# Increased photosystem II stability promotes $H_2$ production in sulfur-deprived *Chlamydomonas reinhardtii*

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**Photobiological $H_2$ production is an attractive option for renewable solar fuels. Sulfur-deprived cells of *Chlamydomonas reinhardtii* have been shown to produce hydrogen with the highest efficiency among photobiological systems. We have investigated the photosynthetic reactions during sulfur deprivation and $H_2$ production in the wild-type and state transition mutant 6 (Stm6) mutant of *Chlamydomonas reinhardtii*. The incubation period (130 h) was dissected into different phases, and changes in the amount and functional status of photosystem II (PSII) were investigated in vivo by electron paramagnetic resonance spectroscopy and variable fluorescence measurements. In the wild type it was found that the amount of PSII is decreased to 25% of the original level; the electron transport from PSII was completely blocked during the anaerobic phase preceding $H_2$ formation. This block was released during the $H_2$ production phase, indicating that the hydrogenase withdraws electrons from the plastoquinone pool. This partly removes the block in PSII electron transport, thereby permitting electron flow from water oxidation to hydrogenase. In the Stm6 mutant, which has higher respiration and $H_2$ evolution than the wild type, PSII was analogously but much less affected. The addition of the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea revealed that ∼80% of the $H_2$ production was inhibited in both strains. We conclude that (*i*) at least in the earlier stages, most of the electrons delivered to the hydrogenase originate from water oxidation by PSII, (*ii*) a faster onset of anaerobiosis preserves PSII from irreversible photoinhibition, and (*iii*) mutants with enhanced respiratory activity should be considered for better photobiological $H_2$ production.**

Solar fuels are an attractive concept for development of future renewable energy systems. Among other fuels, $H_2$ is considered to be one of the most effective and clean fuels (1–3). Solar-driven $H_2$ production by photosynthetic microorganisms (photo-bio-$H_2$ production) is a viable alternative that complements the proposed chemical technologies. Green algae and cyanobacteria can, using water as an electron source via photosynthesis, produce $H_2$ with specific $H_2$-evolving enzymes (hydrogenases) coupled to the photosynthetic machinery (4–6).

Although green algae possess a very active hydrogenase enzyme compared with other organisms (the turnover rate of the algal Fe-Fe hydrogenase is in thousands per second, 100-fold higher than that of other hydrogenases), direct light-to-$H_2$ conversion efficiency is very low (7, 8). Thus, this is not a main metabolic process. Moreover, $H_2$ formation requires anaerobic conditions in the cell because the hydrogenase activity is sensitive to the presence of $O_2$. The consequence is that oxygenic photosynthesis cannot easily be directly coupled to $H_2$ evolution in green algae.

Melis and coworkers reported a two-stage process based on sulfur (S) deprivation in *Chlamydomonas reinhardtii*, which allowed the separation of the photosynthetic reactions from $H_2$ formation (9). Cell cultivation in sealed conditions in media deprived of sulfur allowed light-dependent $H_2$ evolution for several days. The efficiency was the highest for photobiological systems reported so far (2%) (8). Under these conditions photosynthesis is down-regulated and $O_2$ consumption overtakes $O_2$ evolution, creating anaerobic conditions in the cell. This in turn activates hydrogenase expression and activity (10, 11).

Cells of *C. reinhardtii* undergo morphological changes and accumulate starch under S deprivation (12). Both the light and the dark reactions of photosynthesis are down-regulated (9, 12, 13). The amount of Rubisco is drastically reduced during the first 24 h, leading to cessation of the $CO_2$ fixation (12, 14). Photosynthetic electron transfer reactions are also inhibited (9, 15, 16). This is mostly associated with changes in the photosystem II (PSII) complex that are not well characterized. This is unfortunate because a fraction of the electrons delivered to the hydrogenase have been suggested to originate from water oxidation in PSII (17).

PSII is a large multisubunit complex that drives photooxidation of water and reduction of the plastoquinone (PQ) pool, thus initiating the electron transport chain in the thylakoid membrane (18). Apart from light capture and charge separation, PSII possesses a defined sequence of electron and proton transfer reactions (19). In addition, the PSII complex undergoes complex regulation in response to environmental stress factors, especially high light intensities (20, 21). In this study we analyze the changes in the PSII content and activity under $H_2$ production conditions in the wild-type (WT) and the Stm6 mutant of *C. reinhardtii*. The latter was earlier reported to exhibit enhanced $H_2$ yield.* We report and quantify the decrease in the amount of PSII and describe a regulation of the acceptor side reactions in PSII. The latter is controlled by changes in the redox state of the quinone acceptors in PSII and of the PQ pool occurring during S deprivation and subsequent $H_2$ production.

## Results

**$O_2$ and $H_2$ Balance During S Deprivation of WT and Stm6 Mutant Cells.** Under photoheterotrophic conditions, the growth rates of the WT and the Stm6 mutant strains were not different. After the cells were transferred to the S-deprived conditions, the cell number decreased by less than 10% by the end of the experiment. The total amount of chlorophyll (Chl) also decreased by ∼20%, and the Chl *a*/*b* ratio increased from 2.60 to 3.06 for the WT and from 2.30 to 2.94 for the Stm6 mutant. This is explained by partial degradation of the LHCII antenna (12).

In both the WT and the Stm6 mutant cells, the rate of $O_2$ evolution significantly decreased and the rate of respiration increased

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*The Stm6 mutant (state transition mutant 6) with modified light harvesting antenna properties and lacking cyclic electron flow was first isolated and described in (22). The mutant is lacking the *Moc1* gene involved in the assembly of the mitochondrial respiratory chain in the light (22) and has been shown to have extended $H_2$ production under S deprivation (23).

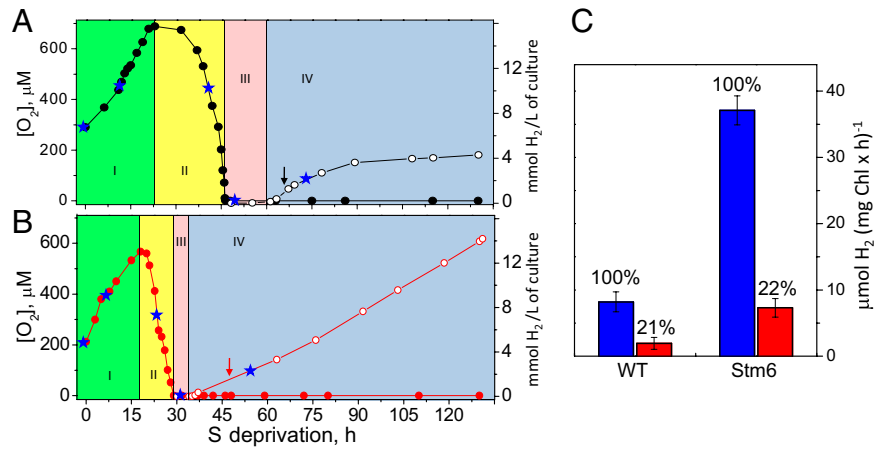


**Fig. 1.** Changes in the concentration of dissolved $O_2$ (filled circles) and produced $H_2$ (open circles) during incubation of WT (*A*, black circles) and the Stm6 mutant (*B*, red circles) of *C. reinhardtii* under S-deprived conditions in sealed flasks. Reflecting the $O_2$ and $H_2$ concentrations, the time course was divided into four phases: I—the $O_2$ evolution phase; II—the $O_2$ consumption phase; III—the anaerobic phase; and IV—the $H_2$ production phase. The blue stars in each phase indicate the time points for EPR and fluorescence experiments. The sample at $t = 0$ was taken as a control. (*C*) The effect of the addition of 20 μM DCMU on the rate of $H_2$ production. The aliquots of the cell culture were taken as described in the text at the time points indicated by arrows in *A* and *B*. The amount of $H_2$ formed in the respective strains in the absence of DCMU (blue) is set as 100%. The fraction of $H_2$ formed in the presence of DCMU (red) was similar in both WT and the Stm6 mutant whereas the net amount of $H_2$ formed was four times greater in the mutant.

in the beginning of S deprivation, leading to establishment of anaerobic conditions in the culture. Interestingly, due to the lower initial rate of $O_2$ evolution and the higher initial rate of respiration in the Stm6 mutant, anaerobiosis was achieved much faster in the Stm6 mutant cells than in the WT cells. Fig. 1 *A* and *B* shows the concentration of dissolved $O_2$ in the medium during S deprivation in the sealed flasks. First, the concentration of $O_2$ in the medium increased due to still photosynthetically active cells during this period. After this, the concentration of $O_2$ started to decrease, finally reaching anaerobicity. The decrease in the $O_2$ concentration reflects mainly a decrease in the $O_2$ evolution capacity from PSII and partly the increased respiration (by 25–30%) in both WT and Stm6 mutant cells. The concentration of $O_2$ remained at zero during the rest of the experiment (up to 130 h, Fig. 1 *A* and *B*). A few hours after the anaerobic conditions were established, $H_2$ evolution was observed in both strains.

Our observed changes in the $O_2$ and $H_2$ concentrations during cultivation of *C. reinhardtii* cells in S-deprived conditions are similar to those observed in an earlier study (24) where the process was dissected in four different phases: the $O_2$ evolution phase (I), the $O_2$ consumption phase (II), the anaerobic phase (III), and the $H_2$ production phase (IV) (Fig. 1 *A* and *B*) (24). All four phases were observed in the WT and the Stm6 mutant. However, the time dependence of the phases was different. First, the anaerobic phase was established in much shorter time for the Stm6 mutant (by 15 h if compared with the WT cells, Fig. 1 *A* and *B*). This reflects a combined effect of the decreased $O_2$ evolution capacity and the increased respiration rate in the mutant. Second, phase III was much shorter in the Stm6 mutant cells; the $H_2$ production was detected only a few hours after the onset of anaerobiosis. Third, $H_2$ production was also much more effective in the Stm6 mutant than in the WT [as shown before (23)] and continued with an increasing rate during the entire 130 h time course of the experiment (Fig. 1*B*).

3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is a specific inhibitor of PSII that disrupts the electron transfer from $Q_A^-$ to $Q_B$ by occupying the $Q_B$ site. It has been reported that the $H_2$ production during S-deprived cultivation of *C. reinhardtii* cells is partially inhibited by the addition of DCMU (12, 17, 23, 25–27), which indicates that electrons from PSII are used by hydrogenase (12, 17, 27). This link between photosynthetic water oxidation and hydrogen evolution, and the mechanism behind it, is very important for the understanding of photobiological hydrogen production and its further development.

We have tested if this DCMU effect occurs also under our conditions and the results are shown in Fig. 1*C*. The samples were withdrawn from the culture at the time points indicated by arrows in Fig. 1*A* (WT) and Fig. 1*B* (Stm6 mutant) and incubated in the presence or absence of DCMU. In both the WT and the Stm6 mutant, ~80% of the rate of $H_2$ evolution was lost upon the addition of DCMU (Fig. 1*C*). Thus, our results indicate a significant involvement of PSII in photosynthetic hydrogen evolution.

The molecular mechanism of how this occurs has been addressed here with selective spectroscopic analysis. Measuring points to assess PSII reactions were selected during the four different phases (indicated by stars in Fig. 1 *A* and *B*), and at each time point samples were anaerobically withdrawn from the main culture for further analysis.

The control point was at the beginning of the S deprivation ($t = 0$). Here the properties of PSII were essentially the same as in samples analyzed just before the S deprivation procedure was commenced. In phase III, the samples were taken out within 10 min after the anaerobic conditions were established but clearly before we observed any production of $H_2$ (11).

**Electron Paramagnetic Resonance Measurements.** The amount of PSII in cells can be directly quantified by electron paramagnetic resonance (EPR) spectroscopy (28). This is done on the basis of the tyrosine $D^{\bullet}$ radical, which amounts to one radical spin per PSII reaction center. The results are shown in Fig. 2 and Table 1. The amount of PSII in the control samples was set as 100%. In the WT cells it decreased to 54% in the $O_2$-evolving phase (phase I) and to 25% in the $O_2$-consumption phase. Interestingly, the concentration of PSII remained at this level during the anaerobic (III) and $H_2$ production (IV) phases (Fig. 2 and Table 1). Thus, the number of PSII centers in the WT cells decreased by ~75% during S deprivation.

The situation was different in the Stm6 mutant. The amount of PSII did not change during first three phases of the S deprivation process (Fig. 2 and Table 1). Moreover, the amount of PSII decreased to only 52% during the $H_2$ production phase in the Stm6 mutant (Fig. 2 and Table 1). Thus, the amount of PSII was much less affected in the Stm6 mutant than in the WT during the S deprivation process. The different remaining PSII content (23% in the WT; 52% in the Stm6 mutant) is clearly very important when the cells have reached the capacity to produce $H_2$ (phase IV).

**Flash-Induced Fluorescence Decay Measurements.** The functional capacity of the PSII centers during the different phases of S deprivation was analyzed by flash-induced fluorescence decay kinetic measurements in WT and Stm6 mutant cells. This method reports on the electron transport properties of PSII and can be used to monitor both the acceptor- and the donor-side reactions (29, 30). Immediately after the flash, the fluorescence rises from the $F_0$ level

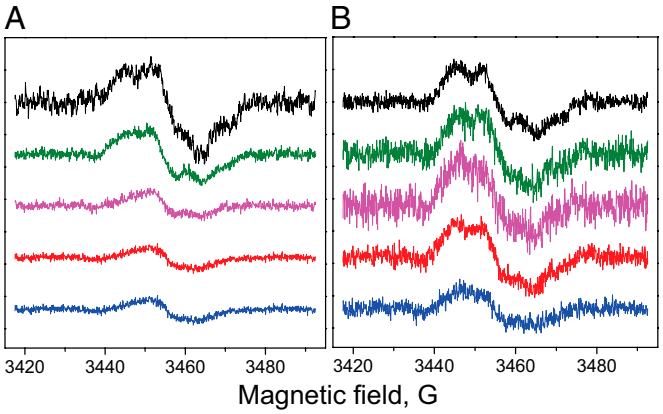


**Fig. 2.** EPR spectra of tyrosine D• taken from intact cells of the WT (*A*) and Stm6 mutant (*B*) of *C. reinhardtii* at different stages of S deprivation: control (time = 0, black); the $O_2$-evolving phase (I, green); the $O_2$ consumption phase (II, magenta); the anaerobic phase (III, red); and the $H_2$ production phase (IV, blue). The spectra were normalized to the same Chl concentration. The full induction of the signal was achieved as described in ref. 28. EPR conditions were: microwave frequency 9.76 GHz, microwave power 8 mW, modulation amplitude of 5 G, and room temperature.

to the $F_{max}$ level (Fig. 3). This reflects the charge separation in PSII and the stabilization of the electron on the first quinone acceptor, $Q_A$. This results in the formation of a high-fluorescence state. The consequent decay of variable fluorescence demonstrates how the electron leaves $Q_A^-$ in the particular sample (29, 30).

In the control sample from the WT cells, more than 80% of the fluorescence decayed in the microsecond to millisecond time range (Fig. 3*A*, black circles). This fast decay of the variable fluorescence is normal and reflects fast and efficient forward electron transfer from $Q_A^-$ to $Q_B$ (resulting in $Q_B^-$) in the majority of the PSII centers. In the oxygen evolution (phase I) and consumption (phase II) phases, this forward electron transfer became sequentially slower. When the cells reached the anaerobic phase (III), the fluorescence decay kinetics were dramatically different (Fig. 3*A*, closed red circles). Instead of the fast decay indicative of forward electron transfer, the decay kinetics were dominated by slow reactions occurring in the hundreds of milliseconds to seconds time range. Such slow decay kinetics were typical for recombination reactions between $Q_A^-$ and the $S_2$ state of the water-oxidizing complex in PSII (29–31). Indeed, very similar kinetics were observed when the flash-induced fluorescence decay was measured in the same sample but in the presence of DCMU, which blocks forward electron transfer between $Q_A^-$ and $Q_B$ (Fig. 3*A*, open red circles). The fluorescence decay kinetics in the presence of DCMU are well understood and reflect recombination between $Q_A^-$ and the $S_2$ state (29–31). Thus, from the almost identical kinetics we conclude that the forward electron transfer from $Q_A^-$ was completely blocked in the anaerobic phase (III) during S deprivation of the WT cells. This indicates that the PQ pool is completely reduced in the anaerobic phase. Furthermore, from the observation of the flash-induced fluorescence we can also conclude that the 22–23% of PSII centers that actually remained (Table 1) were active both in reducing $Q_A$ and in forming the $S_2$ state.

**Table 1. Changes in the PSII amount during different phases of S deprivation in WT and Stm6 mutant cells of *C. reinhardtii***

| | Tyrosine D• (%)* | |
|---|---|---|
| Phase in S deprivation | WT | *Stm6* |
| Control | 100 | 100 |
| $O_2$-evolving phase (I) | 54 | 102 |
| $O_2$ consumption phase (II) | 25 | 102 |
| Anaerobic phase (III) | 22 | 98 |
| $H_2$ production phase (IV) | 23 | 52 |

PSII content was estimated from measurements of tyrosine D•.
*Data are normalized on the basis of the Chl content in the respective sample as described in ref. 28 (*Material and Methods*).

When the cells reached the $H_2$ production phase (IV), the kinetics of the flash-induced fluorescence changed again. In 35–40% of the remaining PSII centers, new decay kinetics occurring in the few millisecond time scale appeared (Fig. 3*A*, blue circles). These kinetics are indicative of rather slow, but existing, forward electron transfer from $Q_A^-$ in PSII. We propose that these kinetics reflect rebinding of $Q_B$ to its site and subsequent electron transfer from $Q_A^-$ to the newly bound $Q_B$ (see refs. 29–32). The consequence of this assignment is that a significant fraction of the PQ pool somehow must have become oxidized in the $H_2$ production phase.

The situation in the Stm6 mutant cells was similar during the first three phases of S deprivation. The control sample showed fast decay of the variable fluorescence, reflecting active PSII with effective forward electron transfer from $Q_A^-$ (Fig. 3*B*, black circles). The kinetics were similar to those in the WT. In the oxygen evolution (I) and consumption (II) phases, this forward electron transfer was still dominant but became sequentially slower. In the anaerobic phase (III), the fluorescence decay kinetics were again dominated by the slow decay (Fig. 3*B*, closed red circles) that here also was similar to the decay in the presence of DCMU (Fig. 3*B*, open red circles). The slow kinetics were indicative of recombination reactions between $Q_A^-$ and the donor side of PSII and reflects blocked forward electron transfer from $Q_A^-$ to $Q_B$ (29–32).

In the $H_2$ production phase (IV) in the Stm6 mutant cells, the opening of the forward electron transfer from $Q_A^-$ was again observed. The forward electron transfer involved a higher fraction of the PSII centers in the Stm6 mutant than in the WT cells and about 60% of the remaining PSII centers showed millisecond fluorescence decay kinetics in the $H_2$ production phase (Fig. 3*B*, blue circles).

## Discussion

The aim of this work was to study how light-driven photosynthesis is regulated in response to $H_2$ production under S deprivation in the green algae *C. reinhardtii*. Despite the fact that this process requires separation of photosynthesis and $H_2$ evolution into different time stages, it remains the most effective known photobiological way of converting solar energy into $H_2$ (4, 7, 8, 33). The separation of $O_2$ evolution (photosynthesis) and $H_2$ evolution, however, is not complete, and partial photosynthetic light reactions still take place under S-deprived conditions as was observed earlier (9, 12, 15, 16, 24, 27). This is of potential importance because, in the ideal photobiological system, electrons and protons for $H_2$ production must originate from the water-splitting reaction, thus leaving PSII as the focus of this study.

**PSII and $H_2$ Evolution in WT cells.** It is known that various photosynthetic reactions are down-regulated to a different extent during the S deprivation procedure. The level of Rubisco became undetectable after 40 h of S deprivation resulting in inhibition of the $CO_2$ fixation (12, 34). PSII is the most affected complex involved in the light reactions as was shown by the decrease in $O_2$ evolution, fluorescence parameters, and the amount of the D1 protein (9, 12, 15, 16, 24). PSI, the cytochrome $b_6f$ complex, and different antenna proteins are less affected (9, 12).

Decrease in $O_2$ evolution and increased respiration rates lead to establishment of anaerobic conditions in cells. This is illustrated in Fig. 1*A* where the concentration of dissolved $O_2$ in the medium first increased due to $O_2$ evolution ($O_2$-evolving phase I) and then decreased when the diminishing $O_2$ evolution by PSII was

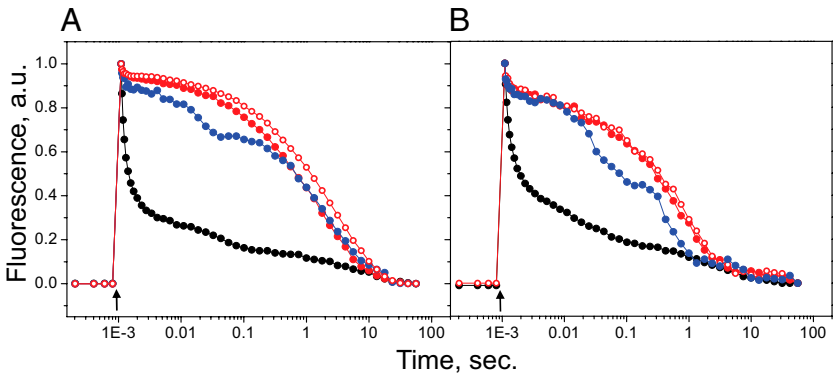


**Fig. 3.** Normalized flash-induced fluorescence decay traces from WT (*A*) and the Stm6 mutant (*B*) of *C. reinhardtii* cells in control samples (I, black circles); samples from the anaerobic phase (III, closed red circles); samples from the anaerobic phase in the presence of 20 μM DCMU (III, open red circles); and samples from the $H_2$ production phase (IV, blue circles). The arrows indicate the time when the flash was applied.

overcome by respiration ($O_2$ consumption phase II). A short time after anaerobiosis was established (III), the light-driven $H_2$ production commenced (Fig. 1*A*, $H_2$ production phase IV). This reflects the expression of the hydrogenase that has been shown to occur within 20 min after the onset of anaerobiosis under conditions similar to ours (11).

It is clear that the metabolic changes that lead to $H_2$ evolution are very dependent on the $O_2$ balance in the cell. Therefore, it is important to follow changes in the PSII properties during the S deprivation procedure. We have used EPR spectroscopy to quantify PSII centers in our samples. The advantage of this method is that it can be applied in vivo on intact cells and that it can be used quantitatively with high precision (28).

Our measurements show that the amount of PSII in the WT cells had already decreased at the beginning of S deprivation (Table 1). The amount of PSII decreased to 54% during the $O_2$ evolution phase and to 25% during the $O_2$ consumption phase and stayed at a similar level during consequent phases. Thus, the amount of PSII was decreased by ∼75% during the first two phases of the S deprivation procedure (Table 1).

The reason for this dramatic decrease in PSII amount during the first 45 h is most probably the distorted balance between photoinhibition and repair of PSII. Our illumination intensity of 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ was chosen for maximal $H_2$ yield at our conditions. Photoinhibition is a steady-state process that takes place even at these moderate light intensities. It results in the inhibition of the electron transport reactions in PSII, disassembly of the complex, and $O_2$-dependent degradation of PSII proteins, most notably, the D1 protein (20, 21, 35, 36). Repair of the photoinhibited PSII centers requires the de novo protein synthesis. It can probably still take place during the first few hours of the $O_2$-evolving phase but will thereafter be completely halted due to the lack of sulfur and inability to synthesize the essential amino acids (12). This eventually results in a substantial (75%) decrease in the amount of PSII during the $O_2$ evolution and consumption phases (Fig. 2 and Table 1).

Surprisingly, in the anaerobic (III) and $H_2$ production (IV) phases PSII remains at ∼25% of the original level (Table 1). The reason for this is probably the anaerobic environment. It is known that D1 protein degradation in normal light is mainly $O_2$-dependent, reflecting interaction between Chl triplets and $O_2$, leading to damaging singlet oxygen formation (35, 36). It is also known that, in the absence of $O_2$, photoinhibition results in an inhibited form of PSII with no bound $Q_B$ and semistable $Q_A^-$ (37, 38) whereas the D1 protein seems unharmed. Such photoinhibition is reversible, and these PSII centers are still able to restore their electron transport capacity, providing that, first, the PQ pool and, then, $Q_A^-$ are somehow reoxidized (37, 38).

The functional state of the remaining 25% of PSII centers needs to be analyzed further, especially with respect to $H_2$ production in the WT *C. reinhardtii* cells. Our experiments with DCMU, which binds to the $Q_B$ site in PSII and blocks forward electron transfer, clearly show that ∼80% of the $H_2$ formation was inhibited in the presence of DCMU (Fig. 1*C*). This effect was known before, and reported values for the DCMU inhibition vary from 20 to 85%, probably reflecting varying experimental conditions (12, 23, 25–27). However, the question of whether the electrons delivered to the hydrogenase during the light-induced $H_2$ evolution really originate from PSII activity has not been fully addressed so far.

Our fluorescence data clarify this question. The strength of the flash-induced fluorescence decay measurements is that it allows monitoring of the electron transfer specifically from $Q_A^-$ after a single flash. These measurements, shown in Fig. 3*A*, indicate that the 25% of the PSII centers that are left in the anaerobic phase are not able to reduce $Q_B$. They are behaving similarly to DCMU-treated centers; i.e., they are not competent in the forward electron transfer from $Q_A^-$ (Fig. 3*A*). These centers behave similarly to the reversibly photoinhibited PSII centers (under anaerobic conditions), which we described briefly above. They are fully competent in charge separation to $Q_A^-$. Moreover, the decay kinetics are indicative of a functional $CaMn_4$ cluster capable of normal S-state advancement when the acceptor side is reactivated again (29–32). The reason why the electron transfer in these remaining PSII centers is blocked beyond $Q_A$ is therefore not because there are modifications within PSII. Instead, it reflects changes in the redox state of the PQ pool, and the fluorescence measurements suggest that the PQ pool is fully reduced in the anaerobic state (III).

It is known that S deprivation changes the redox state in the cells. The redox potential in the TAP-S medium drops from 400 to –100 mV during the anaerobic phase (III) (15, 24). This is the result of the changed metabolism in the S-deprived cells, the decreased activity of Rubisco, accumulation of starch, and contribution of electrons from the starch catabolism to the electron transport chain in the thylakoid membrane. These cumulative changes lead to a very reduced state of the PQ pool. The lack of oxidized PQ results in that the $Q_B$ site in PSII is most probably empty. The result is the complete block of the forward electron transfer from $Q_A^-$, which we observe in the flash-induced fluorescence measurements.

However, this situation is changed with the expression of hydrogenase, which starts shortly after the onset of anaerobiosis (11). Our observations indicate that the PQ pool slowly becomes slightly oxidized. This results in the opportunity for oxidized PQ to rebind to PSII, which, in turn, allows the forward electron transfer from $Q_A^-$ as observed by the appearance of the fast

decay phase in the fluorescence kinetics in samples from the $H_2$ production phase (Fig. 3*A*, blue). This fast decay phase cannot be attributed to normal $Q_A^-$ to $Q_B$ electron transfer. Instead, it is more compatible with the kinetics of electron transfer from $Q_A^-$ to $Q_B$, which first has to bind to PSII (29, 32). This is not surprising because the redox state of the PQ pool is still far from normal under these $H_2$ production conditions. We propose that the electron pressure on the electron transport chain is partially relieved in $H_2$ evolution by hydrogenase-drawing electrons from the PQ pool that have become completely reduced during the earlier phases (I–III, Fig. 1).

This is the first direct experimental evidence that PSII provides electrons to hydrogenase during light-induced $H_2$ evolution in the S-deprived system. Our estimation from the amplitude of this fast phase (Fig. 3*A*, blue) is that only ~9% of the PSII centers that were present from the start of the experiment contribute to $H_2$ production during S deprivation in WT *C. reinhardtii* cells at the time point at which the measurements were conducted (Fig. 1*A*, star in phase IV). The other PSII centers are either degraded during phases I and II (77%, Table 1) or still not active in forward electron transfer from $Q_A^-$ (14%).

**PSII and $H_2$ Evolution in Stm6 Cells.** The situation was similar but also different in the Stm6 mutant. This mutant is reported to have a modified respiratory metabolism, elevated starch reserves, and low $O_2$ evolution. In addition, it was found to be defective in the so-called "state transition" and thus lacking ability for cyclic electron flow around PSI. The mutant was also reported to have more than five times the elevated $H_2$ production rates than the WT cells (23).

We obtained similar results with the Stm6 mutant. The anaerobic condition was attained much faster in the mutant than in the WT. This reflects a combined effect of the lower initial $O_2$ evolution rate and the higher respiration rate. After only 30 h, anaerobiosis was created in the sealed cultures (Fig. 1*B*). Importantly, the $H_2$ formation had already started after less than 5 h of anaerobiosis.

In our experiments, the $H_2$ evolution was about four times higher in the Stm6 mutant when compared with the WT (Fig. 1). We correlate this higher $H_2$ evolution yield to the higher remaining PSII content in the Stm6 mutant. During the $H_2$ production phase (IV), PSII amounted to 52% of its original level (Table 1). Thus, there are more PSII centers left that potentially are able to provide electrons for the $H_2$ production in the Stm6 mutant cells (Table 1). It is interesting to discuss why so much PSII is left in the mutant compared with the WT despite the identical experimental conditions. We propose that this reflects the altered balance between decreased $O_2$ evolution and respiration. This results in a much faster onset of anaerobiosis under which PSII is not irreversibly damaged. Less PSII is consequently damaged during phases I and II, which are much shorter in the mutant. Under anaerobiosis (III), PSII is no longer irreversibly damaged, and, when the electron pressure on the PQ pool is relieved, more PSII centers are able to perform forward electron transport, eventually all of the way to hydrogenase.

Despite the higher rates of $H_2$ evolution and the higher PSII content, ~80% of $H_2$ evolution was inhibited by the DCMU addition (Fig. 1*C*) similar to in the WT. This leads again to the question, what is the functional status of the PSII centers that remain in the $H_2$ production phase of the Stm6 mutant? Our fluorescence measurements showed that PSII was "closed" in the mutant during the anaerobic phase, effectively eliminating the electron supply from $Q_A^-$ to $Q_B$ and the PQ pool. However, following the onset of $H_2$ formation, the opening of the forward electron transport on the acceptor side in PSII was much more effective in the mutant than in WT: as much as 60% of the fluorescence decay became faster and can thus be attributed to the electron flow from $Q_A^-$ to $Q_B$ (Fig. 3*B*). That leaves us with conclusion that ~32% of the original PSII centers (60% of the remaining 52% of PSII) contribute to $H_2$ production during the S deprivation procedure in Stm6 cells. This is about four times more than in the WT (9%) and is consistent with the difference that we have observed in the $H_2$ evolution rates (four times, Fig. 1).

**Conclusions.** We have analyzed the activity of PSII during S deprivation in the WT and the Stm6 mutant of *C. reinhardtii*. In the $H_2$ production phase in particular, we quantified and analyzed the function of the remaining, reactivated PSII centers. They are competent in charge separation and S-state advancement and are able to provide electrons to the PQ pool. Our estimation of the activity of these remaining PSII centers indicates that the water splitting by PSII supplies the majority of electrons for $H_2$ synthesis. This estimation is corroborated by our experiments in which $H_2$ production was inhibited by the addition of DCMU. Our results also indicate that the hydrogenase uses electrons from the PQ pool during the $H_2$ production phase. This partly removes the block in PSII electron transport from $Q_A^-$ to plastoquinone, thereby permitting electron flow from water oxidation in PSII to hydrogen.

Moreover, the rate of $H_2$ production is proportional to the amount of operational PSII present. In the Stm6 mutant where the amount of PSII was four times higher than in the WT during the $H_2$ production phase, the amount of $H_2$ produced was correspondingly four times higher. The higher amount of the remaining PSII centers reflects the modified redox environment in the thylakoid membrane of the Stm6 mutant (23), which leads to an earlier onset of anaerobiosis. The earlier anaerobiosis is an important prerequisite for preservation of PSII during illumination under S-deprived conditions.

Based on our spectroscopic analysis we can also explain how enhanced respiration rates lead to higher rates of $H_2$ production. Enhanced respiration induces a faster onset of anaerobiosis. The absence of oxygen switches off irreversible photoinhibition and D1-protein degradation, which is oxygen dependent (38). This leaves more PSII centers. Under anaerobic conditions these remaining PSII centers exist in the "reversibly inhibited state" that was described in ref. 38. They are competent in all electron transport reactions except the reduction of the PQ pool. Thereby, an earlier anaerobiosis creates conditions that protect PSII centers from irreversible photoinhibition due to D1-protein damage and degradation. When expression and function of the hydrogenase relieve the electron pressure on the PQ pool, these reversibly inhibited PSII centers are reactivated. Therefore, earlier onset of anaerobiosis, as for example in the Stm6 mutant, will preserve more PSII centers capable of water oxidation after reactivation.

This observation is important because it is opens up a way to improve the $H_2$ production in green algae, which is based on enhanced respiratory reactions, similar to Stm6 and some other mutant systems (23, 27, 39). The search for existing mutations or engineering of new ones, which can create earlier and extended anaerobic conditions, will potentially overcome two major problems. First, it will reduce the damaging effect of oxygen on the hydrogenase. Second, the absence of oxygen will protect the D1 protein in PSII against damage. Thereby, the electron source, PSII, is preserved on an elevated level, allowing more efficient photobiological $H_2$ production.

## Materials and Methods

**Culture Growth and S Deprivation.** WT and the Stm6 mutant of *C. reinhardtii* were grown photoheterotrophically in TAP medium in a shaking incubator at 25 °C under cool white fluorescence light of 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity. Cells were collected in the middle of the logarithmic growth phase, washed three times in TAP-S medium (9, 26), and suspended in 300-mL sealed conical flasks (bio-reactors) (25 °C) at the density of ~6–7 × $10^6$ cells/mL. For $H_2$ evolution experiments, the culture in the TAP-S medium was incubated under light of 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ intensity and with constant stirring in the sealed bioreactors for the indicated period and up to 130 h.

**Measurements of Dissolved Oxygen, Respiration, Oxygen, and Hydrogen Production.** The concentration of dissolved oxygen in the liquid culture was measured directly with an oxygen analyzer (MAPK-302T). The rates of

respiration and oxygen evolution in *C. reinhardtii* cells were measured with a Clark electrode at 25 °C. Aliquots of 1 mL were taken from the bioreactor and placed in the Clark electrode cell. After 2 min of adaptation, the rate of the respiration was recorded in the dark for 3 min. Oxygen evolution was measured after the addition of 5 mM $NaHCO_3$ and subsequent 2-min dark incubation with saturating white light for 5 min.

The amount of $H_2$ in the gas phase was determined with a Clarus 500 gas chromatograph complete with a Molecular Sieve 5A 60/80 Mesh column (Perkin-Elmer) and Ar as a carrier gas. The effect of DCMU on the $H_2$ production was measured 20 h after anaerobic conditions had been established. At the position indicated by arrows in Fig. 1 *A* and *B*, samples were anaerobically withdrawn, and 4 mL of the cell culture was transferred into 7-mL gas-tight Ar-flushed glass vials. After 3 h incubation under illumination and constant stirring at 25 °C, 20 μM DCMU was added (this concentration is sufficient to inhibit all PSII centers at the cell density used in our experiments). One hour after the DCMU addition, the amount of produced $H_2$ in the gas phase was determined.

**Variable Fluorescence Decay Kinetics.** Flash-induced variable fluorescence decay kinetics was measured with a FL3000 double modulated fluorometer (Photon Systems Instruments) as described in ref. 31. One mL of the cell suspension (~15 μg of Chl/mL) was taken from the culture at the time points indicated by stars in Fig. 1 and dark-adapted for 5 min before the actinic flash was applied.

**EPR Spectroscopy.** EPR measurements were performed in a flat cell with a Bruker ELEXYS E500 spectrometer equipped with a SuperX EPR049 microwave bridge and a SHQ4122 cavity as described in ref. 28. At each time point indicated by a star in Fig. 1, the entire cell culture from one bioreactor was concentrated to ~200 μg of Chl/mL by centrifugation at 1,620 × *g* and frozen at –80 °C before the use.

For all measurements, in the first two time points indicated by stars in Fig. 1, samples were removed from the bioreactor aerobically. In the following time points in Fig. 1, all procedures were done under anaerobic conditions.

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