

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 that possess hydrogenosomes. Hydrogenosomes are highly specialized 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 multi-gene 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 Müller, 1998). Hydrogenosomes metabolize pyruvate or malate to hydrogen, acetate and CO₂, and they are characterized by their key enzymes hydrogenase and pyruvate:ferredoxin oxidoreductase (PFO) (Yarlett *et al.*, 1986; O'Fallon *et al.*, 1991; Marvin-Sikkema *et al.*, 1993a; Müller, 1993; Trinci *et al.*, 1994). However, our knowledge about hydrogenosomes is mainly based on studies carried out over the last decade of the hydrogenosomes of *Trichomonas vaginalis* and its relatives, and it cannot be excluded that the hydrogenosomes of the various protists have deviating properties (Müller, 1993; Coombs and Hackstein, 1995).

There is evidence that the hydrogenosomes of anaerobic chytrids are different; their ultrastructure exhibits a number of traits that discriminates 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 mol of CO₂ per mol of malate utilized 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; F. G. J. Voncken, unpublished) 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.

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Because 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 in which 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 also that 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.

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.

To obtain the 5' part of this cDNA, we performed a 5' rapid amplification of cDNA ends 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

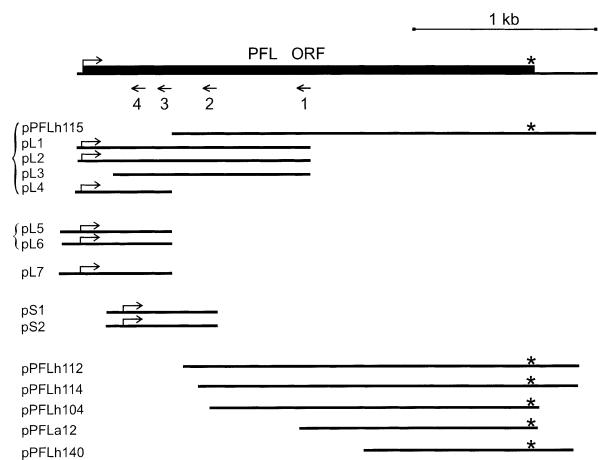


Fig. 1. PFL-encoding cDNA clones from *Piromyces* sp. E2. The scheme of the composite cDNA sequence PFL-L1 (assembled from clones pL1 and pPFLh115) is shown at the top, with the black box indicating the open reading frame. cDNA clones, identified by random screening (designated pPFL) or isolated by 5' RACE (the rest of the clones) are shown by thick lines. Short thick arrows indicate the position of the PFL-specific primers PFLrev1 to PFLrev4 (marked 1–4) used in this study. Thin 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 curly brackets.

obtained with the primer PFLrev3 must be derived from this *pfl* gene. However, the clones pL5, pL6 and 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 that are involved in catalysis, for example 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, in contrast, 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. 2A). This N-terminal extension is enriched in positively charged and hydroxylated amino acids. Because 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

A. PFL N-termini

Piomyces pL1	MESLALSNSVSVLANTVSVNAVAATKVAGVRMAKPSRALHTPAMKTT-LKTSKK--VPAMQAKTYATQAPC	67
Piomyces pL5	MESLTLTQVNNIAIAKSVSVNAVAATKVAGVRSKPSRAIHTTPMTTSLKTSKSSFPSIQTKTYATQAPC	71
Piomyces pL7	MESLTLTQVNNIAIAKSVSVNAVAATKVAGVRSKPSRAMHTTPMTTSLKTSK---VQAMQAKTYATQAPC	68
Piomyces pS1	-----	-
Piomyces pS2	-----	-
C. pasteurianum	-----MFKQWE	6
H. influenzae	-----SELNEMQKLAWA	12
E.coli PFLB	-----SELNEKLATAWE	12
E.coli TdcE	-----MKVDIDTSDKLYADAWL	17
S. mutans	-----MATVKTNTDVFKEAKE	16
L. lactis	-----MKTEVTE-NIFEQAWD	15
Piomyces pL1	ITNDAAAKSEIDVEGWIKKHYPYEGDGSFL-AGPTEKTKKLFAKAEYLAKEERANGGLYDVPHTPSTI	136
Piomyces pL5	ITSDAAAKSEIDVEGWIKKHYPYEGDGSFL-AGPTEKTKKLFAKAEYLAKEERANGGLYDVPHTPSTI	140
Piomyces pL7	ITNDAAAKSEIDVEGWIKKHYPYEGDGSFL-SGPTTEKTKKLFAKAEYLAKEERANGGLYDVPHTPSTI	137
Piomyces pS1	-----MQVIDVAKWIKENYTPYEGDASFLVTDASAKTKDVWNKCCCELRAEEIKTNGCLDVKNTISTV	63
Piomyces pS2	-----MQVIDVAKWIKENHTPYEGDASFLVTDASAKTKDVWNKCCCELRAEEIKTNGCLDVKNTISTV	63
C. pasteurianum	GFQDGEWTVNVRDFIQKNYKEYTGDKSFL-KGPTEKTKKLVWDKAVSLILEE-LKKGILDVDTETISGI	74
H. influenzae	GFAGGDWQENVNRDFIQKNYTPYEGDGSFL-AGPTEATTKLWESVMEGIENRTHAPLDFDEHTPSTI	81
E.coli PFLB	GFTKGDWQENVNRDFIQKNYTPYEGDESFL-AGATEATTTLWDKVMGKLENRTHAPVDFDTAVASTI	81
E.coli TdcE	GFKGTDWKNEINVRDFIQHNYTPYEGDESFL-AEATPATELWEEKVMGIRIENATHAPVDFDTNIATTI	86
S. mutans	GFKGTDWKDRASISRFVQDNYTPYDGGESFL-AGPTERSLHKKKVVEET--KAHYEETRFPMDDT-RITSI	82
L. lactis	GFKGTNWRDKASVTRFVQENYKPYDGESEFL-AGPTERTLKVKKIIEDT--KNHYEEVGFPPFDTRVTSI	82

B. PFL C-termini

Piomyces PFL-L1	LLDGYFTKG-----AHHLNVNLKRETLIDAMAHPENYPNLTIRVSGYAVNFVKLTPOQOKEVIA	747
C. reinhardtii	LLDGYFANG-----GHHLNVNLNRSMLMDAVEHPEKYPNLTIRVSGYAVHFARLTREQQLEVIA	188
C. pasteurianum	IMGGYFGQG-----AHHLNVNLNRETLIDAMNPNPKYPTLTIRVSGYAVNFNRLSKDHQKEVIS	733
H. influenzae	LMDGYFHHEATVEG---GQHLNVNLNREMLLDAMENPKYPQLTIRVSGYAVRFNSLTKEQQQDVIT	762
E.coli PFLB	LMDGYFHHEASIEG---GQHLNVNMNREMLLDAMENPEKYPQLTIRVSGYAVRFNSLTKEQQQDVIT	752
E.coli TdcE	LLDGYFHHEADVEG---GQHLNVNMNREMLLDALIEHPEKYPNLTIRVSGYACASTH-----	744
S. mutans	LLDGYFEGG-----GQHLNVNMDLKDVIYDKIMNGE---DVIIVRISGYCVNTKYLTKQKTELTO	755
L. lactis	LLDGYFTPGALINGTEFAGQNVNVLNMDLKDVIYDKIMRGE---DVIIVRISGYCVNTKYLTPQOQELTE	767
Piomyces PFL-L1	RTFHEKM-----	754
C. reinhardtii	RTFHDTM-----	195
C. pasteurianum	RTFHEKL-----	740
H. influenzae	RTFTESM-----	769
E.coli PFLB	RTFTQSM-----	759
E.coli TdcE	-----	-
S. mutans	RVFHEVLSMDDAATDLVNNK	775
L. lactis	RVFHEVLSNDDEEVMHTSNI	787

Fig. 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 legend to Fig. 3. Glycine residue, involved in catalysis (Wagner *et al.*, 1992) is underlined.

represent a hydrogenosomal targeting signal (see Brondijk *et al.*, 1996; van der Giezen *et al.*, 1997; 1998).

The high similarity between PFL-L1 of *Piomyces* 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

(*Piomyces* sp. E2 and *Chlamydomonas reinhardtii*) do not form a separate clade (Fig. 3).

PFL proteins of Piomyces 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

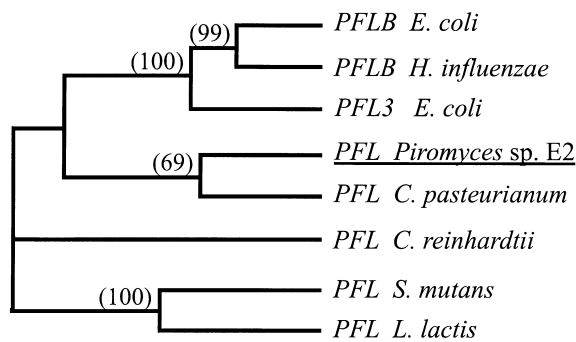


Fig. 3. Phylogenetic analysis of the C-terminal parts of the deduced PFL sequences by using the maximum likelihood method. C-terminal parts of the PFL sequences, corresponding to the sequence fragment available for *Chlamydomonas reinhardtii* (starting from amino acid residue 612 of *Piromyces* sp. E2 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 the PROTML program is shown. A tree with a similar topology was obtained with the neighbour-joining method. The numbers between brackets indicate the bootstrap values obtained for the same clades by using the neighbour-joining method. Database accession numbers are: *Clostridium pasteurianum*, Q46267 (Weidner and Sawers, 1996); *Chlamydomonas reinhardtii*, P37836 (Dumont et al., 1993); *E. coli* PFLB, P09373 (Rödel 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).

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 (Figs 2A 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). Because the PFL probe that was hybridized 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 because introns in the genome of *Piromyces* sp. E2 are rare and small (F. Voncken and H. Harhangi, unpublished).

Because the *pfl-11* gene of *Piromyces* sp. E2 encodes an N-terminal extension that might function as a hydro-genosomal import and targeting signal, the question arose as to whether all *pfl* genes encode such an N-terminus. The latter ones might encode a cytoplasmic PFL, which has been postulated by Marvin-Sikkema et al. (1993a). Therefore, we performed a 5' RACE-PCR with the primer PFLrev2 (see Fig. 1) to identify genes with and without an N-terminal extension. In addition to clones identical to pL1 (possessing the N-terminal extension), two shorter clones (pS1 and pS2) were identified that lacked the first 74 N-terminal amino acids encoded by pL1 (Fig. 2A). Moreover, they differed from each other by four nucleotide substitutions (one of them non-synonymous) (Figs 2A 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. 2A).

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). 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 hybridized to the same Northern blot lanes. The 'long'-type probe hybridized to both transcripts, obviously as a result of a length polymorphism at the 3' end (see Fig. 4B). The 'short'-type probe hybridized only to the 2800 nt long mRNA (Fig. 6B and C). Consequently, it must be concluded that both the 'long' and the 'short' types of *pfl* genes are abundantly expressed.

Fig. 4. Multiple alignments of the 5' terminal (A) and the 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 A. In B, 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); and Y16750 (clone pL3).

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

pL1 -----
 pL5 -----ATAAAAAAGAATTTACTTATTTGTA AAAACAAAAAATCTTAATATACCAATAATATTATGTGAAA
 pL7 TAAATACCTTAAGGAATTTAAATAATCTTTATATACCAAAAAAACTGTGAATTATTATATTTAAACAAACGCAATATATCCTTTTTT
 pS1 -----
 pS2 -----

pL1 -ATTTATATTTTTTTTTTACAAAACAAAATAAAATGGAAGCTTAGCTTTATCCAATGTCAGT--GTTCTTGCCTAACACTGTTTCTGTT 86
 pL5 ATAAAAATTAATTAATATATAATAAAAAAATGGAAGCTTAACCTTTAACTCAAGTTAATAATGCTATTGCCAAGTCAGTTTCAGTT
 pL7 TTATTATTA AAAA AACTTTTTTAAAAACAAAATGGAAGCTTAACCTTTAACTCAAGTTAACAACGCTATTGCCAAGTCAGTTTCAGTT
 pS1 -----
 pS2 -----

pL1 AACCGTGTGGCTGCCACCAAGGTCGCTGGTGTGAGAATGGCCAAGCCATCCCGCGCTCTTCACACCCAGCTATGAAGACTACT---CTT 173
 pL5 AATGCTGTGGCTGCTACTAAAGTTGCTGGTGTAGAAATCAGCAAGCCATCCCGTGCATCCACACTACTCCAATGACTACGACTAGTCTT
 pL7 AATGCTGTGGCTGCTACAAAGGTTGCTGGTGTAGAAATCAGCAAGCCATCCCGTGCATGCCACCACTCCAATGACTACTACTAGTCTT
 pS1 -----TAAGTAAATAATGAGAAAATCAATTTTGCCTAATTTTTAATATTT
 pS2 -----ATAAATACTATTAAAAATATGAGAAATCAATTTTTTGATAATTTTTAATATTT

pL1 AAGACTTC TAAGAAG-----GTCCAGCTATGCAAGCTAAGACCTACGCCACTCAAGCTCCATGCATCACCAACGATGCTGCTGCCAAG 257
 pL5 AAGACTTC TAAGAAGCTTCCTTCCCATCTATCCAAACAAAACCTACGCTACTCAAGCTCCATGCATCACTAGTGTGCGCTGCTAAG
 pL7 AAGACTTC TAAG-----GTTCAAGCTATGCAAGCTAAGACTTACGCCACTCAAGCTCCATGCATCACTAATGATGCTGCTGCTAAG
 pS1 TTTTATATATTTTAAATTTTAAACATATCAAAAATAGTATATTTAAATATTAAGTTATTTTTTATATATTATTAATATAATAAAACATG
 pS2 TTTTATATATTTTAAATTTTAAACATATCAAAAATAGTATATTTAAATATTAAGTTATTTTTTATATATTATTAATATAATAAAACATG

pL1 AGTGAAATCGATGTTGAAGGTTGGATTAAAGAAGCACTACACCCCAACFACGAAGGTGATGGTTCTTTCCCTT---GCTGGTCCAACCTGAAAAG 344
 pL5 AGTGAAATCGATGTTGAAGGTTGGATTAAAGAAGCACTACACCCCAACFACGAAGGTGATGGTTCTTTCCCTT---GCTGGTCCAACCTGAAAAG
 pL7 AGTGAAATCGATGTTGAAGGTTGGATTAAAGAAGCACTACACCCCAACFACGAAGGTGATGGTTCTTTCCCTT---GCTGGTCCAACCTGAAAAG
 pS1 CAAGTTATTGACGTTGCCAAGTGGATCAAGGAAACTACACCCCGTACGAAGGTGATGCTTCTTTCCCTTGTAGCCAGTCTCTGCTAAG
 pS2 CAAGTTATTGACGTTGCCAAGTGGATCAAGGAAACCACACCCCAACFACGAAGGTGATGCTTCTTTCCCTTGTAGCCAGTCTCTGCTAAG

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 GACGGCATGGCCACCAGAAAACCTATCCAAACCTTACTATTCGTTCTCTGGTTACGCTGTCAACCTTTGTTAAGTTAACTCCA 2402
 pPFLh112 GATGCTATGGCTCATCCAGAAAACCTATCCAAACCTTACTATTCGTTCTCTGGTTATGCTGTAACTTTGTTAAGTTAACTCCA
 pPFLh114 GATGCAATGGCACATCCAGAAAACCTATCCAAACCTTACTATTCGAGTTTCAGGATATGCTGTAACTTTGTTCAAATTAACATCCA
 pPFLh140 GATGCTATGGTCCACCAGAAAACCTATCCAAACCTTACTATTCGTTCTCTGGTTACGCTGTCCACTTCACTTCCGTTAACTCCA
 pPFLh104 GATGCTATGGCTCATCCAGAAAACCTATCCAAACCTTACTATTCGTTCTCTGGTTATGCTGTAACTTTGTTAAGTTAACTCCA
 pPFLa12 GACGGCATGGCCACCAGAAAACCTATCCAAACCTTACTATTCGTTCTCTGGTTACGCTGTCAACCTTTGTTAAGTTAACTCCA

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 CAACAACAAAAGGAAGTTATTGCTCCCTACCTTCCACGAAAAGATGTAATTCGCTTCAAAAAGTAATCTTTTGAACCAAAAAGT 2486
 pPFLh112 GCTCAACAAAAGGAAGTTATTGCTCCCTACCTTCCACGAAAAGATGTAATGAATAATATTTAATATACTAGAAATATATTTAA
 pPFLh114 CAACAACAAAAGGAAGTTATTGCTCCCTACCTTCCACGAAAAGATGTAATGAACAAAATAATCAAAAATTTATATATATAAACAA
 pPFLh140 GCTCAACAAAAGGAAGTTATTGCTAGAACCTTCCACGAAAACCTTTAAAGAGATTTAAAGGATATAAAAATTTGTTTTACCATGA
 pPFLh104 CAACAACAAAAGGAAGTTATTGCTCCCTACCTTCCACGAAAAGATGTAAGTTTGGAGAATTGTA AAAAAAAAAAAAAAAAAAAC--
 pPFLa12 GCTCAACAAAAGGAAGTTATTGCTCCCTACCTTCCACGAAAAGATGTAATATTT-----

pPFLh115 TTTTATTCACAAAAGTGTCTGAAAAATCAGTGACCAATTTTTTAACTTTTTTGACTGGGTAAAAATTTTATTTATATAAAC 2570
 pPFLh112 AGAAGAAATACTATCACCTCTTTATTTATTTTATTTTTTTTGGTGTCAAAGAGGTAATAATATATAGTATACTCGAAAAAAT
 pPFLh114 TTA AAAATATATATAAACATAAAAATGAAATGAAATATTA AAAATTTAAAAAATTTAAATTTACTAATCTTAATATAATGCTAA
 pPFLh140 AAAATATATCAATTTTAAATAATTAATATAAAATTTTATGAAAAATATATCATAAATAATTAGCCATCTCCAATCAACCAAAACA
 pPFLh104 -----
 pPFLa12 -----

pPFLh115 CATCCCTCCCAAAAATTTATAAAAATAGTAAAAGATTTTGTTTTTATATTTAAAAGTAAAATTTTAAAAATTAATAATTTTTTTT 2654
 pPFLh112 AAAATAAAAATAAAAATAAAAATAAACGAATATACAATTTAATAGCAACAACAACAACAAAAATAATAATAAACA
 pPFLh114 AATAATTTAGCTTTTACAATAAAGAAAAAATTTATTTATATAATAAAAATACTACATGTAATAAAAAAAAAAACTACTATATAAAA
 pPFLh140 CTATATAAATTTTATAATAATAATAATAATAAAAAAATTAATTTTTTAAATAATCAAAAAAAAAAAAAATAATAATAAATTTAT
 pPFLh104 -----
 pPFLa12 -----

pPFLh115 ATATAAAAATAGTAAAAAATTAATGCAAAATATAAAAAAGTAAATAAATAAATAAATAAATAAATAAATAAATAAATAAATCAAAAT 2733
 pPFLh112 AATAATAACAAAAATAAATAATC-----
 pPFLh114 ATACTATCAATAACT-----
 pPFLh140 TAGTTTAT-----
 pPFLh104 -----
 pPFLa12 -----

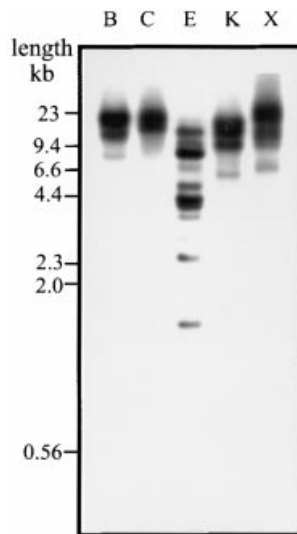


Fig. 5. Southern blot of genomic DNA from *Piromyces* sp. E2. Ten micrograms of genomic DNA digested with *Bam*HI (B), *Cla*I (C), *Eco*RI (E), *Kpn*I (K) and *Xba*I (X) was loaded per lane. cDNA clone pPFLa12, containing the 3' terminal part of the PFL-encoding gene, was used as probe. This probe does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

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.

Expression of PFL at the protein level

The subcellular localization of PFL in *Piromyces* sp. E2

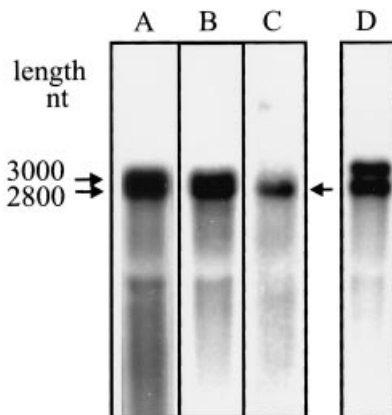


Fig. 6. Analysis of PFL expression by Northern blotting. A. Northern blot with 10 μ g of total RNA from *Piromyces* sp. E2, probed with the pPFLh115 clone. B. The same lane as in A, probed with the 5' part of the pL1 clone ('long'-type specific probe). C. The same lane as in A, probed with the 5' part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

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 double protein band with a molecular mass of \approx 80–82 kDa was recognized 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

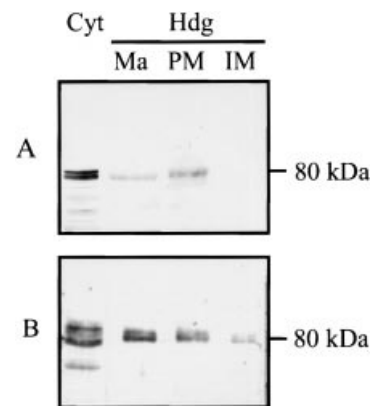


Fig. 7. Western blot analysis of the subcellular distribution of PFL. Western blotting of different subcellular fractions from *Piromyces* sp. E2 (A) and *Neocallimastix* sp. L2 (B). Cyt, cytosolic fraction. Hydrogenosomal fractions (Hdg) are indicated as follows: Ma, hydrogenosomal matrix; PM, peripheral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

because a removal of the putative N-terminal import signal from this isoform could account precisely for the observed difference. However, because we cannot exclude that the 'short'-type ('cytoplasmic') PFLs could also account for a cross-reacting protein with a molecular mass of \approx 81 kDa (if the C-terminal part of the 'short' PFLs does not contain significant deletions or insertions), a confirmation of the hydrogenosomal localization 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

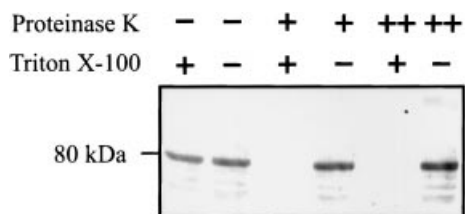


Fig. 8. Proteinase K protection assay with isolated hydrogenosomes. Hydrogenosomes were incubated in the absence (-) or presence of 0.2 (+) or 20 (++) $\mu\text{g ml}^{-1}$ proteinase K and in the presence (+) or absence (-) of detergent (0.1% Triton X-100 and 0.1% deoxycholate). The Western blot was incubated with antiserum against PFL from *E. coli*.

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.

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 localization 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 *et al.* (1994), our measurements revealed that ≈ 1 mol of hydrogen was formed per 1 mol of malate utilized (Fig. 9; see also Marvin-Sikkema *et al.*, 1994). Because acetate and formate were also produced in equimolar amounts, a significant PFO activity in the hydrogenosomes could be excluded. However, only 0.7 mol of acetate and formate was formed per 1 mol of malate consumed. As a concomitant

Table 1. PFL activities in the cytosolic and hydrogenosomal fractions of *Piromyces* sp. E2.

	Enzyme activity ($\mu\text{mol mg}^{-1}$ protein)	
	Cytosol	Hydrogenosomes
PFL	0.111	0.235
PFL + hypophosphite	ND	ND

PFL activity was measured by formate production after 60 min incubation at 39°C. Hypophosphite, a specific inhibitor of PFL activity, was added to a final concentration of 10 mM. ND, not detectable.

accumulation of pyruvate (≈ 0.3 mol per 1 mol 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.

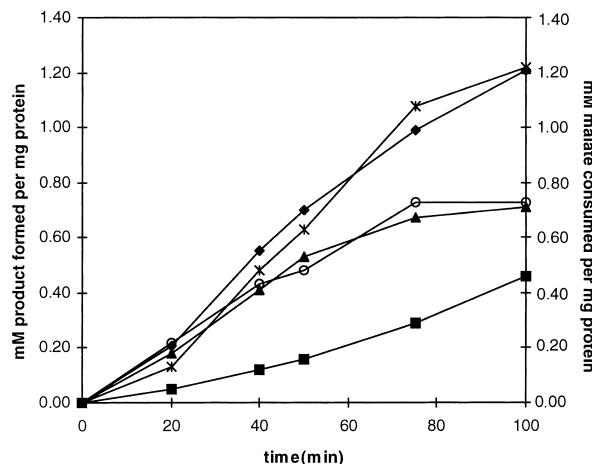


Fig. 9. Consumption of malate and production of hydrogen, acetate, formate and pyruvate by isolated *Piromyces* sp. E2 hydrogenosomes. Suspensions of isolated hydrogenosomes (aliquots containing ≈ 0.55 mg protein) were incubated anaerobically in the assay mixture (see *Experimental procedures*) at 39°C. 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; × 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.*, 1997). 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 PFLs. 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 because the evidence that the extension might be cleaved off after import into

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 only allows the generation of one ATP molecule by substrate level phosphorylation. Compartmentalization of the pyruvate catabolism, i.e. the evolution of hydrogenosomes, might allow the generation of extra ATP molecules 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, i.e. these hydrogenosomes cannot rely on pyruvate for hydrogen formation (see Marvin-Sikkema *et al.*, 1994; K. Hosea, unpublished). 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 also implies that the NAD(P)H that is generated by the hydrogenosomal malic enzyme must be reoxidized by the hydrogenase, either directly or indirectly. For anaerobic chytrids, we do not know yet how NAD(P)H is reoxidized, 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 reoxidizing 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 because a hydrogenosomal PFL requires 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) 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 because gene duplication is an important evolutionary process in eukaryotes, and is 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 (Figs 2, 4 and 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) compartmentalization of a subfamily of PFLs in the hydrogenosomes might provide an explanation as to why PFL and not PFO is present in the hydrogenosomes of anaerobic chytrids. Consequently, the evolution of PFL-possessing hydrogenosomes by gene duplication and compartmentalization 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.

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 (Marvin-Sikkema *et al.*, 1990; Teunissen *et al.*, 1991). Biomass was harvested after 40–48 h 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 previously (Akhmanova *et al.*, 1998a). cDNA clones were picked at random and were 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'-GTCTTGGAATAAGG-TACGAC-3'; PFLrev2, 5'-TA(AG)TCACCAATAATACGAC-CACG-3'; PFLrev3, 5'-TCAAGGTAACCTGGCTTG-3'; PFLrev4, 5'-AGAACCATCACCTTCGTATG-3' (see Fig. 1). The resulting RACE-PCR products were subcloned into pCR 2.1 (Invitrogen) and were 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 analysed 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).

Southern and Northern blotting

Genomic DNA was prepared from *Piromyces* 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, an 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. Hybridization was performed in 0.5 M sodium phosphate buffer, pH 7.0, 7% SDS, 1% BSA and 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 *Piromyces* sp. E2 biomass, essentially as described by Marvin-Sikkema *et al.* (1993a) with the following modification: the potassium phosphate (K_P) buffer in the homogenization medium was substituted for 20 mM K-HEPES buffer, pH 7.4. The hydrogenosomal fraction was subfractionated by carbonate extraction as described previously by Elgersma *et al.* (1996). Proteins were separated on 7.5% polyacrylamide gels with 0.1% SDS and were blotted to polyvinylidenedifluoride membrane by semidry transfer (Bio-Rad) according to the manufacturer's 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 of 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 μ g ml⁻¹) and detergent (0.1% Triton X-100 and 0.1% deoxycholate) in all possible combinations. After 30 min 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 K_P, 100 mM succinate, 5 mM magnesium ADP, 2 mM DTT, 1.3 mM ferriammonium sulphate, 0.12 mM CoA and 30 mM pyruvate. The incubation was stopped after 60 min by heating (5 min, 100°C). Formation of formate was determined with formate dehydrogenase (Boehringer Mannheim). Samples, heated (5 min, 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) to permeabilize

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 K_P buffer, 100 mM succinate, 5 mM magnesium ADP, 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).

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