Water oxidation by photosystem II is the primary source of electrons for sustained H_2 photoproduction in nutrient-replete green algae

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The unicellular green alga *Chlamydomonas reinhardtii* is capable of photosynthetic H_2 production. H_2 evolution occurs under anaerobic conditions and is difficult to sustain due to 1) competition between [FeFe]-hydrogenase (H_2ase), the key enzyme responsible for H_2 metabolism in algae, and the Calvin–Benson–Bassham (CBB) cycle for photosynthetic reductants and 2) inactivation of H_2ase by O_2 coevolved in photosynthesis. Recently, we achieved sustainable H_2 photoproduction by shifting algae from continuous illumination to a train of short (1 s) light pulses, interrupted by longer (9 s) dark periods. This illumination regime prevents activation of the CBB cycle and redirects photosynthetic electrons to H_2ase. Employing membrane-inlet mass spectrometry and $H_2^{18}O$, we now present clear evidence that efficient H_2 photoproduction in pulse-illuminated algae depends primarily on direct water biophotolysis, where water oxidation at the donor side of photosystem II (PSII) provides electrons for the reduction of protons by H_2ase downstream of photosystem I. This occurs exclusively in the absence of CO_2 fixation, while with the activation of the CBB cycle by longer (8 s) light pulses the H_2 photoproduction ceases and instead a slow overall H_2 uptake is observed. We also demonstrate that the loss of PSII activity in DCMU-treated algae or in PSII-deficient mutant cells can be partly compensated for by the indirect (PSII-independent) H_2 photoproduction pathway, but only for a short (<1 h) period. Thus, PSII activity is indispensable for a sustained process, where it is responsible for more than 92% of the final H_2 yield.

green algae | hydrogen production | water splitting | carbon dioxide | hydrogenase

Many species of green algae have [FeFe]-hydrogenases (H_2ases) (1) that catalyze the reversible reduction of protons to molecular hydrogen:

$$2H^+ + 2e^- \rightleftarrows H_2. \quad [1]$$

Since [FeFe]-H_2ases are extremely O_2-sensitive (2), reaction 1 typically proceeds under anoxic conditions. With respect to H_2 metabolism, *Chlamydomonas reinhardtii* is the most studied alga. This alga possesses two [FeFe]-H_2ases in the chloroplast, HYDA1 and HYDA2 (3, 4). In the light, they accept electrons from photosynthetically reduced ferredoxin (FDX1) (5), while in the dark electrons come from the activity of pyruvate ferredoxin oxidoreductase (PFR1) (6). PFR1 catalyzes the oxidation of pyruvate to acetyl-CoA, and its activity is linked to H_2ase via FDX1 (7). Since [FeFe]-H_2ases interact with the photosynthetic electron transport chain at the level of ferredoxin, they may accept electrons originating both from water oxidation via the photosystem II (PSII)-dependent pathway ("direct water biophotolysis") and from the degradation of organic substrates via a PSII-independent mechanism ("indirect water biophotolysis" or "indirect pathway") (8). In the latter case, the reductants are supplied to the plastoquinone (PQ) pool by the type II NADPH dehydrogenase (NDA2), thus bypassing PSII (9, 10).

The release of H_2 leads to a loss of metabolic energy. In healthy, actively growing *C. reinhardtii* cultures, H_2 production is therefore only a temporal phenomenon observed during dark anoxia and upon subsequent onset of illumination (11). In contrast to dark fermentation, H_2 photoproduction is a very efficient process that proceeds for only a short period of time (from a few seconds to a few minutes). Two theories have been developed to explain the short duration. The first is based on the oxygen sensitivity of H_2ases (12, 13). In the light, algae accumulate O_2 that is produced by water oxidation at PSII (14). As a result, H_2 photoproduction may cease over time (14, 15), and the duration of this process is reported to shorten with increased light intensity (16). Because of the negative correlation between the rates of H_2 photoproduction and O_2 evolution, the inhibition of H_2ases by O_2 is frequently quoted as the primary reason for the rapid loss in H_2 photoproduction after the onset of illumination (17).

Alternatively, the loss in the H_2 photoproduction efficiency during illumination could be explained by the light-induced induction of competitive pathways, which may drain reducing equivalents away from the [FeFe]-H_2ase enzyme (18, 19). Candidates for this role are the Mehler-like reaction driven by flavodiiron proteins (FDPs) (15, 20, 21) and the Calvin–Benson–Bassham (CBB) cycle (22). Compelling evidence for the competition between these two

Significance

Photosynthetic H_2 production in the green alga *Chlamydomonas reinhardtii* is catalyzed by O_2-sensitive [FeFe]-hydrogenases, which accept electrons from photosynthetically reduced ferredoxin and reduce protons to H_2. Since the process occurs downstream of photosystem I, the contribution of photosystem II (PSII) in H_2 photoproduction has long been a subject of debate. Indeed, water oxidation by PSII results in O_2 accumulation in chloroplasts, which inhibits H_2 evolution. Therefore, clear evidence for direct water biophotolysis resulting in simultaneous H_2 and O_2 releases in algae has never been presented. This paper demonstrates that sustained H_2 photoproduction in *C. reinhardtii* is directly linked to PSII-dependent water oxidation and brings insights into regulation of PSII activity and H_2 production by CO_2/HCO_3^- under microoxic conditions.

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pathways and H_2 production has been accumulated in recent studies (23–25). As CO_2 fixation provides the strongest sink for photosynthetic reductants, it should play a major role in the cessation of H_2 photoproduction in algae when the CBB cycle is active (19, 22).

For preventing competition between the [FeFe]-H_2ases and the CBB cycle, we recently devised a pulse-illumination protocol that allows H_2 production in nutrient-replete algal cultures for up to 3 d (23). To achieve this, we specifically selected the duration of light pulses in the light/dark sequence to avoid activation of the CBB cycle, thus allowing for the redirection of photosynthetic electrons toward the [FeFe]-H_2ases. Typically, a train of 1- to 6-s light pulses interrupted by 9-s dark periods is sufficient for sustained H_2 photoproduction in *C. reinhardtii* cultures (23, 25). Our protocol thus differs from earlier pulse-illumination approaches that aimed at preventing the accumulation of O_2 in the cultures (26).

While we could demonstrate competition of [FeFe]-H_2ase with FDPs (25), the origin of reductants for H_2 photoproduction in the pulse-illuminated algae remained unclear. The relatively high efficiency of the process suggests the involvement of water oxidation by PSII, and consequently the simultaneous production of H_2 and O_2. Although widely proposed in the current literature (8, 24), the presence of the direct water biophotolysis in H_2-producing green algae has not yet been proven by direct experimental data.

In the present study, we provide clear evidence for the presence of PSII-dependent oxidation of ^{18}O-labeled water ($H_2^{18}O$) with concomitant evolution of $^{16}O_2$ and $^{16,18}O_2$ during H_2 photoproduction in the pulse-illuminated green alga *C. reinhardtii* under anoxic conditions. O_2 evolution is balanced by light-dependent and light-independent respiration that sustains the anoxic condition. We also demonstrate that the loss of PSII activity in algae can be partly compensated by the PSII-independent H_2 photoproduction pathway. Nevertheless, the activity of PSII is indispensable for the sustained process, where it contributes to more than 92% of the final H_2 yield.

Results

Net H_2 Photoproduction, Water Oxidation, and O_2 Exchange. Employing membrane-inlet mass spectrometry (MIMS), we confirmed the induction of sustained H_2 photoproduction in anoxic *C. reinhardtii* cultures by a train of 1-s light pulses interrupted by 9-s dark periods (Fig. 1*A*). The signal exhibited a typical sawtooth wave with the H_2 production transients during pulse illumination and a strong H_2 uptake on the shift to darkness, which is in line with previous data (23). The H_2 uptake is the result of H_2 consumption by the MIMS setup and the reversible action of the H_2ase (as demonstrated in *SI Appendix*, Fig. S1). The response of anoxic algae to the train of light pulses was strain-specific. While in the cell wall-deficient mutant the first H_2 peak appeared already on the first 1-s flash (Fig. 1*C*, CC-4533), the wild-type CC-124 strain required some time before demonstrating the pronounced H_2 photoproduction yield (Fig. 1*A*).

The application of ^{18}O-labeled water ($H_2^{18}O$) to the algae allowed simultaneous monitoring of PSII activity via both nonlabeled and ^{18}O-labeled O_2 isotopologues (27). Thus, as shown in Fig. 1*B*, the injection of $H_2^{18}O$ into the MIMS cell filled with the algal suspension led to the detection of O_2 evolution at *m/z* 32 (for ambient $^{16}O_2$) and *m/z* 34 (for singly labeled $^{16,18}O_2$) signals as a result of the water-oxidizing activity of PSII centers during pulse illumination:

$$2H_2^{16}O \rightarrow O_2^{16} + 4H^+, \quad [2]$$

$$H_2^{16}O + H_2^{18}O \rightarrow O_2^{16,18} + 4H^+. \quad [3]$$

The *m/z* 34 signal showed O_2 evolution at the first 1-s light pulse, and algal cultures reached maximum O_2 production yields after

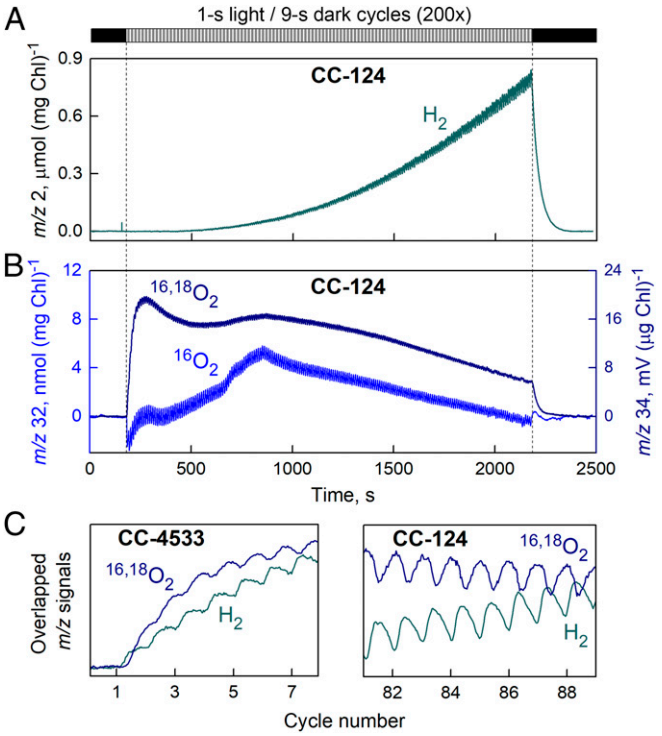

Fig. 1. H_2 photoproduction (*A*) and O_2 exchange (*B*) in pulse-illuminated algae. Anoxia was achieved within 3 min after placement of aerobic algae in the O_2-consuming MIMS chamber under complete darkness. The total (*a* + *b*) chlorophyll (Chl) content in the samples was around 9 mg·L^{-1}. The photochemical reactions were initiated by a train of 1-s light pulses interrupted by 9-s dark periods. (*C*) The behavior of the *m/z* 2 and *m/z* 34 signals at better time resolution for the cell wall-deficient strain (CC-4533) in the beginning of pulse illumination and for the wild-type strain (CC-124) in the middle of H_2 photoproduction. One representative result out of three repeats is presented. Additional experimental data can be found in *SI Appendix*, Fig. S9.

10 to 60 pulses (Fig. 1*B*). Then, the signal slowly declined but kept the sawtooth shape until the end of the pulse-illumination period. The decline in the net O_2 evolution coincided well with the simultaneous increase in the H_2 photoproduction yield (Fig. 1*A*). Surprisingly, $^{16}O_2$ production (*m/z* 32) of midlog-phase *C. reinhardtii* cultures was significantly delayed by strong light-dependent uptake at the beginning of pulse illumination (Fig. 1*B*). Since ambient $^{16}O_2$ dominates in the MIMS chamber and the level of $^{16,18}O_2$ is negligible in the beginning of pulse illumination, the pronounced uptake of $^{16}O_2$ in the first few cycles of pulse illumination indicates the domination of light-dependent respiration over water oxidation during this period. On the contrary, the mature (close to the stationary phase) algae did not show any signs of the light-dependent $^{16}O_2$ uptake (*SI Appendix*, Fig. S2). In the latter case, the *m/z* 32 signal simply repeated the shape of the *m/z* 34 curve. Thus, light-dependent O_2 uptake is not caused by the self-shading effect and activation of respiration in dense cultures. This phenomenon is most likely linked to O_2 photoreduction by FDPs that operate during the dark-to-light (or low-light to high-light) transients under oxic and microoxic conditions (25, 28).

The rise of the H_2 (*m/z* 2) signal occurred almost immediately upon the firing of each light pulse. This was followed by a rise in the O_2 (*m/z* 34) signal with ~3-s delay (Fig. 1*C*). As a result, the H_2 production wave showed maxima at almost the minimums of the *m/z* 34 signal. Since the diffusivity of H_2 in the polydimethylsiloxane membrane used in the MIMS cell (applied to separate the liquid phase of the sample from the high vacuum of the mass spectrometer) is approximately four times faster than for O_2 (29), this

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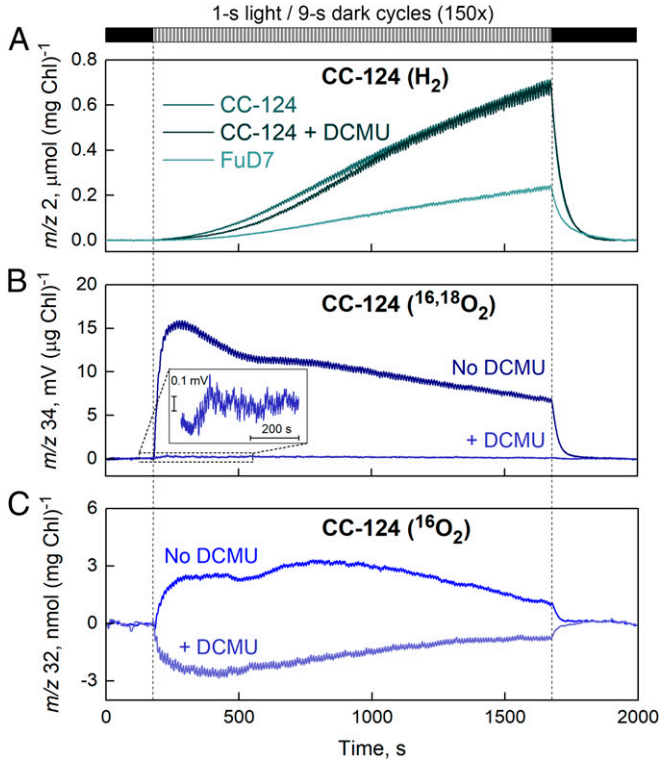

Fig. 2. The effect of DCMU and the *psbA* deletion (FuD7) on H_2 (*A*), $^{16,18}O_2$ (*B*), and $^{16}O_2$ (*C*) yields in pulse-illuminated algae. The experimental conditions were the same as in Fig. 1, except that the total Chl content was increased to ~20 mg·L^{-1} (to distinguish the difference between O_2 production and O_2 consumption in the control and DCMU-treated samples). DCMU was introduced to aerobic algae at a final concentration of 30 µM. Curves are the mean of two to four independent experiments. The H_2 photoproduction of the FuD7 mutant is a result of one measurement with MIMS but was repeated nine times with a H_2 sensor, producing similar results (*SI Appendix*, Fig. S3). Additional experimental data can also be found in *SI Appendix*, Fig. S10.

behavior of H_2 and O_2 signals is not a physiological response but rather a reflection of the detection method. Therefore, we suggest simultaneous photoproduction of these two gases in *C. reinhardtii* cells.

The Role of PSII-Dependent and PSII-Independent Pathways. The addition of 30 µM of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], a specific inhibitor that binds to the Q_B-pocket of PSII and blocks electron transport from PSII to the PQ pool, did not affect H_2 photoproduction much during the first 20 min of pulse illumination (Fig. 2*A*). The *m/z* 2 signal of the DCMU-treated algae (CC-124 + DCMU) was only slightly reduced as compared to the control (CC-124). This response of pulse-illuminated algae to DCMU was confirmed using an H_2 electrode (*SI Appendix*, Fig. S3). In contrast, the presence of DCMU significantly reduced the $^{16,18}O_2$-evolving activity in algae at the start of pulse illumination (Fig. 2*B*), but inhibition was not complete. Thus, as seen in Fig. 2*B*, *Inset* a residual water-oxidizing activity (<1.5% of the control) was observed in the DCMU-treated samples. At the same time, DCMU-treated algae demonstrated no light-induced evolution of nonlabeled $^{16}O_2$ (*m/z* 32) but a strong light-dependent $^{16}O_2$ uptake (Fig. 2*C*).

Since the *m/z* 34 signal represents the net $^{16,18}O_2$ exchange with a significant expected share of light-induced O_2 uptake (as seen from the *m/z* 32 signal in Fig. 2*C*), the contribution of the residual PSII activity in the H_2 photoproduction yield of the DCMU-treated samples remained unclear. Therefore, in the next experiment, a train of light pulses was applied to the PSII-deficient (FuD7) mutant. Due the absence of fully assembled PSII centers in the thylakoid membrane (30), this strain completely lacks water-oxidizing activity (*SI Appendix*, Fig. S4*A*). Nevertheless, FuD7 still produced H_2, albeit at the decreased rate (Fig. 2*A* and *SI Appendix*, Fig. S3). Similar to DCMU-treated algae, the PSII-deficient mutant showed a strong light-dependent $^{16}O_2$ uptake (*SI Appendix*, Fig. S4*B*).

The short-term experiments undertaken with the DCMU-treated wild-type strain and the FuD7 mutant revealed functioning of the PSII-independent H_2 photoproduction pathway when PSII was not available. Therefore, it was worth checking whether this pathway could sustain H_2 photoproduction in the long-term process. It was subsequently observed that the PSII-independent pathway could not substitute the PSII-dependent pathway in the long-term process (Fig. 3*A*). The H_2 photoproduction yields both in the FuD7 mutant and in the DCMU-treated wild-type strain began decreasing during the first hour (Fig. 3 *A*, *Inset*). This result is in good agreement with those of the short-term experiments (*SI Appendix*, Fig. S3). In the CC-124 and CC-5325 strains (Fig. 3 *A* and *B*), which possess full PSII activity, DCMU addition stopped H_2 photoproduction after 6 h, while H_2 production of the FuD7 mutant continued, but at a greatly reduced rate. In all cases, final H_2 yields were only slightly higher than in the dark samples.

In contrast to PSII deficiency, the absence of NDA2 (responsible for nonphotochemical reduction of the PQ pool) (9, 10) in *C. reinhardtii* cells was almost completely compensated for by the PSII-dependent pathway. This was particularly evident in the first 4 h of the experiment (Fig. 3 *B*, *Inset*). Here, the input of the PSII-independent pathway was noticeable only by the end of the

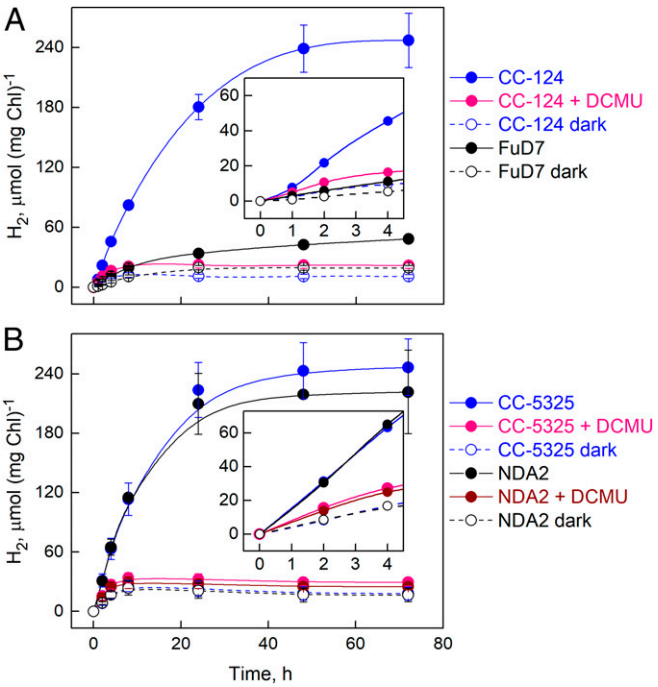

Fig. 3. Long-term H_2 photoproduction by pulse-illuminated algae. The cultures of the wild-type (CC-124) strain and the PSII-deficient (FuD7) mutant (*A*), and NDA2-deficient (NDA2) mutant and its wild-type progenitor (CC-5325) (*B*) were exposed to a train of 1-s light/9-s dark pulses in the absence and in the presence of 30 µM DCMU under an Ar atmosphere. The H_2 production activity of the same strains was also checked under complete darkness. (*Insets*) The same samples in the first 4 h of pulse illumination. Values are mean of 6 to 13 independent replicates (± SD).

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experiment, but even in that case the difference was not significant.

CO_2 Exchange of Algal Cultures Correlates with O_2 Evolution and H_2 Photoproduction. Previously, we demonstrated that *C. reinhardtii* cells exposed to a train of 1-s light pulses interrupted by 9-s dark periods do not fix CO_2 and as a result do not accumulate biomass (23, 25). These previous experiments were performed at low cell densities (<10 mg total Chl per L) to ensure optimal photosynthetic performance. In the current work, we employed sensitive MIMS methodology at a higher cell density of around 20 mg Chl·L^{-1} to improve the resolution of CO_2 analysis. Thus, the culture was observed to release CO_2 during the 1-s light/9-s dark pulse-illumination train (Fig. 4). The ambient (*m/z* 44) CO_2 signal rose within 5 min of engaging the pulse-illumination train and then slowly declined until train cycling was concluded. At this point, the *m/z* 44 signal decayed exponentially to reach the initial state. Along with ambient CO_2 release, *C. reinhardtii* cells also produced ^{18}O-labeled CO_2 isotopologues: $C^{16,18}O_2$ (*m/z* 46) and $C^{18,18}O_2$ (*m/z* 48) (Fig. 4).

Obviously, the release of all CO_2 isotopologues was light-dependent, but the signals did not fluctuate during pulse illumination (Fig. 4). The typical sawtooth shape appeared only when the duration of the light pulse in the light/dark sequence was extended to 8 s. In this regime, a clear sign of CO_2 fixation appeared 5 min after the start of pulse illumination (Fig. 5*A*). All three CO_2 signals, *m/z* 44, *m/z* 46, and *m/z* 48, were affected (Fig. 5*A* and *SI Appendix*, Fig. S5). A similar trend in behavior of *m/z* 44, *m/z* 46, and *m/z* 48 on activation of the CBB cycle indicates that these three signals primarily represent $C^{16}O_2$, $C^{16,18}O_2$, and $C^{18,18}O_2$, but not other possible gases with a similar molecular weight like $N_2{}^{16}O$ and $N^{16}O_2$. For example, the latter two may appear in *C. reinhardtii* as a result of NO detoxification (31).

As expected, prolonging the light pulse in the light/dark sequence led to enhanced O_2 release, but the burst amounts of O_2 were produced only in the 8-s/9-s regime where the CBB cycle was activated (Fig. 5*B*; notice the different scale for the 8-s/9-s pulse protocol). By contrast, *C. reinhardtii* cells exposed to the 5-s light/9-s dark protocol yielded almost the same amount of O_2 as the algae exposed to 3-s/9-s pulse illumination, presumably due to a more pronounced O_2 consumption during the dark phases of the same duration. The enhanced O_2 consumption, however, did not correlate with CO_2 release (Fig. 5*A*). Interestingly, 3-s/9-s and 5-s/9-s pulse-illumination protocols yielded almost the same amounts of H_2 (Fig. 5*C*). By contrast, the 8-s/9-s protocol released H_2 only in the beginning of pulse illumination. Thus, the activation of the CBB cycle immediately led to H_2 uptake (Fig. 5*C*). As seen in Fig. 5 *A* and *C*, the H_2 trace (*m/z* 2) starts to decline at exactly the time when CO_2 signals establish a pronounced sawtooth wave.

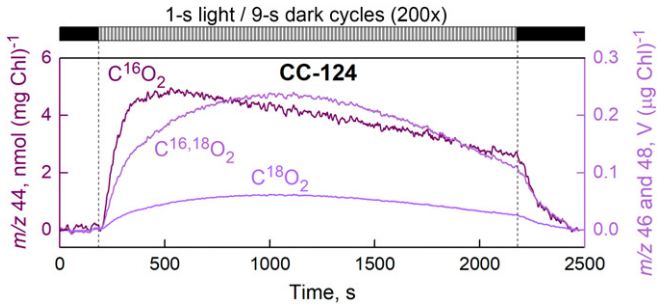

Fig. 4. The release of ambient (*m/z* 44) CO_2 and ^{18}O-labeled (*m/z* 46, *m/z* 48) CO_2 isotopologues during pulse illumination. The experimental conditions were similar to those presented in Fig. 1. The total Chl content was around 20 mg·L^{-1}. A representative experiment is presented. Additional experimental data can be found in *SI Appendix*, Fig. S11.

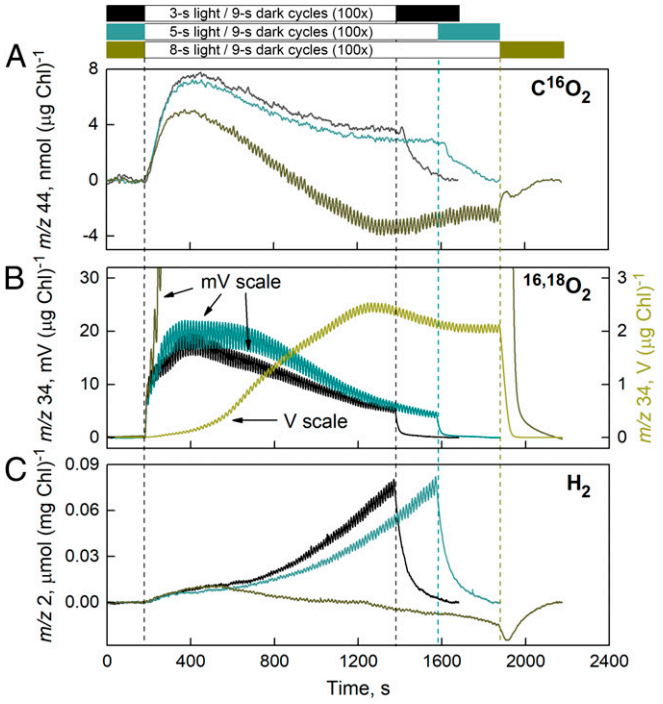

Fig. 5. The effect of light pulse duration in the pulse-illumination sequence on CO_2 exchange (*A*), O_2 evolution (*B*), and H_2 photoproduction (*C*) of algal cultures. In *A*, only ambient (*m/z* 44) CO_2 signals are presented. For behavior of the *m/z* 46 and *m/z* 48 CO_2 signals see *SI Appendix*, Fig. S5. In *B*, *m/z* 34 O_2 signal for the 8-s/9-s illumination regime is also shown at low resolution (V) scale. Additional experimental data can be found in *SI Appendix*, Fig. S12.

Discussion

Clear Evidence for Simultaneous Water Oxidation and H_2 Photoproduction in Green Algae. The role of PSII in H_2 photoproduction in green algae has long been a subject of debate (32). The PSII-dependent net O_2 release of dark-adapted anoxic algae, when exposed to light, appears only after a period of efficient H_2 production (14). Therefore, direct involvement of PSII in H_2 photoproduction is not obvious, especially because DCMU does not completely inhibit H_2 production (33, 34). In sulfur (S)-deprived *C. reinhardtii* cells, where the recovery of PSII reaction centers is affected by stress (35), the involvement of residual PSII activity in the H_2 photoproduction yield has been proposed based on inhibitory analysis, electron paramagnetic resonance spectroscopy, and fluorescence data (36–38). Nevertheless, Clark-type O_2 electrodes used directly in algal suspensions have not indicated water-splitting activity in S-deprived cells (36), either due to low sensitivity of the technique (39) or due to the absence of net O_2 release in the actively respiring cells (40). The situation improved with application of a high-sensitive MIMS approach (15, 22). The publications showed that the release of O_2 in dark-adapted algae occurs a few seconds after appearance of H_2 in the system, suggesting direct involvement of PSII in H_2 photoproduction.

As shown in Fig. 1, the application of the 1-s light/9-s dark protocol to anoxic algae results in sustained H_2 photoproduction and also leads to the simultaneous evolution of O_2 (detected at *m/z* 32 and *m/z* 34). The appearance of both nonlabeled and ^{18}O-labeled isotopologues of O_2 upon enrichment with $H_2{}^{18}O$ is well known for monitoring PSII-driven water oxidation reaction (41). The appearance of photosynthetically produced O_2 in algal cultures should indicate a shift in the environment from anoxic to

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microoxic. However, the situation is not so simple. This is for two reasons: First, light activates strong O_2 uptake, which is observed in midlog-phase cultures (Fig. 1*B*, *m*/*z* 32 signal) in the FuD7 mutant (*SI Appendix*, Fig. S4*B*) and in the DCMU-treated algae (Fig. 2*C*, +DCMU trace); second, the release of photosynthetically produced O_2 occurs simultaneously with mitochondrial respiration (which was subtracted from all signals during data processing; *SI Appendix*, Fig. S6 *A* and *B*). These respiratory processes under normal atmospheric pressure balance photosynthetically produced O_2 to a level undetectable by polarographic techniques (Clark-type O_2 electrode) (23). Nevertheless, the competition of H_2 photoproduction with FDP-driven O_2 photoreduction under such low O_2 levels is still highly possible (25), especially in the beginning of the experiment where extracellular O_2 concentrations are at around 0.1 to 0.5 μM. In this context, the gradually increasing H_2 photoproduction rate (Fig. 1*A*) may reflect a release of FDPs of such competition. Indeed, the level of extracellular O_2 does decrease in the course of the experiment due to its consumption by the instrument and the culture itself (*SI Appendix*, Fig. S6*A*).

Surprisingly, the cell wall-deficient CC-4533 strain showed the production of H_2 on the first light pulse (Fig. 1*C*, CC-4533). The rapid response of cells to the establishment of anoxic conditions (~3 min from fully aerobic environment) suggests the expression of the O_2-sensitive [FeFe]-H_2ase in *C. reinhardtii* cells at the time of aerobic cultivation, as has been previously proposed (42). In agreement with this suggestion, transcripts of *hydA1* and *hydA2* hydrogenases and the *hydEF* and *hydG* maturation factors have been found in aerobic algae (43, 44). Since the HYDA1/HYDA2 proteins are also already available in aerobic *C. reinhardtii* (23), the delay in net H_2 evolution of the CC-124 strain (Fig. 1*A*) is most likely caused by the limitation of H_2 diffusion through the cell wall. Assuming the reversible nature of the H_2ase-driven reaction (45, 46), enhanced intracellular levels of H_2 may increase H_2 consumption during dark periods and decrease the final H_2 yield. In accordance with this suggestion, prolongation of the light pulse duration to above 3 s in the pulse-illumination sequence demonstrates the early appearance of the H_2 signal in the same strain (Fig. 5*C*). Similar to the CC-4533 strain, the periods of H_2 photoproduction in the CC-124 algae coincide well with O_2 evolution (Fig. 1*C*, CC-124). Simultaneous production of H_2 and O_2 in the light indicates the direct involvement of electrons from water oxidation by PSII in H_2 photoproduction.

PSII Plays a Major Role in Sustained H_2 Photoproduction. Reductants for the H_2 photoproduction of *C. reinhardtii* are suggested to be supplied not only by PSII but also by the degradation of stored organic substrates, primarily starch (47). In a similar manner to the PSII-dependent process, the indirect pathway requires active PSI for the donation of electrons to H_2ase. However, by contrast, the indirect process results in CO_2 release with a maximum molar stoichiometry of 1 CO_2 per 2 H_2. It has previously been shown that contribution of the indirect pathway to the H_2 photoproduction yield might be significant in S-deprived cells, where the PSII activity is substantially affected by the stress (48).

The results obtained with DCMU-treated algae and the PSII-deficient mutant (Fig. 2*A* and *SI Appendix*, Fig. S3) clearly show that the PSII-independent H_2 production pathway can partly compensate for the loss of the PSII-dependent H_2 production pathway toward H_2 photoproduction of pulse-illuminated cells. The indirect process, however, could not sustain H_2 production for longer than 6 h (Fig. 3*A*), and the specific rate of H_2 production had already started to decline during the first hour (Fig. 3 *A*, *Inset*). On the contrary, the elimination of NDA2 in *C. reinhardtii*, which is the main player of the PSII-independent pathway (10), shows almost no effect on H_2 photoproduction under pulse-illumination conditions (Fig. 3*B*). It has been previously demonstrated that NDA2 deficiency decreases the H_2 photoproduction activity of S-deprived algae (9, 10). However, S deprivation leads to a significant accumulation of starch reserves in cells during the photosynthetic stage (36). Therefore, it is not surprising that S deprivation increases the contribution of the NDA2-dependent pathway in the H_2 photoproduction yield, especially when the activity of PSII is limited or fully absent (49). Thus, S-deprivation data do not contradict our conclusions, since in actively growing algae, which are used in this study, the starch reserves are limited. In the presence of DCMU, which blocks the electron flow from PSII, the *nda2* deletion mutant produces almost the same amount of H_2 as algae placed in complete darkness. The slightly higher H_2 yield of the DCMU-treated *nda2* deletion mutant might be attributed to 1) the light activation of fermentation, 2) incomplete inhibition of electron flow by DCMU (Fig. 2*B*), or 3) the presence of other player(s) in the PSII-independent pathway. If any, their input in the total H_2 yield is negligible and limited to the beginning of pulse-illumination (Fig. 3 *B*, *Inset*). A full compensation of algal NDA2 elimination by PSII confirms that H_2 photoproduction during 1-s illumination periods proceeds via the most efficient mechanism of direct water biophotolysis. This conclusion is in agreement with fluorescence data showing that PSII activity in dark-adapted *C. reinhardtii* cells is linearly related to the hydrogenase capacity observed during the first seconds of illumination (50).

In the 1-s/9-s pulse-illumination regime, algae spend most of the time in darkness, where fermentation plays a major role. According to the data presented in Fig. 3*A*, up to 4% of the H_2 yield in the CC-124 strain is supported by dark fermentation, while 96% is supported by PSII. In the presence of DCMU, the PSII-independent pathway doubles the H_2 yield in pulse-illuminated algae as compared to the dark samples (Fig. 3*A*). Thus, even if both pathways (PSII-independent and dark) operate in the CC-124 cells during pulse illumination, PSII is still responsible for about 92% of the final H_2 yield. Very similar results (93% of the PSII input) were obtained for the *nda2* deletion mutant, where the contribution of fermentation was close to 7% (Fig. 3*B*). However, the NDA2 deficiency may also enhance fermentative H_2 production in the mutant. Taking into account all data, we conclude that H_2 production during 1-s light pulses is driven primarily or even exclusively by the PSII-dependent pathway.

Efficient H_2 Photoproduction in *C. reinhardtii* Occurs before Activation of the CBB Cycle. The light-dependent water oxidation performed by PSII results in O_2 evolution with a simultaneous release of protons (H^+) into the thylakoid lumen. In addition, protons are pumped into the lumen by the PQ/plastohydroquinone cycle driven by PSII and the cytochrome b_6f complex. The accumulation of H^+ in the lumen builds up the ΔpH across the thylakoid membrane and ensures adenosine 5′-triphosphate (ATP) biosynthesis. Simultaneously, a relatively small release of CO_2, which was first detected by MIMS in PSII membranes isolated from spinach (51), could be observed. This CO_2 is known to be released by PSII and is the result of HCO_3^- reaction with H^+ (produced during water splitting) followed by the subsequent bicarbonate dehydration:

$$H^+ + HCO_3^- \rightarrow H_2O + CO_2. \quad [4]$$

This reaction facilitates the removal of H^+ from PSII. Moreover, the electron-acceptor side of PSII is known to be another source for CO_2 evolution under intensive light illumination (52, 53).

In *C. reinhardtii*, the bicarbonate dehydration reaction (and in general interconversions of inorganic carbon) can be accelerated by a luminal carbonic anhydrase, CrCAH3 (54–56). However, in whole cells under normal conditions, the light-dependent CO_2 release is barely detectable due to the presence of two processes: 1) the consumption of CO_2 by Rubisco (i.e., by CBB cycle) and 2) mitochondrial respiration. Therefore, it is not surprising that we observed CO_2 release in the pulse-illuminated algae, when the CBB cycle is not active (Fig. 4) and mitochondrial respiration

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is restricted by an extremely low level of O_2 in the microoxic environment (because of the efficient O_2 consumption by the MIMS setup; *SI Appendix*, Fig. S6*A*). As shown in Fig. 4, the light-induced rise of *m/z* 44, *m/z* 46, and *m/z* 48 signals occurs simultaneously with O_2 evolution and H_2 photoproduction (Fig. 1). The appearance of *m/z* 46 and *m/z* 48 signals together with ambient CO_2 (*m/z* 44) indicate a CO_2–water interexchange:

$$H_2^{18}O + C^{16}O_2 \rightleftarrows H_2C^{16,16,18}O_3 \rightleftarrows H_2^{16}O + C^{16,18}O_2, \quad [5]$$

$$H_2^{18}O + C^{16,18}O_2 \rightleftarrows H_2C^{16,18,18}O_3 \rightleftarrows H_2^{16}O + C^{18,18}O_2. \quad [6]$$

Alternatively, the inclusion of labeled O_2, which appears as a product of water oxidation (reaction 3), in the organic substrate and its immediate degradation should be expected:

$$n^{16,18}O_2 + \left[CH_2^{16}O\right]_n \rightarrow n\ H_2^{16}O + n\ C^{16,18}O_2, \quad [7]$$

$$n^{16,18}O_2 + \left[CH_2^{16}O\right]_n \rightarrow n\ H_2^{16}O + n\ C^{16,18}O_2. \quad [8]$$

The probability of reaction **7**, and especially reaction **8**, is much lower than reactions **5** and **6**, unless they proceed at the same site as reaction **3**. Since the reactions **7** and **8** are expected to occur in the stroma of chloroplasts, they are unlikely to satisfy kinetics of light-dependent release of CO_2 isotopologues during pulse illumination (Fig. 4).

Although the observed CO_2 evolution could be potentially caused by activation of substrate degradation in the light (photofermentation), the positive correlation between O_2 evolution (*SI Appendix*, Fig. S2) and CO_2 release (Fig. 4), and relatively fast recovery of all three CO_2 signals after conclusion of pulse illumination (Fig. 4) strongly suggest direct involvement of PSII in CO_2 evolution. Since the equilibration rate between labeled water and CO_2 occurs with half-times of $t_{1/2} \approx 30$ s or less (55, 57), any donor-side CO_2 formation or other acidification of the lumen would produce CO_2 isotopolouges reflecting the $H_2^{18}O$ enrichment of the water (given that measurements were initiated about 5 min after $H_2^{18}O$ addition). By contrast, CO_2 release at the acceptor side of PSII, where bicarbonate is bound to the nonheme iron and equilibrates slowly with bulk water, would be predominantly $C^{16}O_2$. Thus, the different time dependence of $C^{16}O_2$ versus $C^{16,18}O_2$ and $C^{18}O_2$ (Fig. 4, *m/z* 46 and *m/z* 48 signals) suggests that light-induced CO_2 release may originate from both the acceptor and donor sides of PSII, as well as the acidification of the lumen. This is further supported by estimating the total amount of released CO_2: If just the acceptor side would contribute, we should expect around 2 nmol of CO_2 released per mg Chl [assuming 1.83 mmol of Q_A per mol of total Chl in photomixotrophic *C. reinhardtii* cells (58)]. Instead, formation of around 4 to 8 nmol CO_2 per mg Chl is typically observed in algal cultures (Figs. 4 and 5 and *SI Appendix*, Fig. S11). These data show PSII-associated CO_2 formation in intact cells, which supports the idea that it has an important regulatory and protective function for PSII in vivo.

The prolongation of light pulses to 8 s in the pulse-illumination sequence gradually activates the CBB cycle, leading to a pronounced CO_2 uptake after 5 min of pulse illumination (Fig. 5*A* and *SI Appendix*, Fig. S5). As shown in Fig. 5, the activation of the CBB cycle immediately results in the burst release of O_2 (Fig. 5*B*) and inhibition of H_2 evolution followed by H_2 uptake (Fig. 5*C*). These data support the previously proposed hypothesis that the primary loss of H_2 evolution activity in *C. reinhardtii* cells is caused by competition between ferredoxin-$NADP^+$ oxidoreductase and H_2ases for reduced ferredoxin, rather than by the sensitivity of hydrogenase to oxygen (19, 22). Besides the activation of the CBB cycle, increasing O_2 levels inside the chloroplast can also enhance the flow of photosynthetic electrons toward FDPs. Such competition indeed becomes more pronounced with prolongation of the light pulse in the sequence (25). In long-term experiments, prolongation of the light pulse results in biomass accumulation (25). These experiments show that the pulse-illumination approach allows a fine tuning of algal metabolism between microoxic H_2 photoproduction and aerobic CO_2 fixation, thus enabling PSII-dependent water-oxidation activity to be maintained at the desired level.

Summary

The illumination of anoxic *C. reinhardtii* cultures with a train of short light pulses interrupted by longer dark periods demonstrates that efficient H_2 photoproduction in algal cells occurs exclusively in the absence of photosynthetic CO_2 fixation, thus when the CBB cycle is not active. The cells produce H_2 via the most efficient mechanism of direct water biophotolysis, where water oxidation at the donor side of PSII provides the electrons for reduction of protons by the [FeFe]-H_2ase enzyme(s) downstream of PSI. Thus, the two reactions occur simultaneously. However, under normal conditions O_2 is not released by the cells due to its consumption by respiration. During short periods of light illumination, the H_2ase activity supports a linear photosynthetic electron flow from PSII to PSI, promoting proton translocation across the thylakoid membrane and ensuring efficient water oxidation by PSII. Thus, [FeFe]-H_2ase, in concert with PSII, creates favorable conditions for O_2 accumulation and ATP biosynthesis to levels sufficient for the activation of mitochondrial respiration and the CBB cycle. If the duration of light pulses is not sufficient to fulfill the above condition, the algae continue to produce H_2. The long-term production of H_2 in the absence of CO_2 fixation suggests the central role of [FeFe]-H_2ase in supporting algal photosynthesis and cell fitness under anoxic conditions.

Materials and Methods

The wild-type *C. reinhardtii* strain CC-124, cell wall-deficient strains CC-4533 and CC-5325, *psbA* deletion (FuD7) mutant CC-4147, and the *nda2* deletion mutant (LMJ.RY0402.257129) were obtained from the Chlamydomonas Resource Center at the University of Minnesota, St. Paul, MN. The *nda2* deletion is characterized as described in *SI Appendix*, Fig. S7. All cultures were maintained, grown, and checked for H_2 photoproduction activity under pulse illumination with H_2 and O_2 microsensors (H2-NP and OX-NP; Unisense A/S) as described in detail by Kosourov et al. (23). Conditions for the long-term experiments were reported in the same publication. All experiments were performed in TAP medium with unstressed, actively growing photomixotrophic algae, which were pipetted into the MIMS chamber or vials just before measurements.

The gas exchange in the suspension of algal cells was studied by time-resolved MIMS setup (*SI Appendix*, Fig. S8) as described previously (59, 60). Briefly, the setup consisted of an isotope ratio mass spectrometer (Delta V Plus; Thermo Fischer Scientific), a cooling trap (−78 °C; dry ice + EtOH), and an in-house-built gas-tight membrane-inlet chamber with 200-μL working volume. Before the measurements, $H_2^{18}O$ (97%; Larodan Fine Chemicals AB) was added to the MIMS chamber to a final enrichment of 4%. Analysis of O_2 reactions (evolution/consumption) was based on the *m/z* 32 ($^{16}O_2$) and *m/z* 34 ($^{16,18}O_2$) signals, with Faraday cup amplification of 3×10^8 and 1×10^{11}, respectively. The signal *m/z* 36 ($^{18,18}O_2$) was not considered due to its low evolution at quite low $H_2^{18}O$-enrichement and contamination of this signal by the presence of ^{36}Ar. Analysis of CO_2 reactions was based on simultaneous monitoring of *m/z* 44 ($^{12}C^{16}O_2$), *m/z* 46 ($^{12}C^{16,18}O_2$), and *m/z* 48 ($^{12}C^{18}O_2$) signals with cup amplification of 1×10^9, 1×10^{11}, and 1×10^{12}, accordingly. The argon signal (*m/z* 40) with cup amplification of 1×10^{11} was used as a control. H_2 photoproduction activity was studied in separate runs by monitoring the *m/z* 2 ($^1H_2$) signal with an amplification of 1×10^{12}. No $H_2^{18}O$ was added for H_2 assays. The white light-emitting diode light pulses (~1,000 μmol photons·m^{-2}·s^{-1}) were applied using the STM32F103 microcontroller board controlled by the OxyHydrogen software. Before each measurement, microoxic environment inside the MIMS chamber was achieved within ~2 min after sealing by degassing the sample with the vacuum pump of the mass spectrometer. The initial levels of oxygen slightly varied from experiment to experiment from 0.1 to 0.5 μM. The final gas exchange

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curves were obtained after correction for the gas consumption by the mass spectrometer during the dark periods in the beginning and in the end of each experiment as demonstrated in *SI Appendix*, Fig. S6. This correction also included the gas diffusion component and consumption/production of the gas by the culture during darkness. The analysis of the collected MIMS signals was done using OriginPro 2019 software. The H_2, $^{16}O_2$, and $^{12}C^{16}O_2$ yields were determined in moles per milligram of total Chl according to the standard calibration. All other signals were normalized to Chl.

Data Availability. All data needed to evaluate conclusions of this paper are present in the paper and in *SI Appendix*.

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