6) which are harbouring the plasmid including the ADP/ATP carrier encoding gene (*hdgaac*). Incubation was carried out with IPTG-induced *E. coli* cells for 4 min. Uptake was terminated by rapid filtration of *E. coli* cells. Data are the mean of three independent experiments.

Functional expression in *E. coli*

The complete ORF encoding the putative hydrogenosomal AAC of *Neocallimastix* sp. L2 has been cloned in the plasmid pET and expressed in the *E. coli* strains BL-21 and C43. After induction, both strains exhibited a substantial uptake of α -[³²P] ADP (Table 1). The K_m for the uptake of ADP has been determined as approximately 165 μ M ADP. Incubation with α -[³²P] ATP did result in a much lower uptake of radioactivity, in clear contrast to the rapid uptake of α -[³²P] ATP by transgenic strains of *E. coli* expressing plastidic or *Rickettsial* ANT_s (Tjaden et al. 1999). Since uninduced cells did not import either α -[³²P] ADP or α -[³²P] ATP at significant rates, it must be concluded that (i) the recombinant *hdgAAC* from *Neocallimastix* sp. L2 is functionally expressed in *E.coli*, (ii) the recombinant protein is inserted into the bacterial membrane, and (iii) the *hdgAAC* from *Neocallimastix* sp. L2 is a functional, mitochondrial-type AAC that imports ADP, and, most likely, exports ATP. Table 1 shows that the *hdgaac* gene product is capable of both ATP and ADP transport. These data are in agreement with results obtained with intact mitochondria. Also in mitochondria, ADP is imported preferentially. In both cases (bacterial cells and intact mitochondria) an electrogenic gradient (positive outside/negative inside) seems to be responsible for the fact that the import of ADP³⁻ exceeds that of ATP⁴⁻ by far (c.f. Klingenberg 1989).

Localisation of the gene product

Homogenates of *Neocallimastix* sp. L2 were subjected to differential centrifugation in order to obtain a 30000x g organellar fraction that is enriched in hydrogenosomes (Marvin-Sikkema et al. 1993b). Western blotting using the organellar fraction and a heterologous antiserum directed against mitochondrial AACs of *Saccharomyces cerevisiae* revealed a cross-reacting membrane protein of 32kD (Fig. 4a,b). This band was absent from the cytosolic fraction (Fig. 4b). The cross-reacting band was excised from the PVDF membrane and subjected to amino-terminal protein sequencing. The first

15 N-terminal amino acids of the protein appeared to be identical with the first 15 deduced amino acids of the cloned *hdgaac* gene (Fig. 1), confirming that the authentic AAC protein was recognised by the antiserum. In order to analyse whether the cross-reacting protein was membrane-associated or an integral component of the hydrogenosomal membranes, the proteins of the hydrogenosomal fraction were subjected to salt extraction procedures and proteinase K treatment (Fig. 4c,d). Incubation of the pellet fraction with 1 M KCl failed to release any AAC protein (lane 4, Fig. 4d). Also, after carbonate treatment most of the protein was retained in the pellet fraction (lane 7, Fig. 4d). Moreover, the protein was also largely protected against proteinase K digestion in the absence of detergent. Under these conditions, proteinase K could only remove a small part of the protein (Fig. 4c). However, AAC was completely removed from the organelle fraction when both detergent and proteinase K were added. These observations led to the conclusion that the cross-reacting protein was an integral constituent of the hydrogenosomal membranes.

Figure 4. Western blot analysis and subcellular localisation of hgdAAC. (A) After SDS-PAGE a single band of the membrane fraction of isolated hydrogenosomes binds a heterologous antiserum against a mitochondrial AAC from yeast (lane 1). Lane 2: Coomassie Brilliant Blue stained membrane proteins. m: marker proteins (kDa). (B) Lane 1: homogenate of the fungal mycelium. The cross-reacting protein is highly enriched in the hydrogenosomal fraction (lane 3), and absent from the cytosolic fraction (lane 2). (C) Proteinase K protection assay localising hgdAAC in the hydrogenosomes: hgdAAC is only digested completely after incubation with proteinase K (pK) and detergent (d, Triton X-100/deoxycholate). "-", no addition of proteinase K or detergent. (D) Differential extraction of hgdAAC from the hydrogenosomal fraction. Lane 1, total hydrogenosomes; lane 2, supernatant containing hydrogenosomal matrix proteins after freeze-thawing of hydrogenosomes; lane 3, pellet of hydrogenosomes after freeze-thawing; lane 4, supernatant containing membrane-associated (1 M KCl - soluble) proteins; lane 5, pellet after high salt (1 M KCl) extraction; lane 6, supernatant containing 0.1 M carbonate (pH 11.5) extractable proteins; lane 7, integral membrane (carbonate resistant) proteins. All data confirm the localisation of the cross-reacting protein in the hydrogenosomal membranes.

Figure 5. Ultrastructure of the hydrogenosomes *Neocallimastix* sp. L2. **A-D:** Immuno-gold labeling by the anti-AAC serum: Mycelia and zoospores of *Neocallimastix* sp. L2. were fixed, embedded, and ultra-thin sectioned. The labeling, indicating the presence of hdgAAC, is confined to the hydrogenosomal membranes, both the internal and external ones. h, hydrogenosomes; r, ribosome globules (Munn et al.1988). Bars represent 0,5 μ m.

Immunocytochemistry

Immuno-gold labelling of ultra-thin sections from zoospores of *Neocallimastix* sp. L2, revealed that the anti-AAC labelling was confined to the hydrogenosomal membranes, both the bounding and the internal ones (Fig. 5). Since the internal membranes were most intensively labelled, we analysed the unusual structure of the hydrogenosomes in more detail. Serial sectioning followed by computer-aided 3D-reconstruction revealed that most of the internal structures were vesiculated (Fig. 6). Up to three vesicles were found inside the hydrogenosomes, both in the hydrogenosomes of fixed zoospores and in

Figure 6. Serial sectioning and 3D reconstruction of the hydrogenosomes of *Neocallimastix* sp. L2. **A-D:** Electron micrographs of 4 of the 16 serial sections of zoospores that were used for the 3D reconstruction shown in **E** and **F**. Bar: 0,5 μ m; h, hydrogenosomes; r, ribosome globules (Munn et al.1988). Asterisk: internal vesicular structures. **E-F:** Computer-aided 3D reconstruction.

the isolated hydrogenosomes obtained after cellular fractionation of mycelial homogenates (see Fig. 7). In order to determine whether two membranes or only a single membrane bounded the organelle, isolated hydrogenosomes were subjected to osmotic treatment in an anaerobic isolation buffer containing 0.0, 0.1, 0.25, 0.5, and 1.0 M of sucrose. After incubation on ice for 30 minutes the hydrogenosomes were fixed and subjected to electron microscopical analysis. Fig. 7 shows that the organelles are sensitive to the osmotic treatment. Hypertonic treatment resulted in a close apposition of the internal and external membranes, whereas hypotonic treatment eventually caused a rupture of the outer membrane. This membrane, but also the inner membranes are single membranes. Both, the bounding and the internal membranes are morphologically indistinguishable, in clear contrast to the hydrogenosomal membranes of *Nyctotherus ovalis* (c.f. Fig. 1 in Akhmanova et al. 1998a, see Appendix).

Also the chemical composition of both hydrogenosomes is different: mass-spectroscopy revealed that the hydrogenosomal membranes of the anaerobic chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2 are devoid of cardiolipin whereas the hydrogenosomes of the anaerobic ciliate *Nyctotherus ovalis* possess cardiolipin (not shown).

Figure 7. Hydrogenosomes obtained by cell fractionation. **A-C:** Osmotic treatment of isolated hydrogenosomes with solutions of 1.0 M (**A**), 0.5 M (**B**), and 0.0 M (**C**) of sucrose. Bar: 0.5 μm. **D:** Median section of a hydrogenosome obtained after cellular fractionation. Bar: 0.5 μm. **E:** An artist's view of the hydrogenosome shown in D. Arrows indicate "single" membranes.

Discussion

We have shown here that the hydrogenosomes of the anaerobic chytridiomycete fungus *Neocallimastix* sp. L2 possess a mitochondrial-type ADP/ATP translocator. The DNA sequence of the gene exhibits all characteristics of a mitochondrial AAC, e.g. a tri-partite structure of approximately 3 x 100 amino acids and the conserved “RRRMMM” motif (Klingenberg 1989, 1992; Palmieri 1994). Phylogenetic analysis places the hydrogenosomal AAC gene of *Neocallimastix* with high bootstrap values into a well-defined, monophyletic cluster of mitochondrial AACs (Fig. 3). DNA sequence analysis also reveals that a number of putative hydrogenosomal AACs of anaerobic ciliates cluster together with the mitochondrial AACs. After expression in *E. coli*, the protein encoded by the *hdgaac* gene of *Neocallimastix* sp. L2 facilitates the preferential import of ADP, as expected for a mitochondrial-type AAC (Table 1; c.f. Tjaden et al. 1999; Winkler and Neuhaus 1999). Lastly, the incubation of isolated hydrogenosomes from *Neocallimastix* with bongkretic acid and carboxy-atractylate, well-characterised inhibitors of mitochondrial AACs (Winkler and Neuhaus 1999), inhibits hydrogen formation by isolated hydrogenosomes of *Neocallimastix*, indicating that a mitochondrial-type AAC plays a crucial role in the hydrogenosomal metabolism (Marvin-Sikkema et al. 1994).

These features discriminate the hydrogenosomal AAC of *Neocallimastix* sp. L2 clearly from a putative ADP/ATP translocator from the hydrogenosomes of *Trichomonas vaginalis* (Dyall et al. 2000). This protein, Hmp31, is one of the most abundant constituents of the hydrogenosomal membranes, and, without any doubt, a member of the family of mitochondrial carrier proteins. However, as Fig. 3 reveals, Hmp31 definitively does not belong to the cluster of mitochondrial AACs. Rather, Hmp31 clusters - albeit with low statistical support - with poorly defined members of the mitochondrial carrier family such as the “Graves disease antigen” from rat and human, and several carriers with unknown function from the yeast *S. cerevisiae* (i.e. YGH2, FLX1; El Moulaj 1997; Nelson et al. 1998). Since also the motif “RRRMMM” (found in all mitochondrial-type ADP/ATP carriers analysed until now, including the hydrogenosomal AAC of *Neocallimastix*) exhibits two exchanges (“RKRMMML”) in the Hmp31 of *T. vaginalis*, it has to be concluded that this protein must be a paralogue of the mitochondrial AACs. In addition, Hmp31 possesses a cleavable N-terminal targeting signal, a feature that is very unusual for mitochondrial AACs (Dyall et al. 2000; Adrian et al. 1986; Müller et al. 1996; Sirrenberg et al. 1996). Also, it has been shown earlier that atractyloside, a highly specific inhibitor of mitochondrial AACs mentioned above, only poorly inhibits adenine nucleotide uptake by isolated hydrogenosomes of *T. vaginalis* (Cerkasov et al. 1978). Notwithstanding, the conservation of a charge-pair network and the lack of a “canonical” AAC in the hydrogenosomes of *T. vaginalis* support the assumption that Hmp31 might function as an ADP/ATP translocator in this organism (Dyall et al. 2000). Given a function of Hmp31 as a hydrogenosomal ATP/ADP carrier in *Trichomonas*, then the hydrogenosomes of *Trichomonas* and “real” mitochondria might have shared a common ancestor that lived before ATP-generating and exporting mitochondria evolved. Alternatively, *Trichomonas* might have lost its mitochondrial-type ADP/ATP carrier and subsequently evolved an alternative nucleotide

carrier in the course of its secondary adaptation to anaerobic environments. On the other hand, there is compelling evidence that the hydrogenosomes of both chytrids and ciliates must have evolved from mitochondria (or mitochondria-like organelles that were able of exporting ATP). Notably, the hydrogenosomes of *Nyctotherus ovalis* retained an organellar genome, and phylogenetic analysis of the hydrogenosomal SSU rRNA genes corroborated the results of the phylogenetic analysis of the hydrogenosomal AACs. Both, the SSU rRNA and AAC data support the conclusion that the hydrogenosomes of *N. ovalis* share a common ancestry with the mitochondria of aerobic ciliates (Fig. 3, Akhmanova et al. 1998a - see Appendix, van Hoek et al. 2000). The hydrogenosomes of the anaerobic chytrids *Neocallimastix* and *Piromyces* did not retain a genome. However, the phylogenetic analysis of both the *hdgac* gene of *Neocallimastix* and the chaperonin *hsp 60* gene of the closely related anaerobic chytridiomycete fungus *Piromyces* (Fig. 8) argues for a common ancestry of their hydrogenosomes with the mitochondria of their aerobic relatives. On the other hand, phylogenetic analysis of Hmp31 and of several hydrogenosomal chaperonins of *Trichomonas* as well, fails to support an origin of these hydrogenosomes from differentiated, "modern" mitochondria (Figs. 3, 8, Bui et al. 1996, Germot et al. 1996, Dyall et al. 2000). Also, the analysis of the hydrogenosomal import machinery of *Trichomonas* suggests a common ancestry with mitochondria, but not a descent from a differentiated mitochondrion belonging to an extant group of eukaryotes (Bradley et al. 1997, Hausler et al. 1997, Plummer et al. 1998, 2000, Dyall and Johnson 2000).

Thus, all data consistently argue for an origin of the hydrogenosomes of *Trichomonas* distinct from that of "modern" mitochondria, but also distinct from the hydrogenosomes of anaerobic ciliates and anaerobic chytrids. The latter ones must have evolved from "differentiated" mitochondria that had co-evolved with their particular hosts, comparable to the "anaerobic mitochondria" of uni- and multicellular animals that retained much more mitochondrial characteristics than hydrogenosomes (Tielens and van Hellemond 1998).

Whereas all molecular data clearly argue for a mitochondrial origin of the hydrogenosomes of the anaerobic chytrids, their ultrastructure reveals no mitochondrial affinities at all (Figs. 5-7). Both, immunocytochemistry and cellular fractionation confirm a membrane localisation of the AAC (Fig. 4,5). However, the occurrence of *hdgAAC* is not restricted to the inner membrane - as both ultrastructure and proteinase K protection essays suggest. The structure of the organelle is very odd, and best described as similar to "peas in the pod" (Fig. 7d,e). The number of gold grains on the "inner" membrane might indicate that the number of AAC molecules in the "inner" membrane is significantly higher than in the "outer" membrane (Fig. 5), one might speculate as to whether the inner structures represent propagation stages. Cristae and other differentiation's characteristic of a mitochondrial inner membrane are absent just as any indication for the presence of a differentiated mitochondrial-type outer membrane. Also, the observed absence of cardiolipin argues for substantial differences between the hydrogenosomal membranes of *Neocallimastix* and *Piromyces*, and those of true mitochondria. The 3-D reconstruction from serial ultra-thin sections of hydrogenosomes from zoospores revealed no principal differences to the hydrogenosomes that had been

Figure 8. Phylogenetic tree of HSP 60. See Figure 3 for methods. Using Neighbor Joining, a phylogenetic tree was constructed. The HSP 60 of the anaerobic chytrid *Piromyces* sp. E2 clusters with high bootstrap values with the mitochondrial HSP60s of aerobic yeasts and fungi. HSP 60 (cpn 60) of *Trichomonas* belongs to a different clade. For the swissprot names or genbank identifiers of the sequences in the tree, see Experimental procedures.

isolated from hyphal homogenates by differential centrifugation (Figs. 6,7). Since also electron microscopy using cryo-fixation and freeze substitution (data not shown) did not provide any evidence for “double” membranes, the claims of a few authors that chytrid hydrogenosomes are “double-walled” might be due to misinterpretations of closely opposed single membranes that might be the result of swelling during the isolation and fixation procedure (Benchimol et al. 1997, van der Giezen et al. 1997, see Fig. 7).

In conclusion, all molecular data support an origin of the hydrogenosomes of *anaerobic* chytrids and of *anaerobic* ciliates from the mitochondria of their respective *aerobic* relatives. The hydrogenosomes of *Trichomonas* must have a different origin. The hydrogenosomes of *Trichomonas* are bounded by double membranes, but they reveal no peculiar ultrastructural similarities with present-day mitochondria (Benchimol et al. 1996). The hydrogenosomes of *Nyctotherus ovalis* and other anaerobic ciliates are double-walled, too, and they look like mitochondria (Fenchel and Finlay 1995, Akhmanova et al. 1998a, see Appendix). Notably, the ultrastructure of the hydrogenosomes of *Neocallimastix* sp. L2 is exceptional. However, these observations do not necessarily provide arguments in favour of a microsomal (peroxisomal) origin of the hydrogenosomes as suggested earlier (Marvin-Sikkema et al. 1993a). Ultrastructural studies on the mitochondria of patients with hereditary mitochondrial diseases have shown that mitochondria can exhibit highly abnormal morphologies (Huizing et al. 1997, Frey and Mannella 2000). Thus, the unusual ultrastructure of the hydrogenosomes of anaerobic chytrids does not exclude a mitochondrial descent. Consequently, the application of “Ockham’s razor” argues strongly for a mitochondrial ancestry of the hydrogenosomes of chytrids - notwithstanding their unique morphology and their peculiar biochemistry (Hackstein et al. 1999). This descent from mitochondria must have been accompanied by a dramatic decrease in energy-gain. At present, we can only vaguely speculate about presumed metabolic necessities or particular advantages for the evolution of hydrogenosomes. Therefore, we have to conclude that we are still very ignorant about the forces and trends that governed the evolution of cellular compartmentalisation under the various environmental constraints.

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Chapter 5

A hydrogenosomal [Fe]-hydrogenase from the anaerobic chytrid *Neocallimastix* sp. L2.

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Summary

Hydrogenosomes of the anaerobic chytridiomycete fungus *Neocallimastix* sp. L2 host a [Fe]-hydrogenase. The enzyme has a molecular mass of approximately 67 kDa. The hydrogenosomal hydrogenase is highly sensitive against carbon monoxide and oxygen. Therefore, it is likely that this enzyme is the sole source for molecular hydrogen in *Neocallimastix* sp. L2. A cDNA has been cloned and sequenced. It encodes a [Fe]-hydrogenase of the proper size which exhibits all the motifs that are characteristic of [Fe]-hydrogenases from both bacterial and eukaryotic origins. The ORF encoding this hydrogenase possesses an N-terminal extension of 38 amino acids that has the potential to function as a hydrogenosomal targeting signal. Phylogenetic analysis has revealed that this hydrogenase clusters together with similar [Fe]-hydrogenases from *Trichomonas vaginalis*, *Nyctotherus ovalis*, *Desulfovibrio vulgaris* and *Thermotoga maritima*. However, low bootstrap values do not allow to reject the hypothesis that the eukaryotic [Fe]-hydrogenases are monophyletic, i.e. the data presented here are compatible with the hypothesis that mitochondria and hydrogenosomes evolved from a common ancestor by differential loss of ancestral enzymes.

Introduction

Hydrogenases are enzymes that - in a reversible reaction - catalyse both the splitting and the formation of hydrogen (Adams 1990, Wu and Mandrand 1993, Adams and Stiefel 1998). With a few exceptions, hydrogenases belong to a large group of multi-subunit metalloproteins that are characterised by the presence of [Fe] or [NiFe], respectively, at their catalytic sites. Most of these enzymes can be classified as either [NiFe] or [Fe] ("iron-only") hydrogenases (Cammack 1995, Volbeda et al. 1995, 1996a,b, Fontecilla-Camps et al. 1997, Casalot et al. 1998, Peters et al. 1998, Nicolet et al. 1999). Both types of hydrogenases are found in a wide variety of anaerobic and aerobic prokaryotes, e.g. methanogens, methylotrophs, sulfur-reducing bacteria, *Clostridia*, aerobic H₂-bacteria, photosynthetic purple bacteria, and cyanobacteria (Adams 1990, Pryzbyla et al. 1992, Wu and Mandrand 1993, Nandi and Sengupta 1998).

Among eukaryotes, the occurrence of hydrogenases is rather limited. Hydrogen-evolving hydrogenases have been found in anaerobic protists, anaerobic chytridiomycete fungi, and unicellular algae (Yarlett et al. 1986, Zwart et al. 1988, Happe and Naber 1993, Happe et al. 1994, Schnackenberg et al. 1993, Marvin-Sikkema et al. 1993a,b, Schultz 1995, Fenchel and Finlay 1995, Appel and Schultz 1998, Horner et al. 2000, Florin et al. 2001). For reasons that are not yet understood completely, the hydrogen-evolving hydrogenases of eukaryotes are found exclusively in membrane-bounded organelles. In unicellular cellular algae, the chloroplast exhibits hydrogenase activity, whereas in anaerobic protists and chytridiomycete fungi, the hydrogenase activity seems to be restricted to an elusive, membrane-bounded organelle that had been named "hydrogenosome" (Muller 1993, Embley et al. 1997, Hackstein et al. 1999, Rotte et al. 2000).

Hydrogenosomes have been identified in various anaerobic unicellular eukaryotes such as, for example, trichomonads, amoeboflagellates, anaerobic ciliates, and chytridiomycete fungi (Muller 1993, Roger 1999). This observation suggests that hydrogenosomes were "invented" several times in evolution (Biagini et al 1997, Embley et al 1997, Martin and Muller 1998, Hackstein et al 1999), and the question arose whether all hydrogenosomes are the same (Muller 1993, Coombs and Hackstein 1995, Embley et al 1997). Albeit that all hydrogenosomes evolved from mitochondria, this happened several times independently in rather unrelated lineages of unicellular eukaryotes - most likely as an adaptation to life under anaerobic conditions (Embley et al 1997, Muller 1998, Akhmanova et al 1998a, Plumper et al 1998, Hackstein et al 1999, Rotte et al 2000, van Hoek et al 2000a). The mechanisms that enabled the evolution of hydrogenosomes are still a matter of debate (cf Martin and Muller 1998, Hirt et al 1999). In particular, the origin of the hydrogenosomal hydrogenases remained unclear until now. Based on the phylogenetic analysis of a small number of genes that encode putative hydrogenases, Horner et al have postulated a polyphyletic origin of eukaryotic [Fe]-hydrogenases implicating an acquisition of the eukaryotic hydrogenases by lateral gene-transfer from different prokaryotic sources (Horner et al 2000). In contrast, the various genome projects have provided evidence for the widespread occurrence of genes in unicellular and multicellular eukaryotes that possess significant sequence identity with canonical [Fe]-hydrogenase genes. Potentially, these genes are monophyletic and cluster with "real" eukaryotic [Fe]-hydrogenases, although they are likely to encode proteins that do not exhibit hydrogenase activity (Horner et al 2000). In the case of the human *Narf*, such a protein has acquired the function of binding nuclear prelaminin A (Barton and Worman 1999). Also, the function of the much better conserved (and putative cytoplasmic) "[Fe]-hydrogenases" of *Entamoeba histolytica* and *Spiromucleus barkhanus* has remained unclear until now (Horner et al 2000).

Notably, the only eukaryotic hydrogenase that has been partially purified to date is a 64 kDa hydrogenosomal hydrogenase of *Trichomonas vaginalis*. It has been characterised as a [Fe]-hydrogenase by its electron paramagnetic resonance (EPR) spectrum and its high sensitivity against carbon monoxide (Payne et al 1993). Two genes encoding putative [Fe]-hydrogenases in *Trichomonas vaginalis* have been isolated and cloned (Bui and Johnson 1996), but their inferred amino acid sequence predicted proteins that were much smaller than the above-mentioned hydrogenosomal hydrogenase. Only recently, an ORF has been identified in *T. vaginalis*, which might encode the hydrogenase that had been partially purified earlier. However, neither the expression of the gene, nor the subcellular localisation of the gene product or its function has been demonstrated yet (Horner et al 2000). Moreover, it remained unclear until now as to whether [NiFe]-hydrogenases, that played a crucial role in the evolution of mitochondrial complex I (Albracht and Hedderich 2000, Friedrich and Scheide 2000), are present in one or the other type of hydrogenosome.

Using immuno-cytochemistry we have been able to show that the hydrogenosomes of the gut-dwelling, anaerobic ciliate *Nyctotherus ovalis* host a [Fe]-hydrogenase (Akhmanova et al 1998a). Its hydrogen-emitting activity had been inferred from the presence of endosymbiotic methanogens and measurements of the methane production

rates of isolated ciliates (van Hoek et al. 2000b). A more detailed biochemical analysis of the ciliate's hydrogenosomes has been precluded by the fact that *Nyctotherus ovalis* cannot be cultured yet. In contrast to *Nyctotherus ovalis*, the anaerobic fungi *Neocallimastix* and *Piromyces* can be cultured axenically (Marvin-Sikkema et al. 1990, 1992; Teunissen et al. 1991). Therefore, we were able to localise the hydrogenase of *Neocallimastix* by subcellular fractionation, to measure hydrogenase activity in isolated hydrogenosomes, and confirm its presence in the hydrogenosome by immunocytochemistry at the electron microscopical level. Also, we identified the gene(s) that are likely to encode this hydrogenosomal hydrogenase. Here, we will show that the anaerobic chytridiomycete fungus *Neocallimastix* sp. L2 possesses a hydrogenase that is highly sensitive against CO and encoded by a gene with substantial homology to both eubacterial and eukaryotic [Fe]-hydrogenases. It will be shown that this enzyme accounts for the vast majority - if not all of the - hydrogenase activity measured in the isolated organelles.

Experimental procedures

Organisms and growth conditions

The chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2 were cultured axenically at 39°C in M2 medium supplemented with 0.35% (w/v) fructose under strictly anaerobic conditions (Marvin-Sikkema et al. 1990, 1992; Teunissen et al. 1991). Biomass for the poly (A)⁺ RNA isolation, genomic DNA preparation, and subcellular fractionation was isolated under anaerobic conditions after 48 hours of growth.

Isolation of genomic DNA and messenger RNA

Genomic DNA was prepared from *Neocallimastix* sp. L2 and *Piromyces* sp. E2 biomass according to the protocol of Brownlee (1994). Total RNA was prepared by the guanidinium-chloride method (Chirgwin et al. 1979). Poly(A)⁺ RNA was isolated from *Neocallimastix* sp. L2 using the Pharmacia[™] QuickPrep mRNA Purification Kit.

Isolation of the [Fe]-hydrogenase encoding cDNA

Adaptor-ligated cDNA was prepared from poly(A)⁺ RNA according to the Clontech[™] Marathon cDNA Isolation Kit. DNA polymerase chain reaction (PCR) was used to amplify DNA from adapter ligated cDNA. Oligonucleotide primers, based on highly conserved amino-acid regions of [Fe]-hydrogenases, were used for the PCR amplification of an internal 402 bp DNA fragment. Their sequences are 5'-ttcatcatgtgtgccaggtgg-3' (hydfor1), and 5'-agcagcttccataacaccacc-3' (hydev1). A cDNA containing the complete ORF was isolated by the Rapid Amplification of cDNA end procedure ("RACE-PCR"; Frohman 1990) as described by the protocol of the Clontech[™] Marathon cDNA Isolation Kit. For the forward and reverse RACE, oligonucleotide primers were based on the DNA sequence of the 402 bp DNA fragment. Their sequences are 5'-ccaggtgggattaatatggttgaaaagagtac-3' (hydF3), 5'-ggtgctgttattaaatcctacttgcctaagaag-3' (hydF4), 5'-accagcagatgaaccaatacctaaggactatc-3' (hydR3),

and 5'-atctggaagttcagctggattaatcttctaag-3' (hydR4). Sequencing was performed with the ABI PRISM Model 310 automatic sequencer, using a dRhodamine terminator cycle sequencing ready reaction DNA sequencing kit (Perkin-Elmer Applied Biosystems). A partial cDNA sequence of *Piromyces* was obtained by PCR using primers based on the *Neocallimastix* sequence and DNA from a lambda ZAP II cDNA bank of *Piromyces* sp. E2 (Akhmanova et al. 1998b; Akhmanova et al. 1999; Hackstein et al. 1999).

Phylogenetic analysis

For the identification of the signature sequences, an alignment of deduced amino acid sequences was assembled with the aid of *GeneDoc* (Nicholas and Nicholas, 1997). The alignment for phylogenetic analysis was created using the clustal-X package (Jeanmougin et al. 1998). Only blocks that are well conserved across all sequences (Castresana 2000) were used for phylogenetic reconstruction. A neighbor-joining tree (Saitou and Nei 1987) was created using clustal-X (Jeanmougin et al. 1998), that uses the Kimura model (Kimura 1980), to estimate evolutionary distances. Maximum likelihood analysis was done with Molphy (Adachi and Hasegawa 1996). With approximate likelihood the 100 best trees were calculated, using the HKY85 model (Hasegawa et al. 1985) to account for biases in substitution rates and nucleotide composition. Furthermore, Quartet Puzzling (Strimmer and von Haeseler 1996), using the HKY85 model, and a uniform model of rate heterogeneity or, alternatively, 8 gamma distributed rate categories, was used. For calculating the best tree exact likelihood was used (Adachi and Hasegawa 1996). Finally, a neighbour-joining tree in which variation among nucleotide frequencies of the various sequences was taken into account using log-determinant distances was generated (Steel 1994).

Southern blotting and Northern blotting

Genomic DNA (10 µg per lane) of *Neocallimastix* sp. L2 and *Piromyces* sp. E2 was digested by different restriction enzymes and separated on a 0.8% agarose gel. Poly (A)⁺ RNA (5 µg per lane) of *Neocallimastix* sp. L2 was separated on a 1.2% agarose-formaldehyde gel. Both gels were blotted to Hybond N+ membranes (Amersham). A 357 bp fragment of the *HydL2* gene (position 1221-1578 of the cDNA) was labelled by PCR with α-[³²P]dATP, and used as a probe. Hybridisation was performed in 0.5 M sodium phosphate buffer pH 7.0, 7% SDS, 1% BSA, 1mM EDTA, at 60°C. Filters were washed stringently with 50 mM sodium phosphate buffer pH 7.0 and 0.5% SDS at 60°C.

Subcellular fractionation and Western blotting

Hydrogenosomal and cytosolic fractions were obtained by differential centrifugation, using the protocol of Marvin-Sikkema et al. (1993b) with minor modifications. Samples of both fractions were separated on a 7.5% polyacrylamide gel with 0.1% SDS (SDS-PAGE), and blotted to a polyvinylidenedifluoride (PVDF) membrane by semi-dry transfer (Biorad), according to the manufacturers protocol. Dr. T. Happe (Happe et al. 1993, 1994; Florin et al. 2001) kindly provided a heterologous polyclonal antiserum, directed against the [Fe]-hydrogenase of *Chlamydomonas reinhardtii*. The [Fe]-hydrogenase of *Chlamydomonas reinhardtii* lacks Fe-S clusters, but it contains the highly conserved H-cluster, the catalytic

centrum (c.f. Florin et al. 2001). The antiserum recognises also the [Fe]-hydrogenases of anaerobic chytrids; it was used in a dilution of 1:1000. Detection was performed using goat-anti-rabbit antibodies conjugated to peroxidase (Boehringer Mannheim).

Enzyme assays

Hydrogenase (EC 1.18.3.1), malic enzyme (EC 1.1.1.38-40), adenylate kinase (EC 2.7.4.3), and hexokinase (EC 2.7.1.1) activities were assayed spectrophotometrically (Declerck and Müller 1987; Kremer et al. 1989; Marvin-Sikkema et al. 1993b; Bergmeyer et al. 1983). For enzyme assays using the hydrogenosomal fraction, the detergent Triton X-100 was added to a final concentration of 0.2% (v/v) in order to permeabilise the organelles. All enzyme assays were performed anaerobically (N_2 gas-phase) at 39°C. For the CO-inhibition experiment, hydrogenosomes were incubated anaerobically with different concentrations of CO ranging between 0 and 100 μ molar, as described by Payne et al. (1993).

Immuno-gold labelling

Immunocytochemistry and electron microscopy, using the heterologous antiserum directed against the [Fe]-hydrogenase of *Chlamydomonas reinhardtii* (Happe et al. 1993, 1994) was performed as described previously by Marvin-Sikkema et al. (1993a).

Results

Subcellular localisation of hydrogenase activity

Axenic cultures of *Neocallimastix* sp. L2 produce substantial amounts of hydrogen. In order to determine the subcellular localisation of the hydrogenase activity, conventional fractionation experiments were performed in order to obtain a 30.000x g organellar fraction that is enriched in hydrogenosomes (Marvin-Sikkema et al. 1993b). After differential centrifugation of homogenates from mycelia of *Neocallimastix* sp. L2, the hydrogenase activity in the pellet of the 30.000x g fraction was approximately 40 times higher than the hydrogenase activity in the cytosolic fraction. Adenylate kinase and malic enzyme activities in the pellet were enriched by a factor of 44.7 and 36.4, respectively (Table 1). Since both adenylate kinase and malic enzyme had been localised in the hydrogenosomes of *Neocallimastix* sp. L2 (see Hackstein et al. 1999 for discussion), it had to be concluded that the hydrogenase activity was restricted to this organelle. The low levels of activity of these enzymes that could be detected in the cytosolic fractions most likely were due to damage and

Table 1. Enzyme activities (U/mg protein) in the cytosolic (Cyt) and hydrogenosomal (Hyd) fractions of *Neocallimastix* sp. L2. F, factor indicating the enrichment of the enzyme activities in the hydrogenosomal fraction, when compared to the enzyme activities in the cytosolic fraction. ND, not detectable within the limits of the assay sensitivity (1 nmol/min/mg protein).

Enzyme	Cyt	Hyd	F
hexokinase	0.91	ND	-
malic enzyme	0.54	19.64	36.4
adenylate kinase	0.11	4.92	44.7
hydrogenase	1.46	58.10	39.8

leakage of the organelles during the isolation procedure. The hydrogenosomal fraction was devoid of hexokinase activity. As expected, the activity of this cytosolic marker enzyme could only be detected in the cytosolic fraction (Table 1).

The hydrogenosomal localisation of the hydrogenase was confirmed by immunocytochemistry: on ultra-thin sections of *Neocallimastix* sp. L2 incubated with an antiserum against the [Fe]-hydrogenase of *Chlamydomonas* and goat-anti-rabbit (GAR)-gold, the labeling was predominantly found in the matrix of the hydrogenosomes (Fig. 1). Western blotting of the 30.000x g pellets with a heterologous antiserum directed against the [Fe]-hydrogenase of *Chlamydomonas reinhardtii* (Happe et al. 1993, 1994), revealed the presence of three cross-reacting proteins in the hydrogenosomal fraction (Fig. 2). The size of the largest cross-reacting protein, which exhibited an apparent molecular weight of approximately 67 kDa, matched with the calculated molecular mass of the mature *hydL2* gene product (66.4 kDa: see below). It remained unclear whether the cross-reacting proteins with apparent molecular masses of 60 and 58 kDa represent additional, smaller hydrogenases or processing/degradation products. Alternatively, the 60 and 58 kDa bands might be due to unspecific interactions of the antiserum with unrelated proteins.

Figure 1. Localisation of the [Fe]-hydrogenase by immunogold-labeling, using the heterologous antiserum directed against the [Fe]-hydrogenase of *Chlamydomonas reinhardtii*, reveals the presence of an [Fe]-hydrogenase in the matrix of the hydrogenosomes (H) of *Neocallimastix* sp. L2. R: ribosome globules (Munn et al. 1988). Bars represent 0.5 μ m. (A): Cluster of hydrogenosomes. The labeling "outside" of the hydrogenosomes is nearly exclusively due to tangential sections through hydrogenosomes. (B): "Peas in a pod" stage of a hydrogenosome. The anti-hydrogenase labeling is confined to the matrix.

Figure 2. Western-blotting of cellular fractions obtained after fractionation by differential centrifugation: (1) Cytosolic fraction; only traces of cross-reacting protein can be identified. (2) The pellet of the 30.000x g fraction is enriched in hydrogenosomes (c.f. Table 1). A heterologous antiserum directed against the [Fe]-hydrogenase of *Chlamydomonas reinhardtii* cross-reacts with 3 proteins of apparent molecular masses of 67 (a), 60 (b) and 58 (c) kDa. The 67 kDa band matches precisely with the predicted molecular mass (66.4 kDa) of the mature [Fe]-hydrogenase encoded by *hydL2*.

Figure 3. Hydrogenase activity of isolated hydrogenosomes. Hydrogenase activity is measured as hydrogen : methylviologen reductase activity. The addition of carbon monoxide causes a dramatic decrease of hydrogenase activity. At a concentration of 3.5 μM CO, an inhibition of the hydrogenase activity by approximately 50% is achieved.

Incubation of isolated hydrogenosomes under anaerobic conditions allowed the measurement of hydrogenase activity over extended periods of time (c.f. Table 1). The addition of carbon monoxide (CO) up to a concentration of 100 μM (Fig. 3) revealed that the chytrid hydrogenase is highly sensitive against CO as other eukaryotic and prokaryotic [Fe]-hydrogenases (Adams 1990; Payne et al. 1993). A CO concentration as low as 5 mM CO caused over 90% inhibition of the enzyme activity. Therefore, it is likely that the hydrogenosomes of *Neocallimastix* sp. L2 host a [Fe]-hydrogenase of a size of approximately 67 kDa that accounts for the vast majority if not all of the hydrogenase activity measured in the hydrogenosomal fraction.

The gene hydL2 encodes a [Fe]-hydrogenase

Using PCR with primers directed against conserved regions of [Fe]-hydrogenases and the rapid amplification of cDNA ends (RACE) procedure, we isolated a 2468 bp cDNA fragment (*hydL2*). DNA sequence analysis revealed the presence of an open reading frame (ORF) of 1908 bp that encodes a protein of 636 amino acids (calculated molecular mass 70.4 kDa). The non-coding sequences upstream and downstream of the ORF are very AT-rich as in other genes of anaerobic chytrids (Fig. 4; Reymond et al. 1992; Durand et al. 1995; Fanutti et al. 1995; Akhmanova et al. 1998b, 1999).

Sequence analysis with the aid of PSORT (<http://psort.nibb.ac.jp:8800>) supported the presence of a mitochondrial targeting signal at the N-terminus of the potential hydrogenase (Fig. 4). PSORT analysis also argued for the presence of a potential boundary of the mitochondrial targeting sequence around position 35 (amino-acid motif GRKFQV). If the “R” indicates the position -2 (c.f. van der Giezen et al. 1997), then the hydrogenase will

5' cDNA sequence of *hydL2*

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hydL2      ataatttatatatttcatttcattcttttttttttttcttcgggattgaaa  51
HydL2

taaatcttaataacaaataaataagtatataaaaaaaaaaagaaaaaaaaaaaaataaaa  111
*          *          *

ttaataaagaggaaaaaagaataataaacactatatttaagtaataagacataaataaaa  171
*          *          *

      ↓
gtagatatatatatttttaagaaaaaATGCTCTATGTTATCATCAGTATTAATAAAGCCGTA  231
*          M  S  M  L  S  S  V  L  N  K  A  V  12

GTTAACCCAAAGTTAACTCGTTCTTTTAGCTACAGCTGCTGCTGAAAAGATGGTCAATATT  291
V  N  P  K  L  T  R  S  L  A  T  A  A  A  E  K  M  V  N  I  32

TCTATTAATGGGCGTAAGTTCCAAGTTAAGCCAAAAACTACCGTTCTTGAAGCTGCCAAA  351
S  I  N  G  R  K  F  Q  V  K  P  K  T  T  V  L  E  A  A  K  52

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3' cDNA sequence of *hydL2*

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CATGAATTATTACATACTCATTATAATGATCGTTCAAAGACTATTTCATGATATGGGTCAT  2091
H  E  L  L  H  T  H  Y  N  D  R  S  K  T  I  H  D  M  G  H  632

CATGAAAGAAGTaaacaaatatatgtattttaataaataaataaaaccgttaattaat  2151
H  E  K  K  *          *          *          636

aattgccaaccttatttatcatccttttttaagcatatataataaacattttatttataaa  2211
*          *

agaaagaaaaaaaggaagaacaaaaggatatttaaaatttttaaaagttatataatttgt  2271
*

ttgaaaaatctaaaatttttatatttttttttttttttttttaaaaaataaagggtgttaag  2331
*

gtattttatataataataccccaataacaatacacacgttatataaaacaagtaaaaaagaat  2391
*  *          *          *

aattaagggaaaaattaagaaaattaaataaacgaaaacaagccaacataaagcaaaaaa  2451
*          *

```

Figure 4. 5' (A) and 3' (B) ends of the cDNA clone *hydL2* of *Neocallimastix* sp. L2. The cDNA clone *hydL2* has a length of 2468 bp. DNA sequence analysis revealed the presence of an open reading frame (ORF) of 1908 bp that encodes a protein of 636 amino acids (calculated molecular mass 70.4 kDa). Both ends of the clone consist of AT-rich, non-coding sequences that possess numerous in frame stop codons (asteriks). The start codon is indicated by an arrow, the putative hydrogenosomal targeting sequence at the N-terminus is underlined. The box indicates the 'boundary motif'. Most likely, the arginine (R) indicates the position -2 of the mature protein.

start at position 39. Given that the N-terminal targeting sequence is cleaved after import into the hydrogenosomes (c.f. van der Giezen et al. 1997; Akhmanova et al. 1998b, 1999), the remaining 598 amino acid residues could account for a mature hydrogenase of a molecular mass of approximately 66.4 kDa. This figure perfectly matches the size of the largest protein (approximately 67 kDa) from the hydrogenosomal fraction that hybridises with the anti-hydrogenase serum (Fig. 2).

Comparison of the deduced amino acid sequences of hydrogenase sequences available to date, revealed a substantial similarity (23%-36% identity) between the *Neocallimastix* hydrogenase and the various [Fe]-hydrogenases of prokaryotic and eukaryotic origin (Table 2). The identity with the various [NiFe]-hydrogenases was below 20% (not shown).

	Organism	Gene	1	2	3	4	5	6	7	8	9	10	11	12
1	N.sp.L2	HydL2	-	36	34	34	36	23	35	23	23	35	27	23
2	C.ace.P	HydA		-	67	66	41	27	34	27	27	30	29	28
3	C.ace.A	HydA			-	70	38	28	33	27	26	28	30	27
4	C.pas.W	HydA				-	39	26	35	26	25	29	30	26
5	D.fru	HydD					-	26	41	28	28	34	27	25
6	D.fru	HydA						-	24	73	70	22	31	31
7	D.vul.H	HydC							-	26	24	33	27	25
8	D.vul.H	HydA								-	78	23	31	32
9	D.vul.M	HydA									-	22	33	32
10	N.ova	Hydnyc										-	25	23
11	T.vag	ThhydA											-	59
12	T.vag	ThhydB												-

Table 2. Pairwise comparison of deduced [Fe]-hydrogenase amino acid sequences. Values calculated in percent of identical amino acids are shown. N.sp.L2, *Neocallimastix* sp. L2; C.ace.P, *Clostridium acetobutylicum* P262; C.ace.A, *Clostridium acetobutylicum* ATCC 824; C.pas.W, *Clostridium pasteurianum* W5; D.fru., *Desulfovibrio fructosovorans*; D.vul.H, *Desulfovibrio vulgaris* Hildenborough; D.vul.M, *Desulfovibrio vulgaris* Monticello; N.ova., *Nyctotherus ovalis*; T.vag., *Trichomonas vaginalis*.

Figure 5. Alignment of the [Fe]-hydrogenase *hydL2* of *Neocallimastix* sp. L2. A multiple alignment of the deduced amino acid sequences of various [Fe]-hydrogenases reveals that *hydL2* encodes a [Fe]-hydrogenase that exhibits all the conserved motifs (bold face at the top of the alignment) that are characteristic of a [Fe]-hydrogenase. Abbreviations: *P. sp. E2*, *Piromyces* sp. E2 (personal communication; B. Boxma); *N. sp. L2*, *Neocallimastix* sp. L2 (AY033895); *N. ova.*, *Nyctotherus ovalis* (CAA76373.1); *C. ace.*, *Clostridium acetobutylicum* P262 (AAA85785.1); *C. the.*, *Clostridium thermocellum* (AAD33071.1); *D. fru.*, *Desulfovibrio fructosovorans* (AAA87057.1); *D. vul.*, *Desulfovibrio vulgaris* Hildenborough (CAA40970.1); *TrichVag3*, *Trichomonas vaginalis* (AAG31037.1); *T.vag.*, *Trichomonas vaginalis* ThhydA (AAC47159); *Th. mar.*, *Thermotoga maritima* HydD (AAD35293.1|AE001705_4), HydA (AAD36496.1|AE001794_12).

[Cxxx

N. sp. L2 HydL2 : MSMLSSVLNKAVNPKLTRSLATAAAEKMVNISINGRKFQVKPK-TTVLEAAKANGYYYIPTLCYHQ-
N. ova. HydNyc : -----MISRLIAKKAPLFLRTFATSEMSLKDGGKIIISVPKG-IMLADAIKKAGANVPTMCYHP-
C. ace. HydA : -----MINIVIDEKTIQVQEN-TTVIQAALANGIDIPSLCYLN-
C. the. HydA : -----MVNVITIDNCKIQVPAN-YTVLEAAKQANIDIPTLCFLK-
D. fru. HndD : -----MSMLTITIDGKTSVPEG-STILDAAKTLDDIPTLCYLN-
D. vul. HydC : -----MNAFINGKEVRCPEG-RTILEAARENGHFIPITLCELA-
TrichVag3 : -----MASTGINSTANILRNITVTVNGKPLEAKKG-ETVLELCDRNNIRIPRLCFHP-
T. vag. TvhydA : -----
Th. mar. HydD : MRRFFKNNLRNLSQNETNSVRRCFALADVTVVINGRTLTPDNLTVIEACEKAGIEIPALCHHP-
Th. mar. HydA : -----MKIYVDGREVIINDERNLLEALKNVGIEIPNLCYLS-

F'-cluster 1

---XXXXXXXXXXXXX-----XXXXXXXXXXC]

[Rxxx

N. sp. L2 HydL2 : ---ELPVA-GNCRCLCLVYAKGS---WKPLTACTTEVWEGMEIETDSPAVIETVRSSLSMMREEHPN
N. ova. HydNyc : ---DLPTSGGICRVCLVESAKSP---GYPIISCRTPVEEGMEIVTQGSKMKEYRQANLAMLRSRHPN
C. ace. HydA : ---ECGN-VGKCGVCAVEIEGK---NNLALACITKVEEGMVVKTNSEKVQERVMRVATLLDKHEF
C. the. HydA : ---DIN-EVGACRMCVVEVKGA---RSLQAACVYPVSEGLEVYTQTPAVREARKVTLELILSNHEK
D. fru. HndD : EALSINNKAAASCRVCLVEVEGR---RNLAPSCATPVTDNMVVKTNLSRLNARRTVLELLLSDDHPK
D. vul. HydC : ---DIGHAPGTCRVCLVEIWRDKEAGPQIVTSCPTVPEGMRIFTREPVRRMQRLQVEILLADHDH
TrichVag3 : ---NLPP-KASCRVCLVECDGKW---LSPACVTTVDGLKIDTKSKNVRDSENNKELLDDCHDE
T. vag. TvhydA : -----
Th. mar. HydD : ---RLGESIGACRVCVVEEGA---RNLQPCVTKVRDGMVITKSSDRVKTARKFNALLLSEHPN
Th. mar. HydA : ---EASI-YGACRMCLVEING---QITTSCTLKPVEGKVKVNTPEIYEMRRNILELILATHNR

F'-cluster 2

xXXXXXxxxxxxC]

[DxxxKxx

N. sp. L2 HydL2 : DCMTGSGNDCEFDLIYRQIDAKHPVRSLLKHK-SKKTNHSITEPCYSPFDNTTFSVARDMNKCV
N. ova. HydNyc : ACLSCTSNCTKTQELSANMNIQCG-----FANATPPKND-----DSYDMTTAIEDRNDKCI
C. ace. HydA : KCGPCPRENCEFLKLVITKAKANK-----PFV-VEDKSQ-----YIDRSKSIIVDRTKCV
C. the. HydA : KCLTCVRSENCLEQLAKLDNVKDIR-----FEGE-----MSNL-----PIDLSPSVVRDPMKCV
D. fru. HndD : DCLVCARSGCELOQLAERFIRESP-----YDGG-----EMSH-----YRKDISASIRDMDKCI
D. vul. HydC : DCAACARHGDCLEQDVAQVGLTGTR-----HHFP-DYA-RSR-----TRDVSSPSVVRDMGKCI
TrichVag3 : TCSACIANHRCQFRDMNVASVKAET-----KEI-----CSEE-----GIDESTNAIRLDTSKCV
T. vag. TvhydA : -----MLA-SSATAMKGFANSLRMKDYSSGTINFDMTKCI
Th. mar. HydD : DCMTCEANGRCFQDLIYKYDVEPI-----FGYG-----TKEG-----LVDRSSPAIVRDLKCI
Th. mar. HydA : DCTTCDRNGSCKLQKYAEDFGIRKIR-----FEAL-----KKE-----HVRDESAPVVRDTSKCI

F-cluster 1

xXXXXXxxxxC]

F-cluster 2

[XXXXCGGXXXXXP]

N. sp. L2 HydL2 : KCGRCIRACHHFQNNILGFINRAGYERVGTMPDRPMNFTKCVCEGQCSQVCPVGAITA-RTEVVVD
N. ova. HydNyc : NCDICVHTCS-LQGLNALGFYNEEGHAVK--SMG-TLDVSECIQCGQCINRCPTGAITE-KSEIRPV
C. ace. HydA : LCGRCEAACTKTGTGAISICKSESGRIVQATGGKCFDDTNCLCGQCVAACPVGAITE-KTHVDRV
C. the. HydA : LCRRCVSMCNVQTVGAIVDTERGFRITVSTAFNKPLSEVPCVNGCQCINVCVPGALRE-KDDIDKV
D. fru. HndD : MCRRCETMCNTVQTCVGLSGVNRGFTAVVAPAFEMNLADTVCTNCGQCVAVCPGALVE-HEYIWEV
D. vul. HydC : RCLRCVAVCRNVQGDALVVTGNGIGTEIGLRHNRSSQASDCVCGGQCTLVCPVGAIALG-RDVERV
TrichVag3 : LCGRCIRACEEAVAGTSIIIFGNRAKMMRIQPTFGVTLOETSCICGQCTLYCPVGAITE-KSQVKEA
T. vag. TvhydA : NCQSCVRACTNIAGQNVKLSLTVNGSKSVQTVTGKPLAETNICSCGQCTLGCPKFTIFEADAINPVK
Th. mar. HydD : KCQRCVRCASELQGMHIIYSMVERGHRTPGTPFDMFVYETDCICGGQCAAFCPPTGAIVE-NSAVKVV
Th. mar. HydA : LCGDCVRVCEEIQGVGVEFAKRGFESVVTAFDTPLETCVLCGQCVAYCPGTGALSI-RNDIDKL

N. sp. L2 HydL2 : LRHLDTK-RKVVCSTAPAIRVAPAEFSTEADFDFT-GKMVAGLRKLGFD-YIFDTNFSADLTIME
N. ova. HydNyc : LDAINI---QORLVFQMAPSIRVAVAEFQIKPGEKILKNEIATALRKLGSNVFLDTNFSADLTIME
C. ace. HydA : KEALEDN-NKHIVVAMAPSIKSMGELFKLGYGVDT--GKLYASMRALGFD-KVFDINFGADMTIME
C. the. HydA : WEALANP-ELHVVVQTPAPAVRVALGEEFGMPIGSRVT--GKMVAALSRLGFK-KVFDTDTAADLTIME
D. fru. HndD : VEALANP-DKVVIVQTPAPAVRAALGEDLVGAPGTSVT--GKMAAALRRLGFD-HVFDTFDAADLTIME
D. vul. HydC : IDYLYDP-EIVTVFQFAPAVRVLGEEFGLPPGSSVE-GQVPTALRLGAD-VVLDTNFAADLTIME
TrichVag3 : LDILANKGKITVVQVAPAVRVALSEAFYKKEGTWTT--GKMVSALKALGFD-LVYDTNFGADLTIME
T. vag. TvhydA : EVLTKNKGR-IAVCQIAPAIRINMAEALGVPGTISL-GKVVTALKRLGFD-YVFDTNFAADMTIME
Th. mar. HydD : LEELEKK-EKILVIVQTPAPSVRAIGEEFGYAGPTIST--QGMVAALRRLGFD-YVFDTNFGADLTIME
Th. mar. HydA : IEALES--DKIVIGMIAPAVRAAIQEEFGIEDVDAMA-EKLVSLFKTIGFD-KVFDVSFGADLTIME

start H-cluster motif 1

FTSCCPxWxxxKxxKxxxxxxSP

N.sp.L2 HydL2 : EGTELIDRLNNG-----GKFPMTSCCPGWINMVEKSYPELSDNLSSCKSPQQMIGAVI
P.sp.E2 HydE2 : INMVEKSYPEIRDNLSSCKSPQQMIGAVI
N.ova. HydnyC : EGHELIERLYRNVGTGKKLLGGDHMPIDLPLMTSCCPGWIMFIEKNYPDLNLSSTCKSPQGMGLALI
C.ace. HydA : EATEFIERVKNN-----GPFPMFTSCCPAWRVQVENYYPEFLNLSAKSPQIFGAAS
C.the. HydA : EGTELINRIKNG-----GKLPILTSCCPGWIKFCEHNYPEFLDNLSSCKSPHEMFAGVL
D.fru. HndD : EGSEFLDRLGKHLAGD---TN-----VKLPILTSCCPGWKVFEEHQFMDLDVSTAKSPQGMFGAIA
D.vul. HydC : EGTELLQRLRGG-----AKLPFLTSCCPGWVNFAEKHLPDILPHVSTTRSPPQCLGALA
TrichVag3 : EAGELVNLRD-----PN-----AKFPMFTSCCPAWVNYEQSAPDFIPNLSSCRSPQGLMSALI
T.vag. TvhydA : EATELVQRLSD-----KN-----AVLPMFTSCCPAWNVVEKSDPLSLIPYELSSCRSPMSLSVI
Th.mar. HydD : EGSEFLERLEKG-----DL-----EDLPMFTSCCPGWVNLVEKVYPELATRLSSAKSPQGLMSAMV
Th.mar. HydA : EAHEFYERLKKG-----ERLPQFTSCCPAWVKHAETHYQYQLQNLSSVKSPPQALGTVI

motif 2

VxxdPCxxKxxKxxR

N.sp.L2 HydL2 : KSYFAKKLG-LSTEDIHVSIMPCTAKKGEAKRPEFV-QKGKNGKDYDIDYVITRELLTLLKKK
P.sp.E2 HydE2 : KTYFAKKIN-AKPEDIHVSIMPCTAKKGEAKRPEFK-RDG-----VPOIDHVIITRELLTLLKKR
N.ova. HydnyC : KGYWAKNIKKMDPKDIVSVSIMPCTAKKAERKPLRGDEG-----YKVDYILTRELLAKMLKQSN
C.ace. HydA : KTYYPQISG-ISAKDVFTVTIMPCTAKKFEADREEMY-NEG-----IKNIDAVLTTRELLAKMIKQAK
C.the. HydA : KSYAQAQNG-IDPSKVFGSIMPCTAKKFEAQRLELS-STG-----YPDVDVLTRELLARMIKETG
D.fru. HndD : KTYADLLG-IPEKLVVSVMPCLAKKYECARPEFS-VNG-----NPQVDIVITRELLAKLVKRMN
D.vul. HydC : KTYLARTMN-VAPERMRVSLMPCTAKKEEAARPEFR-RDG-----VRDVAVLTTRELLARLLREG
TrichVag3 : KNYLPLKLL-VKQEDVLFNSIMPCTAKKDEVERELRTKSG-----LKETDMVLTRELLVEMIKLSN
T.vag. TvhydA : KNVFPPKIG-TTADKIYNVAIMPCTRKKDEIQRSQFTMKDG-----KQETGAVLTRELLAKMIKEAK
Th.mar. HydD : KTYFAEKLG-VKPEDI FHSVIMPCTAKKDEALRQLM-VNG-----VPAVDVLTRELLGKILRMKK
Th.mar. HydA : KKIYARKLG-VPEEKI FLVSIMPCTAKKFEAEREHE-G-----IVDIVLTTRELLAQLIKMSR

motif 3

TGGVMEAAxR

N.sp.L2 HydL2 : INPAELPDDKFD-SPLGIGSSAGNLFGVTGGVMEAAARTAQVITGVENPIP-LGELKAIRGLDGIKA
P.sp.E2 HydE2 : INSELKNEKFD-SPLGIGSSAGNLFGVT
N.ova. HydnyC : IDLAKMEPTPF-D-KVMSGTGAAVIFGVTGGVMEAAALRTANEVITGR-EVPFKNLNIEAVRGMGIR
C.ace. HydA : INFANLEDEQAD-PAMGEYTGAGVIFGATGGVMEAAALRTAKDFVEDK-DLT-DIEYTIQIRG LQGIK
C.the. HydA : IDFNSLPDQFD-DPMGEASGAGVIFGATGGVMEAAALRTVYELLSGK-PAD-KIETYEVRLGDGIK
D.fru. HndD : IDFAGLPDEDFD-APLGASTGAAPIFGVTGGVMEAAALRTAYELATGE-TLK-KVDFEDVRGMDGVK
D.vul. HydC : IDLAGLEPSPCDPMLGRATGAAVIFGTGGVMEAAALRTYVHVLNGK-ELA--PVELHALRGYENVR
TrichVag3 : IDFNPLPDTQFD-NIFGFGSGAGQIFATGGVMEAAASRTAFEVYTGK-KLT--NVNIYVVRGMDGLR
T.vag. TvhydA : INFKELPDTPCD-NFYSEASGGGAIFCATGGVMEAAVASRYKFLTCK-ELA--PIDLQDVRGVASGV
Th.mar. HydD : IPFANLPPEEYD-APLGISTGAALFGVTGGVMEAAALRTAYELTKG-ALP-KIVFEEVRLGKGV
Th.mar. HydA : IDINRVEPQPF-D-RPYGVSSQAGLFGFGKAGGVFSCVLSVLNEEIG---IE--KVDVKSPE--DGI

motif 4

PxExdxCPGGCxxGG

N.sp.L2 HydL2 : ANVPLTK-----DGKEVSVRAAVVSGGANIQFLEKIKNKE-----LEFDFIEMMMCPGGCINGG
N.ova. HydnyC : EAGIKLENVLDKYKAFEGVTVKVAIAHGPNNAKVMIDIKQAKESGKPAPWHFVEVMACPGGCIGGG
C.ace. HydA : EATVEIG-----ENYNVAVINGAANLAEFMNSGKILE-----KNYHFIEVMACPGGCVNGG
C.the. HydA : EASIELDG-----FTLKAVAHGLGNARKLLDKIKAGE-----ADYHFIEIMACPGGCINGG
D.fru. HndD : KAKVKVGD-----NELVIGVAHGLGNARELLKPCGAGE-----T-FAHIEVMACPGGCIGGG
D.vul. HydC : EAVVPLGEG-----NGSVKAVVHGLKAARQMVAELAGK-----ADHVFVEVMACPGGCMDDG
TrichVag3 : IAEILDLDG-----TKLKVAVCHGIANTAKLLDRLREKDP--ELMDIKFIEIMACPGGCVCVG
T.vag. TvhydA : KLAEVDIAG-----TKVKVAVAHGINKAMTLIKKIKSGEE--QFKDVKFVEVMACPGGCVCVG
Th.mar. HydD : EAEILDG-----KKIRIAVHGTANVRNLVEKILRME-----VKYHFVEVMACPGGCIGGG
Th.mar. HydA : VAEVTLKD-----GTSFKGAVIYGLGVKVFLEERKD-----VEIIEVMACNYGCVGGG

end H-cluster

GxP

N.sp.L2 HydL2 : GQPK---SADPE---IVAKKMORMYTMDQAKLRLCHENPEIIVDYKN-FLGEPNSHLAHELLHTH
N.ova. HydnyC : GQPK---PTNLE---IQARTQLTFKEDMDLPLRKSHDNPEIKAIYEN-YLKEPLGHNSHHYLHTT
C.ace. HydA : GQPHV-SAKERKV-DVTRVRAVLYNQDNKLEKRKSHKNTALLNMYYD-YMGAPGGQKAHELLHLK
C.the. HydA : GQPIQ-PSSVRNWKDICERAKAIYEDESLPIRKSHENPKIMLYEE-FFGEPSGHKAHELLHTH
D.fru. HndD : GQPYH---HGDVE---LLKRTQVLYAEDAGKPLRKSHENPYIIELEYK-FLGKPLSERSHQQLHTH
D.vul. HydC : GQPRSKRAYNPN---AQA-RRAALFSLDAENALRQSHNNPLIGKVYES-FLGEPSCNLSHRLHLTR
TrichVag3 : GTPQ---PKNRY---SDONRLAAIYNIIDARMECRKSHENPLIKGVYKE-FLGKPNSHLAHELLHTH
T.vag. TvhydA : GSPK---AKTKK---AVQARLNATYSIDKSSKHRTSQDNQLQLYKESFEKGFGGHVAHELLHTH
Th.mar. HydD : GQPY---SRDPE---ILRKRAEAIYTIIDERMTLKSHENPAIKKLYEE-YLEHPLSHKAHELLHTH
Th.mar. HydA : GQPY---PNDSR---IREHRAKVLRTMGILSKLTSPENLFLMKLYEE-DLKD---EHTRHILHTT


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N.sp L2 HydL2 : YNDRSKTIHDMGHHEKK-- - -----
N.oval. HydnyC : Y-----
C.ace. HydA : YNK-----
C.the. HydA : YEKRENYPV-----
D.fru HndD : YFKRQRL-----
D.vul. HydC : YGDRKSEVAYTMRDIWHEMTLGRRVRGDS-----
TrichVag3 : FKHHPKW-----
T.vag TvhydA : YKNRKVNP-----
Th.mar. HydD : YEDRSRKKRLAVK-----
Th.mar. HydA : YRPRRRYPEKDVLEILPVPNGEKRTVKVCLGTSCYTKGSYEILKKLVDPYKENDMEGKIEVLGTFCVE

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N.sp L2 HydL2 : -----
N.oval. HydnyC : -----
C.ace. HydA : -----
C.the. HydA : -----
D.fru HndD : -----
D.vul. HydC : -----
TrichVag3 : -----
T.vag TvhydA : -----
Th.mar. HydD : -----
Th.mar. HydA : NCGASPNVIVDDKIIGGATFEKVLLEELSKNG

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An alignment of [Fe]-hydrogenase sequences (Fig. 5) revealed that the hydrogenase of *Neocallimastix* sp. L2 belongs to the "long-type" hydrogenases, similar to the hydrogenase from *Nyctotherus ovalis*, the *hydA*, *hydC*, *hydD* and *HndD* encoded hydrogenases of the various anaerobic bacteria of the *Desulfovibrio* and *Clostridium* taxa, and of *Thermotoga maritima* (Fig. 6; Wu and Mandrand 1993; Malki et al. 1995; Akhmanova et al. 1998a; Verhagen et al. 1999). In the N-terminal region of these hydrogenases 7 conserved C residues can be identified. Obviously, they belong to two ferredoxin-like clusters (F' - clusters) that can be characterised by a $CX_{10-11}CxxCX_{14-18}C$ and a $HxxxCxxCxxxxxC$ motif, where x indicates any amino acid (c.f. Malki et al. 1995). There is evidence that these motifs indicate one [2Fe-2S] and one [4Fe-4S] cluster (Malki et al. 1995; Volbeda et al. 1995, 1996). Downstream, they are followed by 2 genuine [4Fe-4S]-ferredoxin-like (F-cluster) motifs that are characteristic for all [Fe]-hydrogenases. The first of these F-clusters has the consensus $DxxKCxxCxxCxxxC$. The second is located 32 amino acids downstream and exhibits a $CxxCxxCxxxCP$ consensus (Fig. 5). The region downstream of position 105 of the *hydA* protein harbours a "H-cluster" that is characteristic for the carboxy-terminal half of hydrogenases. DNA sequence analysis identifies three clusters of strictly conserved motifs (Wu and Mandrand 1993). The first cluster starts at position 301, or 303 of the *hydC* protein of *D. vulgaris*. The *Neocallimastix* sequence exhibits the exact consensus sequence $FTSCCPxWxxxxExxxPxxxxxxSxxxSP$ (cluster 1). At position 355 the next cluster follows with a $VxxMPCxxKKxExxR$ motif (cluster 2). The more downstream located cluster 3 starts at position 476 (*DvhyC*) or 347, respectively, of *DvhydA*. The consensus sequence is $VAVxxGx_{19}FxExMACxGGCx_2GGGxP$ (c.f. Wu and Mandrand 1993). This region also contains five cysteine residues that are invariably conserved at residues 341, 342, 397, 552, and 556 of the deduced *HydL2* amino acid sequence. In addition to these conserved cysteine residues, three methionine residues are strictly conserved in all [Fe]-hydrogenases. Two of these residues are located in the clusters 2 and 3, at amino acids 395 and 550, respectively. The third methionine is present within a conserved AA motif, i.e. $TGGVMEAAxR$, located

Figure 6. Schematic representation of the structure of the various [Fe]-hydrogenases, the motifs are not to scale (A) *Nyctotherus ovalis*, (B) *Thermotoga maritima* HydA, (C) *Neocallimastix* sp. L2 HydL2, *Trichomonas vaginalis* “long” (AAG31037.1), (D) *Clostridium acetobutylicum* HydA, *Desulfovibrio fructosovorans* HndD, (E) *Trichomonas vaginalis* TvhydA, TvhydB, (F) *Desulfovibrio fructosovorans* HydA. The hydrogenosomal hydrogenases possess a (putative) N-terminal targeting signal. Also the bacterial hydrogenases potentially possess cellular sorting signals: F, ferredoxin-like [Fe-S] clusters, F', [Fe-S] clusters, H-cluster, active center. The Nuo E', 'Nuo F' blocks in (A) indicate ORFs with substantial homology to *E. coli* NuoE and NuoF, respectively the 24 kDa and 51 kDa subunits of mitochondrial complex I (see Akhmanova et al 1998a).

at AA position 471 of HydL2. It has been proposed that the methionines present in the H-clusters participate in iron binding. Thus, all motifs and signature sequences strongly suggest that the *hydL2* gene encodes a [Fe]-hydrogenase that shares significant similarities with the [Fe]-hydrogenases *hydC* of *Desulfovibrio vulgaris* and *hndD* of *Desulfovibrio fructosovorans* but also with the hydrogenase of the anaerobic ciliate *Nyctotherus ovalis* (Fig 6).

Genomic organisation and expression of the hydL2 gene

Southern blotting suggested that not more than two hydrogenase genes are present in the genome of *Neocallimastix* sp. L2. Evidence in favour of a single gene is provided by hybridisation with a labelled *hydL2* probe after EcoRI digestion that identified a single, heavily hybridising band (Fig 7a). The coding region contains a single EcoRI cleaving site near to the 5' end of the gene. Since the probe will only detect the downstream part of the

Figure 7. Southern blots with genomic DNA of (a) *Neocallimastix* sp. L2, and (b) *Piromyces* sp. E2. The genomic DNA was digested with the restriction enzymes Clal (C), EcoRI (E), and Xbal (X), and probed with a 357 bp fragment of the *hydL2* cDNA (position 1221-1578 of the cDNA). Since the weak cross-hybridising (*) of the EcoRI digest cannot be interpreted unequivocally, a discrimination between 1 or 2 genes is not possible. Since there are no Clal and Xbal restriction sites in the *hydL2* cDNA, the presence of introns in the coding region of the hydrogenase gene is likely.

Figure 8. Northern blot; poly (A)⁺ RNA of *Neocallimastix* sp. L2 was probed with the 357 bp fragment of the *HydL2* cDNA. The length of the transcript is 3200 nt.

hydrogenase, a minimal length of 1865 bp of the hybridising fragment will be expected. The weak band (indicated by an asterisk) found in the genomic DNA digested with EcoRI might nevertheless be indicative of a second gene. Since the cloned cDNA does not possess Clal and Xbal restriction sites, only sequencing of the complete *hydL2* gene including the upstream and downstream regions can answer the question whether there are one or two closely related hydrogenase genes in *Neocallimastix* sp. L2. Southern blotting of *Piromyces* sp. E2 genomic DNA might also be indicative for the presence of two slightly divergent hydrogenase genes (Fig. 7b), but also here only sequencing of the complete gene including leaders, trailers and potential introns will allow to give the definitive answer. The hybridisation of poly (A)⁺ RNA isolated from *Neocallimastix* sp. L2 with the *hydL2* probe argues for the presence of only one transcribed hydrogenase gene, since Northern blotting revealed the presence of a single band (Fig. 8). However, since the length of this RNA (3200 nucleotides) exceeds the length of the isolated cDNA (2468 bp), the presence of two (similar) transcripts cannot be excluded. The length difference between the ORF and the mRNA is due to transcription of the AT-rich non-coding regions upstream and downstream of the ORF, as in other genes from *Neocallimastix* and *Piromyces* (c.f. Reymond et al. 1992; Durand et al. 1995; Fanutti et al. 1995; Akhmanova et al. 1998b, 1999). The sequence composition of the leaders and trailers precludes cloning of full-length cDNAs in these organisms.

There is no evidence for an operon-like structure of the hydrogenase gene of *Neocallimastix* or the presence of genes encoding potential diaphorases similar to those present in *Nyctotherus ovalis* (not shown).

Phylogenetic analysis

Phylogenetic analysis of the deduced HydL2 amino-acid sequence of *Neocallimastix* sp L2 by the neighbor-joining and maximum-likelihood algorithms (Fig 9) was hampered by notoriously low bootstrap values. Using neighbor joining, only one bootstrap value was higher than 50%: the clustering of the [Fe]-hydrogenases of *Desulfovibrio vulgaris* and *Nyctotherus ovalis* was supported by 57%. The grouping of *Neocallimastix*, *Trichomonas* and *Thermotoga* received only a support of 42%, the clustering of *Neocallimastix* and *Trichomonas* 40% (not shown). Maximum-likelihood analysis using conserved blocks of sequences supports a monophyly of the hydrogenases of *Nyctotherus ovalis* and *Desulfovibrio vulgaris* with a reliability value of 78%, the clustering of *Neocallimastix*, *Trichomonas* and *Thermotoga* is supported by a value of 66% (Fig 9). Notably, the hypothesis assuming polyphyly of the *Neocallimastix*/*Trichomonas*/*Thermotoga* triplet and the *Desulfovibrio*/*Nyctotherus* doublet could not be rejected at the 5% confidence level.

Figure 9. Phylogenetic analysis: a maximum likelihood tree was generated as described in materials and methods. Only bootstrap values above 50% are indicated. Non-monophyly of the *Neocallimastix*/*Trichomonas*/*Thermotoga* triplet and the *Desulfovibrio*/*Nyctotherus* doublet could not be rejected at the 5% confidence level. *Clostridium acetobutylicum* (AAA85785.1), *Clostridium thermocellum* (AAD33071.1), *Desulfovibrio fructosovorans* (AAA87057.1), *Neocallimastix* sp L2 (AY033895), *Trichomonas vaginalis* (AAG31037.1), *Thermotoga maritima* (hydD, AAD35293.1|AE001705_4), *Thermotoga maritima* (hydA, AAD36496.1|AE001794_12), *Desulfovibrio vulgaris* (CAA40970.1), *Nyctotherus ovalis* (CAA76373.1).

Discussion

We have provided evidence for the presence of a [Fe]-hydrogenase in the hydrogenosomes of the anaerobic chytridiomycete fungus *Neocallimastix* sp. L2. This hydrogenase could be localised in the hydrogenosomes by immuno-gold labeling on ultra-thin sections of fixed *Neocallimastix* cells and by subcellular fractionation of the homogenates of axenic cultures (Fig. 1, 2; Table 1). Since the hydrogenase activity of isolated hydrogenosomes is highly sensitive against CO (Fig. 3), it has to be concluded (i) that this hydrogenase belongs to the [Fe]-only type, and (ii) that a significant contribution to the hydrogenosomal hydrogen formation by other hydrogenases can be excluded. [NiFe]-hydrogenases, for example, are not inhibited by CO concentrations of about 3-4 μmol that already cause a 50% inhibition of the hydrogenase activity in isolated hydrogenosomes of *Neocallimastix* sp. L2 (Adams 1991; Payne et al. 1993). Therefore, the inhibition of the hydrogenase activity in isolated hydrogenosomes of *Neocallimastix* sp. L2 for about 90% by less than 5 μmol CO (Fig. 3), excludes any significant [NiFe]-hydrogenase activity in the hydrogenosomes of *Neocallimastix*. Thus, our data strongly suggests that solely the organellar [Fe]-hydrogenase accounts for the observed hydrogenase activity.

The isolation of a gene (*hydL2*) with substantial homology to pro- and eukaryotic [Fe]-hydrogenases corroborates this interpretation (Fig. 4, 5, 6). Molecular genetics failed to provide evidence for the presence of [NiFe]-hydrogenases in anaerobic chytrids: using PCR with degenerated primers directed against conserved regions of [NiFe]-hydrogenases, we were unable to identify any gene with substantial homology to [NiFe]-hydrogenases (not shown). On the other hand, DNA sequence analysis of the gene *hydL2* revealed the presence of all conserved motifs that are characteristic for [Fe]-hydrogenases (Fig. 5, 6; Adams 1990; Wu and Mandrand 1993; Adams and Stiefel 1998). Northern blotting shows that this gene is abundantly expressed (Fig. 8), and Western blotting with a heterologous antiserum against a [Fe]-hydrogenase has confirmed the presence of cross-reacting proteins in the hydrogenosomes of *Neocallimastix* (Fig. 2). The presence of an N-terminal extension exhibiting all characteristics of a hydrogenosomal import signal (van der Giezen et al. 1997, Akhmanova et al. 1998b, 1999; see below) is in agreement with our localisation studies. Since the largest of the cross-reacting proteins has an apparent size of 67 kDa, it is likely that this protein is the mature product of the *hydL2* gene after import into the hydrogenosome and cleaving of the putative signal peptide. It remains to be shown whether the two smaller bands (60 and 58 kDa) observed in Western-blotting represent processing or degradation products of the hydrogenase, or whether they are due to unspecific binding.

The partially purified hydrogenase from *Trichomonas vaginalis* hydrogenosomes possesses a similar molecular mass (approximately 64 kDa as determined by SDS-PAGE) and exhibits a high sensitivity against CO. These data and EPR spectra suggest that this hydrogenase is (i) a [Fe]-hydrogenase and (ii) responsible for the bulk of hydrogenase activity in *Trichomonas vaginalis* (Payne et al. 1993). The two genes *hydA* and *hydB* of *T. vaginalis* encode [Fe]-hydrogenases that might be imported into the

hydrogenosomes, but their calculated molecular mass is too low to account for the predominant hydrogenosomal enzyme (Bui and Johnson 1996). The alignment shown in Figs 5 and 6 reveals that these two hydrogenases belong to the "short type" [Fe]-hydrogenases, and one might postulate that the hydrogenase that has been purified by Payne et al (1993) belongs to the "long type" as the hydrogenosomal hydrogenases of *Neocallimastix* and *Nyctotherus*. These hydrogenases are clearly representatives of the "long" type, i.e. they are characterised by 2 additional F'-clusters. The recently described gene encoding a putative "long-type" hydrogenase of *Trichomonas* has not been characterised in more detail. The sequence is still incomplete, but it might encode a hydrogenase that is larger than 60 kDa (Horner et al 2000).

The ORF encoding the putative hydrogenosomal [Fe]-hydrogenase starts with a N-terminal extension of 38 amino acids, that has the potential to function as a mitochondrial targeting signal (Fig 4, 5). Such N-terminal extensions - albeit shorter ones - have been described for a number of hydrogenosomal proteins of chytrids and, most likely, they function as hydrogenosomal targeting signals (Brondijk et al 1996, van der Giezen et al 1997, 1998, Akhmanova et al 1998b, 1999, Hackstein et al 1999). Therefore, the presence of putative targeting signals is in agreement with our localisation studies (Fig 1, 2).

Notably, the phylogenetic analysis cannot prove a close relationship between the [Fe]-hydrogenase of *Trichomonas* and *Neocallimastix* - even if the alignment includes only residues that are shared by both ORFs. Also, a close relationship with the ciliate hydrogenase and "long"-type hydrogenases from the various *Desulfovibrio* species is evident (Fig 9). Because of the small number of eukaryotic [Fe]-hydrogenase genes, our data can neither support nor reject the hypothesis that the hydrogenases from the hydrogenosomes of anaerobic chytrids and protists might be monophyletic, and potentially share a common ancestor with the hydrogenases of *Desulfovibrio* species that belong to the δ -proteobacteria. The Martin and Muller hypothesis (Martin and Muller 1998) postulates a common descent of hydrogenosomes and mitochondria but the hydrogenases from *Neocallimastix* and *Nyctotherus* are rather unrelated to hydrogenases of *E. coli*, and *Ralstonia (Alcaligenes) eutropha* representatives of proteobacteria that might be the closest relatives of the putative ancestors of the mitochondria (not shown). Since also clostridial hydrogenases are related to the hydrogenosomal hydrogenases from chytrids and ciliates (Fig 9), one might speculate as to whether lateral gene transfer in the course of evolution of the hydrogenosomes has been the cause of this unexpected result (Martin 1999). However, it has to be kept in mind that many bootstrap values in the phylogenetic trees are low, and it remains to be shown with a more extended data set that a common ancestry between δ -proteobacterial hydrogenases and hydrogenosomal hydrogenases might be significant. A similar situation has been described for the eukaryotic pyruvate ferredoxin oxidoreductases (PFOs, Horner et al 1999). Although phylogenetic analyses might favour a common origin for eukaryotic cytosolic and hydrogenosomal PFOs from a single eubacterial source, the available sequences and the unbalanced sampling can neither support an evolutionary origin from the common proteobacterial ancestor of mitochondria and hydrogenosomes nor a common ancestry of the PFO from any other

bacterial source. Thus, the data concerning two key enzymes of anaerobic eukaryotes are still insufficient to resolve a common ancestry of mitochondria and hydrogenosomes.

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Chapter 6

Hydrogenosomal adenylate kinases from the anaerobic chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2.

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Summary

The anaerobic chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2 lack mitochondria. Instead they contain a novel energy-generating organelle, called the hydrogenosome. The hydrogenosomal adenylate kinases (AKs) of these chytrids are encoded by nuclear genes. Phylogenetic analysis of the deduced AK amino acid sequences revealed that they are closely related to the mitochondrial adenylate kinase 2 subfamily. The deduced amino acid sequences lack a N-terminal extension, in contrast to the hydrogenosomal AK of the parabasalid *Trichomonas vaginalis*. Instead, the chytrid hydrogenosomal AKs carry a carboxy-terminal sequence - the tripeptide SKL - which in aerobic eukaryotes functions as a prototypical peroxisomal targeting signal. By expression of wildtype and mutant hydrogenosomal AK in the heterologous host *Hansenula polymorpha*, it is shown that the subcellular sorting of these hydrogenosomal enzymes in yeast indeed depends on the carboxy-terminal PTS1-like motif.

Introduction

Anaerobic eukaryotes lack mitochondria. Instead, many of them possess membrane-bound organelles with unique metabolic functions. Since these organelles convert pyruvate (or malate) to acetate and hydrogen, they are called hydrogenosomes (Müller 1993). The most widely studied hydrogenosomes are those of the parabasalids *Trichomonas vaginalis* and *Tritrichomonas foetus* (Müller 1997). However, hydrogenosomes of anaerobic chytrids differ in some structural and biochemical aspects from those of the trichomonads (Yarlett et al. 1986; Marvin-Sikkema et al. 1993a; Marvin-Sikkema et al. 1993b; Brul and Stumm 1994; Hackstein et al. 1998).

Adenylate kinases (E.C. 2.7.4.3) are nucleotide phosphotransferases which play a central role in the energy metabolism of hydrogenosomes and mitochondria. They are also found in other compartments of the eukaryotic cell, such as the cytosol, chloroplasts, and glycosomes (Schulz 1987; Länge et al. 1994; and references therein). The deduced amino acid sequences of 34 different adenylate kinases (AKs) from eukaryotes, and eubacteria, have been analysed so far. An alignment of these sequences has revealed that AKs can be grouped into two subfamilies, e.g. a 'large' and a 'small' type adenylate kinase (AK) subfamily with an apparent calculated molecular weight of 24 and 21 kDa, respectively (Schulz 1987). In eukaryotes, the smaller isoforms (AK1) are cytosolic, whereas the larger ones are located in the intermembrane space (AK2) and the matrix of mitochondria (AK3). All AKs are encoded by nuclear genes, synthesised in the cytoplasm, and hence must be targeted to their particular subcellular compartments. However, studies on the targeting and import of mitochondrial AKs revealed that the AK2 and AK3 isoforms lack a cleavable N-terminal leader sequence which is responsible for a mitochondrial sorting in the vast majority of the mitochondrial proteins (Hartl et al. 1989; Pfanner and Neupert 1990; Magdolen et al. 1992; Schricker et al. 1992; Bandlow et al. 1998). Most probably, the AK2 and AK3 isoforms are sorted to the mitochondria with the aid of cryptic targeting information

that is present in the primary sequence of these enzymes

Until now, only one hydrogenosomal adenylate kinase has been isolated and characterised, e.g. the hydrogenosomal AK of *Trichomonas vaginalis* (Lange et al 1994). This 24.5 kDa enzyme belongs to the 'large' type AK subfamily. Phylogenetic analyses of the deduced amino acid sequence by various methods, failed to provide unequivocal information about the ancestry of this enzyme within the 'large' type AK kinase subfamily, that also includes the eubacterial AKs. The hydrogenosomal AK of *T. vaginalis* is nuclear encoded, and DNA sequence analysis has revealed that the enzyme possesses a cleavable N-terminal extension of 9 amino acids, that might function as a hydrogenosomal targeting signal (Bradley et al 1997, Hausler et al 1997).

In order to better understand the role of AK in chytrid hydrogenosomes, we have investigated the molecular sequence, localisation, and functional analysis of targeting signals of the hydrogenosomal AKs from the anaerobic chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2.

Experimental procedures

Organism

The chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2 were cultured anaerobically at 39°C in M2 medium supplemented with 0.35% (w/v) fructose (Teunissen et al 1991). Wildtype and transformed cells of the yeast *Hansenula polymorpha* NCYC495 (leu1, ura3) were cultured in mineral medium supplemented with 0.5% glucose or 0.5% methanol as carbon source as described by Veenhuis et al (1979).

Isolation of adenylate kinase encoding cDNAs and gDNA

Biomass of *Neocallimastix* sp. L2 and *Piromyces* sp. E2 was harvested after 48 hours of growth at 39°C. Genomic DNA was prepared according to the protocol of Brownlee (1988). Total RNA was prepared by the guanidinium-chloride method (Chirgwin et al 1979). For preparation of poly(A)⁺ RNA, a mRNA purification kit (Pharmacia) was used. Adaptor-ligated cDNA was prepared according to the Clontech Marathon cDNA Isolation Kit. DNA polymerase chain reaction (PCR) was used to amplify adenylate kinase encoding genes from adaptor-ligated cDNA or genomic DNA. Oligonucleotide primers were based on highly conserved amino-acid regions of adenylate kinases (Schulz 1987, Lange et al 1994). Sequencing was performed with the ABI PRISM Model 310 automatic sequencer, using a dRhodamine terminator cycle sequencing ready reaction DNA sequencing kit (Perkin-Elmer Applied Biosystems).

Northern blotting

Poly(A)⁺ RNA was separated on a 1.2% agarose-formaldehyde gel and blotted to a Hybond N⁺ membrane (Amersham). The hdkAKL2.1 encoding gene (acc. no. AJ224658) was labelled by PCR with α -[32P]dATP and used as a probe. Hybridisation was performed in 0.5 M sodium phosphate buffer pH 7.0, 7% SDS, 1% BSA, 1 mM

EDTA, at 60°C. Filters were washed stringently with 50 mM sodium phosphate buffer pH 7.0, and 0.5% SDS, at 60°C.

Phylogenetic analysis

A database of representative 'large' type adenylate kinase sequences was assembled with ESEE (Schulz 1987; Cabot 1990; Lange et al. 1994). The Genbank and NBRF Protein Identification Resource database accession numbers are - *Saccharomyces cerevisiae* AKY2, A25411; *Schizosaccharomyces pombe* AK2, A33572; *Bos primigenus taurus* AK2, A24804; *Rattus norvegicus* AK2, D13601; *Oryza sativa* AK2, D10334; *Rattus norvegicus* AK3, D13062; *Bos primigenus taurus* AK3, A34442; *Saccharomyces cerevisiae* AKY3, S23568; *Homo sapiens* AK3, S16380; *Trichomonas vaginalis* AK, U07203; *Zea mays* AK, S43039; *Cyprinus carpio* AK1, S00394; *Homo sapiens* AK1, A00679; *Oryctolagus cuniculus* AK1, A00680; *Sus scrofa* AK1, A00682; *Bos primigenus taurus* AK1, A00681; *Gallus domesticus* AK1, A25327; *Schistosoma mansoni* AK1, M80542; *Giardia Lamblia* AK1, U19901; *Saccharomyces cerevisiae* URA6 (AK1), A33572; *Escherichia coli* AK, A24275; *Bacillus stearothermophilus* AK, M88104; *Bacillus subtilis* AK, JS0492; *Haemophilus influenzae* AK, X57315; *Paracoccus denitrificans* AK, S02814; *Streptomyces* sp. AK, xxxxxx; *Lactococcus lactis* AK, S17987; *Micrococcus luteus* AK, X14524; *Mycoplasma capricolum* AK, S02851. All insertions and deletions were removed, thus only positions common to all sequences were considered. A pairwise distance matrix based on residue identities in aligned positions was calculated and analysed with the neighbor-joining (NJ) method of Saitu and Nei (1987; NJ program in the PHYLIP 3.5c package; Felsenstein 1991) with 1000 replications. A representative phylogenetic analysis of adenylate kinases has been published previously (Lange et al. 1994).

Antibody preparation

The hdgAKL2.1 encoding gene (acc. no. AJ224658) of *Neocallimastix* sp. L2 was cloned into the pQE-32 expression vector (Qiagen) and transformed to *Escherichia coli* strain M15. Expression of the fusion-protein (harbouring a C-terminal 6 amino acid histidine tag) was performed as described by the QIAexpress Vector Kit protocol (Qiagen). The fusion protein was isolated under denaturing conditions by a Ni-NTA column and analysed by SDS-PAGE. A single protein band with an apparent molecular weight of 28 kDa was visible after silver-staining. This fusion protein was used for immunisation of a rabbit (Central Animal Laboratory, University of Nijmegen).

Localisation of the adenylate kinase gene product

Hydrogenosomal (Hyd) and cytosolic (Cyt) fractions were prepared anaerobically from *Neocallimastix* sp. L2 and *Piromyces* sp. E2 homogenates by differential centrifugation, according to the protocol of Marvin-Sikkema et al. (1993b) - with the following modification: the potassium phosphate buffer in the homogenisation medium was substituted for 20 mM potassium-4-(2-hydroxyethyl)-1-piperazine-ethane-sulfonic acid (K-HEPES) buffer pH 7.5. Hexokinase was assayed as described by Bergmeyer et

al. (1983). Malic enzyme activity was determined with the method of Kremer et al. (1989). Hydrogenase activity was measured as described by Marvin-Sikkema et al. (1993b). Adenylate kinase activity was assayed according to the method of Declerck and Müller (1987).

For the proteinase K protection experiment, isolated hydrogenosomes (about 0.4 mg protein) of *Neocallimastix* sp. L2 were incubated anaerobically (N₂-gas phase) in 0.5 ml 20 mM HEPES pH 7.4, 250 mM sucrose, and 2 mM DTT. Proteinase K (50 µg per 0.5 ml assay volume) or detergent (0.1% triton X-100 and 0.1% deoxycholate) was added when indicated (see Fig. 4b). After 30 minutes incubation on ice, the proteinase K digestion was terminated by the addition of 0.5 ml 15% (w/v) TCA. Protein was recovered by centrifugation. Aliquots of each incubation were separated on a 12.5% SDS-polyacrylamide gel, blotted to a PVDF membrane (Biorad), and probed with the AK antiserum.

For the high-salt and carbonate extraction analyses, the isolated hydrogenosomal fraction was resuspended in 100 mM Tris-HCL buffer pH 7.5. The hydrogenosomes were lysed by repeated freezing and thawing, and subsequently centrifuged (233000x g for 60 minutes, at 4°C). The resulting supernatant represents the matrix localised protein fraction. The pellet (hydrogenosomal membrane fraction) was extracted with 1 M KCl or 0.1 M sodium-carbonate (pH 11.5), during 60 minutes on ice. Subsequent centrifugation (233000x g for 60 minutes, at 4°C) resulted in a high-salt- or carbonate-extractable protein fraction (supernatants), containing membrane-associated proteins, and a non-extractable protein fraction (pellets), containing the integral membrane proteins. All extraction steps were performed at 4°C, and in the presence of the Complete[™] protease inhibitor mix (Boehringer Mannheim). Equal portions of all fractions were separated on a SDS-polyacrylamide gel, blotted to a PVDF membrane (Biorad), and probed with the AK antiserum.

For the immuno-localisation of AK with the aid of confocal laser scanning microscopy, *Neocallimastix* sp. L2 was cultured in medium M2 as described above and harvested after 24-48 hours of growth. Mycelia was mounted on a slide, shock frozen with liquid nitrogen, and fixed with methanol:acetic acid (95:5). The fixed mycelia was washed with PBS, incubated with the undiluted AK antiserum, washed again with PBS, and labelled with the Rhodamine conjugated goat anti-rabbit antibodies according to the protocol of Boehringer Mannheim. Rhodamine fluorescence was visualised by a confocal laser scanning microscope equipped with the appropriate filters. Immuno-gold labeling using the AK antiserum, and electronmicroscopy in *Neocallimastix* sp. L2, were performed as described previously by Marvin-Sikkema et al. (1993a).

Localisation of hdgakL2.1 and hdgakL2.1ΔSKL in Hansenula polymorpha

The wildtype *hdgAKL2.1* and mutant *hdgAKL2.1ΔSKL*, that lacks the codons for the carboxy-terminal tripeptide SKL, were cloned into the expression vector pX4-HENBSX (Rechinger, K.B., unpublished) behind the alcohol oxidase promoter. *HdgAKL2.1ΔSKL* was obtained by PCR on *hdgAKL2.1*, using a 3' degenerated primer. Electroporation of *H. polymorpha* NCYC495 (leu1, ura3) and synthesis of the *hdgAKL2.1* and *hdgAKL2.1ΔSKL* gene products upon methanol induction were

performed as described by Faber et al. (1994). The expression of the wildtype and mutant AK encoding genes in *H. polymorpha* was analysed by western blotting, using the AK antiserum. Immuno-gold labelling and electronmicroscopy were performed as described by Veenhuis et al. (1979). The transgenic *H. polymorpha* strains expressing the wildtype *hdgakL2.1* or mutant *hdgAKL2.1ΔSKL* gene, were subcellular fractionated by sucrose-gradient centrifugation as described by van der Klei et al. (1998). A total of 23 fractions of 1 ml, harvested from the bottom of the ultracentrifuge tubes, were analysed for protein (Biorad), alcohol-oxidase (Eggeling and Sahm 1978), and cytochrome-oxidase activity (Tolbert 1974). The obtained fractions were probed with the AK antiserum.

Results

Adenylate kinases of chytrids have a mitochondrial ancestry

Using a PCR-based cloning strategy, we isolated and sequenced different cDNAs encoding putative hydrogenosomal AKs from the anaerobic chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2. For *Neocallimastix* sp. L2, two different cDNAs (*hdgAKL2.1* AJ224658; *hdgAKL2.2* AJ224659) could be identified, with open reading frames of 693 bp and 696 bp, respectively. For *Piromyces* sp. E2, one cDNA (*hdgAKE2* AJ224660) could be isolated, with an open reading frame of 693 bp.

The corresponding genomic DNA fragments (see Genbank database acc. no. AY033893, AY033894, and AY033892) were isolated by PCR and sequenced. DNA sequence analysis and Southern blotting of genomic DNA from both chytrids confirmed the presence of two nuclear AK encoding genes in *Neocallimastix* sp. L2 and only one in *Piromyces* sp. E2 (results not shown). Northern blotting revealed that the AK genes from both chytrids were expressed (Fig. 1). For both *Neocallimastix* sp. L2 and *Piromyces* sp. E2, single transcripts could be observed. The estimated lengths of these transcripts were in good agreement with the lengths of the corresponding cDNAs.

Figure 1. Northern blot analysis with 5 µg of poly (A)⁺ RNA from the anaerobic chytrids *Neocallimastix* sp. L2 (**a**) and *Piromyces* sp. E2 (**b**). The *hdgAKL2.1* encoding gene (AJ224658) from *Neocallimastix* sp. L2 was used as a probe.


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hdgAKL2.1      MGCAQSKNSLRM-VIMGPPGSGKGTQAPVKVDITYCICHLATGDMRAAVKAGTPIGME
hdgAKL2.2      M.....-V.....
hdgAKE2        M...R...W...-.....L.
S. cerevisiae  MS.SE.I...-LI...A.....NLQERFHAA.....SQIAK..QL.L.
T. vaginalis   MLSTLAKRFA.GKKD..V.FF...V.....KLEKEFNLYQIS...A...EIRGQ..L.KR

AKKIMDAGGLVSD EIVVNLIKENLDT-KACRNGFILDGFPRTVAQAQKLD EQRNQKLDTAIELTVDDSLLFKRIT
.....-.....K.....
.....-.....E.....
.....Q.....D.M..M..DE.TNNP..K.....IP..E...Q..KEQGTP.EK...K...E..VA...
V.G.IES....D.DTIMDILQACMQKN-TDN..Y.F..I...IG.VE...AL.AKMGTP.THVLY.S.NIDE.RE.VC

GRLVHPASGRSYHKIFNPP-KVEGKDDITGEPLIQRSDDTEAALKRRLVAYHKQTVPVAEYKKKKGIWRGVDASQPPA
.....NQ.....D.....
.....S.-.....I.....A.....D.....
...I.....-EDM...V...A.V...NAD...A...A..E.IVDF...T...A.....
...F..G...V...VT...-KPMT.....I.K...PEVFNQ.MNQYFGTFQPCID..S...LQTFPVDGQ.I

AVWAQMQAIFSERKSQS SKL
.....KNKTN SKL
.....FN SKL
T...DILNKLGKD
D.VHKLHAALQ

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Figure 2. Alignment of the deduced amino acid sequences hdgAKL2.1 (AJ224658) and hdgAKL2.2 (AJ224659) from *Neocallimastix* sp. L2, and hdgAKE2 (AJ224660) from *Piromyces* sp. E2. For comparison, the deduced AA sequences of the hydrogenosomal AK from *Trichomonas vaginalis* (TvagHydAK, U07203), and the mitochondrial AKY2 from *Saccharomyces cerevisiae* (ScerAKY2, A25411) are included in the alignment. The putative carboxy-terminal PTS1-like motif - the tripeptide SKL - is boxed. The N-terminal targeting signal of the hydrogenosomal AK from *Trichomonas vaginalis* is underlined. Dots represent identical residues when compared to the hdgAKL2.1 AA sequence, whereas dashes represent gaps.

The deduced amino acid sequences (shown in Fig. 2) predicted proteins with a molecular weight of approximately 27 kDa. This size identifies the encoded enzymes as ‘large’ type adenylate kinases (Schulz 1987; Länge et al. 1994) which are characteristic for eubacteria, mitochondria, and chloroplasts. A comparison of the deduced AA sequences with the primary sequences of 29 different AKs of cytosolic, mitochondrial, chloroplast, hydrogenosomal and eubacterial origin, showed the highest amino-acid identity (49–60%) to the mitochondrial AK2 subfamily (Table 1).

Phylogenetic analysis of the deduced amino acid sequences by the neighbor-joining method (Fig. 3) revealed that the AKs of both chytrids cluster with the mitochondrial AK2s of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The branching order is supported by high bootstrap values.

Table 1 Positional amino-acid identities for the hydrogenosomal adenylate kinase hdgAKL2.1 of *Neocallimastix* sp. L2 when compared to other AKs. L, large type adenylate kinase; S, 'small' type AK. Deduced amino-acid sequences used for the phylogenetic analysis are indicated by asterisks (see Fig. 3)

Localisation	Organism	Isoform	Type	Identity (%)
mitochondrial	<i>Saccharomyces cerevisiae</i>	AKY2*	L	60
intermembrane space	<i>Schizosaccharomyces pombe</i>	AK2*	L	57
	<i>Bos primigenus taurus</i>	AK2*	L	55
	<i>Rattus norvegicus</i>	AK2*	L	55
	<i>Oryza sativa</i> A	AK2*	L	49
mitochondrial	<i>Rattus norvegicus</i>	AK3*	L	38
matrix	<i>Bos primigenus taurus</i>	AK3*	L	38
	<i>Saccharomyces cerevisiae</i>	PAK3*	L	35
	<i>Homo sapiens</i>	AK3*	L	34
hydrogenosome	<i>Trichomonas vaginalis</i>	AK*	L	36
chloroplast	<i>Zea mays</i>	AK*	L	34
cytosol	<i>Cyprinus carpio</i>	AK1	S	28
of eukaryotes	<i>Homo sapiens</i>	AK1	S	28
	<i>Oryctolagus cuniculus</i>	AK1	S	28
	<i>Sus scrofa</i>	AK1	S	28
	<i>Bos primigenus taurus</i>	AK1	S	28
	<i>Gallus domesticus</i>	AK1	S	27
	<i>Schistosoma mansoni</i>	AK1	S	26
	<i>Giardia lamblia</i>	AK1	S	24
	<i>Saccharomyces cerevisiae</i>	URA6*	S	21

Localisation	Organism	Isoform	Type	Identity (%)
eubacteria	<i>Escherichia coli</i>	AK	L	43
	<i>Bacillus stearothermophilus</i>	AK	L	41
	<i>Bacillus subtilis</i>	AK	L	40
continue eubacteria	<i>Haemophilus influenzae</i>	AK	L	39
	<i>Paracoccus denitrificans</i>	AK	L	39
	<i>Streptomyces sp.</i>	AK	L	36
	<i>Lactococcus lactis</i>	AK	L	36
	<i>Micrococcus luteus</i>	AK	L	30
	<i>Mycoplasma capricolum</i>	AK	L	29

Table 2. Enzyme activities (U/mg protein) in the cytosolic (Cyt) and hydrogenosomal (Hyd) fractions of *Neocallimastix* sp. L2 and *Piromyces* sp. E2.

Organism	Enzyme	Cyt	Hyd	F
<i>Neocallimastix</i> sp. L2	hexokinase	0.91	ND	-
	malic enzyme	0.54	19.64	36.4
	hydrogenase	1.46	58.10	39.8
	adenylate kinase	0.11	4.92	44.7
<i>Piromyces</i> sp. E2	hexokinase	0.83	ND	-
	malic enzyme	0.19	20.60	108.4
	hydrogenase	1.90	172.45	90.8
	adenylate kinase	0.10	11.36	113.6

F, factor indicating the enrichment of the enzyme activities in the hydrogenosomal fraction, when compared to the enzyme activities in the cytosolic fraction; ND, not detectable within the limits of the assay sensitivity (1 nmol/min/mg protein).

Figure 3. Unrooted neighbor-joining (NJ) tree, reconstructed from the deduced *Neocallimastix* sp. L2 and *Piromyces* sp. E2 hydrogenosomal AK sequences, and a representative subset of ‘large’ type adenylate kinase sequences (see Experimental procedures for accession numbers). The distantly related *Saccharomyces cerevisiae* URA6 (AK1) sequence was used as outgroup for the phylogenetic analysis. Bootstrap values at the nodes are based on 1000 replicates. The branching order is supported by high bootstrap values.

The AK genes encode hydrogenosomal proteins

Neocallimastix sp. L2 homogenates were fractionated by differential centrifugation, in order to study the subcellular localisation of AK. Enzyme assays (Table 2) revealed that the AK activity co-fractionated with the activities of the hydrogenosomal marker enzymes hydrogenase (Marvin-Sikkema et al. 1993a) and malic enzyme (van der Giezen et al. 1997a), suggesting that the encoded AKs are present in the chytrid hydrogenosome.

Furthermore, a polyclonal antiserum directed against the recombinant hdgAKL2.1 protein, cross-reacted with a protein (Fig. 4a, lane 1 and 3) which was significantly enriched in the hydrogenosomal fraction (lane 3). The apparent molecular weight of this protein (28 kDa) is within the expected size range of the ‘large’ type adenylate kinases after SDS-polyacrylamide gelelectrophoresis (Schulz 1987; Länge et al. 1994).

The localisation of AK in the hydrogenosomal compartment was confirmed by a proteinase K protection experiment. Western blotting revealed that the hydrogenosomal AK was protected against proteinase K, unless the addition of detergent solubilised the hydrogenosomal membrane (Fig. 4b). Disruption of the hydrogenosomes by repeated freezing and thawing revealed that the majority of the AK is associated with the hydrogenosomal membrane (Fig. 4c, compare lanes 1, 2 and 3). Similar results were found after high salt extraction of the hydrogenosomal membrane (Fig. 4c, compare lanes 3, 4 and 5). However, carbonate extraction analysis of the hydrogenosomal membrane fraction clearly revealed that AK is not an integral membrane protein, since it can be completely extracted from the hydrogenosomal membranes with carbonate (Fig. 4c, compare lanes 3, 6 and 7).

Immunocytochemistry with the aid of confocal laser scanning microscopy showed that the AK labelling was restricted to particles with diameters between 0.5 and 2.5 μm (Fig. 5a). This size-distribution correlates with the size of chytrid hydrogenosomes (Yarlett et al. 1986; Marvin-Sikkema et al. 1992; Munn 1994). Immuno-gold labelling and electronmicroscopy revealed that these organelles were indeed hydrogenosomes and that the labeling with the AK anti-serum was nearly exclusively confined to the hydrogenosomal membranes (Fig. 5c).

Figure 4. Western blot analysis, using the adenylate kinase antiserum, showing the presence of AK gene products in (A) Homogenate, cytosolic, and hydrogenosomal fractions of *Neocallimastix* sp. L2. Lane 1, homogenate; 2, cytosolic protein fraction; and 3, total hydrogenosomal fraction. (B) Hydrogenosomes after incubation with proteinase K and/or detergent (Triton X-100/deoxycholate). +, added; –, omitted. (C) Different protein fractions obtained after lysis (freeze-thawing) of hydrogenosomes, and high salt (1 M KCl) or carbonate extraction (0.1 M carbonate, pH 11.5) of the hydrogenosomal membrane fraction. Lane 1, total hydrogenosomes; 2, hydrogenosomal matrix protein; 3, hydrogenosomal membrane-associated protein; 4, high salt extractable protein; 5, high salt extraction resistant membrane protein; 6, carbonate extractable protein; 7, integral membrane (carbonate resistant) protein.

Figure 5. Immunocytochemical localisation of AK in *Neocallimastix* sp. L2 with the aid of confocal laser scanning microscopy (A, B), and electron-microscopy (C). (A) confocal laser scanning microscopy showed that the anti-AK labeling is restricted to particles (indicated by white arrows) with diameters between 0.5-2.5 μm . The bar represents 10 μm . The corresponding mycelia-section of *Neocallimastix* sp. L2, as seen by light interference microscopy, is shown in panel (B). Black arrow-heads point to mycelium of *Neocallimastix* sp. L2. (C) Immuno-gold labeling and electronmicroscopy revealed that the anti-AK is confined to the hydrogenosomal membranes. Black arrows point to (labeled) internal membrane structures. H, hydrogenosome; R, ribosomal globules (Munn 1994). The bar represents 0.5 μm .

The hydrogenosomal AKs have a putative PTS1-type peroxisomal targeting signal

The hydrogenosomal AKs are nuclear-encoded (see above; Länge et al. 1994; Jaussi 1995). Therefore, they have to be synthesised in the cytoplasm, and imported into the developing hydrogenosomes. The N-terminal part of the deduced AA sequences of the full-length chytrid cDNAs did not provide any indication for the presence of a potential presequence, in clear contrast to the hydrogenosomal AK of *T. vaginalis* (Fig. 2; Länge et al. 1994). Unexpectedly, the deduced AA sequences of all three cDNAs terminated with the tripeptide SKL (Fig. 2), the prototypical peroxisomal targeting signal PTS1 (Gould et al. 1990; Keller et al. 1991; de Hoop and Ab 1992). Since the analysis of the carboxy termini of 11 different mitochondrial AKs (Schulz 1987; Länge et al. 1994) did not provide evidence for peroxisomal targeting signals, the presence of a PTS1-like motif in the hydrogenosomal AKs from two different chytrid species is unlikely to be due to chance.

Hydrogenosomal AK is sorted to the peroxisomes of Hansenula polymorpha

The significance and function of the putative targeting signals can only be studied in a heterologous system, since a transformation system for chytrids is not available. Therefore, cells of the yeast *Hansenula polymorpha* were transformed with plasmids carrying either the wild-type (*hdgAKL2.1*) or a mutant hydrogenosomal AK gene (*hdgAKL2.1ΔSKL*), that lacks the codons encoding the C-terminal SKL motif. Clones were recovered that express the recombinant protein under the control of an alcohol oxidase promotor. Western blotting with the AK antiserum confirmed that the recombinant AK genes were expressed in both yeast strains after the induction with methanol (Fig. 6: indicated by +). Under non-induced conditions (Fig. 6: indicated by -), a weak band of a cross-reacting protein of similar size was identified. Also western blotting of a homogenate derived from a methanol induced *H. polymorpha* wild type strain, revealed a similar result (Fig 6). Most probably, the AK antiserum cross-reacts with an endogenous mitochondrial AK of the host *H. polymorpha*.

Figure 6. Western blot analysis, using the AK antiserum, showing the expression of wild-type *hdgAKL2.1* (A) and mutant *hdgAKL2.1ΔSKL* (B) in homogenates from the respective transgenic *Hansenula polymorpha* strains, grown under non-induced (-) or methanol-induced (+) conditions. (C) Endogenous mitochondrial AK, present in a methanol-grown (+) *H. polymorpha* wildtype strain, cross-reacts weakly with the AK antiserum.

Immuno-gold labelling with the AK antiserum and electronmicroscopy of the different transgenic yeast strains revealed that the adenylate kinase with the C-terminal SKL motif (hdgAKL2.1) is predominantly imported into the matrix of the peroxisomes (Fig. 7a). The AK that lacks the SKL motif (hdgAKL2.1 Δ SKL) is not imported into the peroxisomes; the label accumulates in the cytoplasm and the nucleus (Fig. 7b). A methanol induced *H. polymorpha* wild-type strain, showed only a weak (background) labeling with anti-AK over all compartments of the yeast cell (Fig. 7c). The localisation of the hydrogenosomal AK in the peroxisomes of *H. polymorpha* was confirmed by subcellular fractionation. Homogenates prepared from methanol-grown *H. polymorpha* cells, that express the wild-type *hdgAKL2.1* or the mutant *hdgAKL2.1* Δ SKL gene, were fractionated by sucrose-gradient centrifugation. Enzyme assays and western blotting of the recovered fractions (Fig. 8), revealed that the *hdgAKL2.1* gene product co-sediments with the alcohol-oxidase activity. These results confirmed that the wild-type hydrogenosomal hdgAKL2.1 is localised in the peroxisomes of the transgenic *H. polymorpha* strain.

Figure 7. Immuno-gold labeling of AK in the transgenic (A, B) and wild type (C) *H. polymorpha* strains. (A) In the transgenic *H. polymorpha* strain, expressing the wild-type *hdgAKL2.1*, AK is located almost exclusively in the matrix of the peroxisomes. (B) Expression of *hdgAKL2.1* Δ SKL (lacking the codons encoding the C-terminal tripeptide SKL) in *H. polymorpha*, revealed that labeling of the peroxisomes is abolished. Instead, the cytoplasm and the nucleus are heavily labeled. (C) In the methanol-grown *H. polymorpha* wild-type strain, only a weak (background) labeling over all cellular compartments could be shown. M, mitochondrion; P, peroxisome; N, nucleus; V, vacuole. Bars represent 0.5 μ m.

Figure 8. Enzyme assays and western blotting of the different fractions obtained after sucrose gradient centrifugation of homogenates derived from the transgenic *H. polymorpha* strains expressing the hydrogenosomal *hdgAKL2.1* gene. The peroxisomal (alcohol-oxidase containing) and the mitochondrial (cytochrome-oxidase containing) fractions were identified by enzyme assays. For western blotting of the obtained fractions the AK antiserum was used. ○, protein; ■, alcohol-oxidase; ▲, cytochrome-oxidase. Fraction 1 represents a 'dense' sucrose fraction, whereas fraction 23 represents a 'light' sucrose fraction.

Discussion

In the present paper, we have shown by subcellular fractionation, enzyme assays, immunocytochemical experiments, and molecular genetic studies, that the hydrogenosomes of the anaerobic chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2 possess functional AKs. We have isolated cDNAs and the corresponding nuclear genes that appear to encode these enzymes. Phylogenetic analysis of the deduced amino acid sequences has revealed that the hydrogenosomal AKs have a mitochondrial ancestry (Fig. 3). Such a descent has also been postulated for the hydrogenosomal malic enzyme and the β -subunit of the hydrogenosomal succinyl-CoA synthetase from *Neocallimastix* sp. L2 (Brondijk et al. 1996; van der Giezen et al. 1997a).

Like their mitochondrial orthologues, the genes encoding these hydrogenosomal enzymes are localised on the nuclear genome. The targeting to mitochondria frequently depends on the presence of a N-terminal leader peptide with a length of 20 - 80 amino acids, that is removed upon import (Hartl et al. 1989; Pfanner and Neupert 1990). Also the hydrogenosomal malic enzyme and the β -subunit of succinyl-CoA synthetase possess similar N-terminal extensions, that are cleaved off upon import into the hydrogenosomes (Brondijk et al. 1996; van der Giezen et al. 1997a). Expression- and immuno-localisation studies of the hydrogenosomal malic enzyme in the heterologous host *Hansenula polymorpha* revealed that the cleavable N-terminal extension is sufficient to direct this protein to the mitochondria (van der Giezen et al. 1998). Consequently, it has been postulated that the sorting of proteins to the hydrogenosomes of chytrids is similar to mitochondrial import.

This interpretation is consistent with studies on the hydrogenosomes of trichomonads. All hydrogenosomal proteins of the trichomonads studied so far, including the hydrogenosomal adenylate kinase of *Trichomonas vaginalis* (see Fig. 2), are characterised by short (5-14 amino-acid long) N-terminal leader sequences that are cleaved off upon import into the organelle. Recent *in vitro* import studies have confirmed the anticipated similarities with a "primitive" mitochondrial import machinery (Bradley et al. 1997; Dyall and Johnson 2000; Rotte et al. 2000).

In contrast to the hydrogenosomal AK of *T. vaginalis* (Länge et al. 1994), the hydrogenosomal AKs of *Neocallimastix* sp. L2 and *Piromyces* sp. E2 lack a cleavable N-terminal leader sequence (Fig. 2). The absence of such N-terminal leader sequences is characteristic for mitochondrial adenylate kinases, and underscoring the diversity of AK import signals, which parallels the diversity of AK localisation within different eukaryotes. The import of adenylate kinases into mitochondria of *Saccharomyces cerevisiae* depends on the first 12 N-terminal amino acids and on internal targeting and sorting information (Magdolen et al. 1992; Schricker et al. 1992; Bandlow et al. 1998). However, only 6-8% of the AK2Yp becomes incorporated into the mitochondrion, more than 90% stays in the cytoplasm and the nucleus (Bandlow et al. 1988). We have shown here, that expression and immunocytochemical localisation of the hydrogenosomal AK in the heterologous host *Hansenula polymorpha* (Fig 7) revealed no large degree of import of this enzyme into the mitochondria - regardless whether *H. polymorpha* expressed a wild-type or a truncated (Δ SKL) hydrogenosomal adenylate kinase (Fig. 7a,b). And similar to the AK2Yp in *Saccharomyces cerevisiae*, the vast majority of the hydrogenosomal AK from *Neocallimastix* sp. L2 is present in the cytosol and the nucleus of *H. polymorpha* - if the expressed hdgAK2 lacks the SKL motif (see Fig. 7b). In the presence of the SKL extension, nucleus and cytoplasm are virtually free of AK-labelling, while the peroxisomes are heavily labelled (see Fig. 7a). Thus, the SKL-extension is capable of overriding the internal targeting and sorting information that, in yeasts, seems to cause the characteristic distribution of AK2 in both cytoplasm (and nucleus) and mitochondria. The observed weak anti-AK labelling of mitochondria in both the transgenic and wild-type *H. polymorpha* strains (Fig. 7) is most likely the result of a cross-reaction with the endogenous yeast mitochondrial AK (see Fig. 6). The presence of only a few gold-grains over the mitochondria, do not

allow conclusions about potential differences in mitochondrial targeting between the SKL and Δ SKL variants.

The present data suggest that the sorting and import of AK into the hydrogenosomes of chytrids does not depend on a cleavable N-terminal extension. Furthermore, the nearly exclusive localisation in the hydrogenosomes, and not in the cytoplasm and the nuclei of the chytrids, suggests that the internal targeting and sorting information of the hdgAK is overrun by the carboxy-terminal SKL motif (Fig. 2). A similar phenomenon has been described by Danpure (1996). He was able to show that, in certain mammals, the import of the alanine:glyoxylate aminotransferase into mitochondria (guided by a cleaved N-terminal extension) is completely abolished by the addition of a C-terminal PTS 1 motif.

Unfortunately, these observation cannot answer the question whether this carboxy-terminal motif is necessary and sufficient for the import of the AK into the hydrogenosomes of chytrids. The same argument, of course, is also valid for a judgement of potential mitochondrial-like import of hydrogenosomal proteins by N-terminal extensions (Brondijk et al. 1997; van der Giezen et al. 1998). Therefore, we are unable to conclude whether the hydrogenosomal import of chytrids is of a mitochondrial or a peroxisomal type, or whether both mechanisms exist in these organelles. We also cannot exclude, that the chytrid hydrogenosomes use a hitherto unknown mechanism for sorting and import.

Moreover, immuno-gold labelling and subcellular fractionation studies have shown that hydrogenosomal AK is tightly associated with a hydrogenosomal membrane (Fig. 5c). This is not the case in the heterologous host *H. polymorpha*, where the hydrogenosomal AK is imported into the peroxisomal matrix (see Fig. 7a). However, also in *S. cerevisiae*, the localisation of AK2Y in the intermembrane space of mitochondria is not due to the presence of a specific sorting sequence (Bandlow et al. 1998). It has been speculated whether stable adhesion sites might be present in the intermembrane space which guarantee the proximity of the AK to an ADP/ATP translocator (Brdiczka et al. 1986). Since also a mitochondrial-like ADP/ATP translocator is present in the hydrogenosomal membranes of *Neocallimastix* sp. L2 (see chapter 4 of this thesis), a similar array might be responsible for the membrane localisation of the hydrogenosomal AK.

Until now, there is no information available that 'mitochondrial' chaperones, and other components of a mitochondrial-like import machinery, might be present in chytrid hydrogenosomes. On the other hand, it has been shown by Marvin-Sikkema et al. (1993a) that several hydrogenosomal proteins from *Neocallimastix* sp. L2 cross-react with an antiserum directed against the carboxy-terminal SKL motif. This suggests that, next to the hydrogenosomal AKs discussed in this paper, additional hydrogenosomal proteins might contain carboxy-terminal sequences similar to the prototypical PTS1-type peroxisomal targeting signal. Moreover, preliminary data reveal that chytrids possess and express genes with a significant homology to components of a peroxisomal import machinery, i.e. *pex5* and *pex6* (Voncken et al. unpublished). Therefore, it might be possible that the PTS1 motif is of functional significance for the sorting of proteins to the chytrid hydrogenosomes. Naturally, this

finding does not provide any answer on the question of how the hydrogenosomal proteins with N-terminal extensions are sorted and imported into hydrogenosomes of chytrids.

The experimental evidence presented here and the data published until now, argue for the presence of two different import machinery's in the hydrogenosomes of chytrids - notwithstanding that both "mitochondrial" (N-terminal) and "peroxisomal" (C-terminal PTS 1) targeting signals are found on hydrogenosomal proteins that share a mitochondrial ancestry. There are a number of hypotheses that might be suitable to resolve this apparent contradiction, but it is obvious that more information about the targeting of hydrogenosomal proteins and their import into the hydrogenosomes is required.

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Chapter 7

Hydrogenosomes: eukaryotic adaptations to anaerobic environments

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Summary

Like mitochondria, hydrogenosomes compartmentalise crucial steps of eukaryotic energy metabolism; however, this compartmentalisation differs substantially between mitochondriate aerobes and hydrogenosome-containing anaerobes. Because hydrogenosomes have arisen independently in different lineages of eukaryotic microorganisms, comparative analysis of the various types of hydrogenosomes can provide insights into the functional and evolutionary aspects of compartmentalised energy metabolism in unicellular eukaryotes.

Introduction

Compartmentalisation is one of the most intriguing attributes distinguishing eukaryotes from prokaryotes. Two well known cell compartments - mitochondria and plastids - arose from prokaryotic cells and house oxidative energy metabolism and oxygenic photosynthesis, respectively. Their current manifestation as organelles is the result of a long-lasting evolution that involved symbiotic association, substantial transfer of genes from the ancestral, endosymbiont to the nuclear genome of the host, differential loss of redundant genes and retargeting of the organellar proteins (Herrmann 1997; Danpure 1997; Martin and Herrmann 1998; Martin et al. 1998). Plastids and mitochondria still possess genomes, but ~90% of the proteins contained in these organelles are encoded by nuclear genes (Herrmann 1997; Martin and Herrmann 1998; Gray et al 1999). Obligatory anaerobic (or microaerophilic) unicellular eukaryotes lack plastids and mitochondria and, consequently, do not compartmentalise their energy metabolism (type I anaerobes: Müller 1993; Martin and Müller 1998; Müller 1998). Recent studies suggest that such protists once possessed mitochondria but secondarily lost them (Clark and Roger 1995; Roger et al. 1998; Hashimoto et al. 1998). Other groups of anaerobic protists also lack mitochondria and instead possess hydrogenosomes, organelles that compartmentalise the terminal steps of anaerobic energy metabolism (type II anaerobes: (Müller 1993; Embley et al. 1997; Martin and Müller 1998; Müller 1998).

Compartments for anaerobic energy metabolism

Hydrogenosomes are membrane-bound organelles of approximately 1 µm diameter, which, like mitochondria, produce ATP. However, they cannot use oxygen as an electron acceptor; rather, they reduce protons to molecular hydrogen (Müller 1993; Müller 1998). They have been found only in anaerobic protists such as the parabasalid flagellate *Trichomonas vaginalis*, the amoeboid flagellate *Psalteriomonas lanterna*, the anaerobic ciliate *Nyctotherus ovalis* and the anaerobic chytridiomycetes *Neocallimastix* and *Piromyces*. Recent evidence suggests that hydrogenosomes share a common ancestor with mitochondria (Martin and Müller 1998; Biagini et al. 1997; Embley et al. 1997; Sogin 1997). It has been postulated that the loss of all enzymes related to aerobic energy

	Amitochondriate anaerobes					Mitochondriate aerobes	
	type I Entamoeba	type II Trichomonas		type II Chytrids			
	Cytoplasm	Cytoplasm	Hydrog	Cytoplasm	Hydrog	Cytoplasm	Mitoch
enzyme forming pyruvate	pyruvate-phosphate dikinase	pyruvate kinase	malate DH ?	pyruvate kinase	malic enzyme	pyruvate kinase	malic enzyme
enzyme forming acetyl CoA	PFO	none	PFO	PFL	PFL	isocitrate lyase	PDH
terminal electron acceptor	acetyl CoA, acetaldehyde	pyruvate	ferredoxin H+	pyruvate acetyl CoA fumarate	H+	pyruvate	O ₂
fermentation products	acetate ethanol alanine	ethanol malate succinate lactate alanine glycerol	acetate H ₂ malate ?	acetate formate succinate lactate ethanol	acetate formate H ₂	ethanol lactate	CO ₂ , * H ₂ O
formation of ethanol	from acetyl CoA via acetyl-CoA reductase/ACDH	from acetaldehyde	none	from acetyl CoA via acetyl-CoA reductase/ACDH	none	from acetaldehyde	none
enzymes involved in ATP formation	PGK, acetate thiokinase, pyruvate-phosphate dikinase	PGK, PK, PEPCK	ASCT, STK	PGK, PK, PEPCK	ASCT, STK	PGK, PK	ASCT** STK
[H ₂]-hydrogenase	no	no	yes	no	yes	no	no
electron transport chain	-	-	no	-	no	-	yes
PMF ΔpH	-	-	yes ?	-	yes	-	yes

Table 1. Carbon and energy metabolism of amitochondriate, mitochondriate and hydrogenosome-bearing eukaryotes (adapted from Müller 1998) Abbreviations. Hydrog., hydrogenosome; mitoch., mitochondrion; ACDH, acetaldehyde dehydrogenase, ASCT, acetate-succinate CoA transferase; malate DH, malate dehydrogenase, PDH, pyruvate dehydrogenase; PEPCK, phosphoenolpyruvate carboxykinase, PFL, pyruvate-formate lyase; PFO, pyruvate-ferredoxin oxidoreductase; PGK, phosphoglycerate kinase; PK, pyruvate kinase, STK, succinate thiokinase (also known as succinyl-CoA synthetase); *, end products of oxidative mitochondrial metabolism, **, in Trypanosomatidae and *Fasciola hepatica* (van Hellemond et al 1998)

metabolism from hydrogenosomes was concomitant with the loss of their complete organellar genome (Sogin 1997; Martin and Müller 1998). In most cases, this was accompanied by the compartmentalisation of a characteristic set of enzymes, including hydrogenase and pyruvate : ferredoxin oxido-reductase (PFO). However, hydrogenosomes evolved in evolutionarily distant lines of unicellular eukaryotes and the question arises: are all hydrogenosomes the same? (Biagini et al 1997; Embley et al. 1997). New data are accumulating on the hydrogenosomes of anaerobic chytridiomycete fungi (Marvin-Sikkema et al. 1993a; Marvin-Sikkema et al. 1993b; Marvin-Sikkema et al. 1994; Akhmanova et al. 1998) and it is worthwhile making a comparison with the hydrogenosomes of *T. vaginalis* (Müller 1993), which are the best studied (Table 1).

Hydrogenosomes of anaerobic chytrids are different

Anaerobic chytrids are important symbionts in the gastrointestinal tract of herbivorous mammals (Orpin 1994; Trinci et al. 1994). Various phylogenetic analyses have shown that anaerobic chytrids cluster with their aerobic relatives (Li and Heath 1992; Li et al. 1993; Akhmanova et al. 1998), suggesting that they evolved from aerobic, mitochondriate fungi. Furthermore, phylogenetic analysis of the cytosolic and endoplasmic reticulum (ER)-type heat-shock protein 70 (Hsp70) chaperonins places the anaerobic chytrids among the aerobic yeasts and fungi (Fig. 1). Like all other hydrogenosomes studied to date, the chytrid organelle possesses an [Fe]-hydrogenase (Payne et al. 1993; Bui and Johnson 1996; F.G.J. Voncken et al., see Chapter 5). All known hydrogenosomes produce ATP by substrate-level phosphorylation via acetate-succinate-CoA transferase (ASCT) and succinyl-CoA synthetase (also known as succinate thiokinase, STK; Müller 1993; Marvin-Sikkema et al. 1993) (Table 1). However, the formation of pyruvate and acetate, the key metabolites in the hydrogenosomes of *Neocallimastix* and *Trichomonas*, occurs by different routes and, to some extent, in different compartments. In trichomonads, pyruvate is formed in the cytoplasm by pyruvate kinase. It is then taken up by the hydrogenosome and decarboxylated by PFO to acetyl CoA and CO₂ (Müller 1993). In the chytrid *Neocallimastix* sp. L2, pyruvate can be formed in the cytoplasm from phosphoenolpyruvate, but is also generated in the hydrogenosome by malic enzyme, which decarboxylates imported malate (Marvin-Sikkema 1993a; van der Giezen et al. 1997).

Figure 1. Phylogenetic position of the anaerobic chytrid *Piromyces* sp. E2 based on the comparison of the major cytosolic and endoplasmic reticulum (ER)-type heat-shock protein 70 (Hsp70) from eukaryotes. Phylogenetic analysis was performed using Hsp70.1 and Hsp70.2 from *Piromyces* sp. E2 (accession numbers AJ238886 and AJ238887, respectively) using PHYLIP 3.4. The sequence used corresponds to amino acids 13-359 of *Saccharomyces cerevisiae* SSA1. A Fitch-Margoliash tree was obtained after bootstrapping. The distance matrix sets were generated using PROTDIST. The bootstraps are based on the generation of 100 trees. The sequences were aligned using the CLUSTAL program; minor changes were made by hand to correct misalignments.

In marked contrast to other hydrogenosomes studied, in the hydrogenosomes of *Neocallimastix* sp. L2 and *Piromyces* sp. E2, pyruvate is cleaved by pyruvate-formate lyase (PFL), yielding acetyl CoA and formate (Akhmanova et al. 1999; see Chapter 3). All other hydrogenosomes studied, including that of *T. vaginalis*, use PFO for this step, yielding acetyl CoA, CO₂ and reduced ferredoxin (Müller 1993, 1998). In trichomonad hydrogenosomes, acetate, H₂ and CO₂ are formed directly from pyruvate. Chytrid hydrogenosomes also produce H₂, but the electrons do not come from pyruvate. Further differences between the energy metabolism of trichomonads and chytrids are found in the cytosolic reactions of glycolysis, which are catalysed by different enzymes, and, in some cases, have different ancestries (Fig. 2).

Compartmentalisation of carbon metabolism in chytrids

Figure 2 summarises the current knowledge of the core metabolism of the anaerobic chytrids *Neocallimastix* sp. L2 and *Piromyces* sp. E2. It contains well established, tentative and rather speculative findings and thus should be viewed as a 'grab-bag' of summary and working hypotheses. It also indicates some sequence similarities, without making explicit statements on their evolutionary origins (Martin and Schnarrenberger 1998; Martin and Müller 1998; Martin and Herrmann 1998). It should be mentioned that there have been conflicting results from metabolic studies in chytrids (Yarlett 1986; O'Fallon et al. 1991; Marvin-Sikkema 1993a). These conflicts might be a result of the use of different species (or mutant lines) of chytrids or the well known differences in metabolism in response to different culture media and different glucose concentrations (Akhmanova et al. 1999; F.G.J. Voncken, unpublished). In *Piromyces* sp. E2 and *Neocallimastix* sp. L2, the glycolytic pathway is localised in the cytoplasm but, notably, there are also rudiments of a tricarboxylic-acid (TCA) cycle, as well as acetoxyacid reductoisomerase, which is involved in branched chain amino acid

Figure 2. A schematic representation of glucose catabolism in the anaerobic chytrids *Piromyces* sp. E2 and *Neocallimastix* sp. L2. The inner box represents the hydrogenosome. The metabolic scheme is mainly based on Marvin-Sikkema et al. (1993a, 1993b, 1994), van der Giezen et al. (1997) Akhmanova et al. (1998, 1999), and contains not only well established evidence but also circumstantial and speculative evidence. Question marks indicate enzymes and transporters, the existence of which is uncertain. I indicates putative subunits of mitochondrial complex I, which would allow direct NAD(P)H oxidation by the hydrogenase. The putative origin of the enzymes, based on BLAST searches using partial DNA sequences, is: HK, PFK, GAPDH, PGK, ENOL - strongly related to the corresponding enzymes of aerobic fungi; PEPCK - 'eukaryotic' but without known fungal homologs; PGM - related to plant and bacterial sequences; Malate DH, Aconitase, STK, AAC - mitochondrial; IDH, Malic enzyme - potentially mitochondrial; ADH, ACDH, PFL, Hydrogenase - highly related to bacterial sequences; PK, Fumarase, Fumarate reductase, ASCT, LDH, ATPase - lack of DNA sequence information.

biosynthesis and is typically a mitochondrial enzyme in aerobic fungi (Akhmanova et al. 1998: see Chapter 2). Sequence comparisons indicate that the cytosolic malate dehydrogenase, aconitase and acetohydroxyacid reductoisomerase are of mitochondrial origin. In *Piromyces* sp. E2 (and probably also in *Neocallimastix* sp. L2), these enzymes lack the amino-terminal extensions present in homologues located in the mitochondria of other fungi. In *Neocallimastix* sp. L2 and *Piromyces* sp. E2, these enzymes are not in the hydrogenosomes; rather, they are active in the cytoplasm (Akhmanova et al. 1998, Chapter 2). Additionally, PFO, an otherwise canonical enzyme of hydrogenosomal pyruvate catabolism (Müller 1993, 1998), cannot be detected in the hydrogenosomes, and many attempts to clone a PFO-encoding gene have failed. Instead, PFL isoenzymes are found in both the hydrogenosomes and the cytosol (Akhmanova et al. 1998: see Chapter 3). These isoenzymes are encoded by a multigene family, which includes members with and without an amino-terminal extension, suggesting that they represent the hydrogenosomal and cytoplasmic isoforms of PFL. As has been shown for many enzymes of compartmentalised carbon metabolism in eukaryote (Martin and Schnarrenberger 1997), the likely origin of these compartment-specific isoenzymes is gene duplication.

Biochemistry and evolutionary history

The compartmentalisation of several enzymes of core energy metabolism, the enzymatic steps themselves and the metabolic end products are different in trichomonad and chytrid hydrogenosomes. One important difference is the presence of PFL in the hydrogenosomes of *Piromyces* and *Neocallimastix* and not in trichomonads (Akhmanova et al. 1999). Curiously, a mitochondrial PFL has been reported in *Chlamydomonas* (Kreuzberg et al. 1987; Dumont et al. 1993). Another difference, the lack of PFO activity in the hydrogenosomes of *Piromyces* and *Neocallimastix*, carries the odd biochemical consequence that pyruvate is not oxidised; rather, it is simply cleaved by PFL. Chytrid hydrogenosomes produce H_2 via hydrogenase, using electrons provided by imported malate (Fig. 2). Malate is catabolised by a malic enzyme that is unrelated to the corresponding yeast enzyme, which is a malolactic variant (van der Giezen et al. 1997). In the absence of PFO, it is likely that the NAD(P)H formed by the decarboxylation of hydrogenosomal malate (rather than reduced ferredoxin) is used to transfer electrons to the hydrogenase. In ciliates, it has been shown that the H_2 -generating [Fe]-hydrogenase of the hydrogenosomes is made as an unusual polyprotein that includes components with substantial homology to mitochondrial complex I subunits, which seem to allow the direct regeneration of oxidised NAD (Akhmanova et al. 1998: see Appendix). To date, there is no evidence for the presence of such a polyprotein in chytrids but, by analogy, we can assume that NAD(P)H can also be used by the chytrid hydrogenase to produce H_2 and maintain the redox balance of the organelle (Fig. 2). Despite these obvious differences, substrate-level ATP synthesis is the same in chytrids and trichomonads. It is noteworthy that the ATP-producing step of hydrogenosomal metabolism is catalysed by the two-enzyme system consisting of STK and ASCT. This reaction is also known to

occur in the mitochondria of trypanosomatids and the helminth *Fasciola hepatica* (van Hellemond et al. 1998). The evolutionary basis for these similarities and differences is uncertain. Regarding the origin of the ancestral chytrid PFL-encoding gene, lateral gene transfer is obviously one possibility. However, it is also possible that the putative common ancestor of mitochondria and hydrogenosomes possessed both PFO and PFL, as do many present-day, facultative anaerobic α -proteobacteria. Differential loss of either PFO or PFL might explain the presence of PFO in some hydrogenosomes and PFL in others. The significance of the presence of PFL in the mitochondria of algae (Kreuzberg et al. 1987; Dumont et al. 1993) is uncertain however, and further studies are required to resolve this question. Moreover, the presence of a cytosolic PFL and an adhE-type alcohol dehydrogenase in anaerobic chytrids (Fig. 2, Table 1) might suggest that the present-day hydrogenosomes of chytrids, unlike the hydrogenosomes of trichomonads, evolved secondarily after the eventual loss of mitochondria in their aerobic ancestors and a potential (transient) type I adaptation to anaerobic environments.

Why do hydrogenosomes tend to be membrane bound?

So, why are the terminal steps of energy metabolism separated from the cytosol by a membrane? Without compartmentalisation, glucose fermentation does not generate more than four moles of ATP per mole of glucose. Additionally, in mitochondria, compartmentalisation is absolutely essential for generating a proton motive force (PMF) and, consequently, for driving ATP synthesis. Hydrogenosomal membranes might also eventually allow the generation of ATP via a PMF, but experimental evidence is still lacking in *Trichomonas vaginalis* and is circumstantial in *Neocallimastix* sp. L2. The available evidence for *Neocallimastix* sp. L2 suggests that a proton gradient can be created between the matrix of the hydrogenosomes and the cytosol, which might be used to drive an ATP synthase (Marvin-Sikkema et al. 1994). Moreover, it should be kept in mind that a PMF can be generated in the absence of an electron transport chain. In certain bacteria, a PMF can be generated, for example, by oxalate/formate or aspartate/alanine exchange. Basically, the import of divalent anions in exchange for monovalent anions produces an electrochemical gradient that can be used for ATP synthesis (Anantharam et al. 1989; Abe et al. 1996). The removal of H^+ from the matrix of a hydrogenosome by hydrogen formation generates a pH gradient that could drive an ATP synthase, in a manner similar to that inferred for *Neocallimastix* sp. L2 hydrogenosomes (Marvin-Sikkema et al. 1994). Hydrogenosomes cannot avoid generating a pH gradient for the simple reason that for every molecule of H_2 formed, two protons go into the gas phase and leave the organelle by diffusion. If an ATP-synthase is present, this could lead to a yield of two moles of ATP per three moles of H_2 produced and, hence, to a significant increase in the energy yield of the hydrogenosome (Fig. 2). Such compartmentalisation would improve the energy yield of a type I anaerobe after the loss of mitochondria. One can speculate whether chytrid hydrogenosomes evolved in such a way, as they clearly differ from trichomonad hydrogenosomes, and the presence of mitochondrial enzymes in the cytosol, together with certain type I-like adaptations,

might be indicative of a loss of mitochondria during the adaptation of chytrids to anaerobic environments (Marvin-Sikkema et al. 1993a; Akhmanova et al. 1999) (Fig. 2; Table 1). However, it should not be forgotten that this reasoning cannot easily be applied to the trichomonad hydrogenosomes, as there is a wealth of argument in favour of a common ancestry of these hydrogenosomes with mitochondria (Martin and Müller 1998; Embley et al. 1997; Biagini et al. 1997). This would mean that the adaptation of such proto-mitochondria to anaerobic conditions, which involved the loss of the respiratory electron-transport chain, caused a loss of ~90% of the potential ATP yield from glucose. Therefore, one might reason that the evolution of hydrogenosomes was driven by factors other than the extra energy gain that can be achieved by compartmentalisation. The potential role of hydrogenosomes as cellular calcium stores might have been another evolutionary trend (Benchimol et al. 1982; Biagini et al. 1997), and there could be many others. The recent discovery of a genome in the hydrogenosomes of the anaerobic ciliate *Nyctotherus ovalis* (Akhmanova et al. 1998; see Appendix) might improve our understanding of hydrogenosome evolution. Phylogenetic analysis of the genes encoding the small subunit of the organellar rRNA strongly argues for a mitochondrial ancestry (van Hoek et al. 2000). Moreover, it has been shown that the hydrogenosomes of *N. ovalis* possess an unusual [Fe]-hydrogenase that apparently connects fermentative glucose metabolism with components of a rudimentary mitochondrial electron-transport chain (Akhmanova et al. 1998). The hydrogenase is linked to components of mitochondrial complex I (i.e. NADH-dehydrogenase), thus potentially allowing the reoxidation of reduced NAD(P) and, hypothetically, the maintenance of the proton-expelling capacity of complex I under anaerobic conditions. Among ciliates, the evolution of hydrogenosomes seems to have occurred three or four times during adaptation to anaerobic niches (Embley et al. 1995). A comparable, yet different, adaptation of mitochondria to anaerobic conditions has apparently also occurred in metazoa including parasitic helminths, freshwater snails, mussels, lug-worms and other marine invertebrates exposed to periods of anaerobiosis during their development. This is a peculiar variant of anaerobic respiration called malate dismutation (Tielens 1994; Tielens and van Hellemond 1998). In such mitochondria, endogenous fumarate is reduced to succinate by fumarate reductase; fumarate serves as an electron sink. This process requires the replacement of ubiquinone by rhodoquinone in the mitochondrial electron-transport chain (Tielens 1994; Tielens and van Hellemond 1998), but it also allows the use of mitochondrial complex I for the generation of a proton gradient under anoxic conditions.

Conclusion

Hydrogenosomes have evolved several times in highly disparate eukaryotic lineages, apparently always in connection with an ecological specialisation for anaerobic habitats. One might speculate that the different types of hydrogenosomes are convergent adaptations to anaerobic conditions, that all hydrogenosomes are derived from mitochondria or that they have evolved from a common progenitor of both. The common

denominator seems to be a compartmentalisation of reactions involved in energy metabolism and the use of protons as electron acceptors. It is possible that in some but not all cases, the potential energy gain by the generation of a PMF is one of the driving forces for the evolution of hydrogenosomes.

Questions for future research

Why are electrons for hydrogen formation in chytrids derived from malate and not from pyruvate?

Why do hydrogenosomes tend to be membrane bound?

Why did chytrid hydrogenosomes not retain a genome, unlike the hydrogenosomes of the anaerobic ciliate *Nyctotherus ovalis*?

Will the origin of the cellular compartment that became a hydrogenosome ever be determined in the absence of an organellar genome?

Why does the chytrid hydrogenosome use pyruvate-formate lyase (PFL) and not pyruvate ferredoxin oxidoreductase (PFO)?

Why is there PFL activity in both the cytoplasm and the hydrogenosome?

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Summary

Samenvatting

Summary

Hydrogenosomes are membrane-bound organelles found in a wide variety of unicellular anaerobic eukaryotes. They are characterised by the production of hydrogen. Like mitochondria, hydrogenosomes compartmentalise crucial steps of eukaryotic energy metabolism. However, compartmentalisation differs substantially between mitochondriate aerobes and hydrogenosome-bearing anaerobes. In contrast to mitochondria, hydrogenosomes have arisen independently in different lineages of eukaryotic microorganisms. Comparative analysis of the various types of hydrogenosomes can provide insights into functional and evolutionary aspects of compartmentalised energy metabolism in unicellular eukaryotes.

Chytrid hydrogenosomes seem to be especially suited to address the problem of a potential polyphyletic origin of hydrogenosomes since their ultrastructure and physiology differs in several aspects from the prototypical hydrogenosomes of *Trichomonas*. Therefore, in this thesis, a number of genes encoding hydrogenosomal proteins of anaerobic chytrids, e.g. *Neocallimastix* sp. L2 and *Piromyces* sp. E2, was analysed and compared to their homologues in aerobic eukaryotes, free-living anaerobic eubacteria, and hydrogenosome-bearing anaerobic eukaryotes in order to gain insights into the nature of functional and evolutionary relationships between hydrogenosomes and mitochondria. Moreover, the subcellular localisation of genuine mitochondrial proteins was studied in the chytrids, as well as the presence or absence of potential targeting signals. Also, special attention was paid to the unique ultrastructure of the hydrogenosomes of chytrids.

In Chapter 2 of this thesis we discussed the consequences of a potential loss of mitochondria. We showed that malate dehydrogenase, aconitase and acetohydroxyacid reductoisomerase - without exception enzymes with a mitochondrial ancestry - are located in the cytoplasm and not in the hydrogenosome of *Piromyces* sp. E2. DNA sequence analysis reveals a lack of potential (mitochondrial) N-terminal import signals. Published in *Molecular Microbiology* 30 (5), p. 1017-1027, 1998.

In chapter 3 we showed that, unlike all other hydrogenosomes described to date, a pyruvate formate-lyase (PFL) and not a pyruvate:ferredoxin oxidoreductase (PFO) is crucial for the function of the hydrogenosomes from anaerobic chytrids. The PFL genes of *Piromyces* sp. E2 comprise a multigene family that might be acquired by lateral gene-transfer from eubacterial (perhaps *Clostridium*) ancestors. One subfamily possesses a long N-terminal extension that might function as a targeting and import signal for the hydrogenosomes, whereas the other subfamily consists of members lacking an N-terminal extension. The absence of the putative targeting signal might explain the presence of PFL activity in the cytoplasm. Published in *Molecular Microbiology* 32 (5), p. 1103-1114, 1999.

In chapter 4 we described a hydrogenosomal ADP/ATP carrier (AAC). Phylogenetic analysis has confirmed that this adenine nucleotide transporter belongs to the family of mitochondrial AACs. Like its mitochondrial homologues, it lacks N- and C- terminal extensions that might function as potential targeting signals. Expression of the cDNA in *E. coli* confers the ability to the bacterial host to incorporate preferentially ADP, but also

ATP at significant rates. Biochemical and ultrastructural studies revealed that the ADP/ATP carrier is an integral component of the hydrogenosomal membranes. Notwithstanding the unusual structure of the hydrogenosomes of *Neocallimastix* sp. L2, all data consistently argue for the presence of a functional mitochondrial-type AAC in the hydrogenosomal membranes, and, consequently, a mitochondrial origin of these organelles. Hydrogenosome-containing anaerobic ciliates possess similar, but clearly distinct mitochondrial-type AACs. Since the putative hydrogenosomal nucleotide carrier (Hmp31) of the anaerobic flagellate *Trichomonas vaginalis* is paralogous to the mitochondrial-type AACs of both, ciliate and chytrid hydrogenosomes, a multiple, independent origin of hydrogenosomes is likely. *Submitted*

Chapter 5 was devoted to a hydrogenosomal [Fe]-hydrogenase from the anaerobic chytridiomycete fungus *Neocallimastix* sp. L2. The enzyme has a molecular mass of approximately 67 kDa, and is highly sensitive against carbon monoxide and oxygen. It is likely that this enzyme is the sole source for molecular hydrogen in *Neocallimastix* sp. L2. A cDNA has been cloned and sequenced. It encodes a [Fe]-hydrogenase of the proper size which exhibits all the motifs that are characteristic of [Fe]-hydrogenases from both bacterial and eukaryotic origins. The ORF encoding this hydrogenase possesses an N-terminal extension of 38 amino acids that has the potential to function as a hydrogenosomal targeting signal. Phylogenetic analysis revealed that this hydrogenase clusters together with similar [Fe]-hydrogenases from *Trichomonas vaginalis*, *Nyctotherus ovalis*, *Desulfovibrio vulgaris* and *Thermotoga maritima*. However, low bootstrap values do not allow to reject the hypothesis that the eukaryotic [Fe]-hydrogenases are monophyletic, i.e. the data presented here are compatible with the hypothesis that mitochondria and hydrogenosomes evolved from a common ancestor by differential loss of ancestral enzymes. *Submitted*

In Chapter 6, a hydrogenosomal adenylate kinase from both *Neocallimastix* sp. L2 and *Piromyces* sp. E2 was isolated and characterised. Sequence analysis showed that the enzyme has a mitochondrial origin. The deduced amino acid sequences lack a N-terminal extension, in contrast to the hydrogenosomal AK of the parabasalid *Trichomonas vaginalis*. Instead, the chytrid hydrogenosomal AKs carry a carboxy-terminal sequence - the tripeptide SKL - which in aerobic eukaryotes functions as a prototypical peroxisomal targeting signal. By expression of wildtype and mutant hydrogenosomal AK (lacking the tripeptide SKL) in the heterologous host *Hansenula polymorpha*, it is shown that the subcellular sorting of these hydrogenosomal enzymes in yeast indeed depends on the carboxy-terminal PTS1-like motif. The enzyme is associated with the membranes of the hydrogenosome.

Finally, in Chapter 7 we conclude that hydrogenosomes have evolved several times in highly disparate eukaryotic lineages, apparently always in connection with an ecological adaptation for anaerobic habitats. One might speculate that all hydrogenosomes are derived from mitochondria or that they have evolved from a common progenitor of both. The common denominator seems to be a compartmentalisation of reactions involved in energy metabolism and the use of protons as electron acceptors. It is possible that in some but not all cases, the potential energy gain by the generation of a PMF is one of the driving forces for the evolution of hydrogenosomes. Published in *Trends in Microbiology* 7 (11), p. 441-446, 1999.

Samenvatting

Hydrogenosomen zijn celorganellen van eencellige anaerobe eukaryote organismen die waterstof produceren en daarmee extra energie verwerven voor hun metabolisme. Net als bij mitochondriën zijn de daarbij betrokken enzymen in een apart cellulair compartiment gelokaliseerd. De wijze waarop de extra energie verkregen wordt is sterk verschillend in beide typen celorganellen. Verder zijn hydrogenosomen, in tegenstelling tot mitochondriën, meerdere keren onafhankelijk van elkaar ontstaan in verschillende evolutionaire lijnen van eukaryote micro-organismen.

In dit proefschrift wordt een vergelijkend onderzoek gedaan aan hydrogenosomen van verschillende anaerobe eukaryoten om een inzicht te krijgen in het functioneren en de evolutie van deze bijzondere celorganellen. De hydrogenosomen van anaerobe schimmels (chytridiomyceten) blijken bijzonder geschikt om de polyfyletische oorsprong te onderzoeken. De ultrastructuur en fysiologie van de hydrogenosomen van deze schimmels verschillen in belangrijke mate van de uitvoerig bestudeerde hydrogenosomen van de anaerobe flagellaat *Trichomonas vaginalis*. In het proefschrift worden een aantal genen gekarakteriseerd die coderen voor hydrogenosomale eiwitten van de twee nauw verwante anaerobe chytridiomycete schimmels *Neocallimastix* sp. L2 en *Piromyces* sp. E2. Zij worden vergeleken met verwante genen in aerobe eukaryoten, vrij levende anaerobe bacteriën en anaerobe eukaryoten die in het bezit zijn van hydrogenosomen. Op deze wijze wordt meer inzicht verkregen in de functionele en evolutionaire verwantschap tussen hydrogenosomen en mitochondriën. Daarenboven wordt ook de subcellulaire lokalisatie van mitochondriële eiwitten in de anaerobe schimmels bestudeerd, evenals de aan- of afwezigheid van mogelijke "targeting" en import signalen. Tenslotte wordt ook aandacht besteed aan de bijzondere ultrastructuur van de hydrogenosomen van bovengenoemde schimmels.

In hoofdstuk 2 van dit proefschrift bespreken we de mogelijke consequenties van een verlies van mitochondriën, als gevolg van de mogelijke overgang van een aerobe naar een anaerobe leefwijze van de chytridiomyceten. We laten zien dat malaat dehydrogenase, aconitase en acetoxyhydroxyzuur reductoisomerase, allemaal eiwitten met een mitochondriële oorsprong, in het cytosol van *Piromyces* sp. E2 zijn gelokaliseerd en niet in de hydrogenosomen. Analyse van de aminozuurvolgorde van deze eiwitten laat verder zien dat de typische mitochondriële import signalen aan de N-terminus ontbreken. Gepubliceerd in *Molecular Microbiology* 30 (5), p. 1017-1027, 1998.

In het koolstofmetabolisme speelt pyruvaat een centrale rol. In alle tot nu toe beschreven hydrogenosomen wordt pyruvaat omgezet door het enzym pyruvaat ferredoxine oxidoreductase (PFO). In hoofdstuk 3 tonen we aan dat voor het functioneren van de hydrogenosomen van de anaerobe schimmels niet PFO maar pyruvaat formaat-lyase (PFL) een belangrijke rol speelt. De PFL genen van *Piromyces* sp. E2 vormen een multi-gen familie en zijn tijdens de evolutie mogelijkerwijze verkregen van een bacteriële voorouder (*Clostridium*?) door middel van laterale gen transfer. Een van de in totaal twee PFL subfamilies wordt gekenmerkt door een lange N-terminale verlenging die vermoedelijke functioneert als een "targeting" en import signaal.

voor de hydrogenosomen. De andere subfamilie bestaat uit PFL genen die geen N-terminale verlenging bezitten. De afwezigheid van dit "targeting" en import signaal bij deze laatste subfamilie zou de aanwezigheid van PFL activiteit in het cytosol van *Piromyces* sp. E2 kunnen verklaren. Gepubliceerd in *Molecular Microbiology* 32 (5), p. 1103-1114, 1999.

Bij de energiehuishouding speelt de beschikbaarheid en productie van ATP een essentiële rol. In hoofdstuk 4 wordt een hydrogenosomale ADP/ATP transporter (AAC) beschreven. Een fylogenetische vergelijking toont aan dat deze adenine nucleotide transporter tot de familie van mitochondriële AACs behoort. Net als de mitochondriële AACs heeft het hydrogenosomale AAC geen N- of C-terminale verlengingen die als mogelijke "targeting" en import signalen zouden kunnen dienen. Expressie van het betreffende AAC gen in *Escherichia coli* had als gevolg dat bij voorkeur ADP in de bacteriële gastheer werd opgenomen. Ook ATP wordt in belangrijke mate opgenomen. Biochemische en ultrastructurele studies lieten vervolgens zien dat het ADP/ATP transporteiwit in de hydrogenosomale membranen is gelocaliseerd. Opvallend bij de ultrastructurele studies is de buitengewone ultrastructuur van de hydrogenosomen van *Neocallimastix* sp. L2. Alle gegevens tezamen vormen een sterke aanwijzing voor de aanwezigheid van een functioneel "mitochondrieel" AAC in de hydrogenosomale membranen van anaërobe schimmels en voor een mogelijk mitochondriële oorsprong van deze hydrogenosomen. Ook anaërobe ciliaten bezitten hydrogenosomen met vergelijkbare doch duidelijk verschillende "mitochondriële" AACs. Omdat de hydrogenosomale nucleotide carrier (Hmp31) van de anaërobe flagellaat *Trichomonas vaginalis* paraloog is aan de "mitochondriële" AACs van zowel anaërobe ciliaten als schimmels, is een onafhankelijke oorsprong (polyfylie) van de verschillende hydrogenosomen zeer waarschijnlijk.

Bij de productie van waterstof spelen hydrogenases een essentiële rol. In Hoofdstuk 5 wordt een nieuw hydrogenosomaal [Fe]-hydrogenase van *Neocallimastix* sp. L2 beschreven. Het betreffende eiwit heeft een molecuul gewicht van ongeveer 67 kDa. Dit hydrogenase is extreem gevoelig voor koolmonoxide en zuurstof, en is zeer waarschijnlijk in belangrijke mate verantwoordelijk voor de door *Neocallimastix* sp. L2 geproduceerde waterstof. De geïsoleerde en gekarakteriseerde cDNA kloon codeert voor een [Fe]-hydrogenase met het verwachte molecuulgewicht van 67 kDa. Verder bevat de aminozuurvolgorde geconserveerde motieven die karakteristiek zijn voor [Fe]-hydrogenases van zowel bacteriële als eukaryote oorsprong. De afgeleide aminozuur volgorde bevat een N-terminale verlenging van 38 aminozuren die mogelijk als een hydrogenosomaal "targeting" signaal zou kunnen dienen. Een fylogenetische vergelijking laat zien dat dit hydrogenase binnen een groep valt van vergelijkbare [Fe]-hydrogenases van *Trichomonas vaginalis*, *Nyctotherus ovalis*, *Desulfovibrio vulgaris* en *Thermotoga maritima*. Vanwege de lage "bootstrap" waarden kan men echter niet tot de conclusie komen dat alle eukaryote [Fe]-hydrogenases een monofyletische oorsprong hebben. Wel zou men uit de gepresenteerde gegevens kunnen concluderen dat mitochondriën en hydrogenosomen uit een gemeenschappelijke voorouder zijn ontstaan, door verschillend verlies van de oorspronkelijke enzymen.

De omzetting van ADP in ATP en AMP wordt door het enzym adenylaat kinase gekatalyseerd. In hoofdstuk 6 wordt de isolatie en karakterisering van hydrogenosomale adenylaat kinases (AKs) van *Neocallimastix* sp. L2 en *Piromyces* sp. E2 beschreven. Uit de fylogenetische vergelijking van deze AKs met verschillende typen van adenylaat kinases van andere organismen valt af te leiden dat deze enzymen een mitochondriële oorsprong hebben. In tegenstelling tot het hydrogenosomale adenylaat kinase van de flagellaat *Trichomonas vaginalis* ontbreekt echter een N-terminale verlenging. In plaats daarvan hebben de hydrogenosomale AKs van beide schimmels een C-terminale aminozuurvolgorde (het tri-peptide SKL) dat in aërobe eukaryoten een rol speelt als een typisch peroxisomaal targetting signaal (PTS1). Na expressie van zowel het wildtype alsook het genetisch gemodificeerd hydrogenosomaal AK (zonder SKL) in de heterologe gastheer *Hansenula polymorpha* wordt duidelijk dat de subcellulaire "targeting" en import van dit hydrogenosomale enzym in gist inderdaad berust op de aanwezigheid van het C-terminale PTS1 motief. Verder blijkt dat het betreffende adenylaat kinase in de anaërobe schimmel aan de hydrogenosomale membranen gebonden is.

In het laatste hoofdstuk (7) komen we tot de conclusie dat hydrogenosomen in de evolutie verschillende keren en onafhankelijk van elkaar ontstaan zijn in zeer uiteenlopende takken van de eukaryote stamboom. Blijkbaar gaat dit altijd hand in hand met een ecologische aanpassing in anaërobe milieus. Verder concluderen we dat de verschillende typen hydrogenosomen ontstaan zijn uit mitochondriën of een gemeenschappelijke voorouder van beide. De gemeenschappelijke noemer is waarschijnlijk de compartimentering van reacties die betrokken zijn bij het energie metabolisme en het gebruik van protonen als elektronen acceptoren door de hydrogenosomen. De mogelijkheid van energiewinst, door de vorming van een "proton motive force" (pmf) over de membranen van celorganellen, kan als een van de drijvende krachten achter de evolutie van hydrogenosomen gezien worden. Gepubliceerd in *Trends in Microbiology* 7 (11), p. 441-446, 1999.

Appendix

A hydrogenosome with a genome

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Some anaerobic protozoa and chytridiomycete fungi possess membrane-bound organelles known as hydrogenosomes. Hydrogenosomes are about 1 μm in diameter and are so-called because they produce molecular hydrogen (Müller 1993). It has been postulated that hydrogenosomes evolved from mitochondria by the concomitant loss of their respiration and organellar genomes (Müller 1993; Palmer 1997; Martin and Müller 1998; Fenchel and Finlay 1998), and so far no hydrogenosome has been found that has a genome (Müller 1993; Palmer 1997). Here we provide evidence for the existence of a hydrogenosomal genome of mitochondrial descent, and show that the anaerobic heterotrichous ciliate *Nyctotherus ovalis* possesses a new type of nuclear-encoded 'iron-only' hydrogenase enzyme.

N. ovalis, found in the hindgut of the cockroaches *Periplaneta americana* and *Blaberus* spp. (van Hoek et al. 1998), has numerous hydrogenosomes that are intimately associated with endosymbiotic methane-producing Archaea, which use hydrogen produced by the hydrogenosomes (Fig. 1a). The hydrogenosomes are bounded by distinct double membranes and have an inner membrane with cristae-like projections. The matrix contains ribosome-like particles of the same size as the numerous 70S ribosomes of the endosymbiotic methanogenic Archaea (Fig. 1d).

Weak but consistent immuno-gold labelling was obtained with a commercial antiserum against DNA in more than 80% of the hydrogenosomes we sectioned (Fig. 1b). We labelled the same organelles by using heterologous antisera against an iron-only hydrogenase ([Fe]-hydrogenase; Bui and Johnson 1996) and a hydrogenosomal adenylate kinase of the AK2 type (Fig. 1c, see chapter 6). Electron microscopy and immunocytochemistry indicated that DNA, ribosomes and components of a hydrogenosomal metabolism are present in the hydrogenosomes of *N. ovalis*.

Because all hydrogenosomes studied so far lack a genome (Müller 1993; Palmer 1997), we wondered whether the immuno-reactive organelle DNA in *N. ovalis* is functional. By using the polymerase chain reaction (PCR), with primers directed against conserved regions of the mitochondrial small-subunit (SSU) ribosomal RNA genes from ciliates (Seilhamer et al. 1984; Schnare et al. 1986), we isolated and cloned a fragment of a homologous SSU rRNA gene from *N. ovalis* total DNA. The complete sequence of this rDNA was obtained by rapid amplification of complementary DNA ends, using total RNA from *N. ovalis* as a template. Phylogenetic analysis of conserved regions places the *N. ovalis* sequence with high bootstrap values within the mitochondrial SSU rRNA genes from aerobic ciliates (not shown). Northern blotting reveals that the isolated SSU rRNA gene is abundantly expressed. The cross-hybridising rRNA is fragmented, supporting the idea of descent from a mitochondrial rRNA gene of a ciliate (Fig. 2a; Seilhamer et al. 1984; Schnare et al. 1986).

By using PCR, we identified a nuclear-encoded cDNA 3.6 kilobases long encoding a putative [Fe]-hydrogenase. The cDNA contains a single open reading frame (ORF) consisting of 1198 codons (Fig. 2e). The amino-terminal half of the predicted polypeptide (residues 22–620) shares 35–41% identity with the [Fe]-hydrogenases from the bacterium *Clostridium* and the proteobacterium *Desulfovibrio* (Casalot 1998). The middle part of the ORF (residues 630–810) shares 21–24% identity with the HoxE protein of *Synechocystis* spp., the NADP-reducing hydrogenase subunit HndA of

Figure 1. Electron micrographs of *Nyctotherus ovalis* showing hydrogenosomes.

A. *N. ovalis* from the hindgut of *Blaberus* (van Hoek et al. 1998). The hydrogenosomes (H) are surrounded by endosymbiotic methane-producing Archaea (dark spots); N, macronucleus; n, micronucleus; V, vacuole. Visible by MnO₄ fixation/Epon. **B.** **C.** and **E.** *N. ovalis* from the hindgut of *Periplaneta americana* (van Hoek et al. 1998). Immuno-gold labelling of glutaraldehyde-fixed and Unicryl-embedded sections; m, methanogenic Archaea (endosymbionts). **B.** DNA antiserum (Boehringer) labels the matrix of about 80% of the hydrogenosomes on randomly chosen sections with 3-10 grains. The difference in DNA concentration causes the label over the endosymbiotic methanogens to be heavier. **C.** Immunogold labelling obtained with a polyclonal antiserum against hydrogenosomal adenylate kinase (hdgAK2L2) from the anaerobic chytrid *Neocallimastix* sp. L2 (F.V. and B.B., unpublished). Matrix of hydrogenosomes and endosymbiotic methanogens is labeled. **E.** Labelling of the hydrogenosomes (and methanogens) with an antiserum against an [Fe]-hydrogenase of *Trichomonas vaginalis* (Bui and Johnson 1996). There is about 40% amino-acid sequence identity with the [Fe]-hydrogenase described here. **D.** Electron micrograph of a hydrogenosome of *N. ovalis* from *Blaberus*. OsO₄ fixation/Epon. Cristae are clearly visible. Arrows indicate ribosomes. Scale bars are 1 μm, except for that in A, which represents 10 μm.

E

- Similarity to mitochondrial transit peptides
- Similarity to 'iron-only' hydrogenases
- Similarity to precursors of the 24K subunit of mitochondrial complex I
- Similarity to precursors of the 51K subunit of mitochondrial complex I

- 4Fe-4S motif
- H-cluster
- NAD-binding site
- FMN-binding site

Figure 2. The *N. ovalis* genome

A Northern blot of *N. ovalis* total RNA hybridised to a α -[32 P]-labelled fragment of the mitochondrial SSU ribosomal RNA (positions 550-1660). The largest hybridising RNA species corresponds in size to the sequenced mitochondrial SSU rRNA, at 1701 nucleotides (nt). The smaller RNA fragments on the blot probably represent naturally occurring discontinuities, similar to those described for *Paramecium* and *Tetrahymena* (Seilhamer et al 1984, Schnare et al 1986). **B** Northern blot of *N. ovalis* RNA hybridised to the 3' part of the hydrogenase complementary DNA (positions 1308-3625). An identical result was obtained when the 5' part of the cDNA was used as a probe. **C** Ethidium bromide-stained gel with 5 μ g undigested genomic *N. ovalis* DNA. Most DNA fragments are gene-sized, at 0.5-10 kilobases (kb). **D** Southern blot of the same gel hybridised to the α -[32 P]-labelled hydrogenase cDNA fragment (positions 1308-3625). **E** The open reading frame encoding the putative [Fe]-hydrogenase. Homologies to known proteins and putative functional domains are indicated. Motifs are not to scale.

DDesulfovibrio fructosovorans, and the (nuclear-encoded) NuoE/Nuo5 precursors of the 24K (relative molecular mass 24,000) subunit of complex I (NADH-ubiquinone oxidoreductase) from the respiratory chain. The carboxy-terminal part of the ORF (residues 840-1170) has 28-34% identity with the HoxF genes of *Synechocystis* spp. and *Alcaligenes eutrophus* and, notably, with the nuclear-encoded NuoF/Nuo6 precursors of the 51K subunit of mitochondrial complex I. In all subunits, the NAD-binding, flavin-mononucleotide-binding and [Fe-S] motifs are conserved.

The hydrogenase cDNA hybridises to a 4-kilobase fragment of undigested genomic DNA (Fig. 2d), indicating that the hydrogenase is encoded by gene-sized pieces of *N. ovalis* macronuclear DNA (Fig. 2c). The genomic fragment terminates in a G3 T4 G3 (T4 G4)5 repeat that is very similar to the telomere sequences of hypotrichous ciliates (Hoffman et al 1995). The cDNA start was about 180 base pairs downstream from the telomere. The 16 amino-terminal amino acids of the ORF resemble mitochondrial transit peptides. The length of the *N. ovalis* hydrogenase mRNA is in agreement with the sequence data (Fig. 2b). Our results indicate that *N. ovalis* hydrogenosomes evolved from mitochondria but, contrary to recent predictions (Palmer 1997), they have not relinquished their genome. The evolutionary origin of the chimaeric *N. ovalis* hydrogenase gene remains puzzling. Sequence similarity suggests that the hydrogenase couples hydrogen production to the reoxidation of NADH through a combination of functional components derived from respiratory (complex I modules) and fermentative ([Fe]-hydrogenase module) metabolism. The hydrogenase gene could have been inherited from the common proteobacterial ancestor of mitochondria and hydrogenosomes (Martin and Muller 1998), it may have been acquired through lateral gene transfer from prokaryotes in the *N. ovalis* lineage, or perhaps it was assembled *de novo* in the *N. ovalis* lineage from pre-existing or acquired genetic components.

Hydrogenase expression is clearly a prerequisite for the conversion from mitochondrion to hydrogenosome during the eukaryotic specialisation to anaerobic niches. Because hydrogenosomes have arisen independently several times in mitochondrion-bearing lineages (Muller 1993, Fenchel and Finlay 1995, Embley et al 1995) it is possible that their hydrogenases did as well.

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Sequence data have been deposited in the EMBL database under accession numbers: Y16775, Y16669, and Y16670.

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Curriculum vitae

Franciscus Godfried Josef (Frank) Voncken werd geboren op 12 november 1965 te Heerlen. Hij volgde de Atheneum opleiding aan het 'Sophianum' te Gulpen van 1978 tot 1984. In 1984 begon hij met een studie biologie aan de Katholieke Universiteit van Nijmegen. Het doctoraal examen werd behaald in 1990 met als hoofdvak Microbiologie (prof. dr. ir. G. Vogels), als bijvak Biochemie (prof. dr. W. de Jong) en een "toegepaste" stage bij het Nederlands instituut voor zuivelonderzoek (NIZO, dr. H. Exterkate) te Ede-Wageningen. Tijdens zijn studie behaalde hij tevens het "Deskundigheid Stralingshygiëne niveau C" diploma en leverde hij een bijdrage aan de practica Algemene Microbiologie en Biotechnologie I. Van mei 1990 tot mei 1992 was hij werkzaam als wetenschappelijk medewerker bij de vakgroep Moleculaire Microbiologie (prof. dr. W. Hoekstra) van de Universiteit Utrecht, in opdracht van de Suiker Unie Research te Roosendaal. Van september 1992 tot november 1998 was hij onderzoeker in opleiding (OIO) bij de vakgroep Microbiologie en Evolutiebiologie aan de Katholieke Universiteit van Nijmegen. Hier werd het in dit proefschrift beschreven promotie-onderzoek uitgevoerd, onder leiding van dr. J. Hackstein en prof. dr. ir. G. Vogels. De resultaten van dit onderzoek presenteerde hij op een groot aantal congressen. Tevens organiseerde hij het praktikum Fysiologie van Plant en Mikroorganismen. In 1996 nam hij deel aan de FEBS cursus "Molecular mechanisms of Signaling and Targeting" georganiseerd door het NATO Advanced Study Institute, te Spetsai. Sinds november 1998 is hij werkzaam als wetenschappelijk medewerker bij het "Zentrum für Molekulare Biologie" (ZMBH) te Heidelberg, Duitsland.