Pyruvate:ferredoxin oxidoreductase is coupled to light-independent hydrogen production in Chlamydomonas reinhardtii*

Jens Noth, Danuta Krawietz, Anja Hemschemeier, and Thomas Happe

From the Ruhr Universität Bochum, Fakultät für Biologie und Biotechnologie, AG Photobiotechnologie, 44801 Bochum, Germany

Received for publication, October 22, 2012, and in revised form, December 18, 2012. Published, JBC Papers in Press, December 20, 2012, DOI 10.1074/jbc.M112.429985

In anaerobiosis, the green alga Chlamydomonas reinhardtii evolves molecular hydrogen (H2) as one of several fermentation products. H2 is generated mostly by the [Fe-Fe]-hydrogenase HYDA1, which uses plant type ferredoxin PETF/FDX1 (PETF) as an electron donor. Dark fermentation of the alga is mainly of the mixed acid type, because formate, ethanol, and acetate are generated by a pyruvate:formate lyase pathway similar to Escherichia coli. However, C. reinhardtii also possesses the pyruvate:ferredoxin oxidoreductase PFR1, which, like pyruvate:formate lyase and HYDA1, is localized in the chloroplast. PFR1 has long been suggested to be responsible for the low but significant H2 evolution in the dark because the catalytic mechanism of pyruvate:ferredoxin oxidoreductase involves the reduction of ferredoxin. With the aim of proving the biochemical feasibility of the postulated reaction, we have heterologously expressed the PFR1 gene in E. coli. Purified recombinant PFR1 is able to transfer electrons from pyruvate to HYDA1, using the ferredoxins PETF and FDX2 as electron carriers. The high reactivity of PFR1 toward oxaloacetate indicates that in vivo, fermentation might also be coupled to an anaerobically active glyoxylate cycle. Our results suggest that C. reinhardtii employs a clostridial type H2 production pathway in the dark, especially because C. reinhardtii PFR1 was also able to allow H2 evolution in reaction mixtures containing Clostridium acetobutylicum 2[4Fe-4S]-ferredoxin and [Fe-Fe]-hydrogenase HYDA.

Chlamydomonas reinhardtii is a photoautotrophic eukaryote that is equipped with a repertoire of fermentative enzymes allowing the cells to perform a mixed acid type fermentation (1–4). Of special interest for biotechnological applications is the capability of the cells to generate molecular hydrogen (H2) in the absence of oxygen (O2) (5). H2 is generated by a highly efficient hydrogenase of the [Fe-Fe] type, HYDA1 (6–8), which, despite its extreme sensitivity toward O2 (9–11), is located in the chloroplast (6). The natural electron donor of HYDA1 is the photosynthetic ferredoxin PETF (7, 12), and the highest rates of H2 evolution are observed in the light (4, 13). However, the cells have to be adapted to dark anaerobic conditions to induce hydrogenase activity (7, 14). Upon the shift to illumination, H2 production is only transient, because the hydrogenase enzymes are inactivated by photosynthetically generated O2 (15), and assimilatory photosynthetic electron sinks, the Calvin cycle above all, are reactivated (16, 17). However, a sustained H2 metabolism in illuminated C. reinhardtii cells is induced by sulfur deprivation (18). Sulfur deficiency results in a strongly reduced photosystem 2 activity, mainly because of photo-damage and inadequate recycling of the D1 core subunit of photosystem 2 (19–21). Hence, incubated in sealed flasks, sulfur-deficient algae establish hypoxic conditions despite illumination, because O2 evolution rates drop below respiratory O2 consumption (18). Anaerobiosis elicits hydrogenase gene expression (22, 23) and allows the O2-intolerant enzyme to be active. However, only the diminution of assimilatory electron sinks caused by cessation of cell division (18, 21) allows sustained and relatively high H2 evolution rates (17). Electrons for photosynthetic H2 production originate from residual photosystem 2 activity (24–26) but also from nonphotochemical plastoquinone reduction via plastidic NAD(P)H:plastoquinone oxidoreductase NDA2 (27–29). Electrons for this photosystem 2-independent, so-called indirect pathway result from oxidative starch and possibly protein degradation (18, 24, 26). It is generally accepted that in sulfur-deprived C. reinhardtii cells, H2 production serves as an alternative electron sink, allowing photosynthetic electron transport and thus energy generation to continue while preventing an over-reduction of the photosynthetic machinery (18, 25).

H2 production in nutrient-deficient green algae, however, is not the only pathway allowing the cells to maintain an energy and redox balance. Rather, the cells accumulate formate and ethanol simultaneously (30–32). The excretion of nongaseous fermentation products prevails in anaerobic Chlamydomonas cells in the dark (4). The generation of formate, ethanol, and acetate in a ratio of 2:1:1 in dark-incubated algae resembles...
mixed acid fermentation of prokaryotes like the enterobacterium Escherichia coli (33). The presence of a pyruvate:formate lyase (PFL1), catalyzing the thiolcatic cleavage of pyruvate to formate and acetyl-CoA in the eukaryotic alga, was therefore proposed (34) and genetically and biochemically proven (2, 30, 35). Because C. reinhardtii does not contain genes for a formate-hydrogen lyase complex that is responsible for formate-dependent H₂ generation in fermenting E. coli (33), light-independent H₂ generation in the green algae was proposed to result from pyruvate:ferredoxin oxidoreductase (PFOR)² activity (36). A Chlamydomonas PFOR (PFR1) was identified on the genetic (2) and protein levels, and the protein is located in the chloroplast of the cells (37, 38). PFOR enzymes oxidatively decarboxylate pyruvate to yield acetyl-CoA and CO₂, simultaneously transferring electrons to flavodoxin or ferredoxin (39). Thereby, ferredoxin reduction by plastidic C. reinhardtii PFR1 might couple fermentative pyruvate catabolism to H₂ generation in a way typical for strict anaerobic bacteria of the genus Clostridium (40–43).

In this study we show that C. reinhardtii PFR1 heterologously produced in E. coli is active in pyruvate- and oxaloacetate-dependent methyl viologen reduction. Moreover, PFR1 enables methyl viologen- or ferredoxin-dependent H₂ production by isolated Chlamydomonas HYDA1, which proves that the long postulated pathway of dark H₂ generation in the green alga is biochemically possible.

**EXPERIMENTAL PROCEDURES**

Organisms and Growth Conditions—E. coli strain DH5α MCR (Novagen) was used for cloning procedures. Heterologous expression of C. reinhardtii PFR1 and ferredoxin encoding cDNAs was done in E. coli BL21 (DE3) ΔiscR (44, 45). E. coli strains were grown according to standard procedures as described before (30). Clostridium acetobutylicum ATCC 824 was used for heterologous synthesis of C. reinhardtii HYDA1 and homologous expression of C. acetobutylicum [Fe-Fe]-hydrogenase HYDA and ferredoxin CAC0303.

C. reinhardtii strain CC124 (137c, mt- nit1 – nit2 –) was grown in Tris acetate-phosphate (TAP) medium (46) on a shaker with bottom-up illumination of 100 μmol of photons·m⁻²·s⁻¹ at 20 °C. For determination of in vivo H₂ production rates in the light or in the dark, C. reinhardtii cultures were grown until they reached a chlorophyll (Chl) content of 15 μg·ml⁻¹, harvested by mild centrifugation (2,000 × g, 3 min, 20 °C), and resuspended in fresh TAP medium to reach a final Chl concentration of 110 μg·ml⁻¹. The cell suspension was transferred to a shaded flask and purged with nitrogen gas for 4 h. In vitro hydrogenase activity was determined as described before (47) to ensure the anaerobic induction. Afterward, 2-ml aliquots of the cell culture were withdrawn using a syringe and transferred to sealed and O₂-free head space bottles. Half of the withdrawn 2-ml aliquots were incubated in the dark and half in the light (100 μmol of photons·m⁻²·s⁻¹). The H₂ amount in the head space was analyzed after various time points of incubation by gas chromatography as reported previously (35).

² The abbreviations used are: PFOR, pyruvate:ferredoxin oxidoreductase; Chl, chlorophyll; TPP, thiamine pyrophosphate; PETF, ferredoxin PETF/FDX1.
Pyruvate-dependent H₂ Production in Green Algae

VOLUME 288 • NUMBER 6 • FEBRUARY 8, 2013

ratating SDS-PAGE according to standard techniques (51). PFR1 activity tests were conducted immediately after purification.

Heterologous production of C. reinhardtii [Fe-Fe]-hydrogenase HYDA1, as well as homologous production of HYDA in C. acetobutylicum and subsequent purification via Strep-tag II was done as described before (52, 53). Recombinant Chlamydomonas [2Fe2S]-ferredoxins PETF and FDX5 were obtained as published previously (12, 54). C. reinhardtii FDX2 was produced accordingly by amplifying the FDX2 coding region without the N-terminal transit peptide encoding sequence using oligonucleotides 5'-AGGGTCCTACGACCCACTTTTAAGGTCAGTTTAAAGACC-3' and 5'-AGGGTCCTACATACAGCCTTGACCTCGTGTT-3' (BsaI restriction sites are indicated by bold letters). The C. acetobutylicum ferredoxin CAC0303 encoding region was amplified from the E. coli expression vector pET21c0303 (55) using hot start Pfu DNA polymerase (Stratagene) with oligonucleotides 5'-GAGGGAATATCTCGCATATTTGTAAATAACAGACGCTTGTG-3' and 5'-AAGCGGGGCCACCGAGCTGAATTCT-3'. The bold letters mark the BamHI and EcoH restriction sites used for ligation with the clostridial expression vector pThydaC_{CRI}.C-tag after excision of its insert (52). All further steps (transformation and cultivation of C. acetobutylicum, as well as protein purification) were done as described before (53).

PFR1 Activity Assays Using Methyl Viologen as Artificial Electron Acceptor—The enzymatic activity of recombinant C. reinhardtii PFR1 was determined by following the reduction of methyl viologen spectrophotometrically at λ = 604 nm (56) using a 96-well plate reader (Beckmann, Paradigm 1113) operated in an anaerobic tent and connected to a PC running multimode analysis software. The molar extinction coefficient of methyl viologen used was ε_{604} = 13.6 mmol⁻¹ cm⁻¹ (57). The standard reaction mixture contained 1.4 μM recombinant PFR1, 100 mM Tris-HCl, pH 8.0, 10 mM sodium pyruvate, 2 mM sodium CoA, 5 mM TPP, 10 mM methyl viologen, and 16 mM dithioerythritol in a final volume of 100 μl. The reaction was started by adding PFR1 and conducted at room temperature. Absorbance was measured every 30 s until saturation was reached. The value obtained after 6 min, which was in the late linear phase, was used for determining activity. Kinetics were performed varying the concentration of one substrate while keeping the concentrations of all other substrates constant and saturating. The Kₘ and Vₘₐₓ values were determined in each reaction and calculated using GraphPad Prism® software.

In Vitro Reconstitution of PFR1-coupled H₂ Production—For analyzing the H₂ producing capacity of [Fe-Fe]-hydrogenases upon electron delivery by PFR1-catalyzed ferredoxin reduction, recombinant enzymes and proteins were mixed in various combinations. The standard reaction mixture contained 0.7 μM recombinant PFR1, 40 μM ferredoxin, 0.01 μM hydrogenase, 10 mM sodium pyruvate, 2 mM sodium CoA, 5 mM TPP, and 16 mM dithioerythritol in 200 μl of 100 mM potassium-phosphate buffer, pH 6.8. The reactions were carried out in sealed 2-ml reaction vessels. Before incubation, the reaction mixtures were purged with argon for 3 min to reset the system. After incubation for 30 min at 37 °C in a shaking water bath, 400 μl of the head space were injected in a gas chromatograph (GC-2010 (Shimadzu), equipped with a PLOT fused silica coating mol-

![FIGURE 1](image-url) In vivo H₂ evolution rates of C. reinhardtii cultures in the light or in the dark. Concentrated cell suspensions were flushed with nitrogen for 4 h until they had reached an in vitro hydrogenase activity of 109 ± 18 nmol of H₂·μg Chl⁻¹·h⁻¹. Then culture aliquots were withdrawn, transferred to gas tight head space bottles, and incubated in the light (white bars) or the dark (gray bars) until the indicated time points before determining the H₂ concentration of the head space by gas chromatography. The results shown are the mean values from three independent experiments carried out as technical duplicates. The error bars indicate the standard deviation.

RESULTS

Recombinant C. reinhardtii PFR1 Has Typical PFOR Activity—As described in the introduction, in vivo H₂ production in C. reinhardtii is higher in the light, because electrons are provided by photosynthetic activity. We compared the light-dependent and -independent H₂ evolution rates of anaerobically adapted Chlamydomonas cell suspensions in a setup moderately modified from those reported before (as in Ref. 35, for example) (Fig. 1). In cells transferred from anaerobic conditions in the dark to illumination, H₂ accumulated to 2.13 nmol of H₂·μg Chl⁻¹ within the first 10 min, whereas cells kept in the dark produced only 0.05 nmol of H₂·μg Chl⁻¹ in the same time period (Fig. 1). In the following 50 min, the cells exposed to light produced additional 0.92 nmol of H₂·μg Chl⁻¹ (plus 43.2%) and shaded cells generated 0.22 nmol of H₂·μg Chl⁻¹ (plus 437.5%). In both illuminated and dark-incubated C. reinhardtii cell suspensions, H₂ generation rates slowed down in the following hour, because the former evolved 0.078 nmol of H₂·μg Chl⁻¹ and the latter evolved 0.152 nmol of H₂·μg Chl⁻¹ (Fig. 1). To analyze whether the low but significant H₂ production in dark-adapted algae might be driven by pyruvate oxidation via PFOR activity, Chlamydomonas PFR1 was heterologously produced.

The annotated gene models of the C. reinhardtii PFR1 gene have changed considerably from the first Chlamydomonas genome version to the most recent annotation on Phytozome v8.0, C. reinhardtii v5.3. Although most parts of the primary sequences are the same in the newest gene models (Cre11.g473950.t1.1 and g1910.t2 on Phytozome v8.0, C. reinhardtii v4.3 and v5.3, respectively, and au5.g2553_t1 and SKA_Chlr2_kg.scaffold_62000019 on JGIv4), a region starting at position 940 in the Cre11.g473950.t1.1 protein model is highly variable. We aligned all available PFR1 models, as well as the sequence translated from the PFR1 cDNA amplified in this study with bacterial enzymes and concluded that the cDNA and
Protein sequences, respectively, obtained here are correct (i.e., amino acids AKKWVLFCARLLTQ starting at position 940 in Cre11.g473950.t1.1 are actually missing). Therefore, we used the protein sequence deduced from our cDNA for the alignment shown in Fig. 2. The alignments revealed that the C. reinhardtii PFR1 polypeptide sequence contains all sequence motifs known to be essential for PFOR enzyme activity (marked in Fig. 2). It features an N-terminal conserved 2-oxoacid-binding motif (YPITP), as well as three [4Fe-4S]-cluster coordinating motifs and the region homologous to TPP-binding sites, which are underlined. Note that the third [4Fe-4S]-cluster-binding site is atypical and consists of the CXXC motif at positions 942–945 and two separated cysteines at positions 970 and 1221 (60, 61). The first amino acid of recombinant PFR1 is marked by an asterisk.

**FIGURE 2.** Stacked polypeptide alignment of pyruvate:ferredoxin oxidoreductase primary sequences. Eight PFOR sequences were used for an alignment using ClustalW2 and WebLogo 3 (89, 90). These sequences were C. reinhardtii PFR1 derived from the cDNA obtained in this study, Volvox carteri f. nagariensis (Phytozome v8.0, V. carteri Vocar20008508m), Chlorella variabilis (GenBank™ EF55341.1), C. acetobutylicum PFO (GenBank™ NP_348846.1), Clostridium pasteurianum (GenBank™ AAD55756.1), Desulfovibrio africanus POR (GenBank™ CA70873.1), E. coli YdbK (GenBank™ YP.002699180.1), and Synechococcus sp. PCC.7002 NifJ (GenBank™ ACA99434.1). The conserved YPITP substrate-binding site (58), as well as the [4Fe-4S]-clusters coordinating motifs and the region homologous to TPP-binding sites (59, 91) sites, are underlined. Note that the third [4Fe-4S]-cluster-binding site is atypical and consists of the CXXC motif at positions 942–945 and two separated cysteines at positions 970 and 1221 (60, 61). The first amino acid of recombinant PFR1 is marked by an asterisk.
ing site (YPITP) (58), a C-terminal TPP-binding site (59), and three [4Fe-4S]-cluster-binding signatures, two of which are typical for 2[4Fe-4S]-ferredoxins and one of which is atypical (60, 61) (Fig. 2). The comparisons with bacterial enzymes revealed that the *C. reinhardtii* PFR1 sequence contains an N-terminal extension that is not homologous to other PFOR proteins. Because PFR1 was shown to be localized in the chloroplast (37), we assumed that the first VXA amino acid motif (starting at position 24 of Cre11.g473950.t1.1) might represent a chloroplast targeting sequence cleavage site (48). Therefore, we excluded the respective region encoding these first 24 residues from the cDNA used for heterologous production of PFR1.

After heterologous expression of the truncated *PFR1* cDNA in *E. coli* and subsequent purification of the His-tagged protein via nickel-nitritolatric acid chromatography, a protein of the expected size (144 kDa) could be eluted. Activity assays using pyruvate and CoA as substrates and methyl viologen as artificial electron acceptor resulted in a specific activity of 0.45 ± 0.01 units·mg⁻¹ (1 unit was defined as the conversion of 1 μmol of pyruvate or CoA and the reduction of 2 μmol of methyl viologen, respectively, per minute) (Fig. 3). The *K*ₘ values obtained for methyl viologen, pyruvate, and CoA in this assay were 2.3, 0.7, and 1.7 mM, respectively (Fig. 3).

*Chlamydomonas PFR1 Allows Pyruvate-dependent H₂ Production*—To analyze whether PFR1-catalyzed pyruvate oxidation would allow H₂ production by *C. reinhardtii* HYDA1, reconstitution assays were performed in which various combinations of electron carriers (methyl viologen, and ferredoxins) were mixed. The combination of recombinant PFR1 and *C. reinhardtii* HYDA1 in the presence of pyruvate, CoA, and methyl viologen as artificial electron carrier resulted in a H₂ production rate of 400 ± 66 nmol of H₂·min⁻¹·mg PFR1⁻¹, which was only slightly higher than the rate obtained with *Chlamydomonas* ferredoxin PETF (Fig. 4A; 338 ± 32 nmol of H₂·min⁻¹·mg PFR1⁻¹). The *K*ₘ value of PFR1 for PETF was determined as 2.4 ± 0.34 μM (Fig. 4B), which is considerably lower than the *K*ₘ value of HYDA1 for PETF (20–30 μM) (7, 12). We also examined whether two further ferredoxins, FDX2 and FDX5, would allow PFR1-dependent H₂ evolution. FDX2 (62) and especially FDX5 transcripts and FDX5 protein (54, 63, 64) have been shown to accumulate in anaerobic *Chlamydomonas* cells, which makes them candidates for being involved in pathways specific for anaerobiosis. Furthermore, both ferredoxin isoforms are localized in the plastid (54, 62) together with HYDA1 and PFR1. Using FDX2 as electron carrier, a PFR1-dependent H₂ evolution rate of 287 ± 15 nmol of H₂·min⁻¹·mg PFR1⁻¹ could be observed, whereas no activity was determined using FDX5 (Fig. 4A). Notably, recombinant *C. reinhardtii* PFR1 also allowed H₂ generation of *C. acetobutylicum* HYDA1 in the presence of the clostridial ferredoxin CAC0303 in a rate of 118 ± 35 nmol of H₂·min⁻¹·mg PFR1⁻¹ (Fig. 4A).

Using the amount of recombinant *C. reinhardtii* HYDA1 enzyme as a basis, the PFR1- and methyl viologen-mediated H₂ production rates (80 ± 13 μmol of H₂·min⁻¹·mg HYDA1⁻¹) were only 16% of those determined with sodium dithionite-reduced methyl viologen (516 ± 42 μmol of H₂·min⁻¹·mg HYDA1⁻¹; Table 1). However, in the presence of PETF as the electron carrier, the reaction driven by 0.7 μM PFR1 reached 42% of the reaction in which sodium dithionite served as chemical electron donor (68 ± 7 versus 160 ± 17 μmol of H₂·min⁻¹·mg HYDA1⁻¹; Table 1). In this reaction mixture, 0.7 μM PFR1 was close to saturation because the rates obtained using 0.35 μM PFR1 were 61 ± 9, and those in assays containing 0.9 μM PFR1 were 72 ± 8 μmol of H₂·min⁻¹·mg HYDA1⁻¹.

**PFR1 Is Able to Use Oxaloacetate, but Not α-Ketoglutarate as a Substrate**—Some bacterial PFOR enzymes have been reported to be able to oxidize various substrates such as 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus* species strain 7 or *Sulfolobus solfataricus* P1 (58, 65), whereas others such as PFOR from *E. coli* can only oxidize pyruvate (66). We examined the methyl viologen reducing activity of *C. reinhardtii* PFR1 in the presence of oxaloacetate and α-ketoglutarate. When oxaloacetate was included in the reaction mixture, a specific activity of 0.26 ± 0.03 U·mg⁻¹ was observed, but no methyl viologen reduction could be detected using α-ketoglutarate (Fig. 5A). In a reconstitution assay using the same substrates but using the electron carrier PETF and the [Fe–Fe]-hydrogenase HYDA1 to examine substrate-dependent H₂ evolution, oxaloacetate was almost as suitable as pyruvate, whereas no H₂ production could be observed in the presence of α-ketoglutarate (Fig. 5B).

**DISCUSSION**

*C. reinhardtii* has long been known for its complex mixed acid fermentative metabolism, which has more similarities to fermentation of bacteria or strictly anaerobic protists than to plant or animal anaerobic pathways (1, 2, 4, 34). In addition to PFL1, which is mainly known from enterobacteria such as *E. coli* (33), a cDNA encoding pyruvate:ferredoxin oxidoreduc-
HYDA1 (36, 63) in analogy to fermentative H$_2$ production in clostridia (40, 42, 67).

The capacity of PFR1 to reduce methyl viologen (A) and to drive H$_2$ evolution by C. reinhardtii HYDA1 (B) using oxaloacetate or $\alpha$-ketoglutarate was examined. The reaction mixtures contained 10 mM pyruvate, oxaloacetate, or $\alpha$-ketoglutarate and 2 mM CoA in 100 mM potassium phosphate buffer, pH 6.8, and additionally 1.4 $\mu$M PFR1 and 10 mM methyl viologen (A) or 0.7 $\mu$M PFR1, 40 $\mu$M PETF, and 0.01 $\mu$M HYDA1 (B). A methyl viologen reduction was determined spectrophotometrically. B, $H_2$ evolution rates were determined by gas chromatography as described in the legend of Fig. 4. The results shown are the means and standard deviations from two independent experiments carried out as technical duplicates.

Pyruvate-dependent $H_2$ Production in Green Algae

FIGURE 4. $H_2$ generation in reconstitution assays of recombinant C. reinhardtii PFR1 and [Fe-Fe]-hydrogenases. Table 1. H$_2$ evolution rates of C. reinhardtii HYDA1 using electrons provided by sodium dithionite (NaDT) or pyruvate oxidation via PFR1 related to the amounts of hydrogenase protein.

All of the reactions contained 100 mM potassium phosphate buffer and 0.01 $\mu$M C. reinhardtii HYDA1. PFR1-containing reactions included 10 mM pyruvate and 2 mM CoA. The values shown were derived from three independent PFR1 and HYDA1 preparations ± standard deviation.

| Reaction components | $H_2$ min$^{-1}$mg HYDA1$^{-1}$ $\mu$mol |
|---------------------|------------------------------------------|
| 100 mM NaDT, 10 mM methyl viologen | 516 ± 42 |
| 50 mM NaDT, 40 $\mu$M PETF | 160 ± 17 |
| 0.7 $\mu$M PFR1, 10 mM methyl viologen | 80 ± 13 |
| 0.7 $\mu$M PFR1, 40 $\mu$M PETF | 68 ± 7 |

In this study we show that PFR1-driven H$_2$ production is biochemically plausible, because recombinant C. reinhardtii PFR1 is able to allow H$_2$ generation by isolated [Fe-Fe]-hydrogenases. Although these results cannot prove that this reaction occurs in living Chlamydomonas cells, they indicate that the PFR1-dependent pyruvate to H$_2$ pathway can be operable in vivo (Fig. 6). The biochemical properties of the purified PFR1 protein are similar to PFOR enzymes isolated and characterized from other organisms regarding pyruvate- and CoA-dependent methyl viologen reduction (72, 73). The $K_m$ values for pyruvate and methyl viologen were in the range of the $K_m$ values determined for other PFOR enzymes, whereas the $K_m$ for CoA was higher (74–76). Also, the specific activity of our PFR1 preparations were at the lower range when compared with other PFOR enzymes (74, 77, 78). This might support the physiological data obtained so far, which all speak for PFL1 being the major fermentative enzyme in C. reinhardtii wild type cultures (4, 30, 35). However, we cannot exclude that the protein solution contained inactive PFR1 enzymes. We observed a marked instability of the enzyme, despite its isolation and examination under strictly anaerobic conditions sufficient for the analysis of the extremely O$_2$-sensitive [Fe-Fe]-hydrogenase of C. reinhardtii (9–11). Instability of isolated PFOR enzymes has been reported before (79), and besides destruction by O$_2$ (73, 80), loss of the TPP factor has been proposed to be one reason for this phenomenon (75).
In reaction mixtures combining PFR1 with C. reinhardtii HYDA1 and ferredoxin PETF, which is the most suitable redox partner for HYDA1 known so far (12), a pyruvate-dependent H₂ production could be observed, showing that the postulated reaction is possible. Notably, PFR1-driven H₂ generation via PETF was only 2.5-fold lower than H₂ generation using sodium dithionate-reduced PETF as an electron donor for HYDA1. This indicates that the capacity of PFR1-coupled H₂ production is quite high, despite the low specific activity of PFR1. We assume that both the low Kₘ value of PFR1 for PETF and the high specific activity of HYDA1 contribute to the high efficiency of the coupled system. The low rates of in vivo H₂ evolution in dark-incubated algal cells are therefore probably limited by PFR1 substrate supply rather than by PFR1 activity.

PFR1-mediated H₂ production by HYDA1 was also possible using FDX2 as an electron carrier (Fig. 6), and the rates obtained were only moderately lower than with PETF. Notably, FDX2 lacks one phenylalanine residue that, in PETF, is essential for proper interaction with HYDA1 (12, 81). The capability of HYDA1 to generate H₂ using FDX2 as an electron donor in the PFR1-driven system might therefore indicate a different interaction mechanism. FDX2 was suggested to be specifically involved in nitrite reduction. The protein can hardly be detected in C. reinhardtii cells incubated in ammonium-containing medium but accumulates in cells growing on nitrate and allows a higher catalytic activity of Chlamydomonas nitrite reductase than PETF (62). However, because FDX2 was also as efficient as PETF for FNR catalytic activity and even better regarding affinity (62), FDX2 might in general be used for reactions that can also employ PETF.

The results obtained with FDX2 indicated that the electron transfer reaction coupling PFR1 and HYDA1 might be different from the other electron delivering reactions analyzed so far. Therefore we analyzed whether FDX5 was able to shuttle electrons between PFR1 and HYDA1, although FDX5 is unable to drive H₂ generation activity upon artificial reduction (54). The FDX5 gene is strongly induced upon anaerobiosis (54, 63) but also in copper-deficient C. reinhardtii cells (62, 64, 82). FDX5 is regulated by CRR1 (the copper response regulator 1) under both conditions (64). Notably, it was shown recently that the HYDA1 gene is also activated by the absence of copper (82) and partially regulated by CRR1 (83). Although a connection between FDX5 and HYDA1 might be suggested from these findings, the data presented here confirm that a direct metabolic interaction does not take place.

PFOR-mediated H₂ generation is central to anaerobic energy generation in several strict anaerobes such as amitochondriates (69) and clostridia (67). Notably, C. reinhardtii was able to allow C. acetobutylicum HYDA activity in the presence of clostridial 2[4Fe-4S]-ferredoxin. This indicates that PFR1 kept the basic features of the evolutionary old PFOR protein (68, 84), despite the fact that C. reinhardtii and clostridial PFOR sequences were calculated to be evolutionary distant (85). A similar promiscuity regarding redox partners was observed for other PFOR enzymes, such as Clostridium thermoaceticum (74) and Rhodobacter capsulatus PFORs (86).

Recombinant C. reinhardtii PFR1 had methyl viologen reducing and H₂ driving activity also in the presence of oxaloacetate as substrate, whereas α-ketoglutarate did not result in measurable activity. Both metabolites are intermediates of the TCA cycle, whereas the branch via α-ketoglutarate and succinyl-CoA is absent in the glyoxylate cycle. An anaerobically operating glyoxylate cycle is active in the alga (87), and the oxidation of malate was suggested to contribute to H₂ photo-production (88). Pyruvate and oxaloacetate are furthermore end products of the catabolism of several amino acids. It might be assumed that oxaloacetate degradation by PFR1 supports the anaerobic operation of the glyoxylate cycle or parts thereof (Fig. 6).

The physiological role of PFR1 in fermenting C. reinhardtii cells might therefore become important during long-term fermentation. A 2:1:1 ratio of formate:ethanol:acetate production observed in algae after 4–6 h of anaerobiosis (4, 34, 35) is typical for pyruvate:formate lyase activity. However, formate production only prevails in the first hours of anaerobiosis, whereas ethanol and especially CO₂ generation rates increase thereafter, simultaneously to a slowdown of starch degradation (34). Although speculative, a scenario might be envisioned in which PFL1 is mostly responsible for short-term fermentation using starch and glucose, respectively, as substrate. In long-term anaerobiosis, PFR1 activity would allow Chlamydomonas to utilize acetyl-CoA derived from fatty acids and end products of amino acid catabolism as energy sources (Fig. 6). Furthermore, the coupling to the hydrogenase is a means to dispose of electrons via the nontoxic and highly diffusible H₂ molecule.

Acknowledgments—We are thankful for the gift of pET21c0303 from Laurence Girbal (Laboratoire d’Ingénierie des Systèmes Biologiques et des Procédés, Toulouse, France). We also thank Annika Brünje and Simone Schmidt for technical support during cloning of CAC0303 and FDX2.
REFERENCES

1. Hemschemeier, A., and Happe, T. (2005) The exceptional photofermentative hydrogen metabolism of the green alga Chlamydomonas reinhardtii. Biochem. Soc. Trans. 33, 39–41

2. Atteia, A., van Lis, R., Gieß-Dietrich, G., Adrait, A., Garin, J., Joyard, J., Rolland, N., and Martin, W. (2006) Pyruvate formate-lyase and a novel route of eukaryotic ATP synthesis in Chlamydomonas mitochondria. J. Biol. Chem. 281, 9909–9918

3. Atteia, A., van Lis, R., Tieles, A. G., and Martin, W. F. (2013) Anaerobic energy metabolism in unicellular photosynthetic euakaryotes. Biochim. Biophys. Acta 1827, 210–223

4. Gfeller, R. P., and Gibbs, M. (1984) Fermentative metabolism of Chlamydomonas reinhardtii. I. Analysis of fermentative products from starch in dark and light. Plant Physiol. 75, 212–218

5. Hemschemeier, A., and Happe, T. (2011) Alternative photosynthetic electron transport pathways during anaerobiosis in the green alga Chlamydomonas reinhardtii. Biochim. Biophys. Acta 1807, 919–926

6. Happe, T., Mosler, B., and Naber, J. D. (1994) Induction, localization and metal content of hydrogenase in the green alga Chlamydomonas reinhardtii. Eur. J. Biochem. 222, 769–774

7. Happe, T., and Naber, J. D. (1993) Isolation, characterization and N-terminal amino acid sequence of hydrogenase from the green alga Chlamydomonas reinhardtii. Eur. J. Biochem. 214, 475–481

8. Kamp, C., Silakov, A., Winkler, M., Reijerse, E. J., Lubitz, W., and Happe, T. (2008) Isolation and first EPR characterization of the [FeFe]-hydrogenases from green algae. Biochim. Biophys. Acta 1777, 410–416

9. Goldet, G., Brandmayr, C., Stripp, S. T., Happe, T., Cazava, C., Fontecilla-Camps, J. C., and Armstrong, F. A. (2009) Electrochemical kinetic investigations of the reactions of [FeFe]-hydrogenases with carbon monoxide and oxygen. Comparing the importance of gas tunnels and active-site electronic/redox effects. J. Am. Chem. Soc. 131, 14979–14989

10. Lambertz, C., Leidel, N., Havelius, K. G., Noth, J., Chernev, P., Winkler, M., Happe, T., and Haumann, M. (2011) O2 reactions at the six-iron active site (H-cluster) in [FeFe]-hydrogenase. Proc. Natl. Acad. Sci. U.S.A. 108, 39–41

11. Subramanian, V., Posewitz, M. C., and Seibert, M. (2011) Multiple facets of anoxic metabolism and hydrogen production in the unicellular green alga Chlorella fusca. Biochim. Biophys. Acta 1812, 78–87

12. Atteia, A., van Lis, R., Tieles, A. G., and Martin, W. F. (2013) Anaerobic energy metabolism in unicellular photosynthetic euakaryotes. Biochim. Biophys. Acta 1827, 210–223

13. Atteia, A., van Lis, R., Tieles, A. G., and Martin, W. F. (2013) Anaerobic energy metabolism in unicellular photosynthetic euakaryotes. Biochim. Biophys. Acta 1827, 210–223
butanol yields. *Appl. Environ. Microbiol.* **48**, 764–770
42. Calusinska, M., Happe, T., Joris, B., and Wilmotte, A. (2010) The surprising diversity of clostridial hydrogenases. A comparative genomic perspective. *Microbiology* **156**, 1575–1588
43. Demueez, M., Cournac, L., Guerrini, O., Soucaille, P., and Girbal, L. (2007) Complete activity profile of *Clostridium acetobutylicum* [FeFe]-hydrogenase and kinetic parameters for endogenous redox partners. *FEMS Microbiol. Lett.* **275**, 113–121
44. Akhtar, M. K., and Jones, P. R. (2008) Deletion of iscr stimulates recombinant clostridial Fe-Fe hydrogenase activity and H₂-accumulation in *Escherichia coli* BL21(DE3). *Appl. Microbiol. Biotechnol.* **78**, 853–862
45. Schwartz, C. J., Giel, J. L., Patschkowski, T., Luther, C., Ruzicka, F. I., Beinert, H., and Kiley, P. J. (2001) IsCR, an Fe-S cluster-containing transcription factor, represses expression of *Escherichia coli* genes encoding Fe-S cluster assembly proteins. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 14895–14900
46. Harris, E. H. (1989) *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use*, Academic Press, San Diego.
47. Hemschemeier, A., Melis, A., and Happe, T. (2009) Analytical approaches to photobiological hydrogen production in unicellular green algae. *Photosynth. Res.* **102**, 523–540
48. Franzen, L. G., Rochaix, J. D., and von Heijne, G. (1990) Chloroplast transit peptides from the green alga *Chlamydomonas reinhardtii* share features with both mitochondrial and higher plant chloroplast presequences. *FEBS Lett.* **260**, 165–168
49. Sambrook, J., and Russell, D. W. (2006) Transformation of *E. coli* by Electroporation. *CSPH Protoc.* 2006, piii
50. Vogel, H. J., and Bonner, D. M. (1956) Acetylornithinase of *Escherichia coli*. Partial purification and some properties. *J. Biol. Chem.* **218**, 97–106
51. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
52. Girbal, L., von Abendroth, G., Winkler, M., Benton, P. M., Meynial-Salles, I., Croux, C., Peters, J. W., Happe, T., and Soucaille, P. (2005) Homologous and heterologous overexpression in *Clostridium acetobutylicum* and characterization of purified clostridial and algal Fe-Fe hydrogenases with high specific activities. *Appl. Environ. Microbiol.* **71**, 2777–2781
53. von Abendroth, G., Stropp, S., Silakov, A., Croux, C., Soucaille, P., Girbal, L., and Happe, T. (2008) Optimized over-expression of [FeFe] hydrogenases with high specific activity in *Clostridium acetobutylicum*. *Int. J. Hydrogen Energy** **33**, 6076–6081
54. Jacobs, J., Puddeleit, S., Hemschemeier, A., and Happe, T. (2009) A novel, anaerobically induced ferredoxin in *Chlamydomonas reinhardtii*. *FEBS Lett.* **583**, 325–329
55. Guerrini, O., Burlat, B., Léger, C., Guigliarelli, B., Soucaille, P., and Girbal, L. (2007) Complete activity profile of *Clostridium acetobutylicum* [FeFe]-hydrogenase and kinetic parameters for endogenous redox partners. *FEMS Microbiol. Lett.* **275**, 113–121
56. Akhtar, M. K., and Jones, P. R. (2008) Deletion of iscr stimulates recombinant clostridial Fe-Fe hydrogenase activity and H₂-accumulation in *Escherichia coli* BL21(DE3). *Appl. Microbiol. Biotechnol.* **78**, 853–862
44. Akhtar, M. K., and Jones, P. R. (2008) Deletion of iscr stimulates recombinant clostridial Fe-Fe hydrogenase activity and H₂-accumulation in *Escherichia coli* BL21(DE3). *Appl. Microbiol. Biotechnol.* **78**, 853–862
57. Mayhew, S. G. (1978) The redox potential of dithionite and SO₂ from...
acterization of the pyruvate-ferredoxin oxidoreductase from Clostridium acetobutylicum. Arch. Microbiol. 152, 244–250
80. Cavazza, C., Contreras-Martel, C., Pielue, L., Chahrière, E., Hatchikian, E. C., and Fontecilla-Camps, J. C. (2006) Flexibility of thiamine diphosphate revealed by kinetic crystallographic studies of the reaction of pyruvate-ferredoxin oxidoreductase with pyruvate. Structure 14, 217–224
81. Winkler, M., Hemschemeier, A., Jacobs, J., Stripp, S., and Happe, T. (2010) Multiple ferredoxin isoforms in Chlamydomonas reinhardtii. Their role under stress conditions and biotechnological implications. Eur. J. Cell Biol. 89, 998–1004
82. Castruita, M., Casero, D., Karpowicz, S. J., Kropat, J., Vieler, A., Hsieh, S. I., Yan, W., Cokus, S., Loo, J. A., Benning, C., Pellegrini, M., and Merchant, S. S. (2011) Systems biology approach in Chlamydomonas reveals connections between copper nutrition and multiple metabolic steps. Plant Cell 23, 1273–1292
83. Pape, M., Lambertz, C., Happe, T., and Hemschemeier, A. (2012) Differential expression of the Chlamydomonas [FeFe]-hydrogenase-encoding HYDA1 gene is regulated by the copper response regulator 1. Plant Physiol. 159, 1700–1712
84. Embley, T. M., van der Giezen, M., Horner, D. S., Dyal, P. L., Bell, S., and Foster, P. G. (2003) Hydrogenosomes, mitochondria and early eukaryotic evolution. IUBMB Life 55, 387–395
85. Hug, I. A., Stechmann, A., and Roger, A. J. (2010) Phylogenetic distributions and histories of proteins involved in anaerobic pyruvate metabolism in eukaryotes. Mol. Biol. Evol. 27, 311–324
86. Yakunin, A. F., and Hallenbeck, P. C. (1998) Purification and characterization of pyruvate oxidoreductase from the photosynthetic bacterium Rhodobacter capsulatus. Biochim. Biophys. Acta 1409, 39–49
87. Gibbs, M., Gefeller, R. P., and Chen, C. (1986) Fermentative metabolism of Chlamydomonas reinhardtii. III. Photoassimilation of acetate. Plant Physiol. 82, 160–166
88. Willeford, K. O., and Gibbs, M. (1989) Localization of the enzymes involved in the photoevolution of H₂ from acetate in Chlamydomonas reinhardtii. Plant Physiol. 90, 788–791
89. Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., and Higgins, D. G. (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948
90. Crooks, G. E., Hon, G., Chandonia, J. M., and Brenner, S. E. (2004) WebLogo. A sequence logo generator. Genome Res. 14, 1188–1190
91. Muller, Y. A., Lindqvist, Y., Furey, W., Schulz, G. E., Jordan, F., and Schneider, G. (1993) A thiamin diphosphate binding fold revealed by comparison of the crystal structures of transketolase, pyruvate oxidase and pyruvate decarboxylase. Structure 1, 95–103
92. Magneschi, L., Catalanotti, C., Subramanian, V., Dubini, A., Yang, W., Mus, F., Posewitz, M. C., Seibert, M., Perata, P., and Grossman, A. R. (2012) A mutant in the ADH1 gene of Chlamydomonas reinhardtii elicits metabolic restructuring during anaerobiosis. Plant Physiol. 158, 1293–1305

Pyruvate-dependent H₂ Production in Green Algae

FEBRUARY 8, 2013 • VOLUME 288 • NUMBER 6