Pyruvate : NADP$^+$ Oxidoreductase from the Mitochondrion of *Euglena gracilis* and from the Apicomplexan *Cryptosporidium parvum*: A Biochemical Relic Linking Pyruvate Metabolism in Mitochondriate and Amitochondriate Protists

Carmen Rotte, Frantisek Stejskal,†[1] Guan Zhu,†[2] Janet S. Keithly,† and William Martin**

*Institut für Botanik III, Universität Düsseldorf, Düsseldorf, Germany; and †Wadsworth Center, New York State Department of Health, David Axelrod Institute, Albany, New York

Most eukaryotes perform the oxidative decarboxylation of pyruvate in mitochondria using pyruvate dehydrogenase (PDH). Eukaryotes that lack mitochondria also lack PDH, using instead the O_2-sensitive enzyme pyruvate : ferredoxin oxidoreductase (PFO), which is localized either in the cytosol or in hydrogenosomes. The facultatively anaerobic mitochondria of the photosynthetic protist *Euglena gracilis* constitute a hitherto unique exception in that these mitochondria oxidize pyruvate with the O_2-sensitive enzyme pyruvate : NADP$^+$ oxidoreductase (PNO). Cloning and analysis of Euglena PNO revealed that the cDNA encodes a mitochondrial transit peptide followed by an N-terminal PFO domain that is fused to a C-terminal NADPH-cytochrome P450 reductase (CPR) domain. Two independent 5.8-kb full-size cDNAs for Euglena mitochondrial PNO were isolated; the gene was expressed in cultures supplied with 2% CO_2 in air and with 2% CO_2 in N_2. The apicomplexan *Cryptosporidium parvum* was also shown to encode and express the same PFO-CPR fusion, except that, unlike *E. gracilis*, no mitochondrial transit peptide for *C. parvum* PNO was found. Recombination-derived remnants of PNO are conserved in the genomes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* as proteins involved in sulfite reduction. Notably, *Trypanosoma brucei* was found to encode homologs of both PFO and all four PDH subunits. Gene organization and phylogeny revealed that eukaryotic nuclear genes for mitochondrial, hydrogenosomal, and cytosolic PFO trace to a single eubacterial acquisition. These findings suggest a common ancestry of PFO in amitochondriate protists with Euglena mitochondrial PNO and Cryptosporidium PNO. They are also consistent with the view that eukaryotic PFO domains are biochemical relics inherited from a facultatively anaerobic, eubacterial ancestor of mitochondria and hydrogenosomes.

Introduction

Three basic eukaryotic cell types are known (Müller 1998) that differ with respect to (1) the compartmentation of their ATP-synthesizing pathways and (2) the metabolic fate of pyruvate—the end product of the glycolytic (Embden-Meyerhof) pathway. In most eukaryotes, pyruvate is oxidatively decarboxylated in mitochondria by the pyruvate dehydrogenase (PDH) complex to yield CO_2, acetyl-CoA, and NADH. Acetyl-CoA enters the Krebs cycle, and electrons of NADH enter the respiratory chain whereby fumarate (Tielens and Van Hellemond 1998), nitrate (Kobayashi et al. 1996) or, more commonly, O_2 serves as final acceptor.

Protists that lack mitochondria generate ATP by anaerobic fermentations. Those studied to date oxidize pyruvate with the O_2-sensitive enzyme pyruvate : ferredoxin oxidoreductase (PFO; EC 1.2.99.-) (Müller 1998; Hrdy and Müller 1995) and are classified either as type I or type II on the basis of their compartmentalized energy metabolism (Müller 1998). PFO yields CO_2, acetyl-CoA, and reduced ferredoxin (Fd_{red}). Type I protists (such as *Giardia lamblia*) lack organelles involved in core energy metabolism. Here, PFO is a cytosolic enzyme and electrons from Fd_{red} are transferred to acetyl-CoA, yielding ethanol as a major waste product (Müller 1998). In type II protists (such as *Trichomonas vaginalis*), PFO is localized within hydrogenosomes—double-membrane-bounded organelles of anaerobic ATP synthesis (Müller 1993)—whereby electrons from Fd_{red} are transferred to protons by an [Fe] hydrogenase yielding H_2 characteristic of the organelle.

Due to their anaerobic lifestyle, fermentative energy metabolism, and tendency to branch deeply in molecular phylogenies, type I and type II protists were long viewed as members of the earliest-branching, and hence most ancient, eukaryotic cell lineages. However, newer findings are at odds with this interpretation.

First, neither type I nor type II amitochondriate protists represent natural taxonomic groups (Biagini, Finlay, and Lloyd 1997; Embley and Hirt 1998; Müller 1998; Roger 1999)—with type I organisms occurring among such disparate eukaryotic lineages as diplomonads (Roger et al. 1998), entamoebids (Tovar, Fischer, and Clark 1999), amoeboflagellates (Hannaert et al. 2000), and microsporidia (fungi) (Hirt et al. 1999) and type II protists occurring among trichomonads (Müller 1993), ciliates (Embley et al. 1995), heteroloboseans (Broers et al. 1993), and chytridiomycetes (fungi) (Hackstein et al. 1999). Second, a wealth of data indicate a common ancestry of mitochondria and hydrogenosomes from a common ancestral organelle (reviewed in Biagini, Fin-

[1]Present address: First Faculty of Medicine, Charles University, Prague, Czechoslovakia.

[2]Present address: Texas A&M University, College Station, Texas.

Abbreviations: aa, amino acids; CPR, NADPH-cytochrome P450 reductase; Fd, ferredoxin; PDH, pyruvate dehydrogenase; PFO, pyruvate : ferredoxin oxidoreductase; PNO, pyruvate : NADP$^+$ oxidoreductase.

Key words: pyruvate : ferredoxin oxidoreductase, hydrogenosomes, energy metabolism, endosymbiosis, mitochondria, sulfite reductase.

Address for correspondence and reprints: William Martin, Institut für Botanik III, Universität Düsseldorf, Universitätsstrasse 1, 40225 Düsseldorf, Germany. E-mail: w.martin@uni-duesseldorf.de.

Mol. Biol. Evol. 18(5):710–720. 2001

lay, and Lloyd 1997; Embley and Hirt 1998; Roger 1999; Dyall et al. 2000; Rotte et al. 2000). Third, molecular data indicate that all protists studied thus far that lack ATP-producing organelles descend from mitochondrion-bearing ancestors (Müller 1993; Embley et al. 1995; Martin and Müller 1998; Roger et al. 1998; Gray, Burger, and Lang 1999; Hirt et al. 1999; Roger 1999; Tovar, Fischer, and Clark 1999). In light of data indicating a single origin of the α-proteobacterial symbiont from which mitochondria and hydrogenosomes descend (Gray, Burger, and Lang 1999), it seems that all contemporary eukaryotes possessed such a symbiont in their evolutionary past, whereby some have subsequently lost the organelle through reductive evolution (Embley and Hirt 1998; Martin and Müller 1998; Müller 1998; Roger 1999).

Given multiple independent origins of type I and type II protists, as well as the pivotal role of PFO in their anaerobic energy metabolism (Müller 1993, 1998; Fenchel and Finlay 1995; Biagini, Finlay, and Lloyd 1997), the evolutionary origin of this enzyme bears heavily on views concerning the metabolic lifestyle of the earliest eukaryotic cells (Martin and Müller 1998). In principle, three alternative explanations could currently account for the distribution of PFO among eukaryotes and its evident correlation with the amitochondriate phenotype.

The first possible explanation is that the host of mitochondrial symbiosis might have already possessed a PFO gene. Since this host descended in some manner from the archaebacteria, as molecular data from ribosomes (Jain, Rivera, and Lake 1999), RNA polymerases (Langer et al. 1995), DNA replication genes (Tye 2000), histones (Bailey et al. 2000), and small nucleolar RNAs (Omer et al. 2000) indicate, eukaryotic PFO in this case should be more similar to archaebacterial than to eubacterial homologs. Yet, initial studies have indicated that the converse is true, suggesting that eukaryotic PFO, like most enzymes involved in eukaryotic energy metabolism (Martin and Müller 1998; Hannaert et al. 2000), is an acquisition from eubacteria (Hrdy and Müller 1995; Rosenthal et al. 1997; Horner, Hirt, and Embley 1999).

The second possible explanation is that type I and type II protists might have acquired their genes for PFO (and other enzymes essential to anaerobic ATP synthesis) via independent lateral transfers from anaerobic prokaryotes as adaptations to hypoxic habitats (Rosenthal et al. 1997; Andersson and Kurland 1999). In this case, PFO genes in different eukaryotic lineages should branch with different prokaryotic homologs. A previous analysis of PFO sequences from three eukaryotic lineages (Rosenthal et al. 1997) has suggested multiple origins from eubacteria, whereas the analysis of four eukaryotic PFOs has suggested a single eubacterial origin (Horner, Hirt, and Embley 1999), indicating that additional data might help to resolve this issue.

The third possible explanation is that the common ancestor of mitochondria and hydrogenosomes might simply have been a facultatively anaerobic α-proteobacterium that possessed the genes for both aerobic and anaerobic energy metabolism typical of eukaryotes today, including both PDH and PFO (Martin and Müller 1998). In this case, several predictions follow: (1) eukaryotic PFO should reflect a single eubacterial origin (Horner, Hirt, and Embley 1999), (2) some extant eukaryotes should possess genes for both PFO and PDH, (3) ATP-producing organelles with energy metabolism intermediate to that in typical hydrogenosomes and mitochondria should be found, and (4) these should harbor biochemical relics of a facultatively anaerobic past (Martin and Müller 1998).

The mitochondrion of the photosynthetic protist *Euglena gracilis* is a facultatively anaerobic organelle that produces ATP in the presence and absence of O_2 (Buetow 1989; Kitaoka et al. 1989). However, in contrast to typical mitochondria, it does not use PDH; rather, it uses an unusual O_2-sensitive enzyme for the oxidative decarboxylation of pyruvate—pyruvate : $NADP^+$ oxidoreductase (PNO) (Inui et al. 1985, 1987, 1991; Buetow 1989; Kitaoka et al. 1989). Under aerobiosis, acetyl-CoA from PNO enters a modified Krebs cycle, which drives oxidative phosphorylation using O_2 as the terminal electron acceptor (Buetow 1989). Under anaerobiosis, PNO is the key enzyme of a unique wax ester fermentation. Acetyl-CoA from PNO is used both as primer and as C2-donor for fatty acid synthesis in mitochondria, thereby consuming the electrons stemming from glucose breakdown (Inui et al. 1985; Kitaoka et al. 1989). Fatty acids are reduced and esterified, yielding waxes that accumulate in the cytosol (Buetow 1989). Upon return to aerobic conditions, these waxes undergo β-oxidation and oxidative phosphorylation.

Here, we report the cloning, phylogeny, and expression under aerobic and anaerobic conditions of nuclear-encoded mitochondrial PNO from *E. gracilis.* This gene is shown to have arisen through an ancient fusion of a eubacterial gene for PFO and for NADPH-cytochrome P450 reductase (CPR). In addition, a homolog of PNO is shown to be conserved in structure and to be expressed during different stages in the life cycle of the apicomplexan *Cryptosporidium parvum*, an opportunistic parasite of humans.

Materials and Methods

Organisms

Euglena gracilis strain SAG 1224-5/25 was cultured in 5 liters of Euglena medium with minerals (Schlösser 1997) under continuous light and was harvested 4 days after inoculation with 50 ml preculture. Aerobic cultures were supplied with 2 liters/min of 2% CO_2 in air, and anaerobic cultures were supplied with 2% CO_2 in N_2. For dark treatment, cells were grown using 2% CO_2 in N_2 for 2 days in the light, subjected to darkness, and harvested after 2 additional days. Cells were harvested by centrifugation and immediately frozen in liquid N_2. *Cryptosporidium parvum* IOWA strain sporozoites were excysted as described (Zhu and Keithly 1997). Intracellular stages of the KSU-1 strain were obtained by in vitro cultivation in human ileocecal epithelial HCT-8 (ATCC CCL 244) cell lines as before (Upton 1997).

Molecular Methods

Euglena gracilis nucleic acid isolation, cDNA synthesis, cloning in λ ZapII, and standard molecular methods were performed as described (Henze et al. 1995; Hannaert et al. 2000). A hybridization probe for PNO from Euglena was isolated by PCR with total DNA using combinations of degenerate primers designed against the conserved amino acid (aa) motifs LFEDNEFG(F/W/Y)G and GGDGWAYDIG(F/Y) identified from alignments of prokaryotic and eukaryotic PFO extracted from the database. PCR was performed using standard protocols. The primers pno1F953 (5′-TITTYGARGAYAAYGCIGARTTYGGITTYGG-3′) and pno2R1095 (5′-AAICCDATRTCRTAIGCCCAICCRTCICC-3′) yielded a ~700-bp amplicon that was used to screen a cDNA library constructed with mRNA from aerobically light-grown cells.

Two gene fragments of *C. parvum* encoding sequences similar to PFO (AQ023783, AQ023784) identified from a random survey of the *C. parvum* genome (Liu et al. 1999) were used as probes for library screening. The open reading frame was deduced from five overlapping clones isolated from *Eco*RI and *Hin*dIII *C. parvum* KSU-1 genomic libraries constructed in pBluescriptSK+. Total RNA was isolated both from free sporozoites and from uninfected or *C. parvum*–infected HCT-8 cells using a commercial kit (Qiagen). Reverse-transcription (RT) PCR was performed with the Access RT-PCR kit (Promega) using two different primer pairs. One pair spanned the conserved FeS-centers and thiamine pyrophosphate (TPP)–binding motifs: pfo1F1927 (5′-GAAACGGGAATAAAGACCAAG-3′) and pfo1R2716 (5′-ATCCGTATTCCGCATTATCTTC-3′), yielding a 789-bp amplicon. A second pair spanned the C-terminal region of PFO into the CPR coding region: pnoF22941 (5′-AAATATCAAAGTTGCTTGAGG-3′) and pnoR23967 (5′-CTAAATTCAATGAGCTGGATG-3′), yielding a 1,020-bp amplicon. Identity of the RT-PCR products was confirmed by sequencing.

Phylogenetic Methods

Database searching, sequence handling, and alignment were performed with programs of the GCG package, version 9.1 (Genetics Computer Group, Madison, Wis.). Alignments were reinspected and manually adjusted. Analyses excluded highly gapped regions and regions of uncertain alignment, leaving 1,336 and 480 aa positions, respectively, in the PFO and CPR alignments. Phylogenetic inference used the protein maximum-likelihood (ML) method with PROTML of the MOLPHY package (Adachi and Hasegawa 1996) and with Puzzle (Strimmer and von Haeseler 1997). *Trypanosoma brucei* PFO homologs were found by searching http://www2.ebi.ac.uk/blast2/parasites.html.

Results and Discussion

Euglena Mitochondrial PNO: A Functional Fusion of PFO and CPR

PFO is an O_2-sensitive enzyme that is widespread among anaerobic and facultatively anaerobic prokaryotes and eukaryotes, where it functions both in pyruvate metabolism and in pathways where strong electron-donating potentials are required. Among eubacteria, on the one hand, PFO functions in H_2-producing fermentations (Clostridium) (Menon and Ragsdale 1997), in lactate utilization during sulfate reduction (Desulfovibrio) (Pieulle et al. 1995), in N_2-fixation as pyruvate : flavodoxin oxidoreductase (NifJ—Anabaena and proteobacteria) (Bauer, Scappino, and Haselkorn 1993; Steibl, Siddavattam, and Klingmüller 1995), or in CO_2-fixation as pyruvate synthase (Chlorobium) (Yoon et al. 1999). On the other hand, among eukaryotes, PFO is only known to function in oxidative pyruvate decarboxylation in amitochondriate protists, where the electrons are donated to Fd_{ox} in hydrogenosomes or the cytosol.

In contrast to PFO, PNO donates electrons to $NADP^+$ rather than to Fd_{ox}. Nevertheless, PNO purified from *E. gracilis* mitochondria is more similar to PFO in subunit size, stoichiometry, and O_2-sensitivity than it is to PDH of typical aerobic mitochondria (Inui et al. 1987, 1991), suggesting that PNO might possess a functionally conserved PFO domain. Using degenerate primers against conserved PFO regions, a 695-bp fragment of *E. gracilis* DNA was amplified that spanned 300 bp of PFO coding region and two introns of 221 and 174 bp. With this probe, clones pEgPNO3 (5,812 bp) and pEgPNO12 (5,840 bp) were isolated and found to encode a protein of 1,803 aa. The N-terminal 1,239 aa had extensive similarity with PFO. The C-terminal 564 aa had extensive similarity with NADPH-cytochrome P450 reductases (CPR). Since pEgPNO12 and pEgPNO3 are independent cDNAs for the same mRNA, the PFO-CPR fusion was not a cloning artifact. Furthermore, a cDNA for Euglena PNO has very recently been reported (Nakazawa et al. 2000) that differs from pEgPNO3 at 6 nt, resulting in five amino acid differences.

Very importantly, the deduced polypeptide of pEgPNO3 (EgPNOmt) contained two peptides that were previously determined by direct sequencing of the active enzyme purified from Euglena mitochondria (Inui et al. 1991). This is shown in figure 1*A*. The first 12 N-terminal residues of active mitochondrial Euglena PNO (Inui et al. 1991) are identical to those starting from Thr_{38} of the pEgPNO3 translation, revealing both the functional identity of the clone and the mitochondrial transit peptide processing site (fig. 1*A*). The 15 residues determined from the N-terminus of the smaller tryptic ''NADPH diaphorase active'' fragment (Inui et al. 1991) prepared from purified PNO were identical to the pEgPNO3 protein starting from Ala_{1240} within the region with similarity to CPR (fig. 1*A*).

A Southern blot of total Euglena DNA probed with pEgPNO3 and washed at low stringency (55°C in 2 × SSPE, 0.1% SDS) indicated the presence of one to three genes in the genome (fig. 1*B*). Northern hybridization revealed that Euglena mitochondrial PNO was expressed under both aerobic and anaerobic conditions. Steady-state PNO mRNA levels in dark-grown cells under 2% CO_2 in N_2 were ~twofold higher than in cells grown

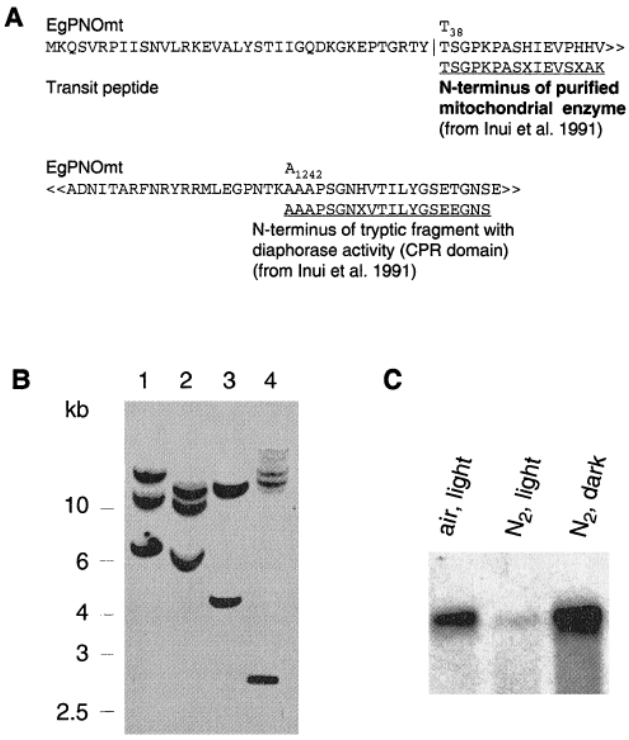

FIG. 1.—Mitochondrial PNO from *Euglena gracilis*. *A*, Comparison of pEgPNO3 with peptide sequences (underlined) determined from the protein purified from Euglena (Inui et al. 1991). The processing site of the mitochondrial transit peptide is indicated by "|." *B*, Southern blot of *E. gracilis* DNA (20 μg per lane) using pEgPNO3 as a probe: *Hin*dIII (lane 1), *Kpn*I (lane 2), *Eco*RI (lane 3), and *Sal*I (lane 4). *C*, Northern blot of *E. gracilis* polyA+ mRNA (5 μg per lane) extracted from cells grown under the conditions indicated, probed with pEgPNO3. The band is 5.8 kb; no additional bands were detected.

under 2% CO_2 in air, whereas cells grown anaerobically in the light had reduced PNO mRNA levels (fig. 1*C*).

Cryptosporidium Encodes and Expresses a Conserved PFO : CPR Fusion

An intronless *C. parvum* gene encoding a protein of 1,934 aa was isolated that displayed the same PFO : CPR fusion as that of EgPNOmt. The N-terminal PFO domain was separated from the C-terminal CPR domain by a 42-aa linker that lacked detectable similarity in BLAST searches. Unlike EgPNOmt, the deduced *C. parvum* protein (CpPNO) is not preceded by a transit peptide. The gene encoding CpPNO is expressed in both sporozoites and intracellular stages of the life cycle, as shown by RT-PCR (fig. 2). A 790-bp fragment from the CpPFO transcript was detected in sporozoites (fig. 2*A*), and a 1,026-bp RT-PCR product was detected both in sporozoites (fig. 2*A*) and in intracellular stages of *C. parvum* propagated in 12–72 h infected HCT-8 cells (fig. 2*B*). These intracellular stages include an asynchronous mixture of both first- and second-generation meronts and merozoites, as well as some gametocytes and gametes (Upton 1997).

CpPNO is structurally similar to EgPNOmt, but its physiological function and localization are not yet clear. Indeed, whether *C. parvum* even possesses a mitochondrion has been a subject of debate, although recent data suggest this to be the case. These data include (1) the description of a double-membrane-bounded acristate organelle in *C. parvum* (Riordan et al. 1999), (2) the finding that growth of this organism can be effectively reduced by inhibitors known to block mitochondrial respiration (Riordan et al. 1999; Kayser et al. 2001), and (3) the uptake of mitochondrion-specific fluorescent dyes (Mito-tracker Green FM, Rhodamine B, Rhodamine 123, and $DiOC_6$ [Molecular Probes]) by this organelle, revealing the presence of a membrane potential and cardiolipin (unpublished data). However, the function of this organelle is not yet known, since previous biochemical data have indicated that core ATP synthesis in *C. parvum* stems from glycolysis only (Entrala and Mascaro 1997). Although a typical N-terminal mitochondrial targeting signal is lacking in CpPNO, nothing is currently known about *C. parvum* protein import. Indeed, N-terminal targeting sequences may be lacking in *C. parvum* proteins involved in electron transport (Lill, Nargang, and Neupert 1996).

Sequence Similarity Among PFO and CPR Domains of PNO

Database searching with EgPNOmt and CpPNO and their constituent PFO and CPR domains revealed complex patterns of sequence similarity, shared domains among proteins, gene fusions, and recombination events, as summarized in figure 3. The functional domains of PNO correspond to structural domains I–VII determined from the crystal structure of *Desulfovibrio africanus* PFO (Charon et al. 1999) (fig. 3*A*). The FMN-, FAD-, and NADP-binding domains of rat microsomal NADPH-cytochrome P450 reductase indicated in figure 3*A* were also taken from the crystal structure (Wang et al. 1997).

Typical eubacterial and eukaryotic PFOs are colinear and are organized like the *D. africanus* enzyme (Horner, Hirt, and Embley 1999), yet they lack domain VII, which is specific to *D. africanus* PFO (Charon et al. 1999) (fig. 3*B*). By contrast, the enzyme from archaebacteria, Thermotoga, and Helicobacter consists of several independent proteins designated α-, β-, γ-, and δ-subunits (Horner, Hirt, and Embley 1999), which span the conserved functional domains (Charon et al. 1999) (fig. 3*C*). EgPNOmt and CpPNO are colinear with eubacterial PFO but are fused to a C-terminal CPR domain (fig. 3*D* and *E*). EgPNOmt and CpPNO are 40% identical at the aa level and share 30%–35% aa identity with eukaryotic and eubacterial PFO but only 20%–25% identity with archaebacterial PFO.

Although the fusions of a complete PFO protein with NADPH-cytochrome P450 reductase in EgPNOmt and CpPNO are unique among sequences reported to date, fusions of subdomains of both proteins are observed among eukaryotes. Regions homologous to PFO domain III and a portion of domain II plus the FAD and NADP domains of CPR are found in the *Saccharomyces cerevisiae* MET10 gene product (L26504), which encodes the α-subunit of assimilatory sulfite reductase

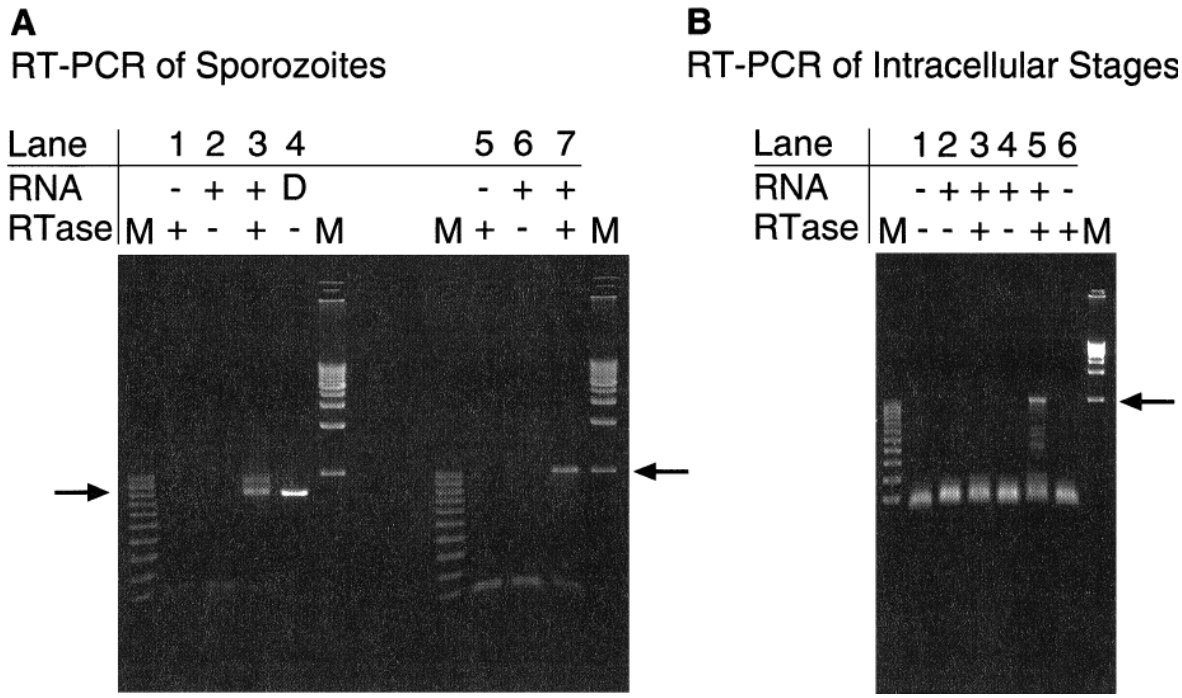

FIG. 2.—RT-PCR expression analysis of CpPNO. Presence and absence of *C. parvum* RNA and reverse transcriptase (RTase) in the reactions is indicated. *A,* RNA from *Cryptosporidium parvum* sporozoites. Arrows denote the 790-bp (lane 3) and 1,026-bp (lane 7) bands expected for primers F1/R1 and F2/R2, respectively. The identity of each RT-PCR amplicon was confirmed by sequencing. D: DNA instead of RNA as substrate; M: marker; lane 4 (DNA): positive control using a plasmid containing cloned CpPNO. *B,* RNA from intracellular stages used as substrate with primers F2/R2. Substrate RNA was isolated from uninfected cells (lanes 2 and 3) and from 72 h *C. parvum*–infected HCT-8 cells which included an asynchronous mixture primarily of first- and second-generation meronts and merozoites, as well as some gametocytes and gametes (lanes 4 and 5), respectively. An arrow denotes the 1-kb band expected for primer pair F2/R2.

(SRα) (Hansen, Cherest, and Kielland-Brandt 1994), and in the *Schizosaccharomyces pombe* homolog (T41439) (fig. 3*G*). Furthermore, consistent with findings of Horner, Hirt, and Embley (1999), residual sequence similarity was found using DOTPLOT between PNO and a different fungal protein, annotated as a putative sulfite reductase (designated here as PuSR) from *S. cerevisiae* (P47169) and *S. pombe* (O14167) (fig. 3*F*). PuSR encodes a translational fusion of PFO domains I, II (partial), and VI with the FMN domain of CPR, which, in turn, is fused to a hemoprotein domain (fig. 3*F*). Sequence similarity between *C. parvum* ESTs, PFO, and CPR was previously detected (Horner, Hirt, and Embley 1999). The PFO domains of PuSR share 30% identity in conserved regions with eubacterial PFO. The FMN domain shares 30% identity with FMN domains from eubacterial and eukaryotic CPR, yet only 20%–25% identity with the FMN domain of EgPNOmt and CpPNO. The C-terminal hemoprotein domain of PuSR shares 40% identity with the hemoprotein components of eubacterial sulfite reductase and 25% identity with eubacterial nitrite reductases. Notably, in their analysis of yeast SRα, Hansen, Cherest, and Kielland-Brandt (1994) predicted the presence of a flavodoxin domain in the yeast sulfite reductase β-subunit, which would appear to be borne out by the present data for PuSR.

CPR catalyzes the redox metabolism of numerous compounds (Wang et al. 1997). The CPR domain of EgPNOmt and CpPNO shares similarity with the α-subunit of NADPH sulfite reductase (CysJ, fig. 3*H*) from Salmonella and with CPR proper (fig. 3*I*). The cognate substrate of CPR is typically cytochrome P450 (Wang et al. 1997), which is fused to the CPR domain in the fatty acid hydroxylase P450BM-3 from *Bacillus megaterium* (Govindaraj and Poulos 1997) and in an identically organized homolog from the fungus *Fusarium oxysporum* (AB030037) (fig. 3*J*). Finally, the CPR domain also occurs in metazoan nitric oxide synthases (fig. 3*K*) and as individual proteins (Paine et al. 2000), including flavodoxin (fig. 3*L*), to which PFO donates electrons as NifJ, and ferredoxin : $NADP^+$ reductase (fig. 3*M*), which, like PNO, transfers electrons to $NADP^+$.

Clearly, the complex families of protein fusions shown in figure 3 interconnect enzymes of differing electron-donating and -accepting potentials. As Govindaraj and Poulos (1997, p. 7915) previously surmised regarding CPR fusions and their constituent domains: ''Apparently, once nature discovers a useful functional unit, the various units are used in multiple ways by covalently tethering the domains via a gene fusion mechanism resulting in redox proteins with novel activities.''

Phylogeny of the PFO and CPR Domains of PNO

Eukaryotic PFO and PNO share only 20%–25% aa identity with heteromeric PFO from archaebacteria and eubacteria (fig. 3*C*) and with PFO domains in SRα and PuSR of fungi (fig. 3*F* and *G*). Because of this low sequence identity, heteromeric PFO and sulfite reductases were excluded from phylogenetic analysis. Using

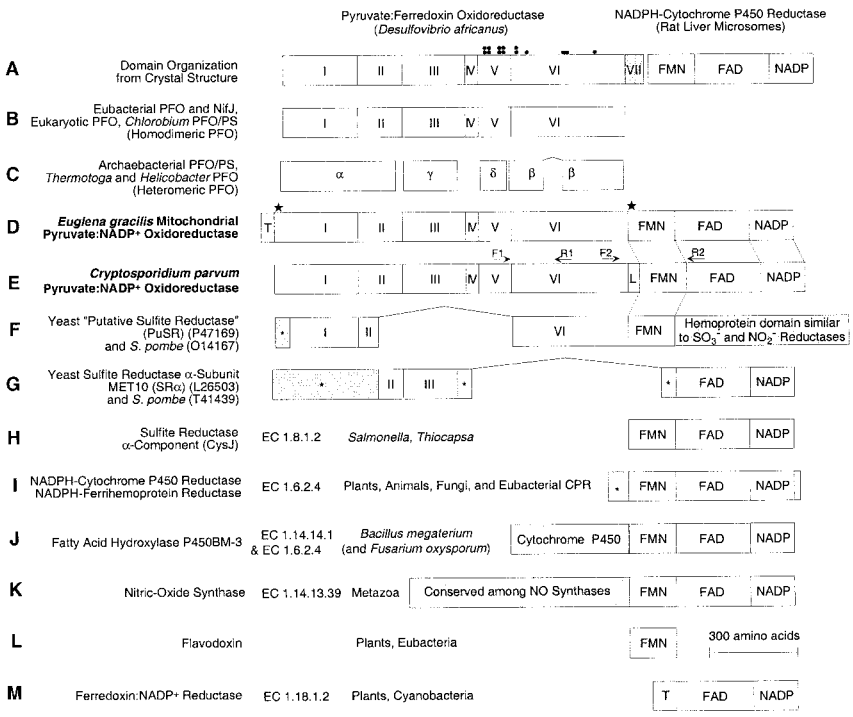

FIG. 3.—Sequence similarity and domain organization among PFO, CPR, PNO, and related proteins. Asterisks denote regions with no BLAST hits to other proteins. Gray boxes indicate domains with no detectable similarity to other proteins in the figure. Lines connect contiguous domains. *A,* Desulvovibrio PFO (Charon et al. 1999) and NADPH-cytochrome P450 reductase from rat liver microsomes (Wang et al. 1997); domains taken from crystal structures. Black dots: conserved Cys residues of iron-sulfur clusters; Small black box: conserved TPP-binding site. *B,* Homodimeric eukaryotic PFO, eubacterial PFO, NifJ, and Chlorobium pyruvate synthase. *C,* Heteromeric PFO/pyruvate synthase from archaebacteria, Thermotoga, and Helicobacter. *D* and *E,* Euglena and Cryptosporidium PNO. T: mitochondrial transit peptide; L: linker region. Stars denote locations of peptides (fig. 1) sequenced from the purified protein (Inui et al. 1991). Arrows indicate positions of primers used for RT-PCR (fig. 2). *F,* Conservation between PNO and putative sulfite reductase from *Saccharomyces cerevisiae* (PuSR; P47169) and *Schizosaccharomyces pombe* (014167). *G,* Sulfite reductase α-subunit (MET10) from yeast (L26504) and *S. pombe* (T41439). *H,* NADPH sulfite reductase (α-subunit) from Salmonella and Thiocapsa. *I,* CPR from eubacteria, fungi, plants, and animals; NADPH : ferrihemoprotein reductase from fungi, plants, and animals. *J,* Fatty acid hydroxylase (P450BM-3) from *Bacillus megaterium* (Govindaraj and Poulos 1997) and *Fusarium oxysporum* (AB030037). *K,* Metazoan nitric-oxide synthetase. *L,* Eubacterial and plant flavodoxin. *M,* Cyanobacterial and plant ferredoxin : $NADP^+$ reductase (see refs. 51, 52).

PROTML with local rearrangements and the JTT-F substitution matrix starting from the neighbor-joining tree of ML distances, eukaryotic PFO proteins were found to share a common branch with low support (fig. 4*A*). Using the quicksearch option of PROTML, 1,000 trees were generated and evaluated by the approximate likelihood criterion (Adachi and Hasegawa 1996). In the best tree found by the ML criterion, the branch bearing the sequences from Trichomonas, Spironucleus, and Giardia was positioned basally on the branch bearing the eubacterial homologs from Treponema to Chlorobium. However, of 1,000 trees so examined, none were significantly better ($P = 0.95$), and 465 were not significantly worse ($P = 0.95$), than the starting topology (fig. 4*A*) using the Kishino-Hasegawa test (Adachi and Hasegawa 1996). Of the 465 trees that were not significantly worse than the topology shown in figure 4*A*, 162 indicated monophyly of eukaryotic PFO sequences.

However, PROTML performs more efficiently when the amino acid compositions of individual sequences do not significantly differ from that of the data set as a whole (Adachi and Hasegawa 1996). Using PUZZLE (Strimmer and von Haeseler 1997), the aa compositions of PFO sequences from Escherichia, Salmonella, Yersinia, Rhodobacter, Rhodospirillum, Klebsiella, and Clostridium were found to be significantly different ($P = 0.95$). Exclusion of these sequences (25-OTU data set) and reanalysis as above revealed that the best of 1,000 trees examined indicated monophyly of eukaryotic PFO. Of 95 other trees found that were not significantly worse ($P = 0.95$) than the best tree with the 25-OTU data, 43 indicated monophyly of eukaryotic PFO.

Furthermore, using PUZZLE with the JTT-F matrix and a gamma distribution for the variability of substitution rate across sites and 16 categories of variability, no branch was detected that disrupted the monophyly of eukaryotic PFO for either the complete data set ($\alpha = 0.81$) or the 25-OTU data ($\alpha = 0.86$). Thus, notwithstanding the fact that the ML criterion in topology searching is not infallible (Nei, Kumar, and Takahashi 1998), these ML analyses indicate monophyly of eukaryotic PFO, albeit with weak support.

In all analyses, PFO and NifJ homologs from eubacteria fell into the two groups previously designated as clusters I and II (Horner, Hirt, and Embley 1999): PFO homologs from the γ-proteobacteria *Escherichia coli* and Salmonella fell into cluster I; NifJ from the γ-proteobacterium *Klebsiella pneumoniae* (Arnold et al.

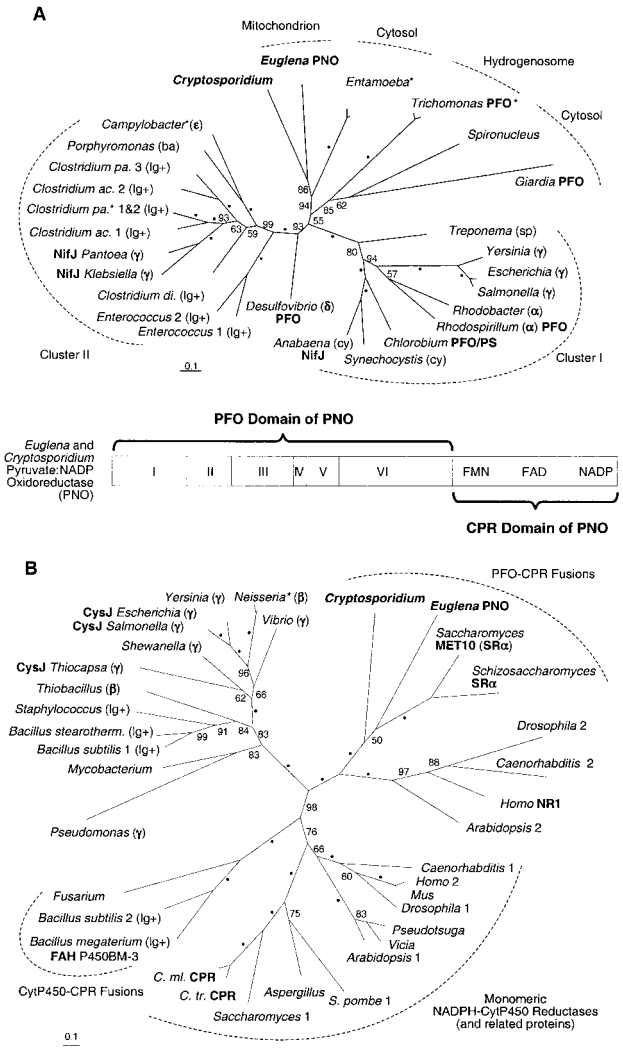

FIG. 4.—Protein phylogenies for PFO and CPR domains of PNO. RELL bootstrap proportions (BPs) (Adachi and Hasegawa 1996) are indicated next to branches; BPs of 100 are indicated by dots. The scale bar at the lower left indicates substitutions per site. An asterisk indicates that two very closely related sequences from the same genome reside on the branch. Sequences were retrieved from GenBank and from finished and unfinished genome projects through TIGR (http://www.tigr.org/tdb/) and NCBI (http://www.ncbi.nlm.nih.gov). Alignments and accession numbers for all sequences analyzed here are available from ftp.uni-duesseldorf.de/pub/science/. *A,* PFO phylogeny. Localization of the eukaryotic proteins is indicated. Higher eubacterial taxa are indicated in parentheses: α, γ, δ, ϵ, proteobacteria; sp, spirochaetes; lg+, low GC Gram positive; ba, bacteroides; cy, cyanobacteria. Known physiological functions of PFO sequences are indicated in bold next to taxon names: Desulfovibrio PFO (crystal structure known) (Charon et al. 1999); Pantoea (Steibl, Siddavattam, and Klingmüller 1995) and Klebsiella (Arnold et al. 1988) NifJ (encoded in *nif* operons); *Anabaena* NifJ (essential for growth in medium depleted of Fe and N) (Bauer, Scappino, and Haselkorn 1993); Rhodospirillum (mutants lack PFO activity) (Lindblad et al. 1996); Chlorobium PFO/pyruvate synthase, where the purified enzyme (Yoon et al. 1999) is encoded by the PFO sequence available from the Chlorobium genome project (F. R. Tabita, personal communication). Clostridium species abbreviations: *pa., pasteurianum*; *ac., acetobutylicum*; *di., difficile. B,* CPR phylogeny. *C. ml., Candida maltosa*; *C. tr., Candida tropicalis.* Other symbols are as in *A.*

1988) fell into cluster II. Desulfovibrio PFO fell into neither cluster.

A representative subset from over 200 identified CPR-related proteins from eubacteria and eukaryotes, encompassing those most similar to *E. gracilis* and *C. parvum,* was analyzed with PROTML as above. The CPR domains of EgPNOmt and CpPNO branched robustly with the fused FAD- and $NADP^+$-binding domains from the C-terminus of *S. cerevisiae* SRα and the *S. pombe* homolog (fig. 4*B*). The monophyly of eukaryotic CPR homologs is disrupted by the common branching of the cytochrome P450-CPR fusion from Fusarium with the homologous fatty acid hydroxylase fusion (FAH) from *B. megaterium* (P450BM-3) and *Bacillus subtilis* (figs. 3*J* and 4*B*). This indicates that lateral gene transfer involving sequences on this branch might have occurred in one direction or the other.

No CPR homologs from archaebacteria were found. The FMN domains of *S. cerevisiae* and *S. pombe* PuSR share the greatest sequence identity (~40%) with EgPNOmt and CpPNO. Together with the pattern of domain conservation (see fig. 3), the CPR phylogeny indicates that the FMN-, FAD-, and NADP-binding domains of *S. cerevisiae* and *S. pombe* PuSR and SRα stem from the same fusion event as that which generated Euglena and *C. parvum* PNO.

Evolutionary Significance of Eukaryotic PFO Fusions and Phylogeny

Euglena PNO establishes that active PFO, which is otherwise specific to type I and type II protists, occurs in mitochondrion-bearing eukaryotes, albeit as a fusion with CPR, and furthermore that it functions in the organelle. *Euglena gracilis* mitochondria thus assume an intermediate position between hydrogenosomes and mitochondria in that oxidative decarboxylation via PFO—as a domain of PNO—is coupled with a Krebs cycle. Several proteins provide phylogenetic links between hydrogenosomes and typical mitochondria, including Hsp60, Hsp70, Hsp10 (Embley and Hirt 1998; Roger 1999), succinyl-CoA synthase (Lahti, Bradley, and Johnson 1994), and the ADP-ATP translocator (Dyall et al. 2000) (see Biagini, Finlay, and Lloyd 1997; Embley and Hirt 1998; Müller 1998; Roger et al. 1998; Roger 1999; Tovar, Fischer, and Clark 1999; Rotte et al. 2000). Euglena PNO thus provides a functional and phylogenetic link between energy metabolism in facultatively anaerobic mitochondria, hydrogenosomes, and the cytosol of type I protists, compatible with the view that these pathways share a common origin (Martin and Müller 1998).

Conservation of the PFO-CPR fusion in the apicomplexan *C. parvum,* as well as in the photosynthetic flagellate *E. gracilis,* suggests that PNO was present in the common ancestor of either the eukaryotic host or the eukaryotic algae that gave rise to the plastids of apicomplexans and euglenids, groups that arose through secondary endosymbiosis. The conserved PFO-CPR fusion boundary in *S. cerevisiae* and *S. pombe* PuSR (fig. 3*F*) indicates that the fungal sulfite reductase subunits

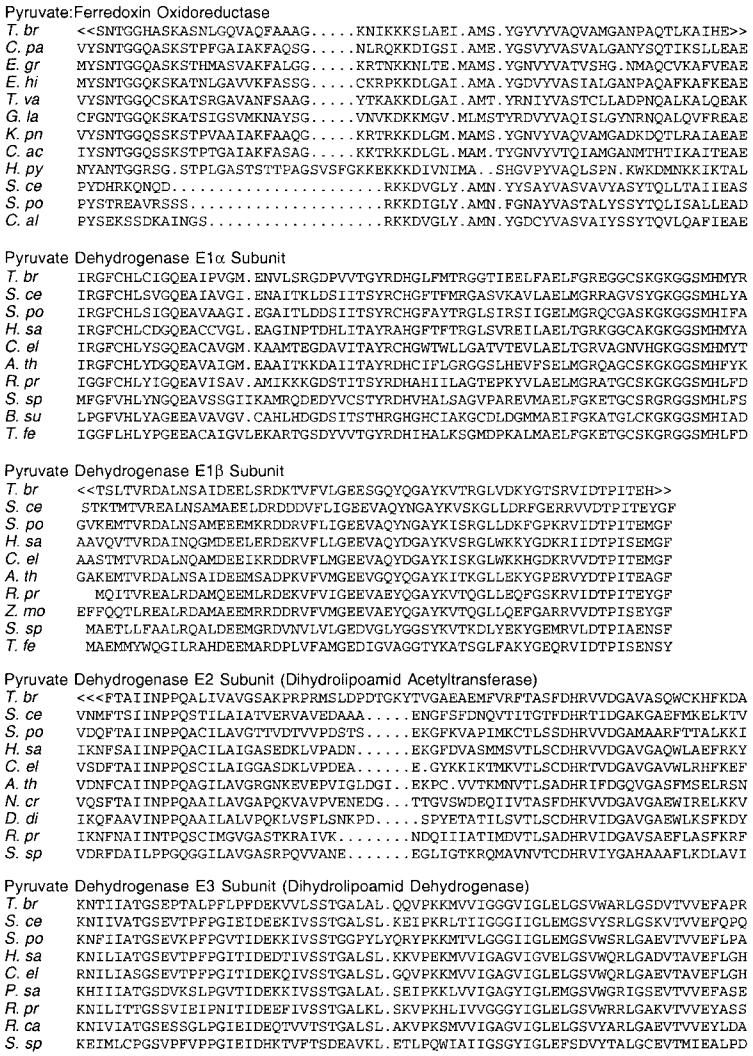

FIG. 5.—Alignments of amino acid sequences for PFO and for the E1α, E1β, E2, and E3 subunits of PDH with homologs from genome sequencing data of *Trypanosoma brucei*. *A.th. = Arabidopsis thaliana; B.su. = Bacillus subtilis; C.ac. = Clostridium acetobutylicum*; *C.al. = Candida albicans*; *C.el. = Caenorhabditis elegans*; *C.pa. = Clostridium pateurianum*; *D.di. = Dictyostelium discoideum*; *E.gr. = Euglena gracilis*, *E.hi. = Entamoeba histolytica*; *G.la. = Giardia lamblia*; *H.py. = Helicobacter pylori*; *H.sa. = Homo sapiens*; *K.pn. = Klebsiella pneumoniae*; *N.cr. = Neurospora crassa*; *P.sa. = Pisum sativum*; *R.ca. = Rhodobacter capsulatus*; *R.pr. = Rickettsia prowazekii*; *S.ce. = Saccharomyces cerevisiae*; *S. po. = Schizosaccharomyces pombe*; *T.br. = Trypanosoma brucei*; *T.fe. = Thiobacillus ferrooxidans*; *T.va. = Trichomonas vaginalis*; *Z.mo. = Zymomonas mobilis.* GenBank accession numbers of *T. brucei* genes are as follows: PFO, EMBL:B13566; E1α, EMBL:AQ953618; E1β, EMBL:AQ639015; E2, EMBL:AQ656033; E3 EMBL:X70646.

arose through fragmentation of the same ancestral PNO gene as that found in *E. gracilis* and *C. parvum.*

The phylogeny of eukaryotic PFO (fig. 4*A*) suggests that the common ancestor of diplomonads, trichomonads, Entamoeba, euglenids, apicomplexans, and fungi (by virtue of the CPR fusion in SRα; figs. 3 and 4*B*) possessed one and the same commonly inherited eubacterial PFO gene. Hence, PFO (Horner, Hirt, and Embley 1999) and the PFO domain of PNO seem to have been acquired only once, rather than multiple times independently as would be expected if eukaryotes had acquired their PFO genes through lateral gene transfers not involving the origin of mitochondria (Rosenthal et al. 1997; Andersson and Kurland 1999).

One conceivable explanation to account for the monophyly of eukaryotic PFO is that eukaryotes acquired their ancestral PFO gene from a eubacterial host that engulfed an archaebacterial endosymbiont, which gave rise to the nucleus prior to the origin of mitochondria, as envisaged by Lopez-Garcia and Moreira (1999) and as discussed by Horner, Hirt, and Embley (1999). This possibility is unlikely for three reasons: (1) This model derives a primitively amitochondriate eukaryote (an archezoon)—a hypothetical organism for whose existence there is no evidence (Roger 1999). (2) Arguments that the nuclear compartment derives from an endosymbiont are themselves problematic (Martin 1999*a*). (3) This model entails the notion that the α-proteobacterial ancestor of mitochondria was an anaerobic methane oxidizer, whereas newer data indicate anaerobic methane oxidation to be a property of methanogens, not of eubacteria (Boetius et al. 2000; DeLong 2000).

Alternatively, it is possible that eukaryotic PFO is an acquisition from the cyanobacterial antecedent of plastids. However, by inference, that would mean that the PFO-bearing amitochondriate eukaryotes (and fungi by virtue of the PFO-CPR fusion in sulfite reductase) sampled here would have once also possessed plastids, which they subsequently lost—an interpretation that cannot be excluded but for which there is currently no strong supporting evidence.

A more straightforward interpretation is that the eubacterial PFO donor could have simply been the free-living α-proteobacterial ancestor of mitochondria and hydrogenosomes. This would be compatible with the findings that the type I amitochondriate lineages sampled here (Giardia, Spironucleus, and Entamoeba) possessed a mitochondrial (hydrogenosomal) symbiont in their evolutionary past, but subsequently lost the organelle (Roger et al. 1998; Gray, Burger, and Lang 1999; Roger 1999). It would furthermore be compatible with the contemporary localization of PNO and PFO in mitochondria and hydrogenosomes.

But why does eukaryotic PFO/PNO then not branch specifically with the available α-proteobacterial homolog? Horizontal gene transfer is well known to occur at appreciable rates among free-living prokaryotes today (Doolittle 1999; Ochman, Lawrence, and Groisman 2000). Therefore, we should assume it also to have occurred in the distant past. Indeed, the interweaving of PFO sequences from bacterial groups (particularly γ-proteobacteria; fig. 4*A*) indicates that some degree of lateral PFO gene transfer between prokaryotes has occurred. If the free-living descendants of the α-proteobacterial ancestor of mitochondria and hydrogenosomes exchanged genes with other eubacteria subsequent to the origins of organelles, which is likely, it is possible—if not probable—that no single contemporary α-proteobacterium contains exactly the same set of genes as the mitochondrial symbiont did (Martin 1999*b*).

Of course, ancient paralogy and differential loss, rather than lateral transfer, could be invoked to account for the unexpected eubacterial PFO relationships. However, if differential loss were invoked to explain all of the discrepancies observed among all trees for all eubacterial genes, the size of the ''ancestor of all genomes'' so inferred would vastly exceed that known for any contemporary prokaryote. By contrast, lateral gene transfer is an observable phenomenon (Doolittle 1999; Ochman, Lawrence, and Groisman 2000) and would not require an ''ancestor of all genomes'' to ever have existed within a single cell. Hence, notwithstanding the vagaries of phylogenetic inference using limited numbers of sites (Nei, Kumar, and Takahashi 1998), as in the case of the present PFO data, lateral gene transfer seems preferable to ancient paralogy and differential loss to account for these branching discrepancies.

If eukaryotic PFO was indeed acquired from a facultatively anaerobic mitochondrial symbiont (Martin and Müller 1998), it follows that some eukaryotes should have retained functional genes both for PFO and PDH, as have many facultatively anaerobic eubacteria, such as Rhodobacter. Through database searching, we found that a relative of Euglena, the kinetoplastid *Trypanosoma brucei,* possesses homologs of all four subunits of the PDH complex—E1α, E1β, E2, and E3—and a genomic fragment encoding a protein with 57% aa identity across 61 residues to PFO domain VI of EgPNOmt (fig. 5). PDH activity has been reported for insect (procyclic) forms of both *T. brucei* (Else et al. 1994) and *Trypanosoma congolense* (Obungu et al. 1999).

Due to the facultatively anaerobic lifestyle of many euglenozoa (Van Hellemond, Opperdoes, and Tielens 1998), their mitochondria may harbor further biochemical relics of ancestral eukaryotic energy metabolism. In line with this view is the finding that aerobically growing insect-form trypanosomatid mitochondria contain high activities of acetate : succinate CoA transferase (Van Hellemond, Opperdoes, and Tielens 1998), an enzyme that among eukaryotes is otherwise specific to anaerobic energy metabolism in hydrogenosomes (Müller 1998).

Supplementary Material

Sequences reported in this paper have been submitted to GenBank under the following accession numbers: *E. gracilis,* AJ278425; *C. parvum,* AF208233.

Acknowledgments

We thank M. Abrahamsen (University of Minnesota) for the two *C. parvum* gene fragments of PFO (AQ023783, AQ023784) used for initial library screening; the molecular core facility at the Wadsworth Center for support; M. Müller (Rockefeller University), T. M. Embley, and D. S. Horner (NHM, London) for critical comments on the manuscript; P. A. M. Michels for valuable discussions; and F. R. Tabita for communicating results prior to publication. This research was supported in part by NIH grant AI 40320 (J.S.K.), by a Fogarty Fellowship (F.S.), by grants from the DFG (W.M.), and by a Ph.D. stipend from the Studienstiftung des deutschen Volkes (C.R.).

LITERATURE CITED

ADACHI, J., and M. HASEGAWA. 1996. MOLPHY version 2.3: programs for molecular phylogenetics based on maximum likelihood. Comput. Sci. Monogr. **28**:1–150.

ANDERSSON, S. G. E., and C. G. KURLAND. 1999. Origins of mitochondria and hydrogenosomes. Curr. Opin. Microbiol. **2**:535–541.

ARNOLD, W., A. RUMP, W. KLIPP, U. B. PRIEFER, and A. PÜHLER. 1988. Nucleotide sequence of a 24,206-base-pair DNA fragment carrying the entire nitrogen fixation cluster of *Klebsiella pneumoniae.* J. Mol. Biol. **203**:715–738.

BAILEY, K. A., S. L. PEREIRA, J. WIDOM, and J. N. REEVE. 2000. Archaeal histone selection of nucleosome positioning sequences and the procaryotic origin of histone-dependent genome evolution. J. Mol. Biol. **303**:25–34.

BAUER, C. C., L. SCAPPINO, and R. HASELKORN. 1993. Growth of the cyanobacterium *Anabaena* on molecular nitrogen: *NifJ* is required when iron is limited. Proc. Natl. Acad. Sci. USA **90**:8812–6881.

BIAGINI, G. A., B. J. FINLAY, and D. LLOYD. 1997. Evolution of the hydrogenosome. FEMS Microbiol. Lett. **155**:133–140.

BOETIUS, A., K. RAVENSCHLAG, C. J. SCHUBERT, D. RICKERT, F. WIDDEL, A. GIESEKE, R. AMANN, B. B. JÙRGENSEN, U. WITTE, and O. PFANNKUCHE. 2000. A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature **407**:623–626.

BROERS, C. A. M., H. H. M. MEIJERS, J. C. SYMENS, C. K. STUMM, and G. D. VOGELS. 1993. Symbiotic association of *Psalteriomonas vulgaris* n. spec. with *Methanobacterium formicicum.* Eur. J. Protistol. **29**:98–105.

BUETOW, D. E. 1989. The mitochondrion. Pp. 247–314 *in* D. E. BUETOW, ed. The biology of *Euglena,* Vol. 4. Subcellular biochemistry and molecular biology. Academic Press, San Diego.

CHARON, M.-H., A. VOLBEDA, E. CHABRIERE, L. PIEULLE, and J. C. FONTECILLA-CAMPS. 1999. Structure and electron transfer mechanism of pyruvate : ferredoxin oxidoreductase. Curr. Opin. Struct. Biol. **9**:663–669.

DELONG, E. F. 2000. Resolving a methane mystery. Nature **407**:578–579.

DOOLITTLE, W. F. 1999. Phylogenetic classification and the universal tree. Science **284**:2124–2128.

DYALL, S. D., C. M. KOEHLER, M. G. DELGADILLO-CORREA, P. J. BRADLEY, E. PLÜMPER, D. LEUENBERGER, C. W. TURCK, and P. J. JOHNSON. 2000. Presence of a member of the mitochondrial carrier family in hydrogenosomes: conservation of membrane-targeting pathways between hydrogenosomes and mitochondria. Mol. Cell. Biol. **20**:2488–2497.

ELSE, A. J., J. F. CLARKE, A. WILLIS, S. A. JACKMAN, D. W. HOUGH, and M. J. DANSON. 1994. Dihydrolipoamide dehydrogenase in the *Trypanosoma* subgenus, *Trypanozoon.* Mol. Biochem. Parasitol. **64**:233–239.

EMBLEY, T. M., B. J. FINLAY, P. L. DYAL, R. P. HIRT, M. WILKINSON, and A. G. WILLIAMS. 1995. Multiple origins of anaerobic ciliates with hydrogenosomes within the radiation of aerobic ciliates. Proc. R. Soc. Lond. B Biol. Sci. **262**: 87–93.

EMBLEY, T. M., and R. P. HIRT. 1998. Early branching eukaryotes. Curr. Opin. Genet. Dev. **8**:624–629.

ENTRALA, E., and C. MASCARO. 1997. Glycolytic enzyme activities in *Cryptosporidium parvum* oocysts. FEMS Microbiol. Lett. **151**:51–57.

FENCHEL, T., and B. J. FINLAY. 1995. Ecology and evolution in anoxic worlds. Oxford University Press, Oxford, England.

GOVINDARAJ, S., and T. L. POULOS. 1997. The domain architecture of cytochrome P450BM-3. J. Biol. Chem. **272**: 7915–7921.

GRAY, M. W., G. BURGER, and B. F. LANG. 1999. Mitochondrial evolution. Science **283**:1476–1481.

HACKSTEIN, J. H. P., A. AKHMANOVA, B. BOXMA, H. R. HARHANGI, and G. J. VONCKEN. 1999 Hydrogenosomes: eukaryotic adaptations to anaerobic environments. Trends Microbiol. **7**:441–447.

HANNAERT, V., H. BRINKMANN, U. NOWITZKI, J. A. LEE, M.-A. ALBERT, C. W. SENSEN, T. GAASTERLAND, M. MÜLLER, P. MICHELS, and W. MARTIN. 2000. Enolase from *Trypanosoma brucei*, from the amitochondriate protist *Mastigamoeba balamuthi,* and from the chloroplast and the cytosol of *Euglena gracilis*: pieces in the evolutionary puzzle of the eukaryotic glycolytic pathway. Mol. Biol. Evol. **17**:989–1000.

HANSEN, J., H. CHEREST, and M. C. KIELLAND-BRANDT. 1994. Two divergent MET10 genes, one from *Saccharomyces cerevisiae* and one from *Saccharomyces carlsbergensis,* encode the alpha subunit of sulfite reductase and specify potential binding sites for FAD and NADPH. J. Bacteriol. **176**: 6050–6058.

HENZE, K., A. BADR, M. WETTERN, R. CERFF, and W. MARTIN. 1995. A nuclear gene of eubacterial origin in *Euglena gracilis* reflects cryptic endosymbiosis during protist evolution. Proc. Natl. Acad. Sci. USA **92**:9122–9126.

HIRT, R. P., J. M. LOGSDON JR., B. HEALY, M. W. DOREY, W. F. DOOLITTLE, and T. M. EMBLEY. 1999. Microsporidia are related to Fungi: evidence from the largest subunit of RNA polymerase II and other proteins. Proc. Natl. Acad. Sci. USA **96**:580–585.

HORNER, D. S., R. P. HIRT, and T. M. EMBLEY. 1999. A single eubacterial origin of eukaryotic pyruvate : ferredoxin oxidoreductase genes: implications for the evolution of anaerobic eukaryotes. Mol. Biol. Evol. **16**:1280–1291.

HRDY, I., and M. MÜLLER. 1995. Primary structure and eubacterial relationship of the pyruvate : ferredoxin oxidoreductase of the amitochondriate eukaryote *Trichomonas vaginalis.* J. Mol. Evol. **41**:388–396.

INUI, H., K. MIYATAKE, Y. NAKANO, and S. KITAOKA. 1985. The physiological role of oxygen-sensitive pyruvate dehydrogenase in mitochondrial fatty acid synthesis in *Euglena gracilis.* Arch. Biochem. Biophys. **237**:423–429.

INUI, H., K. ONO, K. MIYATAKE, Y. NAKANO, and S. KITAOKA. 1987. Purification and characterization of pyruvate : $NADP^+$ oxidoreductase in *Euglena gracilis.* J. Biol. Chem. **262**: 9130–9135.

INUI, H., R. YAMAJI, H. SAIDOH, K. MIYATAKE, Y. NAKANO, and S. KITAOKA. 1991. Pyruvate : $NADP^+$ oxidoreductase from *Euglena gracilis*: limited proteolysis of the enzyme with trypsin. Arch. Biochem. Biophys. **286**:270–276.

JAIN, R., M. C. RIVERA, and J. A. LAKE. 1999. Horizontal gene transfer among genomes: the complexity hypothesis. Proc. Natl. Acad. Sci. USA **96**:3801–3806.

KAYSER, O., W. R. WATERS, S. J. UPTON, and J. S. KEITHLY. 2001. Evaluation of *in vitro* activity of aurones and related compounds against *Gyptosporidium parvum*. Planta Medica (in press).

KITAOKA, S., Y. NAKANO, K. MIYATAKE, and A. YOKOTA. 1989. Enzymes and their functional location. Pp. 2–135 *in* D. E. BUETOW, ed. The biology of *Euglena,* Vol. 4. Subcellular biochemistry and molecular biology. Academic Press, San Diego.

KOBAYASHI, M., Y. MATSUO, A. TAKIMOTO, S. SUZUKI, F. MARUO, and H. SHOUN. 1996. Denitrification of a novel type of respiratory metabolism in fungal mitochondrion. J. Biol. Chem. **271**:16263–16267.

LAHTI, C. J., P. J. BRADLEY, and P. J. JOHNSON. 1994. Molecular characterization of the alpha-subunit of *Trichomonas vaginalis* hydrogenosomal succinyl CoA synthetase. Mol. Biochem. Parasitol. **66**:309–318.

LANGER, D., J. HAIN, P. THURIAUX, and W. ZILLIG. 1995. Transcription in Archaea: similarity to that in Eucarya. Proc. Natl. Acad. Sci. USA **92**:5768–5772.

LILL, R., F. E. NARGANG, and W. NEUPERT. 1996. Biogenesis of mitochondrial proteins. Curr. Opin. Cell Biol. **8**:505–512.

LINDBLAD, A., J. JANSSON, E. BROSTEDT, M. JOHANSSON, U. HELLMAN, and S. NORDLUND. 1996. Identification and sequence of a *nifJ*-like gene in *Rhodospirillum rubrum*: partial characterization of a mutant unaffected in nitrogen fixation. Mol. Microbiol. **20**:559–568.

LIU, C., V. VIGDOROVICH, V. KAPUR, and M. S. ABRAHAMSEN. 1999. A random survey of the *Cryptosporidium parvum* genome. Infect. Immun. **67**:3960–3969.

LOPEZ-GARCIA, P., and D. MOREIRA. 1999. Metabolic symbiosis at the origin of eukaryotes. Trends Biochem. Sci. **24**: 88–93.

MARTIN, W. 1999*a*. A briefly argued case that mitochondria and plastids are descendants of endosymbionts, but that the nuclear compartment is not. Proc. R. Soc. Lond. B Biol. Sci. **266**:1387–1395.

———. 1999*b*. Mosaic bacterial chromosomes: a challenge en route to a tree of genomes. BioEssays **21**:99–104.

MARTIN, W., and M. MÜLLER. 1998. The hydrogen hypothesis for the first eukaryote. Nature **392**:37–41.

MENON, S., and S. W. RAGSDALE. 1997. Mechanism of the *Clostridium thermoaceticum* pyruvate : ferredoxin oxidoreductase: evidence for the common catalytic intermediacy of the hydroxyethylthiamine pyropyrosphate radical. Biochemistry **36**:8484–8494.

MÜLLER, M. 1993. The hydrogenosome. J. Gen. Microbiol. **139**:2879–2889.

———. 1998. Enzymes and compartmentation of core energy metabolism of anaerobic protists—a special case in eukaryotic evolution? Pp 109–131 *in* G. H. COOMBS, K. VICKERMANN, M. A. SLEIGH, and A. WARREN, eds. Evolutionary relationships among protozoa. Kluwer, Dordrecht, The Netherlands.

NAKAZAWA, N., H. INUI, R. YAMAJI, T. YAMAMOTO, S. TAKENAKA, M. UEDA, Y. NAKANO, and K. MIYATAKE. 2000. The origin of pyruvate : $NADP^+$ oxidoreductase in mitochondria of *Euglena gracilis.* FEBS Lett. **479**:155–156.

NEI, M., S. KUMAR, and K. TAKAHASHI. 1998. The optimization principle in phylogenetic analysis tends to give incorrect topologies when the number of nucleotides or amino acids used is small. Proc. Natl. Acad. Sci. USA **95**:12390–12397.

OBUNGU, V. H., J. K. KIAIRA, N. K. OLEMBO, and M. R. NJOGU. 1999. Pathways of glucose catabolism in procyclic *Trypanosoma congolense.* Indian J. Biochem. Biophys. **36**: 305–311.

OCHMAN, H., J. G. LAWRENCE, and E. S. GROISMAN. 2000. Lateral gene transfer and the nature of bacterial innovation. Nature **405**:299–304.

OMER, A. D., T. M. LOWE, A. G. RUSSELL, H. EBHARDT, S. R. EDDY, and P. P. DENNIS. 2000. Homologs of small nucleolar RNAs in Archaea. Science **288**:517–522.

PAINE, M. J. I., A. P. GARNER, D. POWELL, J. SIBBALD, M. SALES, N. PRATT, T. SMITH, D. G. TEW, and C. R. WOLF. 2000. Cloning and characterization of a novel human dual flavin reductase. J. Biol. Chem. **275**:1471–1478.

PIEULLE, L., B. GUIGLIARELLI, M. ASSO, F. DOLE, A. BERNADAC, and E. C. HATCHIKIAN. 1995. Isolation and characterization of the pyruvate : ferredoxin oxidoreductase from the sulfate-reducing bacterium *Desulvovibrio africanus.* Biochim. Biophys. Acta **1250**:49–59.

RIORDAN, C. E., S. G. LANGRETH, L. B. SANCHEZ, O. KAYSER, and J. S. KEITHLY. 1999. Preliminary evidence for a mitochondrion in *Cryptosporidium parvum*: phylogenetic and therapeutic implications. J. Eukaryot. Microbiol. **46**:S52–S55.

ROGER, A. J. 1999. Reconstructing early events in eukaryotic evolution. Am. Nat. **154**:S146–S163.

ROGER, A. J., S. G. SVÅRD, J. TOVAR, C. G. CLARK, M. W. SMITH, F. D. GILLIN, and M. L. SOGIN. 1998. A mitochondrial-like chaperonin 60 gene in *Giardia lamblia*: evidence that diplomonads once harbored an endosymbiont related to the progenitor of mitochondria. Proc. Natl. Acad. Sci. USA **95**:229–234.

ROSENTHAL, B., M. ZHIMING, D. CAPLIVSKI, S. GHOSH, H. DE LA VEGA, T. GRAF, and J. SAMUELSON. 1997. Evidence for the bacterial origin of genes encoding fermentation enzymes of the amitochondriate protozoan parasite *Entamoeba histolytica.* J. Bacteriol. **179**:3736–3745.

ROTTE, C., K. HENZE, M. MÜLLER, and W. MARTIN. 2000. The origin of hydrogenosomes and mitochondria. Curr. Opin. Microbiol. **3**:481–486.

SCHLÖSSER, U. G. 1997. SAG-Sammlung für Algenkulturen at the University of Göttingen. Bot. Acta **107**:111–186.

STEIBL, H. D., D. SIDDAVATTAM, and W. KLINGMÜLLER. 1995. Self-transmissible nif plasmid (pEA9) of *Enterobacter agglomerans* 339: molecular cloning and evidence for the existence of similar *nif* clusters on dissimilar plasmids in *Enterobacter* strains. Plasmid **34**:223–228.

STRIMMER, K., and A. VON HAESELER. 1997. Likelihood-mapping: a simple method to visualize phylogenetic content of a sequence alignment. Proc. Natl. Acad. Sci. USA **94**:6815–6819.

TIELENS, A. G. M., and J. J. VAN HELLEMOND. 1998. The electron transport chain in anaerobically functioning eukaryotes. Biochim. Biophys. Acta **1365**:71–78.

TOVAR, J., A. FISCHER, and C. G. CLARK. 1999. The mitosome, a novel organelle related to mitochondria in the amitochondrial parasite *Entamoeba histolytica.* Mol. Microbiol. **32**: 1013–1021.

TYE, B. K. 2000. Insights into DNA replication from the third domain of life. Proc. Natl. Acad. Sci. USA **97**:2399–2401.

UPTON, S. J. 1997. Pp. 163–180 *in* R. FAYER, ed. *Cryptosporidium* and cryptosporidiosis. CRC Press, Boca Raton, Fla.

VAN HELLEMOND, J. J., F. R. OPPERDOES, and A. G. M. TIELENS. 1998. Trypanosomatidae produce acetate via a mitochondrial acetate : succinate CoA transferase. Proc. Natl. Acad. Sci. USA **95**:3036–3041.

WANG, M., D. L. ROBERTS, R. PASCHKE, T. M. SHEA, B. S. S MASTERS, and J. P. KIM. 1997. Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. Proc. Natl. Acad. Sci. USA **4**:8411–8416.

YOON, K.-S., R. HILLE, C. HEMANN, and F. R. TABITA. 1999. Rubredoxin from the green sulfur bacterium *Chlorobium tepidum* functions as an electron acceptor for pyruvate : ferredoxin oxidoreductase. J. Biol. Chem. **274**:29772–29778.

ZHU, G., and J. S. KEITHLY. 1997. Molecular analysis of a P-type ATPase from *Cryptosporidium parvum.* Mol. Biochem. Parasitol. **90**:307–136.

GEOFFREY MCFADDEN, reviewing editor

Accepted December 11, 2000