Pyruvate-Phosphate Dikinase of Oxymonads and Parabasalia and the Evolution of Pyrophosphate-Dependent Glycolysis in Anaerobic Eukaryotes†

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In pyrophosphate-dependent glycolysis, the ATP/ADP-dependent enzymes phosphofructokinase (PFK) and pyruvate kinase are replaced by the pyrophosphate-dependent PFK and pyruvate phosphate dikinase (PPDK), respectively. This variant of glycolysis is widespread among bacteria, but it also occurs in a few parasitic anaerobic eukaryotes such as Giardia and Entamoeba spp. We sequenced two genes for PPDK from the amitochondriate oxymonad Streblomastix strix and found evidence for PPDK in Trichomonas vaginalis and other parabasalia, where this enzyme was thought to be absent. The Streblomastix and Giardia genes may be related to one another, but those of Entamoeba and perhaps Trichomonas are distinct and more closely related to bacterial homologues. These findings suggest that pyrophosphate-dependent glycolysis is more widespread in eukaryotes than previously thought, enzymes from the pathway coexist with ATP-dependent more often than previously thought and may be spread by lateral transfer of genes for pyrophosphate-dependent enzymes from bacteria.

Adaptation to anaerobic metabolism is a complex process involving changes to many proteins and pathways of critical function to the cell, but it is found in several distantly related eukaryotic lineages (25, 26), raising questions of how it evolved. Some of these anaerobic protists were previously thought to be ancient lineages that retained anaerobic metabolism as a primitive, premitochondrial trait, but extant eukaryotic anaerobes are now nearly all known to have evolved from mitochondrion-bearing ancestors (8, 10, 15), so the acquisition of anaerobiosis-related traits is most likely secondary. Embden-Meyerhof-Parnas glycolysis is the starting point of the core carbon metabolism in eukaryotes. In aerobic eukaryotes, glycolysis is a minor source of energy, since ca. 95% of ATP production comes from subsequent tricarboxylic acid cycle reactions and oxidative phosphorylation. In contrast, anaerobic organisms rely almost exclusively on glycolysis and fermentation for ATP production, so optimizing the energy output from glycolysis may be subject to strong selective pressure (21, 22). One significant variation of the standard glycolytic pathway that has been described in some anaerobic protists is pyrophosphate-dependent glycolysis, which uses pyrophosphate (PP) instead of ATP as a phosphate donor (21). This version of glycolysis has been best studied in Entamoeba histolytica (29, 30). In this parasite, two key glycolytic enzymes, phosphofructokinase (PFK) and pyruvate kinase (PK), have been replaced by the PP-dependent versions, PFK and pyruvate-phosphate dikinase (PPDK), respectively (for a comparison of these reactions, see reference 21). Energy efficiency seems to be the chief reason for adopting a PP-dependent glycolysis, since PFK-dependent glycolysis can in theory yield five ATP molecules instead of the two yielded by standard glycolysis. This increase of 2.5-fold can be crucial under severe energy-limiting conditions (21, 22).

PPDK catalyzes the interconversion between phosphoenolpyruvate (PEP) and pyruvate. Unlike the reaction catalyzed by PK, this reaction has a small ΔG and is reversible, so it can proceed either in the catabolic or the gluconeogenic directions (34). This highly versatile enzyme has been appointed to many functions in different eukaryotes, although its coexistence in some cells with the ATP-dependent enzyme PK makes it difficult to address its role (21, 26). Involvement of PPDK in glycolysis has been shown or suggested in Giardia (28), Entamoeba (30), and Phytophthora (18). PPDK also works in the opposite direction in chloroplasts of C4 plants, where it catalyzes the regeneration of PEP in the stroma of leaf mesophyll cells (6). A cytoplasmic isozyme of unclear function also exists in C3 and C4 plants, and it has been suggested that it may have a glycolytic role, especially in oxygen-starved tissues (22). Trypanosomatids target PPDK to glycosomes where it fulfills a thermodynamic role of pyrophosphate recycling in place of pyrophosphatase (1).

At present, studies on the molecular evolution of PP-linked glycolysis have focused almost exclusively on PFK and PFP (3, 27). These proteins are homologous, and the gene family has had a complex evolutionary history including multiple events of duplication, loss, and lateral gene transfer (3, 27). In contrast, the phylogeny of PPDK has not been extensively explored. Previous studies using limited taxon sampling addressed the relationships of certain eukaryotic PPDKs, but whether eukaryotic enzymes originated in common or independently has not been clear (it has been shown that a plant
cluster is separated from other eukaryotes (17, 28)). In addition, it is not known whether PPDK and PFP are always found together, or if some organisms use one PPi-dependent enzyme in glycolysis but not the other. In Giardia and Entamoeba both PPi-dependent enzymes are used, but in Trichomonas vaginalis, PPi-specific PFK and ATP-specific PK have been found, but no PPDK activity has been detected (24). Interestingly, neither PK nor PPDK activities have been detected in the related Trichomonas fetus (14, 21).

One group of amitochondriates of which we know relatively little are the oxymonads. These anaerobes are found almost exclusively in association with animals and are generally not available in cultivation. Oxymonad molecular and biochemical data are scarce because they are typically part of a complex community of eukaryotes from which it is difficult to determine which genes or proteins come from which species. We have used the recent documentation of a noncanonical genetic code in one oxymonad, Streblomastix strix, to circumvent this problem (16). S. strix is found exclusively in the hindgut of the Pacific damp-wood termite Zootermopsis angusticollis, an environment it shares with at least six species of parabasalia (37). From this environment we isolated PPDK genes and used the noncanonical genetic code to identify S. strix homologues. We also identified other protist PPDK genes and, comparing them to T. vaginalis genomic data identified the first parabasalian PPDK genes, overturning the notion that T. vaginalis lacks this enzyme. A comprehensive phylogenetic analysis of PPDK shows several independent clades of eukaryotes, overall suggesting at least some lateral gene transfer may have occurred.

MATERIALS AND METHODS
cDNA library construction and EST sequencing. Termites were collected from a rotten log in Point Grey, Vancouver, Canada. The whole hindgut content of about 60 individuals of Z. angusticollis from a single colony was collected and total RNA was extracted using standard TRIzol procedure (Invitrogen). A cDNA library was constructed (Ampliprime Express) and 5,337 clones were sequenced from the 5' end. Expressed sequence tags (ESTs) were trimmed for vector and quality, and assembled into 2,595 clusters by PEPdb (http://amoebidia.bcm.umontreal.ca/public/pepdb/agrm.php).

Characterization of S. strix PPDK. S. strix sequences were recovered from EST sequences by scanning BLASTX outputs to identify putative protein coding sequences containing in-frame TAA and TAG stop codons. Two truncated ESTs with high identity scores to PPDK were recovered according to this criterion. The C-terminal portion of both was acquired by 3' RACE (Ambion) using specific primers and total hindgut RNA. Most of the 5' portion of the gene was obtained by reverse transcription-PCR (RT-PCR) amplification using a degenerate primer designed from a conserved block located at about 90 amino acids from the start of the G. intestinalis PPDK and gene-specific primers designed from the EST sequences. In all cases, PCR products were cloned by using TOPO cloning system (Invitrogen). Sequences were assembled with Sequencher, and the sequence was confirmed to come from a single gene by amplifying from DNA using primers at the ends of the assembled contig. Parabasalian PPDK and PFP sequences from the EST data were identified by comparing them to the T. vaginalis genome project at TIGR, we found gene predictions on eight categories of rates, with gamma and proportion of invariant sites estimated from the data. Support of the nodes was estimated by using 100 bootstrap replicates.

RESULTS AND DISCUSSION
Identification of PPDK genes from the oxymonad S. strix and parabasalian symbionts. From an EST sequence survey of the eukaryotic fauna from the hindgut of the termite Z. angusticollis, we identified three ESTs matching the PPDK family and other related PEP-utilizing enzymes. Two sequences contained several in-frame, TAA and TAG stop codons, identifying them as belonging to the flagellate S. strix, which has an alternate genetic code where TAA and TAG encode glutamine (Q) instead of translational stops (16). One of these sequences was extended by using a combination of RT-PCR with degenerate primers and 3' RACE so that 88% of the gene was ultimately characterized. Attempts to recover the extreme 5' end of the gene by 5' RACE were unsuccessful, probably due to the highly complex nature of the sample and the large size of the gene (the G. intestinalis homologue is 2,655 bp). These procedures resulted in a nucleotide sequence encoding an open reading frame of 780 codons. The second S. strix sequence differed only slightly at the nucleotide level, indicating that at least two gene copies or alleles of this gene exist in this S. strix population and so was not characterized further. Since ppdk belongs to a gene family with at least three functionally differentiated clusters, we wanted to identify which group the S. strix sequences belong to. Phylogenetic analyses of the S. strix gene, including members of the PPDK, water dikinase, and glucon dikinase groups of the family, clearly revealed that the S. strix sequences belong to the PPDK family (not shown).

The third EST we identified was not highly similar to the S. strix genes and did not contain in-frame stop codons. The other major eukaryotic constituents of this environment are several species of parabasalia, which are thought not to contain PPDK (24). To determine whether this PPDK could come from a parabasalid, we performed extensive BLAST searches against NCBI databases and found 13 ESTs from T. vaginalis with high similarity to PPDK. Twelve partially overlapping ESTs assembled to form an open reading frame corresponding to the last 531 amino acids of the PPDK. The remaining EST encompassed a nonoverlapping stretch from positions 25 to 286 (according to C. symbiosum PPDK numbering). Searching the T. vaginalis genome project at TIGR, we found gene predictions that perfectly matched these ESTs. Interestingly, the T. vaginalis project has at least two PPDK sequences, a finding consistent with previous findings that the T. vaginalis genome contains multiple copies of several genes for metabolic enzymes. Comparing the T. vaginalis PPDKs to our EST data showed that the two were very similar, so we infer that this sequence belongs to one of the six species of parabasalia present in Z. angusticollis (37), and tentatively named it “parabasalian endosymbiont.” The sequence of this EST was extended by RT-PCR to 1,680 bp encoding 653 amino acids.

Conservation of key structural features in S. strix and T. vaginalis PPDKs. Certain enzymes can change their substrate specificity with a few substitutions at catalytically active sites. A clear example is PFK, which has been shown to have switched from ATP to PP, specificity several times independently (3, 23, 27). This transformation requires only a few substitutions that
control the entry of ATP into the active site, so that the position of a sequence in a phylogeny does not necessarily predict its function for these enzymes (3). Although functional assays are needed to ultimately demonstrate the actual substrate specificity, careful mapping of the critical positions are usually helpful to predict the substrate specificity, provided that sufficient information on the structure and physicochemical properties of the enzyme exists. Although no cross-substrate specificity has been reported between the ATP and PP, pyruvate kinases, we examined the S. strix and T. vaginalis sequences and looked for conservation of the residues involved in substrate interaction and enzymatic function. We aligned these with PPDks from the protists Giardia, Entamoeba, Trypanosoma, along with maize and C. symbiosum. The latter has been subject to detailed mutagenesis and structural analysis, from which the positions essential for catalytic function have been identified (4, 19, 20, 32, 33, 35, 36, 38). All but two positions identified as catalytically important in C. symbiosum are included in the known S. strix sequences, and every one is conserved in S. strix and T. vaginalis sequences, including the reactive center His455 (Fig. 1). One notable difference found between the parbasalian and other PPDks enzymes is two residues up-

![Sequence alignment of PPDks from several eukaryotes and C. symbiosum showing conservation of important sites for catalytic function. Numbers follow the residue position in C. symbiosum. Arrows indicate positions that have shown to be involved in nucleotide and substrate binding. The dot shows the active site histidine, and the asterisk indicates the site involved in regulation of PPDK activity in plants (see the text).](image)

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| S. strix 1 | 312 | 553 | 762 |
| S. strix 2 | 348 | 567 | 781 |
| G. intestinalis | T. cruzi | T. vaginalis 95394 | T. vaginalis 85781 |
| E. histolytica | M. balamuthi | A. thaliana | C. reihrardi |
| T. vaginalis 95394 | T. vaginalis 85781 | PPDKs from the protists Giardia, Entamoeba, Trypanosoma, along with maize and C. symbiosum. The latter has been subject to detailed mutagenesis and structural analysis, from which the positions essential for catalytic function have been identified (4, 19, 20, 32, 33, 35, 36, 38). All but two positions identified as catalytically important in C. symbiosum are included in the known S. strix sequences, and every one is conserved in S. strix and T. vaginalis sequences, including the reactive center His455 (Fig. 1). One notable difference found between the parbasalian and other PPDks enzymes is two residues up-
stream of the catalytic His458, where a threonine is conserved in all but the parabasalia (Fig. 1 and Fig. S1 in the supplemental material). In C4 plants, this threonine is directly involved in a particular form of light-dependent regulation by a bifunctional protein kinase/phosphatase (5, 6). Briefly, nocturnal inactivation of the enzyme is achieved by ADP-dependent phosphorylation of Thr453 by the PPDK regulatory protein (RP), whereas the opposite (dephosphorylation) is catalyzed by RP in light conditions. Although this residue is not directly involved in catalysis, and site-directed mutagenesis has shown that some substitutions of Thr453 have little or no negative effect on activity, it is an important potential control point (5, 7). The T. vaginalis and the “parabasalian symbiont” sequences have an alanine at this position (Fig. 1 and Fig. S1 in the supplemental material), and experiments of mutagenesis in maize indicate that activity is lost when bulky or strongly charged residues such as phenylalanine, tyrosine, and the acidic amino acids replace threonine, but not when serine and the aliphatic valine are assayed in place of Thr453 (5, 7).

Although functional characterization is ultimately needed, the evidence suggests that the Thr-Ala substitution seen in the aliphatic valine are assayed in place of Thr453 (5, 7).

Phylogeny and evolution of PPDK. Although they share many metabolic similarities, eukaryotic microbes with PPi-dependent glycolysis are not all closely related to one another (26). This was noted some time ago (25, 26), and it was debated whether their metabolism was retained from an ancient ancestor or was a derived adaptation to anaerobic environments. Current data seem to favor the latter interpretation (3). PFK has been extensively sampled (3, 23, 27) and is now known to have a very complex history due to a combination of early duplications and multiple independent losses, as well as some lateral gene transfer events. The phylogeny of PPDK has not been explored in great detail, so we analyzed PPDK sequences from the three domains of life to determine whether the eukaryotic homologues share a common ancestor. A preliminary analysis, including about 100 sequences of purported PPDKs and another member of the extended gene family (water dikinase), confirmed the monophyly of PPDK enzymes (not shown). PPDK genes were subsequently analyzed alone to allow more positions to be included. The overall level of resolution for this phylogeny is not very high, but several nodes are well supported. The maximum-likelihood tree topology (Fig. 2) is generally not consistent with the known relationships within and among different groups of bacteria, archaea, and eukaryotes. For the most part this is probably due to lack of resolution in the phylogeny, but there may also be a case for some ancient duplication events and the incongruent distribution of taxa in some of the well-supported nodes suggests that at least one event of lateral gene transfer probably also occurred (Fig. 2).

PPDK is broadly represented in eubacteria, although its representation varies from group to group. For example, PPDK is scarce among proteobacteria in general but ubiquitous in the alpha subdivision. In archaea, PPDK is restricted to a few groups from both crenarchaeotes and euryarchaeotes, even though more than 40 complete genomes are known. This, and the fact that the archaeal sequences do not cluster together are suggestive of lateral acquisition from eubacteria (Fig. 2). Some bacterium-only groupings in the PPDK tree contain unrelated species, but most of these nodes have low bootstrap support (<50), making it impossible to infer anything about their evolution, although it is noteworthy that some groups such as firmicutes, actinobacteria, and proteobacteria show discontinuous distribution (Fig. 2).

Eukaryotic PPDK sequences do not form a monophyletic group. The largest eukaryotic clade comprises plant and green algae, as well as Phytophthora and Thalassiosira sequences. This grouping is well supported by all reconstruction methods used. Phytophthora and Thalassiosira are heterokonts, which diverged from the green lineage deep in eukaryotic history. Two explanations for this observation are possible: plant and heterokont PPDK derive from an ancestral gene in early eukaryotes that was lost elsewhere, or they were independently acquired from related sources. It is tempting to postulate some link to plastid evolution, given its involvement in photosynthesis in some plants and that the diatom is also photosynthetic and plastid-related genes have been found in Phytophthora (2).

No cyanobacteria has yet been shown to possess a related PPDK gene; however, and none of these genes encode plastid-targeted proteins, so this link is perhaps coincidence. The sequences from the trypanosomatid parasites T. cruzi and T. brucei branch together, as expected, but show no well-supported association to any other organism in the present sample. The trypanosomatid PPDKs are glycosomal and used to recycle pyrophosphate accumulated from various biosynthetic reactions (1). A controversial hypothesis that trypanosomatids evolved from a plastid-harboring ancestor (13) suggested that PPDK was acquired from the phototrophic endosymbions (12). However, the trypanosomatid PPDK shows no association with the plant-algal cluster as would have been expected, much like other genes proposed to be endosymbiont-derived (9).

Among amitochondriate eukaryotes, the only relationship that is consistently found by different reconstruction methods is the branch unifying Giardia and Streblomasix. This node is moderately supported by bootstrap (69%); however, adding the PPDK from a sister-lineage to oxymonads, Trimaolisa pyriformis (A. J. Roger, unpublished data), this value increases to 76% (not shown). Common ancestry between the Giardia and Streblomasix sequences is consistent with the hypothetical eukaryotic supergroup Excavata (11, 31). The PPDK from T. vaginalis, which is also an excavate, does not branch with these sequences but rather with actinobacteria and Thermus spp. with moderate bootstrap support. Finally, PPDK from the amitochondriate amoebae E. hystolytica and M. balamuthi branch within a strongly supported clade containing the gamma-proteobacterium Methylococcus and the crenarchaeon Pyrobculum. Overall, there is no support for a single origin of amitochondriate PPDK, although the G. intestinalis and S. strix genes likely branch together, and we cannot rule out a common origin with the T. vaginalis gene; the PPDK genes from amoebae have an origin apparently independent from those of other eukaryotes.

Evolution of PPi-dependent glycolysis in amitochondriate eukaryotes. Three of the main questions surrounding the evolution of PPi-dependent glycolysis are (i) whether the presence of PFP and PPDK in glycolysis is correlated with one another, or if only one or the other may be used; (ii) whether or how analogous PPi- and ATP-dependent enzymes performing
the same reaction in one organism are functionally differentiated; and (iii) where did the PP_i-dependent enzymes come from and how widespread are they?

Previously nothing was known of oxymonads, and it was thought that parabasalia used PFP but not PPDK. We have now shown that oxymonads and parabasalia both possess PPDK. It is unknown if oxymonads use PFP or PFK to phosphorylate fructose-6-phosphate, or if they also encode PK, but it is possible that their glycolysis is at least partially PP_i dependent. In the case of parabasalia, PPDK was undetected in enzyme assays from *T. vaginalis*, and it was assumed that glycolysis was mixed, such that the phosphorylation of fructose-6-phosphate by PFP was the only PP_i-dependent step. However, *T. vaginalis* and at least one other parabasalian do contain genes for PPDK, further supporting the correlated presence of PFP and PPDK. By analogy to *G. intestinalis*, *E. histolytica*, and now also *T. vaginalis*, we hypothesize that PFP will eventually be found in oxymonads as well. However, *G. intestinalis*, *E. histolytica*, and now also *T. vaginalis*, we hypothesize that PFP will eventually be found in oxymonads as well.

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**FIG. 2.** Maximum-likelihood tree showing the relationships among bacterial, archaeal, and eukaryotic PPDKs. Eukaryotic sequences are in boldface, and the sequences from *S. strix* are marked with black rectangles. Taxonomic groups are indicated with black, gray, and white triangles for eukaryotes, bacteria, and archaea, respectively. Numbers represent bootstrap values (higher than 50%). The bootstrap support for the node uniting *S. strix* and *G. intestinalis* was 69% in the analysis shown but higher (76%) when the unpublished *T. pyriformis* sequence was included (see text).
histolytica, and now also T. vaginalis possess both cytosolic PPDK and PK, so these two analogous enzymes clearly can also coexist in the same compartment, raising questions about whether they are functionally distinct. In plants and trypanosomatids PPDK and PK are sequestered in two different compartments, so this is not an issue. It also suggests that other amitochondriates, such as oomycnads, may be found to possess PK as well as PPDK. This growing list of eukaryotes where both kinds of enzyme are found also suggests that a reevaluation of why these enzymes exist in anaerobes and their possible contribution to energy efficiency may be necessary. Biochemical dissection of the role of ATP- versus PPi-dependent enzymes is growing.

As for the origin of the PPi-dependent enzymes, phylogenetic analyses show that ADP-PFK and PPi-PFP are not mutually exclusive and PFK has been shown to have switched phosphate-donor specificity several times (3, 27); thus, PFP originated several times independently. In contrast, the phylogeny of PPDK is less complex but lacks the resolution needed to conclusively determine whether there is a single origin for the eukaryotic protein. The PPDK of amitochondriates G. intestinalis, S. strix, and T. vaginalis may have originated in common, but this is not clearly supported by the phylogeny, and the support for an independent origin of at least the amoebozoan PPDK is very strong. If several independent lateral gene transfers explain the distribution of PPDK in amitochondriates, then perhaps further sampling of prokaryotes will show this more clearly. Until then, only tentative conclusions can be drawn. Overall, however, analyses of PFP and PPDK suggest that PPi-linked glycolysis is a derived adaptation to specific environments or lifestyles rather than a primitive eukaryotic trait, although the known distribution of PPi-dependent enzymes is growing.

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