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Pyruvate Formate-lyase and a Novel Route of Eukaryotic ATP Synthesis in *Chlamydomonas* Mitochondria*[S]

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Pyruvate formate-lyase (PFL) catalyzes the non-oxidative conversion of pyruvate to formate and acetyl-CoA. PFL and its activating enzyme (PFL-AE) are common among strict anaerobic and microaerophilic prokaryotes but are very rare among eukaryotes. In a proteome survey of isolated *Chlamydomonas reinhardtii* mitochondria, we found several PFL-specific peptides leading to the identification of cDNAs for PFL and PFL-AE, establishing the existence of a PFL system in this photosynthetic algae. Anaerobiosis and darkness led to increased *PFL* transcripts but had little effect on protein levels, as determined with antiserum raised against *C. reinhardtii* PFL. Protein blots revealed the occurrence of PFL in both chloroplast and mitochondria purified from aerobically grown cells. Mass spectrometry sequencing of *C. reinhardtii* mitochondrial proteins, furthermore, identified peptides for phosphotransacetylase and acetate kinase. The phosphotransacetylase-acetate kinase pathway is a common route of ATP synthesis or acetate assimilation among prokaryotes but is novel among eukaryotes. In addition to PFL and pyruvate dehydrogenase, the algae also expresses pyruvate:ferredoxin oxidoreductase and bifunctional aldehyde/alcohol dehydrogenase. Among eukaryotes, the oxygen producer *C. reinhardtii* has the broadest repertoire of pyruvate-, ethanol-, and acetate-metabolizing enzymes described to date, many of which were previously viewed as specific to anaerobic eukaryotic lineages.

Conversion of pyruvate into acetyl-coenzyme A (acetyl-CoA) is a crucial step in carbon energy metabolism. In most eukaryotes studied to date, pyruvate undergoes oxidative decarboxylation in mitochondria via pyruvate dehydrogenase complex (EC 1.2.4.1). In many prokaryotes and in some anaerobic eukaryotes, conversion of pyruvate into acetyl-CoA can occur via pyruvate:ferredoxin oxidoreductase (PFO; EC 1.2.7.1)[2] or via pyruvate formate-lyase (PFL; EC 2.3.1.54). PFO, an iron-sulfur protein, oxidizes pyruvate, yielding 2 mol of reduced ferredoxin, 1 mol of CO_2, and 1 mol of acetyl-CoA per mol of pyruvate (1). By contrast, PFL does not oxidize pyruvate, but uses a radical-based homolytic mechanism (2), yielding 1 mol of formate and 1 mol of acetyl-CoA per mol of pyruvate. Both PFO and PFL are oxygen-sensitive enzymes. The evolutionary origins of such enzymes for the anaerobic lifestyle in eukaryotes remains heavily debated. Either (i) the eukaryote common ancestor was a strict aerobe and anaerobic biochemistry in eukaryotes was acquired secondarily via lateral gene transfer (3, 4) or (ii) it was a facultative anaerobe, with its descendant lineages having undergone specialization and differential loss (5–8).

PFL plays a central role in anaerobic glucose fermentation of a number of obligatory or facultative anaerobic bacteria. The *Escherichia coli* PFL system has been investigated in detail. Following the shift from aerobic to anaerobic conditions, *pfl* transcription is induced 12–15-fold (9, 10), whereas PFL protein levels increase by about 5–10-fold (11). PFL is converted post-translationally from an inactive to a catalytically active form by a 20-kDa iron-sulfur protein called pyruvate formate lyase-activating enzyme (PFL-AE). PFL-AE introduces a radical on the ultimate glycine residue of the PFL, in an *S*-adenosyl-L-methionine- and flavodoxin-dependent reaction (12). The radical-containing protein is extremely sensitive to oxygen: exposure of activated *E. coli* PFL to air results into its cleavage in two polypeptides of ~82 and 3 kDa (2, 13). Fragmentation of activated PFL by oxygen has also been reported in the ruminal bacterium *Streptococcus bovis* (14) and in the lactic acid bacterium *Lactococcus lactis* (15). Oxygenolytic cleavage of PFL appears to be a drastic means to inactivate an enzyme that would produce less energy than its aerobic counterpart, pyruvate dehydrogenase. Some bacteria possess a mechanism for the reversible deactivation of PFL. In *E. coli*, PFL deactivase activity is harbored by the bifunctional enzyme aldehyde/alcohol dehydrogenase (ADHE), which catalyzes the sequential conversion of acetyl-CoA (a product of PFL activity) into acetaldehyde and ethanol. Quenching of the radical present on activated PFL by ADHE (16, 17) occurs via a yet unknown mechanism. However, ADHE does not always exhibit the PFL deactivase activity, as shown in *L. lactis* (15).

Reports on eukaryote PFL are limited to a few anaerobic protists and some green algae. PFL activity has hitherto been measured in two amitochondriate eukaryotes, the chytridomycetes *Neocallimastix* sp. L2 and *Piromyces* sp. E2 (18). In these anaerobic fungi, PFL was localized to the hydrogenosomes (18), which are anaerobic, hydrogen-producing mitochondria (8). Upon a shift to dark anaerobic conditions, a few unicellular photosynthetic algae such as *Chlamydomonas reinhardtii* and *Chlorella fusca* ferment their starch into formate, ethanol, and acetate in a molar ratio of 2:1:1, whereas the production of H_2 and CO_2 remains low (19). The pattern of fermentation end products together with the sensitivity of formate production to oxygen were interpreted as the

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. I–IV.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ620190 (ADHE), AJ620191 (PFL), and AJ620192 (PFL-AE).

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[2] The abbreviations used are: PFO, pyruvate:ferredoxin oxidoreductase; ACK, acetate kinase; ADHE, aldehyde/alcohol dehydrogenase; EST, expressed sequence tag; PFL, pyruvate formate-lyase; PFL-AE, pyruvate formate-lyase activating enzyme; PTA, phosphotransacetylase; TAP, Tris acetate phosphate; MS, mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; Mes, 4-morpholineethanesulfonic acid.

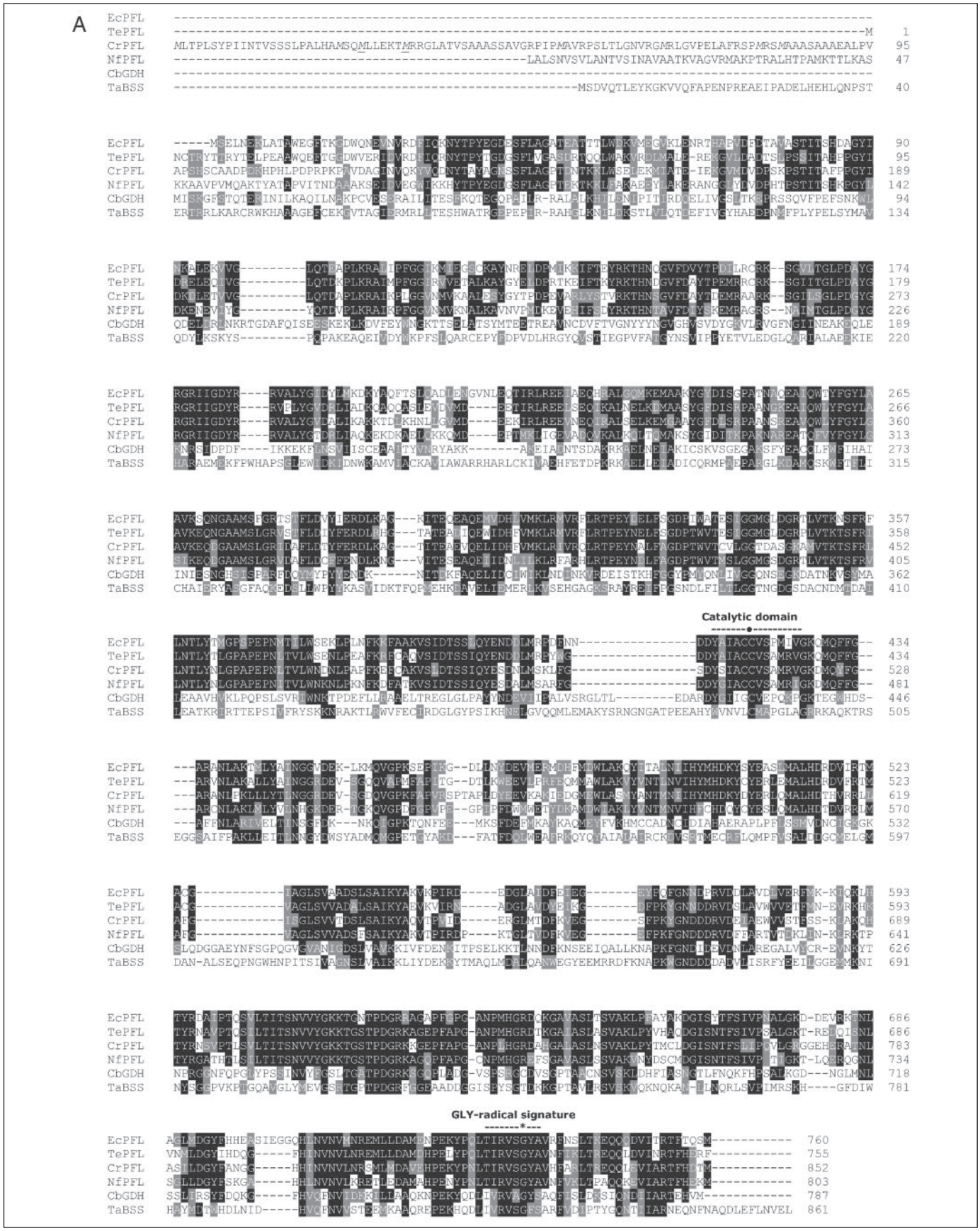

FIGURE 1. *A*, alignment of *C. reinhardtii* PFL with known and predicted glycyl radical enzymes. Identical residues are *shaded black*, conservative replacements are *shaded gray*. At its N terminus, *C. reinhardtii* PFL exhibits eight Met residues (*italics*), of which the third and the fourth (*underlined*) are the most likely to be the initiation Met based on translation context (47). At its C terminus, *C. reinhardtii* PFL exhibits a typical glycine radical signature (Prosite PS00850, (STIV)*X*R(IVT)(CSA)G*Y*X(GACV)); *, glycine residue that is activated into an organic

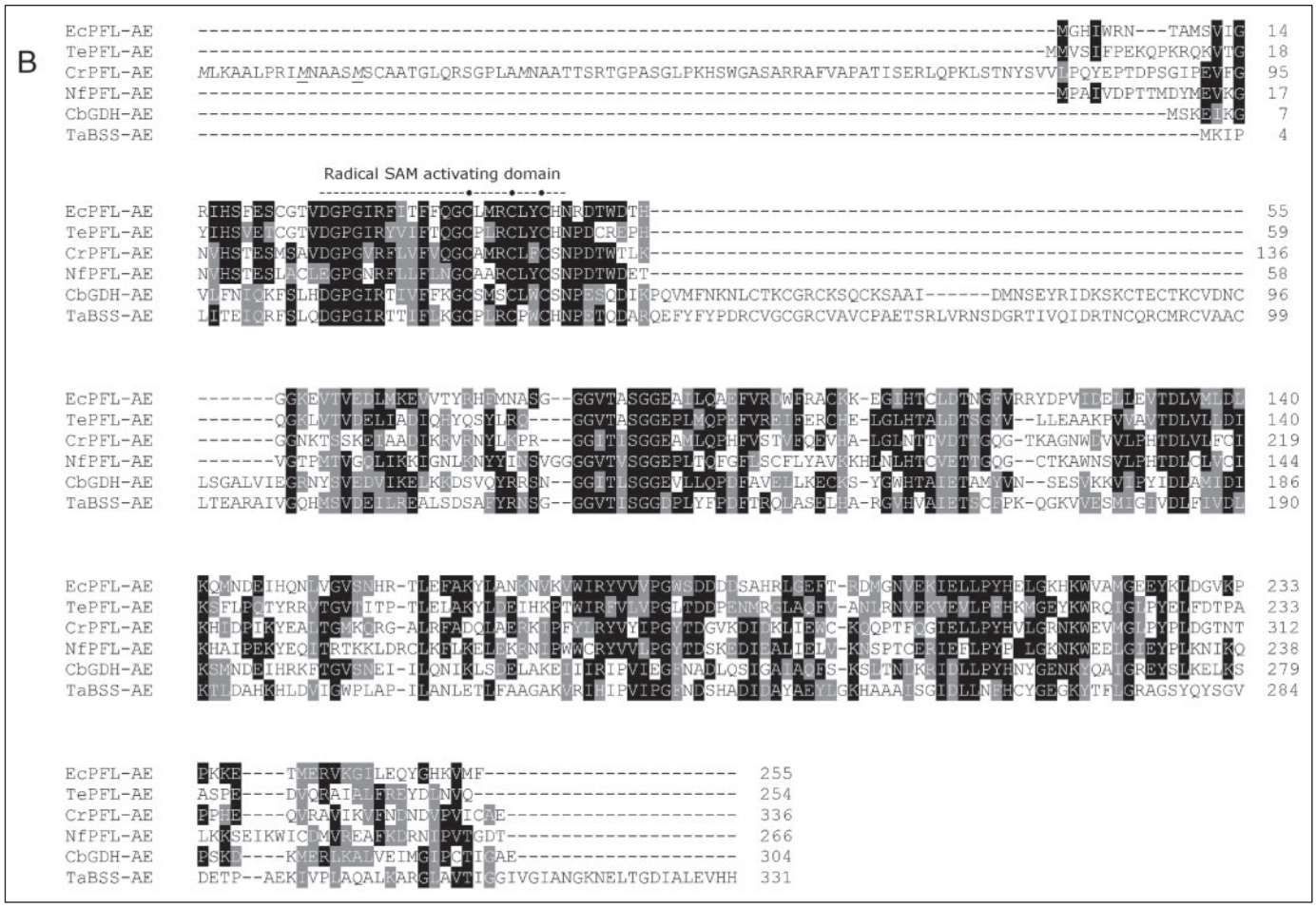

FIGURE 1—*continued*

presence of a PFL-like protein in the algae (19, 20). Neither PFL activity nor PFL genes have been reported in animals or plants.

Here we report the expression and compartmentalization of the PFL system (ADHE, PFL, and PFL-AE) in *C. reinhardtii* with mass spectrometry, immunological, and molecular techniques. Furthermore, using mass spectrometry on the soluble fraction of isolated mitochondria, backed by the available *C. reinhardtii* genome sequence, we report the occurrence of a phosphotransacetylase-acetate kinase (PTA-ACK) pathway, which is yet undescribed but, as we show, apparently not unique among eukaryote genomes investigated to date.

MATERIALS AND METHODS

Strains and Culture Conditions—*C. reinhardtii* wild-type strain 11.32a and cell wall-less strain 83.82 (collection of algae Göttingen) were maintained on Tris acetate phosphate (TAP) medium (21) solidified with 1.5% (w/v) agar. TAP medium and TAP medium supplemented with 34 mM acetate, adjusted to pH 7.2 with KOH (H3 medium), were used for liquid cultures. For anaerobic cultures, cells were transferred into a Falcon tube that was introduced in a jar containing Anaerocult®A (Merck). Anaerobiosis was typically achieved within 30 min. The jar was placed on a rotary shaker, under light or dark conditions. Following incubation under either aerobic or anaerobic conditions at 22 °C, the algal cells were harvested by centrifugation at 2,000 × *g* for 5 min and immediately frozen. XL1-Blue MRF' *E. coli* strain (Stratagene) was grown anaerobically on LB medium in the presence of 0.4% (w/v) glucose and 20 mM MOPS, pH 7.0.

Library Screening for C. reinhardtii cDNAs—Specific probes for *C. reinhardtii* ADHE, PFL, and PFL-AE were obtained by amplification reactions, using as template a cDNA library in λZAPII phagemid (Stratagene) made with mRNAs isolated from cells grown in light with 5% CO_2. The primers used were: *ADHE*, 5′-GCCACCCCCCATGCTGAGGTG-3′ and 5′-GTTGATCTTGGAGAAGAACTC-3′; *PFL*, 5′-GACGCGGGCATCAACGTCCAG-3′ and 5′CATGGTGTCGTGGAAGGTGCG-3′; *PFL-AE*, 5′-GTTTTCGGAAACGTGCATTCA-3′; and 5′-CTCGGCGCAGATGACGGGAAC-3′. The resultant PCR products were cloned in pGEM-T Easy vector (Promega), and sequenced. Specific probes were non-radioactively labeled using a DNA-digoxigenin labeling kit (Roche), and used to screen the same cDNA library. Isolated cDNAs were excised from the λ phages and retrieved in pBluescript SK(−) (Stratagene).

radical by an AE. ●, cysteine residue absolutely conserved in the active site. The first two characters of the abbreviated names refer to the organism: *Cb*, *C. butyricum*; *Cr*, *C. reinhardtii*; *Nf*, *N. frontalis*; *Te*, *Thermosynecchococcus elongatus*. GenBank accession numbers: *CbGDH*, glycerol dehydratase (AAM54728), *CrPFL* (AJ620191), *EcPFL*, *E. coli* (P09373); *NfPFL*, *N. frontalis* (Q6RFH7), *TaBSS*, *T. aromatica* benzylsuccinate synthase (CAA05052); *TePFL* (Q8DK76). *B*, alignment of *C. reinhardtii* PFL-AE with known and predicted activating components of glycyl radical enzymes. *Black* and *gray shadings* are as described in *A*. The position of the radical activating enzyme signature is indicated: (GVPS)*X*(GKS)*X*(KRS)*X*(3)(FL)*X*(2)G*X*(0,1)C*X*(3)C*X*(2)C*X*(NLF); ●, conserved cysteines involved in the [4Fe-4S] cluster binding (48). Of the four N-terminal Met residues (*italics*), the second and the third (*underlined*) are predicted to be the initiation Met. GenBank accession numbers and organisms: *CbGDH-AE*, AAM54729; *CrPFL-AE*, AJ620192; *EcPFL-AE*, NP_752967; *NfPFL-AE*, Q6RFH6; *TePFL-AE*, Q8DM95; and *TaBSS-AE*, CAA05050.

Protein Overexpression and Antibody Production—A partial sequence of *C. reinhardtii PFL* cDNA (coding for Leu^{236}–Val^{677}; tPFL) was amplified by PCR using oligonucleotide primers containing the BamHI and HindIII restriction sites (underlined) as follows: 5′-GACGGATCCCTGTACAG-CACGGTGCGC-3′, and 5′-GTCAAGCTTCACCCACTCGGCGA-TCTCGTC-3′. The PCR product was cloned in pGEM-T Easy (Promega) and recloned in the BamHI/HindIII sites of the overexpression vector pQE30 (Qiagen). Following the same strategy, the nucleotide sequence corresponding to the atypical C-terminal extension (Val^{496}–Lys^{574}) of subunit β of *C. reinhardtii* mitochondrial ATPase (22) was amplified by PCR and cloned into the expression vector pET15b (Novagen). The primers used were: 5′-GAATTCCATATGGTGGAGAAGGCCGACAAGCTG-3′, and 5′-ATGCTGCTCGAGTTACTTCTTGGGCAGGGGCAC-3′. The resultant constructs were introduced into XL1 Blue MRF' or BL21 *E. coli* strains to produce the recombinant proteins. His-tagged proteins were purified under denaturing conditions using Ni-NTA matrix (Qiagen), as recommended by the supplier. Antibodies against tPFL were produced at Eurogentec (Leuven, Belgium), and antibodies against the C-terminal extension of subunit βF_1-ATPase were produced at Charles River Laboratories.

RNA Blot Analysis—Total RNA from *C. reinhardtii* cells was isolated with NucleoSpin®RNAII (Macherey-Nagel) and transferred onto Hybond N^+ nylon transfer membrane (Amersham Biosciences). RNA was analyzed by gel blot hybridization in 6× SSC, 5× Denhardt's solution, 0.5% (w/v) SDS, 100 μg/ml denatured sheared herring sperm DNA, and 50% (v/v) formamide, at 42 °C. Following hybridization, membranes were washed twice in 2× SSC, 0.5% (w/v) SDS at 48 °C, followed by 2 washes in 0.2× SSC, 0.5% (w/v) SDS, at 48 °C. The PFL probe was a 450-bp amplified PCR fragment corresponding to nucleotides 1571–2031 of the isolated *PFL* cDNA, *PFL-AE* probe was a 550-bp fragment released after PstI digestion of *PFL-AE* cDNA, and *ADHE* probe was a 1.5-kb fragment (corresponding to the 5′-end of the open reading frame) released after digestion of *ADHE* cDNA with SacI. Probes were purified with QIAquick gel extraction columns (Qiagen) and labeled with [^{32}-P]dCTP by random priming.

Isolation of C. reinhardtii Chloroplasts and Mitochondria—Cell wall-less *C. reinhardtii* cells were grown on TAP medium to late exponential phase, harvested at 2,000 × *g* in a GSA rotor (Sorvall) for 8 min, and resuspended in breaking buffer (0.25 M sorbitol, 50 mM Tris, 50 mM Mes, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM EDTA, pH 7.2, KOH). Cell suspension was passed through a cell disruptor (BioNeb, Glas-Col, Terre Haute, IN) with N_2 gas pressure at 20 p.s.i. Cell lysate was centrifuged shortly to 5,000 rpm in a GSA rotor (Sorvall). The pellet was used to prepare intact chloroplasts (23). Mitochondria, in the supernatant, were pelleted at 11,000 rpm for 10 min in SS34 rotor (Sorvall) and further purified on Percoll gradient (24).

Protein Analysis—Frozen cells were thawed and resuspended in 50 mM dithiothreitol and 50 mM Na_2CO_3. For protein concentration determination, an aliquot of cells was precipitated with 80% (v/v) acetone and resuspended in 0.4% (w/v) SDS. Protein content was determined using the BCA Assay reagent kit (Pierce). For protein gels, cells were solubilized in 2% (w/v) SDS and 1 mM β-mercaptoethanol and heated at 90 °C, for 2 min. Proteins were separated by SDS-PAGE using a 10% acrylamide (w/v) gel and subsequently transferred onto Hybond C nitrocellulose membranes (Amersham Biosciences) for immunodetection. Blots were incubated for 1 h with primary antibodies as follows: 1:5000 for anti-*C. reinhardtii* PFL (this work), 1:20000 for anti-*C. reinhardtii* βF1-ATPase (this work), and 1:50000 for anti-*C. reinhardtii* LHC proteins (Dr. O. Vallon, IBPC, France). Immunodetection was carried out using the BM chemiluminescent protein blot kit (Roche Diagnostics) according to the manufacturer's instructions. To reprobe the blots, the Restore Western blot Stripping Buffer (Pierce) was used. TMBZ/H_2O_2 method was used (25) for in-gel cytochrome detection. Dual color precision plus protein standards (Bio-Rad) and the Benchmark™ pre-stained protein ladder (Invitrogen) were used to estimate molecular mass.

Mass Spectrometry and Protein Identification—Mitochondria were resuspended in 50 mM MOPS, pH 7.4, in the presence of 0.1 mM phenylmethylsulfonyl fluoride and 1 mM ε-amino caproic acid to a protein concentration of 15 mg/ml and sonicated 4 times for 10 s. Soluble and membrane components were fractionated by ultracentrifugation. The soluble fraction was run on a 12% (w/v) acrylamide SDS-PAGE. Gel pieces were excised from the Coomassie Blue-stained gel and subjected to tryptic digestion (26). Samples were injected into a nanoLC system directly coupled to a QTOF Ultima mass spectrometer (Waters). MS and MS/MS data were acquired and processed automatically using Masslynx 4.0 software. Data base searching was performed with Mascot 2.0, using NCBInr, the JGI *C. reinhardtii* version 2.0 gene models and EST databases. Proteins identified with at least 2 peptides showing scores higher than 40 were validated automatically. Peptides with scores between 18 and 40 were checked manually to confirm or cancel Mascot suggestion.

Sequence Analysis—Expressed sequence tag (EST) clones of *C. reinhardtii* were obtained from the EST databases. A draft of the *Chlamydomonas* genome is available. Sequence alignments were done with ClustalW 1.82 and refined manually. Motif search was done using the Prosite Data base of the protein family and domains. Predictions for intracellular targeting were performed using Predotar version 1.03, TargetP version 1.01, and PSORT.

Networks—Homologues were retrieved by BLAST searches against sequenced genomes and the nonredundant protein data base at GenBank®. Sequences were aligned using ClustalW (27). Gapped positions were excluded from phylogenetic analysis. A protein LogDet distance matrix (28) for each alignment was calculated with LDDist (29). Splits were determined by Neighbor-Net (30), a variant of the Neighbor-Joining algorithm (31), and plotted as planar graphs with Splitstree (32). For calculating the LogDet distances among ACK sequences, amino acids were recorded as Dayhoff classes, yielding six possible character states instead of 20 (7).

RESULTS

C. reinhardtii Expresses PFL, PFL-AE, and ADHE—A mass spectrometry proteomics survey of highly purified *Chlamydomonas* mitochondria revealed numerous peptides matching PFL data base entries (see below). Searching the *C. reinhardtii* EST databases identified a number of clones for PFL along with homologues for its radical-activating (PFL-AE) and putative deactivating (ADHE) enzymes. These ESTs were used to produce specific DNA probes and to isolate full-length cDNAs for the proteins of interest. The sequence of the longest *PFL* cDNA clone (3379 bp) contains an open reading frame of 2559 bp coding for a putative protein of 852 amino acids. The inferred PFL amino acid sequence shares extensive similarity with glycyl radical enzymes, an emerging class of anaerobic enzymes. These enzymes use a radical chemistry for carbon-carbon bond formation or cleavage (33–35). Fig. 1*A* shows sequence alignment of predicted *C. reinhardtii* PFL with different members of the larger PFL family (34). The highest sequence identity with prokaryote enzymes was found with the well characterized PFL of *E. coli* (56%), whereas the identity with other members of the family of PFL-related enzymes was significantly lower: 21% with the glycerol dehydratase from *Clostridium butyricum* and only 11% with benzylsuccinate synthase from *Thauera aromatica*. *C. reinhardtii* PFL exhibits

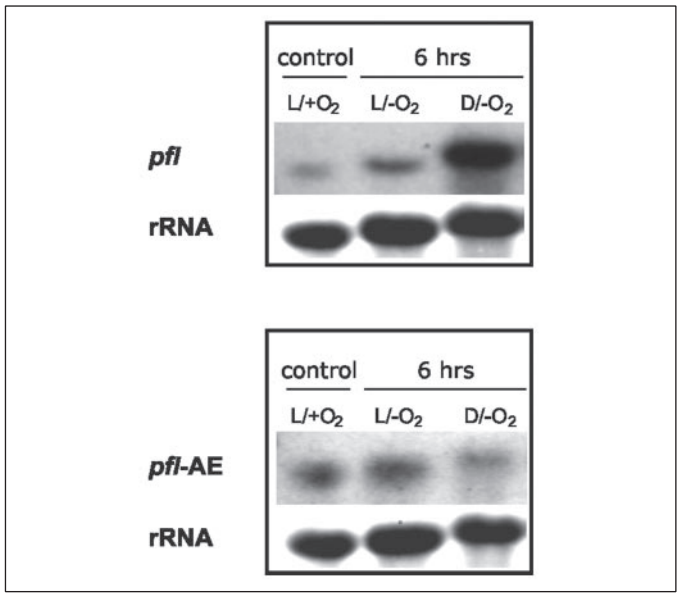

FIGURE 2. **RNA blot analysis of *PFL* and *PFL-AE* transcripts in *C. reinhardtii*.** Cells were grown on TAP medium and transferred to darkness in the presence ($+O_2$) or absence ($-O_2$) of oxygen for 6 h. *L* and *D*, refer to continuous light and darkness, respectively. Ten micrograms of total RNA were loaded in each lane. The ribosomal 28 S rRNA band as seen on a nylon membrane stained with methylene blue is shown as a loading control *below* each RNA blot. Predicted transcript sizes were of 3.3 kb for *PFL* and 2 kb for *PFL-AE*, in agreement with the sizes predicted from the isolated cDNAs.

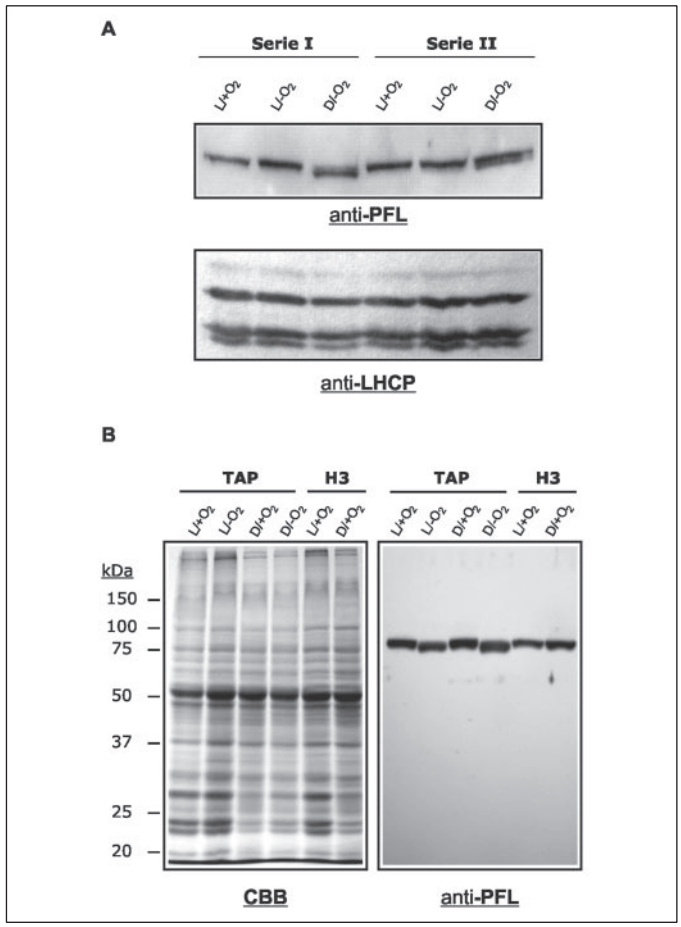

FIGURE 3. **Immunoblots to compare PFL levels in *C. reinhardtii* cells exposed to an oxygen-depleted environment.** *A*, effect of a short anaerobiosis on PFL levels. A culture of *C. reinhardtii* wild-type strain cells grown on TAP medium to 2×10^6 cells/ml was divided in three aliquots. Two aliquots were transferred to an anaerobic jar ($-O_2$) under light (*L*) or dark (*D*) conditions. The third aliquot, maintained in aerobic conditions ($+O_2$), was used as control. After 6 h of incubation under agitation, the cells from two independent experiments (*Series I* and *II*) were harvested, and prepared for SDS-PAGE analysis. Proteins (40 μg) were run on a 10% (w/v) acrylamide gel, transferred to nitrocellulose, and probed with anti-PFL antiserum or anti-light harvesting complex protein antiserum. Note that the ratio between the two PFL bands varied from preparation to preparation. *B*, a culture of *C. reinhardtii* wild-type cells grown on TAP medium (2×10^6 cells/ml) was divided into six aliquots. Cells in the aliquots were harvested, and resuspended in fresh culture medium as indicated. Cells were then exposed to either light (*L*) or darkness (*D*), in the presence ($+O_2$) or absence ($-O_2$) of oxygen for 24 h. Cells were then pelleted and resuspended in gel loading buffer for protein analysis. Proteins, separated on a 10% (w/v) acrylamide SDS-PAGE, were either stained with Coomassie Blue (*CBB*) or transferred to nitrocellulose for further immunodetection using anti-*C. reinhardtii* PFL antiserum (*anti-PFL*).

the two adjacent cysteinyl residues (Fig. 1*A*, Cys^{513}–Cys^{514}), which are present in all PFLs shown to catalyze homolytic pyruvate cleavage (2). As compared with bacterial PFLs, *C. reinhardtii* PFL exhibits a long N-terminal extension (Fig. 1*A*), suggestive of organelle targeting. This extension contains many Met residues, and computer-based subcellular localization predictions vary according to the assumed intiation Met.

The *C. reinhardtii PFL-AE* cDNA clone (2007 bp) contains an open reading frame of 978 bp coding for a protein of 326 amino acids. The predicted protein exhibits a radical activating enzyme signature (Prosite PS01087) (Fig. 1*B*), characteristic of all radical *S*-adenosylmethionine enzymes (36). The highest identity was found with PFL-AE homologues (35% identity with *E. coli*), whereas the activating enzymes of *C. butyricum* glycerol dehydratase and *T. aromatica* benzylsuccinate synthase were more distantly related (23 and 18% identity, respectively). *C. reinhardtii* PFL-AE exhibits a long N-terminal extension (Fig. 1*B*) that is predicted to serve as a chloroplast targeting peptide by PSORT, Predotar, and TargetP.

The isolated *C. reinhardtii ADHE* cDNA (3804 bp) has an open reading frame of 2865 bp encoding a precursor protein of 954 amino acids, sharing 62% identity with ADHE from the cyanobacterium *Thermosynecchococcus elongatus* (Q8DM94). Conservation with eukaryote enzymes is to some extent lower with 49% identity with *Piromyces* sp. ADHE (Q6WJD5) and 53% identity with ADHE of its nonphotosynthetic counterpart *Polytomella* sp. (Q70YJ9) (37). *C. reinhardtii* ADHE has a long N-terminal extension predicted to target the protein to the mitochondrion.

Effect of Anaerobiosis and Darkness on ADHE, PFL, and PFL-AE mRNA Levels—Kreuzberg (19) reported that formate production in *C. reinhardtii* increased during the first hours of anaerobiosis, reaching its highest levels after 6 h. Accordingly, transcript levels for ADHE, PFL, and PFL-AE in cells exposed to aerobiosis and to 6 h of anaerobiosis were compared by RNA blot analysis. *PFL* transcripts were hardly detectable in cells grown in aerated cultures (Fig. 2). Oxygen removal increased *PFL* mRNA levels, which were highest in cells kept in darkness (Fig. 2). *PFL-AE* transcripts were low in cells grown in aerated cultures and were not significantly influenced by anaerobiosis, in contrast to *PFL* transcripts (Fig. 2). No *ADHE* mRNAs could be detected in the RNA samples used to follow *PFL* and *PFL-AE* transcripts (not shown).

Effect of Anaerobiosis and Darkness on PFL Levels—Truncated *C. reinhardtii* PFL protein (tPFL; Leu^{236}–Val^{677} of the precursor protein) was expressed in *E. coli* and used for antibody production. The produced antibody recognized *C. reinhardtii* PFL as a protein of ~78 kDa as well as PFL from *E. coli* and *Neocallimastix frontalis* (supplemental Fig. I). Protein blots were carried out to determine the effect of 6 h of anaerobiosis on PFL levels. As revealed by immunoblotting, no significant changes in PFL levels occurred under anaerobiosis in either light or dark conditions (Fig. 3*A*). The antiserum detected a single band of ~78 kDa in protein extracts from light-exposed cells (with or without

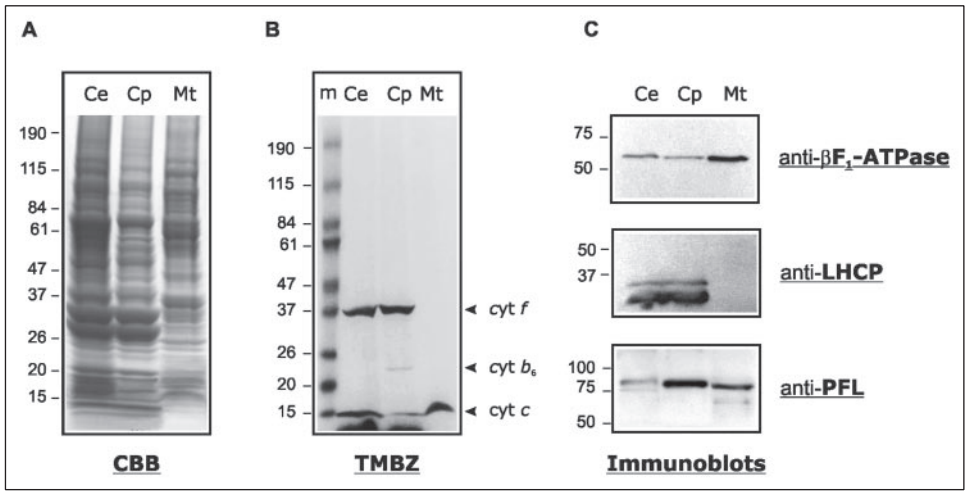

FIGURE 4. **Subcellular localization of PFL in *C. reinhardtii*.** Analysis of purified chloroplast (*Cp*) and mitochondria (*Mt*) from mixotrophically grown cells (*Ce*). Proteins (60 μg) were loaded on a 4–20% SDS-PAGE (*A* and *B*) or 10% SDS-PAGE (*C*). *A*, Coomassie Blue (*CBB*)-stained gel; *B*, protein gel stained for hemes using TMBZ: *cyt f*, *cyt* b_6, cytochromes of the chloroplast b_6f complex; *cyt c*, mitochondrial cytochrome c_{550}; *m*, molecular mass standards; *C*, immunoblots showing the distribution of light-harvesting complex proteins, of subunit β of the mitochondrial F_0F_1-ATPase, and of PFL in isolated organelles.

oxygen), but detected two bands of close molecular mass in protein extracts from cells maintained in anaerobiosis in the dark.

The effects of prolonged darkness or anaerobiosis on PFL accumulation were also investigated. TAP-growing cells were harvested in their exponential growth phase, resuspended in fresh culture medium, and incubated for 24 h in the conditions of interest. The medium for resuspension was either TAP medium (TAP) or a TAP-derived medium that contains 3-fold more acetate (H3). Cells were then harvested and prepared for protein analysis. Protein blots showed the presence of PFL in all cells analyzed, although two forms of close M_r were observed. The larger form of ~78 kDa was found in cells from aerated cultures, regardless of the amount of acetate or the illumination, whereas the smaller form of ~75 kDa was detected in cells kept under anaerobic conditions, in continuous darkness or continuous light (Fig. 3*B*). PFL levels appeared to be slightly lower in cells grown on H3 medium relative to the cells grown on TAP medium (Fig. 3*B*). Further work will determine whether acetate influences PFL steady-state levels in the algae growing under aerobic conditions.

Intracellular Localization of PFL in C. reinhardtii—PFL localization protein was investigated by protein blot analysis. TAP-grown *C. reinhardtii* cells were harvested, and disrupted by nebulization. Chloroplasts and mitochondria were fractionated by differential centrifugation and further purified on Percoll gradients. To ascertain the purity of the organelle fractions, each was tested for the enrichment of specific marker proteins. Heme staining showed a low contamination of chloroplasts by mitochondrial cytochrome c_{550}, whereas chloroplast cytochrome *f* and cytochrome b_6 were not detected in mitochondrial fractions (Fig. 4*B*). Furthermore, immunoblot analyses revealed that purified mitochondria lacked detectable amounts of light-harvesting complex proteins, whereas chloroplasts contained traces of mitochondrial F_0F_1-ATPase (βF_1-ATPase) (Fig. 4*C*). These data indicated that purified mitochondria were devoid of chloroplasts while chloroplasts were slightly contaminated with mitochondria. The same protein blots probed for PFL revealed the occurrence of the protein in both organelles (Fig. 4*C*). The electrophoretic mobility of the chloroplast and mitochondrial PFL proteins differs by ~3 kDa.

Protein Identification by Mass Spectrometry—Protein blot analysis of soluble and membrane fractions of *C. reinhardtii* mitochondria indicated that PFL is a soluble protein (not shown). Therefore, the soluble mitochondrial fraction was run on one-dimensional SDS-PAGE, discrete bands were excised from the gel (Fig. 5*A*) and treated for protein identification by tandem mass spectrometry (26). In the 60–75-kDa region, 22 peptides that matched the predicted PFL sequence were identified. These peptides cover a large part of the full-length PFL, although no peptide corresponding to residues Met^1–Lys^{107} was identified (Fig. 5*B*). The coverage of the PFL sequence is comparable with that obtained for the mitochondrial aconitase, indicating that PFL is an abundant protein.

Proteome analysis also uncovered peptides matching proteins that form the PTA-ACK pathway, which in a number of bacteria reversibly interconverts acetyl-CoA and acetate (38). ACK (EC 2.7.2.1) phosphorylates acetate to acetyl phosphate, which is then converted into acetyl-CoA by phosphotransacetylase (PTA, EC 2.3.1.8). Three tryptic peptides that match a putative PTA were identified in the 70–85-kDa range (Fig. 5*A*). All peptides are specific to annotated PTA1 (JGI C_870001), whereas no peptide corresponding to a second putative PTA, annotated PTA2 (C_330071), was obtained. The molecular mass range from PTA1 is 10–20 kDa higher than the mass of 60 kDa calculated for annotated PTA1, suggesting that the gene model may be incomplete. In the 38–42-kDa range, six tryptic peptides that match an ACK sequence were identified. In the *C. reinhardtii* genome, two genes are annotated as acetate kinase. Whereas one of the six tryptic peptides is common to both ACK1 (C_170112) and ACK2 (C_330070), the other five are specific to ACK2 (Fig. 5*C*). The molecular mass of ACK2 (Fig. 5*A*) is in agreement with the mass of 43 kDa calculated from the gene model. No peptide specific to PFL-AE or ADHE were identified in the fraction of soluble proteins, suggesting either that these proteins are present in very low amounts in the sample analyzed or that they are localized to another subcellular fraction.

DISCUSSION

Occurrence of PFL and PFL-AE in a Photosynthetic Eukaryote—Oxygen-sensitive formate production in *C. reinhardtii* cells under dark, anaerobic conditions had suggested the activity of pyruvate formate-lyase (19), an enzyme rare among eukaryotes. The present identification of PFL peptides in *C. reinhardtii*, along with cDNAs for PFL and PFL-AE, indicate the existence of a PFL system in the algae. Identified *C. reinhardtii* PFL and PFL-AE are well conserved with their counterparts in bacteria and amitochondriate protists.

In its natural habitats, such as soil and fresh water ponds, *C. reinhardtii* is exposed to anaerobiosis even more so in darkness. Short anaerobiosis in the dark (6 h) led to a clear increase of *PFL* transcript levels, but not of protein levels. Ambient levels of PFL in the algae may ensure readiness for immediate adaptation to fermentative conditions,

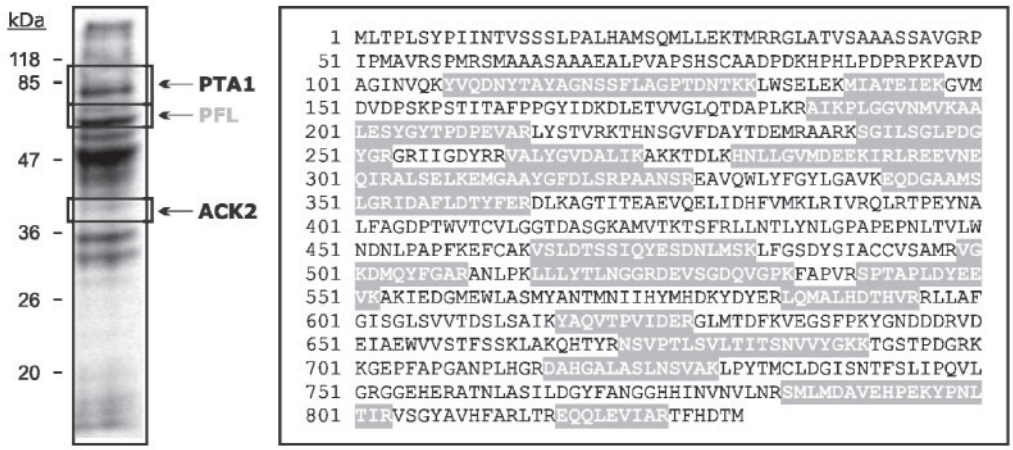

FIGURE 5. **Identification by mass spectrometry of three atypical eukaryote enzymes in *C. reinhardtii* mitochondria.** *A*, Coomassie Blue-stained SDS-PAGE used for mass spectrometry analysis. The gel pieces in which peptides for the proteins of interest were identified are indicated. *B*, PFL sequence that shows the peptides identified are shaded in *gray*. *C*, atypical eukaryotic enzymes identified in *C. reinhardtii* mitochondria. Tryptic peptides that match gene models for an acetate kinase and a phosphotransacetylase in *C. reinhardtii* are listed. The peptides specific to the putative proteins are indicated in *bold*.

without the need for *de novo* protein synthesis. More detailed studies will be required to fully describe the regulation of the PFL at RNA, protein, and activity levels.

Compartmentalization of the PFL System—Anaerobic formate production in *C. reinhardtii* was previously measured in fractions enriched in chloroplasts and mitochondria (20), which suggested the presence of a PFL in both organelles. Here, immunoblots revealed the occurrence of PFL in chloroplasts and mitochondria purified from aerobically grown *C. reinhardtii* cells. Library screening identified several *PFL* cDNAs corresponding to a single mRNA, and searching the draft of the *C. reinhardtii* genome sequence and the EST databases provided no evidence for two *PFL* genes and only one *PFL* transcript was detected in RNA blots. This may suggest that PFL is dual targeted to chloroplasts and mitochondria. Dual targeting is not uncommon in higher plants (39) but has not been previously reported in the green algae.

The ~3-kDa difference observed between the chloroplast and the mitochondrial PFL forms might be explained by differential processing of the cytosolic precursor. Alternatively, the 3-kDa difference might be explained by differential activation and radical-induced cleavage at the C-terminal end. However, it is not currently known whether *C. reinhardtii* PFL may be subject to oxygenolytic cleavage as its counterparts in bacteria (2, 14, 15). In our mitochondrial proteome analysis, one peptide that matches with the C-terminal sequence of PFL was identified. This peptide stems from (i) a protein of high molecular mass (~60–75 kDa) and (ii) a region of the PFL that is C-terminal to the glycine residue to be activated into a glycyl radical. In *E. coli*, PFL is a homodimer that is post-translationally activated by introduction of a radical on the ultimate Gly residue of only one of the two monomers (2, 15). Whether only one or both PFL subunits are activated in *C. reinhardtii* is yet unknown.

The rationale behind the differential compartmentalization of PFL in *C. reinhardtii* is not obvious. Indeed, PFL produces formate, a toxic (but freely diffusible) end product. Plants, which do not possess PFL, have a formate dehydrogenase that oxidizes formate into CO_2 in the presence of NAD^+. Plant formate dehydrogenase is localized to the mitochondria (40). No biochemical or molecular evidence for the presence of a formate dehydrogenase has yet been reported in the green algae, which would agree with a role of PFL in core energy metabolism and the status of formate as a genuine end product.

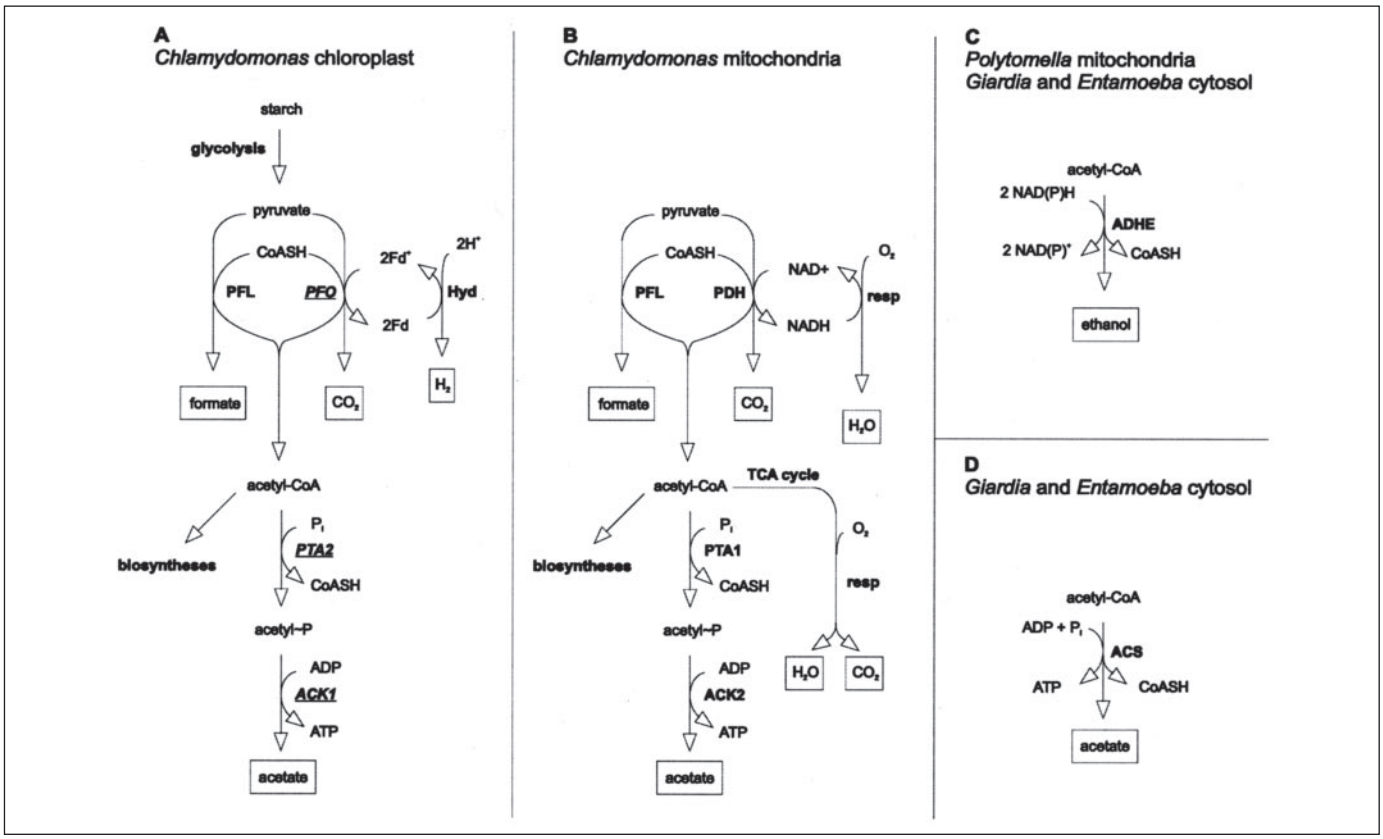

FIGURE 6. **Localization and suggested functions of PFL, PFO, PTA, and ACK in *C. reinhardtii* and for ADHE in its close nonphotosynthetic relative *Polytomella* sp.** Starch is accumulated inside the chloroplast during the light phase and is degraded in the dark. Starch breakdown is greater under anaerobic conditions than in aerobic conditions. In the absence of the functional TCA cycle (anaerobiosis), acetyl-CoA produced either via PFL or PFO enters the acetate-generating ACK-PTA route. The roles for PTA, ACK, and ADHE shown are dissimilatory. Nevertheless, a primarily assimilatory role for PTA-ACK and/or ADHE under some physiological conditions cannot currently be excluded because acetate is a carbon source for *C. reinhardtii* and ethanol is a source for *Polytomella* sp. (see text). Chloroplast pyruvate dehydrogenase is not represented. Enzymes for which the localization is not directly demonstrated are *underlined*. End products are *boxed*. *Fd*, ferredoxin; *ACS*, ADP-forming acetyl CoA synthase; *PDH*, pyruvate dehydrogenase.

Recycling Coenzyme A in C. reinhardtii Mitochondria—In *E. coli*, acetyl-CoA resulting from PFL activity is converted into either ethanol or acetate (38). Acetate dissimilation via the PTA-ACK pathway generates one ATP molecule per acetyl-CoA but does not consume reducing equivalents. Ethanol, produced by ADHE, reoxidizes two molecules of NADH per acetyl-CoA without ATP production. In the anaerobe eukaryotes *Giardia* and *Entamoeba*, fermentative production of ethanol is catalyzed by ADHE, whereas acetate is produced by ADP-forming acetyl-CoA synthase (EC 6.2.1.13) (1) (Fig. 6*D*). The ethanol to acetate ratio depends upon oxygen tension (1). In this sense, the ACK-PTA route, which produces ATP and acetate from acetyl-CoA, P_i, and ADP in most bacteria would be functionally equivalent to the single enzyme acetyl-CoA synthase in amitochondriate eukaryotes.

Predicted *C. reinhardtii* ADHE exhibits features typical of ADHE from amitochondriate eukaryotes and bacteria. Under the conditions investigated here, *i.e.* light *versus* dark and aerobiosis *versus* anerobiosis, we failed to detect ADHE transcripts and protein (supplemental Fig. II). In this respect, the green algae differs from its colorless counterpart *Polytomella* sp. where ADHE is a major protein of the mitochondrial matrix in aerobically grown cells (37) (Fig. 6*C*). The physiological conditions that lead to expression and activity of ADHE in the green algae are not known. Whether *C. reinhardtii* ADHE is involved in the anaerobic production of ethanol, and concomitant regeneration of NAD^+, remains to be determined.

Several peptides from a PTA-ACK pathway were identified in the soluble fraction of mitochondria isolated from aerobically grown algae. These peptides were specific to PTA1 and ACK2 but distinct from PTA2 and ACK1 whose genes are adjacent to *HYDA2* (C_330072) encoding the chloroplast hydrogenase of the algae (41). This gene organization as well as the lack of peptides specific to PTA2 and ACK1 in isolated mitochondria suggest that these proteins are located to the chloroplast (Fig. 6). Phylogenetic networks for *C. reinhardtii* ACK1 (Fig. 7) and PTA2 (supplemental Fig. III) indicate a common ancestry for both enzymes with the corresponding homologues from the oomycete *Phytophthora sojae*, suggesting that the two-step pathway that reversibly converts acetyl-CoA into acetate was present in the common ancestor of oomycetes and plants (42). An ACK but no PTA was found in the amitochondriate eukaryote *Entamoeba histolytica* genome sequence (Fig. 7). The PTA-ACK system is widespread among prokaryotes (38). Notwithstanding the activity measurements by Kreuzberg *et al.* (20), ACK and PTA represent novel and typically eubacterial enzymes among eukaryotes. *C. reinhardtii* is capable of growing heterotrophically on acetate and it is commonly accepted that acetate is assimilated by an acetyl-CoA synthase and metabolized to triose following entry into the glyoxylate cycle (21, 43). The mitochondrial PTA-ACK pathway in *C. reinhardtii* may represent an alternative route for acetate assimilation.

Pyruvate Degrading Systems in the Green Algae C. reinhardtii-PFL Versus PFO—A gene coding for a putative pyruvate:ferredoxin oxidoreductase (1303 amino acids) was identified in the *C. reinhardtii* genome (C_140055). Search of EST databases identified only two PFO clones, which correspond to a conserved N-terminal domain (clone BP093588; His^{241}–Ala^{305}) and to the predicted C terminus (BQ821311; Lys^{1216}–His^{1303}). The PFO sequence inferred from the gene model exhibits four highly conserved PFO motifs including two [4Fe-4S] ferredoxins, and the iron-sulfur binding region signature (Prosite PS00198)

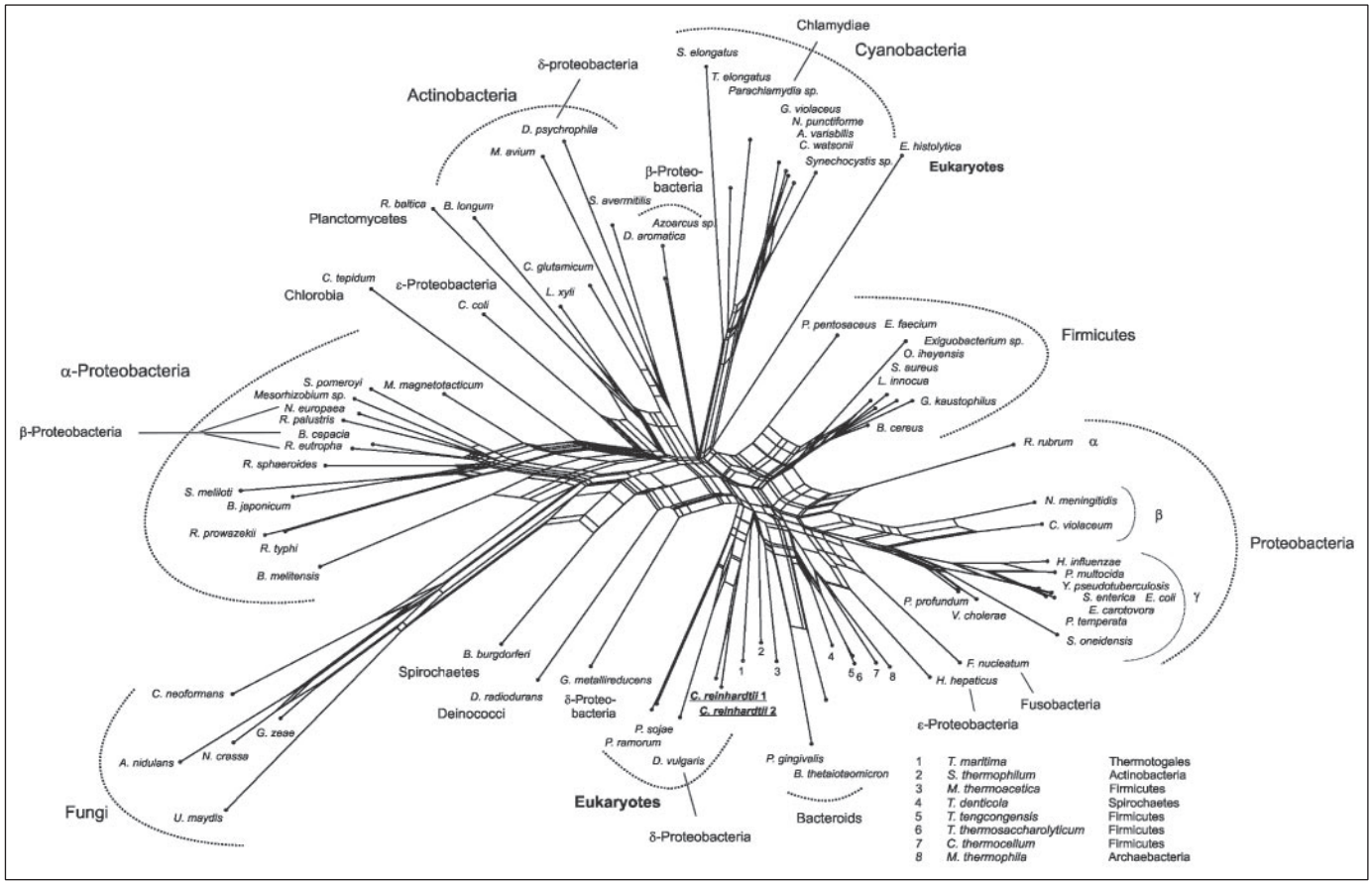

FIGURE 7. **Neighbor-Net analysis of ACK sequences.** Sources of sequences are as given. *Phytophthora ramorum* (Contig 21 in Scaffold 4 nucleotides 413776–415071), *P. sojae* (Contig 24 in Scaffold 12 reverse complement of nucleotides 795216–796527), *Anabaena variabilis* (ZP_00160102), *Aspergillus nidulans* (EAA60992), *Azoarcus sp.* (YP_159859), *Bacillus cereus* (NP_834343), *Bacteroides thetaiotaomicron* (AAO78798), *Bifidobacterium longum* (NP_696143), *Borrelia burgdorferi* (NP_212756), *Bradyrhizobium japonicum* (NP_770098), *Brucella melitensis* (AAL54122), *Burkholderia cepacia* (ZP_00217734), *Campylobacter coli* (ZP_00366841), *Chlorobium tepidum* (NP_662410), *Chromobacterium violaceum* (AAQ59206), *Clostridium thermocellum* (ZP_00311922), *Corynebacterium glutamicum* (BAC00146), *Crocosphaera watsonii* (ZP_00175966), *Cryptococcus neoformans* (EAL17325), *Dechloromonas aromatica* (ZP_00150711), *Deinococcus radiodurans* (AAF12139), *Desulfotalea psychrophila* (YP_064295), *D. vulgaris* (YP_012241), *E. histolytica* (EAL50605), *Enterococcus faecium* (ZP_00287449), *Erwinia carotovora* (YP_051129), *E. coli* (NP_754724), *Exiguobacterium sp.* (ZP_00182864), *Fusobacterium nucleatum* (AAL95367), *Geobacillus kaustophilus* (YP_148638), *Geobacter metallireducens* (ZP_00299464), *Gibberella zeae* (EAA70606), *Gloeobacter violaceus* (NP_923946), *Haemophilus influenzae* (ZP_00154390), *Helicobacter hepaticus* (AAP77905), *Leifsonia xyli* (YP_061463), *Listeria innocua* (NP_470952), *Magnetospirillum magnetotacticum* (ZP_00053788), *Mesorhizobium sp.* (ZP_00197262), *Methanosarcina thermophila* (B49338), *Moorella thermoacetica* (ZP_00331156), *Mycobacterium avium* (NP_962820), *Neisseria meningitidis* (AAF41874), *Neurospora crassa* (CAE76499), *Nitrosomonas europaea* (NP_842138), *Nostoc punctiforme* (ZP_00111397), *Oceanobacillus iheyensis* (NP_693112), *Parachlamydia sp.* (YP_008361), *Pasteurella multocida* (NP_245641), *Pediococcus pentosaceus* (ZP_00323037), *Photobacterium profundum* (YP_130972), *Photorhabdus temperata* (AAN08359), *Porphyromonas gingivalis* (AAQ66195), *Ralstonia eutropha* (ZP_00202877), *Rhodobacter sphaeroides* (ZP_00007961), *Rhodopirellula baltica* (NP_869003), *Rhodopseudomonas palustris* (NP_949900), *Rhodospirillum rubrum* (ZP_00269820), *Rickettsia prowazekii* (NP_220502), *Rickettsia typhi* (YP_066996), *Salmonella enterica* (NP_804384), *Shewanella oneidensis* (NP_718485), *Silicibacter pomeroyi* (AAV93446), *Sinorhizobium meliloti* (AAD24358), *Staphylococcus aureus* (BAB57873), *Streptomyces avermitilis* (BAC70535), *Symbiobacterium thermophilum* (YP_076414), *Synechococcus elongatus* (ZP_00165089), *Synechocystis sp.* (NP_440508), *Thermoanaerobacter tengcongensis* (NP_623096), *Thermoanaerobacterium thermosaccharolyticum* (CAA95986), *Thermosynechococcus elongatus* (NP_683130), *Thermotoga maritima* (NP_228087), *Treponema denticola* (NP_971543), *Ustilago maydis* (EAK84551), *Vibrio cholerae* (AAF94257), and *Yersinia pseudotuberculosis* (YP_071107).

(Cys^{766}–Cys^{777} and Cys^{822}–Cys^{833}), which participate in electron transfer to ferredoxin (44). *C. reinhardtii* PFO shares 50% identity with PFOs of the strictly anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough (YP_012236), and the anaerobic parasite *E. histolytica* (EAL51636) and 49% identity with mitochondrial pyruvate:NADP$^+$ oxidoreductase (PNO) of *Euglena gracilis* (Q94IN5). In the phylogenetic network, *C. reinhardtii* PFO shares common ancestry with other eukaryote PFO homologues (supplemental Fig. IV).

In contrast to *Euglena* pyruvate:NADP$^+$ oxidoreductase (44), *C. reinhardtii* PFO does not exhibit a C-terminal NADPH-generating domain, suggesting that the algal enzyme probably accepts electrons from ferredoxin like its homologues from amitochondriate protists (1, 45). In *C. reinhardtii*, ferredoxin is present in the stroma where it donates electrons to ferredoxin:NADPH oxidoreductase but also to a hydrogenase (under anaerobic conditions) (46). We thus propose that *C. reinhardtii* PFO is localized to the stroma where under anaerobic conditions its activity might be coupled to that of the hydrogenase via ferredoxin (Fig. 6*A*). Under anaerobiosis in the dark, *C. reinhardtii* cells produce large amounts of formate, whereas production of CO_2 is low (19), which might indicate that under these conditions PFO is poorly involved in the survival of the algae. The anaerobic enzymes PFL and PFO in the green algae represent an unexpected biochemical and evolutionary link between energy metabolism in amitochondriate and mitochondriate protists.

C. reinhardtii possesses genes for pyruvate dehydrogenase, PFL, and PFO. This 3-fold pyruvate-metabolizing repertoire is not uncommon in prokaryotes (38) but it is hitherto unique among eukaryotes. Together with the presence of ADHE and HYD, the enzymatic repertoire typical of eukaryote anaerobes (1) is almost completely present in *C. reinhardtii*. The only exception is that ADP-forming acetyl-CoA synthase is lacking, whereby the functionally equivalent PTA-ACK route is present. The large variety of metabolic abilities evidenced in this work are likely

helping the photosynthetic algae to optimize its metabolism to the ever changing environmental conditions.

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