Pyruvate formate-lyase and a Novel Route of Eukaryotic ATP Synthesis in Chlamydomonas Mitochondria

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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 mRNAs 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 antisera 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, acetyl-CoA synthetase, and acetate kinase. The phosphotransacetylase-acetate kinase pathway is a common route of ATP synthesis or acetate metabolism. Pyruvate derived from carbohydrate fermentation 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. 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 deactivating 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 deactivating activity, as shown in L. lactis (15).

Reports on eukaryotic PFL are limited to a few anaerobic protists and some green algae. PFL activity has hitherto been measured in two amitochondriate eukaryotes, the chytridfmycetes Neocallimastix sp. L2 and Pirromyces 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 H2 and CO2 remains low (19). The pattern of fermentation end products together with the sensitivity of formate production to oxygen were interpreted as the
FIGURE 1. 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)(GIVT)(CSA)(GYX)(GACV)); *, glycine residue that is activated into an organic donor. Typical Eubacterial Enzymes in *C. reinhardtii* Mitochondria

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**Catalytic domain**

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**GLY-radical signature**

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*Typical Eubacterial Enzymes in *C. reinhardtii* Mitochondria*
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 Go¨ttingen) 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/H2 3041 (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 ZAPII phagemid (Stratagene) made with mRNAs isolated from cells grown in light with 5% CO2. 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’-CTCGCGCAGATGACGGGAAC-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 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, *Thermosynechococcus 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(KGS)X(KRS)X3(FIX)X2(GIX)0,1(CX)3(CX)2(CX)(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, AJ620191; EcPFL-AE, NP_752967; NfPFL-AE, Q6RFH6; TePFL-AE, Q8DM95; and TaBSS-AE, CAA05050.
Typical Eubacterial Enzymes in C. reinhardtii Mitochondria

Protein Overexpression and Antibody Production—A partial sequence of C. reinhardtii PFL cDNA (coding for Leu3/Va16; tPFL) was amplified by PCR using oligonucleotide primers containing the BamHI and HindIII restriction sites (underlined) as follows: 5’-GACGGATCCCTGTACAGCAGGGTGCGC-3’ and 5’-GTCGACTCCACCCACTCGGCGATCCTC-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 (Val195–Lys574) 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’-GAATTCATATGGTGGAGAAGGCCGACAAGCTG-3’ and 5’-ATGCCTCTAGAATTCCTTTGCAGAGGGCACC-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), and antigens against the C-terminal extension of subunit βF2-ΔTAPase were produced at Charles River Laboratories.

RNA Blot Analysis—Total RNA from C. reinhardtii cells was isolated with NucleoSpin® RNA II (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% (v/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% (v/v) SDS at 48 °C, followed by 2 washes in 0.2× SSC, 0.5% (v/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 wallless C. reinhardtii cells were grown on TAP medium to late exponential phase, harvested at 2,000 x 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 MgCl2, 1 mM MnCl2, 2 mM EDTA, pH 7.2, KOH). Cell suspension was passed through a cell disruptor (BioNeb, Glas-Col, Terre Haute, IN) with N2 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). 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 Na2CO3. 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 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 BF1-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/H2O2 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 e-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 NCBI nr, 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 database 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 database at GenBank®. Sequences were aligned using ClustalW (27). Gapped positions were excluded from phylogenetic analysis. A protein LogDet distance (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 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 glycol radical enzymes, an emerging class of anaerobic enzymes. These enzymes use a radical chemistry for carbon-carbon bond formation or cleavage (33–35). Fig. 1A 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 benzylsuccinic synthase from Thauera aromatica. C. reinhardtii PFL exhibits
Typical Eubacterial Enzymes in C. reinhardtii Mitochondria

The isolated C. reinhardtii ADHE cDNA (3804 bp) has an open reading frame of 2865 bp encoding a precursor protein of 954 amino acids. The predicted protein exhibits a radical activating enzyme signature (Prosite PS01087) (Fig. 1A), characteristic of all radical S-adenosylmethionine enzymes (36). The highest identity was found with PFL-AE homologues (35% identity with PFL from the cyanobacterium E. coli, and 23% identity with PFL from the cyanobacterium Neocallimastix frontalis (37). Polytomella sp. (Q70YJ9), and were not significantly influenced by anaerobiosis, in contrast to PFL-AE transcripts in C. reinhardtii grown on TAP medium and transferred to darkness in the presence (O2) or absence (O2) 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.

The two adjacent cysteinyl residues (Fig. 1A, Cys513-Cys514), 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) 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. 1B) that is predicted to serve as a chloroplast targeting peptide by PSORT, Predotar, and TargetP.

The isolated C. reinhardtii ADHE 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. 1B), 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. 1B) that is predicted to serve as a chloroplast targeting peptide by PSORT, Predotar, and TargetP.

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).

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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 (O2) or absence (O2) 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 (O2) under light (L) or dark (D) conditions. The third aliquot, maintained in aerobic conditions (O2), 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 mg) 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), 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), 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.
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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 b6, cytochromes of the chloroplast b6f complex; cyt c, mitochondrial cytochrome c550, m, molecular mass standards; C, immunoblots showing the distribution of light-harvesting complex proteins, of subunit β of the mitochondrial F0F1-ATPase, and of PFL in isolated organelles.

Typical Eubacterial Enzymes in C. reinhardtii Mitochondria

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, 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. 3B). PFL levels appeared to be slightly lower in cells grown on H3 medium relative to the cells grown on TAP medium (Fig. 3B). 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-growing C. reinhardtii cells were harvested, and disrupted by nebulization. Chloroplasts and mitochondria were fragmented 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 c550, whereas chloroplast cytochrome f and cytochrome b6 were not detected in mitochondrial fractions (Fig. 4B). Furthermore, immunoblot analyses revealed that purified mitochondria lacked detectable amounts of light-harvesting complex proteins, whereas chloroplasts contained traces of mitochondrial F0F1-ATPase (βF1-ATPase) (Fig. 4C). 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. 4C). 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. 5A) 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 Met1–Lys107 was identified (Fig. 5B). 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. 5A). All peptides are specific to annotated PTA1 (gi|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. 5C). The molecular mass of ACK2 (Fig. 5A) 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, oxygen, but detected two bands of close molecular mass in protein extracts from cells maintained in anaerobiosis in the dark.

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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 CO₂ 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.

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**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.
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. 6D). 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, P₇₅ 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 aerobic versus anaerobic, 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 aerobiosis (32). The mitochondrial PTA-ACK pathway in C. reinhardtii 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.
(Cys
<sup>766</sup>–Cys
<sup>777</sup> and Cys
<sup>822</sup>–Cys
<sup>833</sup>), which participate in electron transfer to ferredoxin (44). C. reinhardtii PFO shares 50% identity with PFOs from the strictly anaerobic, sulfate-reducing bacterium Desulfovibrio vulgaris Hildenborough (YP_012236), and the anaerobic parasite Entamoeba histolytica (AAO78798), the enzymatic repertoire typical of eukaryote anaerobes (1) is almost completely present in C. reinhardtii mitochondria (44).

In contrast to Euglena pyruvate-NADP<sup>+</sup> oxidoreductase (44), C. reinhardtii PFO does not exhibit a C-terminal NADP<sup>+</sup>-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 hydro-
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helping the photosynthetic algae to optimize its metabolism to the ever-changing environmental conditions.

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