Iron-Dependent Hydrogenases of Entamoeba histolytica and Giardia lamblia: Activity of the Recombinant Entamoebic Enzyme and Evidence for Lateral Gene Transfer

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Abstract. Entamoeba histolytica and Spironucleus barkhanus have genes that encode short iron-dependent hydrogenases (Fe-hydrogenases), even though these protists lack hydrogenosomes. To understand better the biochemistry of the protist Fe-hydrogenases, we prepared a recombinant E. histolytica short Fe-hydrogenase and measured its activity in vitro. A Giardia lamblia gene encoding a short Fe-hydrogenase was identified from shotgun genomic sequences, and RT-PCR showed that cultured entamoebas and giardias transcribe short Fe-hydrogenase mRNAs. A second E. histolytica gene, which encoded a long Fe-hydrogenase, was identified from shotgun genomic sequences. Phylogenetic analyses suggested that the short Fe-hydrogenase genes of entamoeba and diplomonads share a common ancestor, while the long Fe-hydrogenase gene of entamoeba appears to have been laterally transferred from a bacterium. These results are discussed in the context of competing ideas for the origins of genes encoding fermentation enzymes of these protists.

Introduction

One of the great recent discoveries in the cell biology of protists is that the hydrogenosome of Trichomonas vaginalis, cause of vaginitis, is a modified mitochondrion, in which the enzymes of oxidative phosphorylation have been replaced by fermentation enzymes that produce hydrogen gas (Müller, 1993, 1998; Bui et al., 1996; Horner et al., 1996; Andersson and Kurland, 1999; Rotte et al., 2000). Proof of this idea includes the presence of mitochondrion-like chaperones and a mitochondrion-like ATP/ADP transporter within the hydrogenosome, as well as organelle-targeting sequences at the N-termini of hydrogenosomal proteins that are encoded in the nucleus (Johnson et al., 1990; Hrdy and Müller, 1995; Bui et al., 1996; Bui and Johnson, 1996; Horner et al., 1996; Bradley et al., 1997; Dyall et al., 2000).

The common origin of hydrogenosomes and mitochondria is included in a new biochemical explanation for the origin of mitochondria, called the hydrogen hypothesis (Martin and Müller, 1998). The hydrogen hypothesis, a revision of the widely accepted endosymbiont hypothesis (Gray et al., 1999), suggests that the α-proteobacterium, which became the mitochondrion, was a facultative anaerobe that was selected for its ability to produce hydrogen in a methanogenic archaeal host. Consistent with this idea, multiple hydrogenosomal fermentation enzymes—including ferredoxin, succinyl-CoA synthetase, and malic enzyme—resemble their counterparts in mitochondria (Johnson et al., 1990; Hrdy and Müller, 1995b). One alternative hypothesis suggests that the mitochondrial endosymbiont was selected for its ability to consume oxygen and thus protect the proto-eukaryote from oxidative damage (Andersson and Kurland, 1999). Another alternative hy-
pothesis suggests that the endosymbiosis was based upon cycling of sulfur (Searcy, 1992).

An iron-dependent hydrogenase (Fe-hydrogenase), which gives the hydrogenosome its name, transfers electrons from reduced ferredoxin to two protons to make hydrogen gas (Lindmark and Müller, 1973; Müller, 1993; Payne et al., 1993; Bui and Johnson, 1996; Horner et al., 2000). Fe-hydrogenase activity, which is detected using hydrogen gas and methyl viologen as a reporter, has been shown in extracts of eukaryotes with hydrogenosomes or plastids (Payne et al., 1993; Wunschiers et al., 2001). Fe-hydrogenases, which are also present in strictly anaerobic bacteria, are designated as long or short depending upon the number of N-half ferredoxin-like domains that are adjacent to a conserved C-half hydrogenase domain (Cammark, 1992). The structures of a short Fe-hydrogenase of Desulfovibrio desulfuricans and a long Fe-hydrogenase of Clostridium pasteurianum have been solved, and each revealed two ferredoxin-like [4Fe-4S] iron-sulfur centers and a hydrogenase active-site composed of a [4Fe-4S] center bridged to a [2Fe] cluster (Peters et al., 1998; Nicolet et al., 1999).

The reduced ferrodoxin is produced within the hydrogenosome by pyruvate:ferredoxin oxidoreductase (PFOR), which decarboxylates pyruvate to acetyl-CoA and CO₂ (Lindmark and Müller, 1973; Müller, 1993; Payne et al., 1993; Hrdy and Müller, 1995a; Horner et al., 1999). It has been difficult to determine the phylogeny of the genes that encode Fe-hydrogenase and PFOR, because these iron-sulfur proteins are absent from mitochondria, α-proteobacteria, and most other eubacteria. The pfB genes of T. vaginalis, Entamoeba histolytica (cause of amoebic dysentery), and Giardia lamblia (a cause of diarrhea) appear to share a common ancestry, although no bacterial donor has been identified, and a gene encoding a second keto-iron oxidoreductase of G. lamblia appears to have a distinct ancestry (Reeves, 1984; Hrdy and Müller, 1995a; Rosenthal et al., 1997; Brown et al., 1998; Müller, 1998; Horner et al., 1999; Huston and Petri, 2001). The Fe-hydrogenase genes of T. vaginalis, E. histolytica, and Spironucleus barkhanus (a diplomonad similar to G. lamblia), each of which encodes a short Fe-hydrogenase, also appear to share a common ancestry, although no bacterial donor was identified (Horner et al., 2000; van Hoek et al., 2000). In contrast, the long fe-hydrogenase gene of the microaerophilic ciliate Nyctotherus ovalis appears to have a distinct ancestry.

The Fe-hydrogenase results are surprising for three reasons (Horner et al., 2000). First, the fermentation enzymes of E. histolytica and G. lamblia are present in the cytosol rather than in an organelle (Reeves, 1984; Müller, 1993; Brown et al., 1998; Mai et al., 1999; Ghosh et al., 2000). Entamoebas have mitochondrion-derived organelle called the crypton or mitosome, while the giardial gene encoding a 60-kDa chaperonin appears to be endosymbiont-derived, but no organelle has been identified (Clark and Roger, 1995; Roger et al., 1998; Mai et al., 1999; Tovar et al., 1999; Ghosh et al., 2000). Second, entamoebas and diplomonads have long been thought not to produce hydrogen gas in culture, although a recent report suggests giardias may produce hydrogen under anaerobic conditions (Lindmark and Müller, 1973; Reeves, 1984; Brown et al., 1998; Müller, 1998; Lloyd and Harris, 2002). Third, short fe-hydrogenase genes of E. histolytica and S. barkhanus appear to share a common ancestor, even though these two protists are not closely related to each other in phylogenies drawn with rRNA or protein sequences (Sogin and Silberman, 1998; Horner et al., 2000; Baptiste et al., 2002). The latter result suggested the possibility of lateral transfer of Fe-hydrogenase genes between these protists. Recent phylogenetic studies suggest that numerous genes encoding fermentation enzymes and other proteins of E. histolytica, G. lamblia, and T. vaginalis may have been laterally transferred from prokaryotes (Rosenthal et al., 1997; Doolittle, 1998, 1999; de Koning et al., 2000; Field et al., 2000; Nixon et al., 2002).

With the goal of understanding better the biochemistry and evolution of Fe-hydrogenases of entamoebas and diplomonads, we performed the following studies. (1) The E. histolytica short Fe-hydrogenase 1 was expressed as a glutathione-S-transferase (GST) fusion-protein in E. coli, and its activity was measured in vitro. (2) The G. lamblia fe-hydrogenase gene was identified from shotgun genomic sequences, and mRNAs encoding short Fe-hydrogenases were detected in cultured giardias and entamoebas. (3) An E. histolytica gene encoding a long Fe-hydrogenase 2 was identified from genomic sequences and compared with other long Fe-hydrogenases. (4) Phylogenetic analyses were repeated with the addition of Fe-hydrogenases of G. lamblia, E. histolytica, the green alga Chlamydomonas reinhardtii, and the eubacterium Megasphaera elsdenii.

Materials and Methods

Cloning of the G. lamblia fe-hydrogenase and E. histolytica genes and identification of mRNAs encoding Fe-hydrogenase genes from cultured entamoebas and giardias. An E. histolytica EST (GenBank AB002772), which encodes the N-terminus of a putative short Fe-hydrogenase 1, was identified from GenBank using BLASTP and an amebic [4Fe-4S] ferredoxin sequence (Altschul et al., 1997; Nixon et al., 2002). The 3' end of the E. histolytica fe-hydrogenase 1 gene was isolated using 3' RACE (FirstChoice RLM-RACE kit, Ambion Inc., Austin, Texas), and the entire entamoebic fe-hydrogenase 1 gene was cloned, sequenced on both strands, and deposited in GenBank under accession number AAG09783. A second Entamoeba fae-hydrogenase gene, which encodes a long Fe-hydrogenase, was identified from assemblies of E. histolytica genome sequences at The Institute for Genomic Research. The E. histolytica fe-hydroge-
nase 2 gene was deposited in GenBank under accession number AF172963.

A shotgun clone from the G. lamblia genome sequencing project, which contained the 5' end of a putative Fe-hydrogenase gene, was used to identify the entire Fe-hydrogenase gene from a G. lamblia genomic DNA library made in Lambda Zap (McArthur et al., 2000). The G. lamblia Fe-hydrogenase gene was sequenced on both strands and deposited in GenBank under accession number AAK28337. The N-termini of predicted entamoebic and giardial Fe-hydrogenases were examined with MITOP to determine whether organelle-targeting sequences might be present (Claros and Vincens, 1996).

Total RNA was prepared by lysing cultured entamoebas and giardias in a guanidinium isothiocyanate solution and by centrifuging the lysate through a cesium chloride gradient (Chocznynski and Sacchi, 1987). Reverse-transcriptase and polymerase chain reaction (RT-PCR) were performed with these RNAs and with primers specific for the E. histolytica and G. lamblia genes encoding short Fe-hydrogenase, malic enzyme, alcohol dehydrogenase E (ADHE), and ferredoxin I (entamoebas only) (Rosenthal et al., 1997; Nixon et al., 2002). For negative controls, PCR was performed without RT, and RT-PCR products were identified on agarose gels.

Expression of a recombinant short E. histolytica Fe-hydrogenase and measurement of its activity. A recombinant glutathione-S-transferase (GST) fusion-protein containing a short Fe-hydrogenase at its C-terminus was made by cloning the E. histolytica Fe-hydrogenase 1 coding region into the pGEX-6T vector (Smith and Johnson, 1988). The GST-Fe-hydrogenase 1 construct was transfected into Escherichia coli strain BL21, which was grown anaerobically and induced with isopropyl β-D-thiogalactopyranoside (IPTG). Bacteria were lysed by freezing and thawing, and the hydrogenase activities of the supernatant and of bacterial lysate expressing a GST-Fe-hydrogenase 1 fusion-enzyme (Table 1) (Smith and Johnson, 1988). The activity of the recombinant entamoebic Fe-hydrogenase 1 was measured without RT, and RT-PCR products were identified on agarose gels.

Results and Discussion

A recombinant short Fe-hydrogenase 1 of E. histolytica is active. The amino-terminus of the E. histolytica Fe-hydrogenase 1 did not contain an organelle-targeting sequence (Claros and Vincens, 1996; Horner et al., 2000). Indeed, an epitope-tagged Fe-hydrogenase 1 is present within the cytosol of transfected E. histolytica (Ghosh et al., 2000). The activity of the entamoebic Fe-hydrogenase 1 was measured in lysates of E. coli, which were expressing a GST-entamoebic Fe-hydrogenase 1 fusion-enzyme (Table 1) (Smith and Johnson, 1988). The activity of the recombinant entamoebic Fe-hydrogenase 1 was present when hydrogen was bubbled into the medium but absent when nitrogen (negative control) was bubbled into the medium (Payne et al., 1993). There was no Fe-hydrogenase activity in control E. coli, which were overexpressing a GST-chitinase fusion-protein. Recombinant entamoebic Fe-hydrogenase 1, which was purified on glutathione-agarose beads, had a decreased specific activity (data not shown). This was likely caused by exposure to oxygen during the purification procedure that probably inactivated the Fe-hydrogenase 1 iron-sulfur centers (Cammark, 1992; Payne et al., 1993; Horner et al., 2000). The specific activity of the GST-Fe-hydrogenase 1 fusion enzyme was about 10 times that of the native entamoebic Fe-hydrogenase 1 overexpressed in transfected E. histolytica (Table 1). Interestingly, the specific activity of
the *T. vaginalis* Fe-hydrogenase was greater than that of the recombinant entamoebic GST-entamoebic Fe-hydrogenase and was inhibited by a lysate of non-transfected entamoebas (Table 1). This may explain why it was difficult to detect Fe-hydrogenase activity in lysates of nontransfected entamoebas, even though Fe-hydrogenase 1 mRNAs were identified from them by RT-PCR (next section).

**Cultured entamoebas and giardias express mRNAs encoding short Fe-hydrogenases.** We isolated an *fe-hydrogenase* gene of *G. lamblia*, because we have frequently compared the fermentation enzymes of this diplomonad with those of *E. histolytica* (Rosenthal et al., 1997; Field et al., 2000; Nixon et al., 2002). A search of the contigs predicted from the *G. lamblia* shogun sequences suggested that this gene, which predicts a short Fe-hydrogenase, is the only hydrogenase gene present within the giardial genome. Like the entamoebic Fe-hydrogenase 1, the predicted giardial Fe-hydrogenase lacked an N-terminal organelle-targeting sequence and had two ferredoxin-like iron-sulfur centers and a hydrogenase iron-sulfur center like those present in the short Fe-hydrogenases of *T. vaginalis*, *Desulfovibrio* sp., and *Clostridia* sp. (Cammark, 1992; Thompson et al., 1994; Bui and Johnson, 1996; Horner et al., 1996; Nicolet et al., 1999). RT-PCR showed that cultured entamoebas and giardias contain mRNAs, which encode short Fe-hydrogenases (Fig. 1A, B). Negative controls without RT showed that the RT-PCR was not amplifying DNA from the extracts of cultured entamoebas and giardias. Because the giardial contigs predicted only one Fe-hydrogenase, which is expressed, it is likely that the hydrogenase activity recently detected in cultures of giardias derives from this enzyme (Lloyd and Harris, 2002). In contrast, entamoebas appear to have a second long hydrogenase (see next section), so if entamoebic hydrogenase activity is present, it might derive from one or more enzymes. These results suggest the possibility that entamoebas and giardias use protons as electron acceptors

### Table 1

**Activities of Entamoeba histolytica and Trichomonas vaginalis Fe-hydrogenases**

| Sample                                           | Hydrogenase activity (nmol/min/mg of protein)* |
|--------------------------------------------------|-----------------------------------------------|
| Bacteria transformed with *E. histolytica* Fe-hydrogenase | 36 (2)                                         |
| Transfected *E. histolytica* with Fe-hydrogenase   | 3.5 ± 0.7 (3)                                  |
| *Trichomonas vaginalis*                           | 167 ± 32 (9)                                   |
| *T. vaginalis* + 0.47 mg *E. histolytica* lysate   | 114 (2)                                        |

* Averages +/- standard deviations, where possible. Number of determinations in parentheses.

* Calculated $K_i = 0.56$ mg (amount of *E. histolytica* lysate in mg of protein to cause 50% reduction of *T. vaginalis* hydrogenase activity).

Figure 1. Agarose gels of ethidium-stained RT-PCR products from entamoebic and giardial mRNAs. Images are reversed for clarity of reproduction. (A) RT-PCR of amoebic mRNAs encoding malic enzyme (lane 1), Fe-hydrogenase (lane 2), alcohol dehydrogenase E (lane 3), and ferredoxin (lane 4). Size markers are shown in lane 5. Lanes 6–9 are the negative controls for malic enzyme, hydrogenase, ADHE, and ferredoxin, respectively. (B) RT-PCR of giardial mRNAs encoding ADHE (lane 3), Fe-hydrogenase (lane 4), and malic enzyme (lane 5). A negative control (no RT) for Fe-hydrogenase is shown in lane 6. A positive control for Fe-hydrogenase, using *Giardia lamblia* WB strain DNA, is shown in lane 2. Size markers are shown in lane 1.
when the organisms are growing under strictly anaerobic conditions in the bowel lumen (Brown et al., 1998; Huston and Petri, 2001; Lloyd and Harris, 2002).

E. histolytica has a hydrogenase 2 gene encoding a long Fe-hydrogenase. The assemblies of the shotgun sequences of the E. histolytica genome predicted a long Fe-hydrogenase 2 (Fig. 2) in addition to the short Fe-hydrogenase 1. The entamoebic Fe-hydrogenase 2 was 504 amino acids long and had an N-terminal sequence, which included positively charged Lys and Arg that are often present at the N-termini of organellar proteins (Claros and Vincens, 1996). In addition, the N-terminus of Fe-hydrogenase 2 contained Ser and Leu residues, which are present at the N-termini of crypton and hydrogenosomal proteins (Bui et al., 1996; Mai et al., 1999). However, in the absence of experimental evidence, we cannot be sure that the entamoebic long Fe-hydrogenase is targeted to the crypton.

The entamoebic Fe-hydrogenase 2 was much more similar (>38% amino acid identities) to predicted long Fe-hydrogenases of Bacteroides fragilis and Treponema denticola than to short Fe-hydrogenases of entamoebas, giardias, trichomonads, and other anaerobic bacteria (<28% amino acid identities; Fig. 2). The entamoebic Fe-hydrogenase 2 and the predicted long Fe-hydrogenases of B. fragilis and T. denticola were much more similar (28% identities) than to short Fe-hydrogenases of entamoebas, giardias, trichomonads, and other anaerobic bacteria (28% identities; Fig. 2).
and T. denticola each contained Cys residues that likely coordinate two ferredoxin-like [4Fe-4S] iron-sulfur centers (marked with X’s in Fig. 2) and hydrogenase iron-sulfur centers (marked with o’s), which have previously been identified in structures of short and long Fe-hydrogenases (Peters et al., 1998; Nicolet et al., 1999). In addition, the predicted entamoebic Fe-hydrogenase 2 had eight other N-terminal Cys residues, which aligned with those of the bacteroides and treponema long Fe-hydrogenases (marked with asterisks). Although these Cys residues probably coordinate other iron-sulfur centers, they remain unidentified, because they do not align with the N-terminal iron-sulfur centers of the long Fe-hydrogenase of C. pasteurianum, which has been crystallized (Peters et al., 1998).

The entamoebic and giardial short Fe-hydrogenase 1 genes appear to share a common ancestry, while the entamoebic long Fe-hydrogenase 2 gene appears to have been laterally transferred from a prokaryote. Phylogenetic trees of Fe-hydrogenases from eubacteria and eukaryotes are star-shaped and contain few basal nodes that are strongly supported (Fig. 3). This result suggests that the Fe-hydrogenases are widely divergent and that little phylogenetic signal remains. For example, Fe-hydrogenases of closely related eukaryotes—either trichomonads, green algae (Chlamydomonas reinhardtii, Scenedesmus obliquus, and Chlorella fusca), or chytrid fungi (Piromyces sp. and Neocalimastix frontalis)—each grouped together, but Fe-hydrogenases of unrelated eukaryotes did not group together. In particular, our analysis does not support recent conclusions that hydrogenases of trichomonads are monophyletic with those of chytrid fungi (Voncken et al., 2002) or with those of E. histolytica and S. barthanus (Horner et al., 2000).

The short Fe-hydrogenase genes of G. lambia, S. barthanus, and E. histolytica appear to share a most recent common ancestry, although a particular bacterial donor was not identified. Remarkably, the short Fe-hydrogenase of G. lambia was more similar to that of E. histolytica than to that of S. barthanus. Because G. lamblia and S. barthanus are diplomonads, which share a recent common ancestor in phylogenetic trees of RNA and proteins (Sogin and Silberman, 1998), a possible explanation of these results is that the E. histolytica Fe-hydrogenase gene was laterally transferred from a diplomad (Rosenthal et al., 1997; Doolittle, 1998, 1999; Müller, 1998; de Koning et al., 2000; Field et al., 2000; Nixon et al., 2002). This lateral gene transfer would not have occurred recently, because the Fe-hydrogenases of entamoebas and giardias showed only a 40% amino acid identity with each other, and each Fe-hydrogenase gene has the codon usage of its host. Alternatively, the diplomad-E. histolytica sub-clade could be incorrectly rooted by the long branch connecting it to the remainder of the tree.

The common ancestry of genes encoding the E. histolytica long Fe-hydrogenase 2 and those of B. fragilis and T. denticola is strongly supported. This appears then to be an example of lateral gene transfer, as Entamoeba is not a close relative of either of these eubacteria (Rosenthal et al., 1997; Doolittle, 1998, 1999; Müller, 1998; de Koning et al., 2000; Field et al., 2000; Nixon et al., 2002). There was weak support for the pairing of Fe-hydrogenases of the ciliate N. ovalis and Desulfovibrio sp., as has been previously noted (Horner et al., 2000; Voncken et al., 2002). This suggests that the ciliate hydrogenase was derived by lateral gene transfer, but does not prove it.

Conclusions

This is the first time that an Fe-hydrogenase from a protist has been expressed as a GST-fusion protein in bacteria. This is also the first time that an Fe-hydrogenase gene (encoding the long hydrogenase of entamoebas) has been inferred to have been laterally transferred from a bacterium, although numerous genes encoding fermentation enzymes (e.g., alcohol dehydrogenases, malic enzyme, and acetyl-CoA syn...
thases) appear to have been laterally transferred from prokaryotes to amoebas and giardias (Rosenthal et al., 1997; Field et al., 2000; Nixon et al., 2002). Although the evidence is weak, this may also be the first time that a gene (encoding the short hydrogenase of entamoebas) has been inferred to have been laterally transferred from another protist. Because the hypothesized lateral gene transfer would probably have occurred after the acquisition of the fe-hydrogenase gene by the diplomonal lineage, this particular result does not disprove the hydrogen hypothesis (Martin and Müller, 1998). However, the failure to demonstrate that the eukaryotic Fe-hydrogenases share a common ancestry, or to identify an α-proteobacterial donor for these eukaryotic fe-hydrogenase genes (Horner et al., 2000), dampens our enthusiasm for the hydrogen hypothesis. These results suggest that the mitochondrial endosymbiont was selected for a property other than hydrogen production (e.g., its ability to consume oxygen) (Andersson and Kurland, 1999) and that the presence of Fe-hydrogenases and other fermentation enzymes of microaerophilic eukaryotes may reflect a secondary adaptation to their anaerobic environment (Rosenthal et al., 1997; Doolittle, 1998, 1999; de Koning et al., 2000; Field et al., 2000; Lloyd and Harris, 2002; Nixon et al., 2002).

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