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REGULAR PAPER

Photoproduction of hydrogen by sulfur-deprived *C. reinhardtii* mutants with impaired Photosystem II photochemical activity

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Abstract Photoproduction of H_2 was examined in a series of sulfur-deprived *Chlamydomonas reinhardtii* D1-R323 mutants with progressively impaired PSII photochemical activity. In the R323H, R323D, and R323E D1 mutants, replacement of arginine affects photosystem II (PSII) function, as demonstrated by progressive decreases in O_2-evolving activity and loss of PSII photochemical activity. Significant changes in PSII activity were found when the arginine residue was replaced by negatively charged amino acid residues (R323D and R323E). However, the R323H (positively charged or neutral, depending on the ambient pH) mutant had minimal changes in PSII activity. The R323H, R323D, and R323E mutants and the pseudo-wild-type (pWt) with restored PSII function were used to study the effects of sulfur deprivation on H_2-production activity. All of these mutants exhibited significant changes in the normal parameters associated with the H_2-photoproduction process, such as a shorter aerobic phase, lower accumulation of starch, a prolonged anaerobic phase observed before the onset of H_2-production, a shorter duration of H_2-production, lower H_2 yields compared to the pWt control, and slightly higher production of dark fermentation products such as acetate and formate. The more compromised the PSII photochemical activity, the more dramatic was the effect of sulfur deprivation on the H_2-production process, which depends both on the presence of residual PSII activity and the amount of stored starch.

Keywords *Chlamydomonas reinhardtii* · D1-Arg323 · Site-directed mutagenesis · H_2 Photoproduction · Photosystem II · Sulfur deprivation · Starch · Fermentation products · Algae

Abbreviations

Chl	Chlorophyll
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea
F	The fluorescence level at time *t*
$(F-F_0)/F_0$	Fluorescence yield at a particular time
F_0	Fluorescence emitted by a sample at low-light levels prior to actinic-flash excitation
F_i	The initial peak of a fluorescence induction curve
F_{max}	Maximum fluorescence yield following actinic-flash excitation
F'_m	Maximum fluorescence level under the ambient light induced by a saturating light pulse

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F_p	The saturating level of a fluorescence induction curve
F_t	The steady-state level of fluorescence measured under ambient light prior to a saturating light pulse
$\frac{\Delta F}{F'_m} = (F'_m - F_i)/F'_m$	Measure of the photochemical conversion efficiency (or photochemical activity) of PSII
F_v	Variable fluorescence level ($F_m - F_0$)
OEC	O_2-Evolving complex
PAM	Pulse amplitude modulated
PAR	Photosynthetically active radiation
PQ	Plastoquinone
PSII	Photosystem II
pWt	Pseudo-wild-type
Q_A	The primary quinone acceptor of PSII
Q_B	The secondary quinone acceptor of PSII
TAP	TRIS-acetate-phosphate medium
Y_D	Tyr-160 of the D2 polypeptide

Introduction

Sustained, light-dependent hydrogen production by *Chlamydomonas reinhardtii* can be achieved by incubating algal cells under sulfur-deprived conditions. Sulfur deprivation causes a progressive and specific decrease in the photosynthetic O_2-evolving capacity of the cells, due to the lack of photosystem II (PSII) repair function (Wykoff et al. 1998; Melis et al. 2000), and this leads to the establishment of anaerobiosis in sealed photobioreactors (Ghirardi et al. 2000; Melis et al. 2000). The enzymes catalyzing H_2-photoproduction, reversible [FeFe]-hydrogenases, are activated transcriptionally in the absence of O_2 (Happe and Kaminski 2002; Forestier et al. 2003), but their activity is promptly inactivated by O_2 (Ghirardi et al. 1997). The establishment of anaerobiosis in the photobioreactor with sulfur-deprived algae depends mostly on the uptake of acetate, while maintenance of anaerobiosis during the H_2-photoproduction stage depends on the degradation of endogenous substrates (Kosourov et al. 2007). Starch (Tsygankov et al. 2002; Zhang et al. 2002) and protein (Melis et al. 2000; Kosourov et al. 2002) stored during oxygenic photosynthesis provide reductants for aerobic respiration, which removes any residual photosynthetically generated O_2. The photochemical activity of PSII decreases precipitously at the exact time that the culture becomes anaerobic, but the beginning of volumetric H_2 production a few hours later coincides with the partial recovery of PSII photochemical activity (Antal et al. 2003). This observation indicates that at least some PSII activity is required to sustain the H_2-photoproduction process. Inhibitor experiments, demonstrating that the vast majority of the electrons driving H_2 production at the beginning of the H_2-production period originates from water oxidation, confirmed this supposition (Kosourov et al. 2003). Additional reductants are provided by the initial oxidation steps of starch or protein degradation in the chloroplast, which are re-directed to the plastoquinone pool and contribute to H_2 photoproduction (Ghirardi et al. 2000; Tsygankov et al. 2002; Happe and Kaminski 2002; Zhang et al. 2002; Kosourov et al. 2003; Antal et al. 2003). Thus, substrate degradation plays a dual role in H_2-photoproduction. First, in the consumption of the O_2 evolved by residual PSII activity, and second as a source of additional electrons to the photosynthetic electron transport chain.

Hydrogen gas is only one of the products of anaerobic substrate degradation in illuminated sulfur-deprived cultures. Depending on the pH of the cultivation medium (Kosourov et al. 2003) and the particular type of green algal strain used (Happe et al. 2002), one detects different ratios of excreted formate, acetate, and ethanol, which are the major by-products of anaerobic degradation in *C. reinhardtii* (Gfeller and Gibbs 1984; Kreutzberg 1984; Ohta at al. 1987). The relative amounts of organic products compared to H_2 gas must be dependent on the relative rates of starch degradation compared to photosynthetic electron transport, since only the latter can sustain H_2 production in the light.

The usefulness of sulfur deprivation in inducing H_2 photoproduction is, primarily, as a method to partially (Melis et al. 2000) and reversibly (Kosourov et al. 2005) inhibit photosynthetic O_2 evolution. We reasoned that *C. reinhardtii* mutants that were already partially impaired in PSII function might become anaerobic and photoproduce H_2 gas more rapidly upon sulfur deprivation. On the other hand, PSII mutants are also expected to be affected in starch accumulation, and the work of Posewitz et al. (2004) suggests that if the capacity of the mutants to store starch were decreased, this might compromise the length of the H_2-production phase. The analysis of the H_2-production activities of a set of mutants with progressively more impaired PSII activity and starch-storage capacity should provide us with additional information regarding the balance between these two functions and H_2 production in sulfur-deprived *C. reinhardtii*.

There have been numerous publications showing the effects of site-directed mutagenesis of positively charged arginine residues on PSII activity. Residues D1-R257, D1-R269, D2-Arg-233 and D2-Arg-251 were shown to stabilize bicarbonate/formate binding in PSII (Cao et al. 1991; Xiong et al. 1997, 1998). Also, the alteration of an arginine residue (R305) in the CP43 PSII protein caused a defect in its ability to utilize chloride in support of efficient O_2 evolution in PSII (Young et al. 2002). Arginine R448 in CP47 was suggested to participate in the chloride-binding

domain of PSII and/or in the functional interaction with the 33kDa extrinsic protein of PSII (Bricker et al. 2001). Recently, it was also shown that mutations of the basic arginine residue R334 in the D1 protein (located close to Mn-binding amino acids H332 and E333) leads to unusual S2 state properties in *Synechocystis* sp. (Li and Burnap 2002). These data demonstrate the importance of arginine residues in PSII water oxidation activity.

Besides R334, the D1 polypeptide also contains an arginine residue at position 323, where it can potentially participate in the coordination sphere of the Mn_4/Ca complex (probably through ionic interactions) and/or the binding of substrate (hydroxyl ions) or cofactors associated with the catalytic center (e.g., Cl^-). There are several more specific arguments indicating the potential significance of this residue in the function of the OEC, including: (a) D1-R323 is located near (16–18 Å) the metal cluster (Loll et al. 2005); (b) D1-R323 is conserved in the PSII reaction centers of all examined O_2-evolving organisms (Svensson et al. 1991); and (c) there is similarity between the amino acid sequences of the D1 and D2 polypeptides at their C-terminal ends, including D1-R323 and D2-R327 (Semin and Parak 1997), which, together with the Mn-binding amino acids, D1-H332 and D1-E333, and the D2-H337/D2-E338 motif, provide symmetry between COOH-ends of the reaction center polypeptides.

As a consequence, we hypothesized that replacement of the positively charged arginine in position D1-R323 by either uncharged or negatively charged amino acids might alter the structure of the OEC and inhibit O_2-evolving activity in *C. reinhardtii* cells. Therefore, we substituted R323 with histidine (positively charged or neutral, depending on its local environment), asparagine (uncharged), aspartic acid and glutamic acid (negatively charged), or leucine (contains no polar atoms on its side chain). The result was that the photochemical activity was increasingly affected in mutants WT < R323H < R323N < R323D < R323E < R323L (details about the intrinsic properties of the different mutants are provided in the Appendix). We suggest that the observed phenotypes are the result of increasingly impaired interactions between the OEC and the reaction center photochemistry of PSII. It is possible that the replacement of positively charged D1-R323 affects local ion-binding domains that could lead to the observed decreases in photochemical activity. Nevertheless, as seen below, loss of photochemical activity did not translate into high rates of H_2 photoproduction following sulfur deprivation. On the contrary, decreased photochemical activity resulted in lower starch accumulation during the initial stages of sulfur deprivation. This in turn translated into longer times until H_2 appeared, lower rates of H_2 production, shorter durations of the H_2-photoproduction stage, and, in some cases, higher accumulation of alternative fermention products, such as acetate and formate (which does not depend on photosynthetic electron transport).

Materials and methods

Generation of site-directed mutants

Site-directed mutations were introduced into the *C. reinhardtii psb*A gene using the method of Kunkel et al. (1987). A modified *psbA* gene (coding for the D1 protein) was generated in plasmid pBA155 (Minagawa and Crofts 1994) and introduced into the *psbA* deletion mutant strain, CC-741, using a particle gun. The pseudo-wild-type (pWt) control strain was created by re-introducing an unmodified, wild-type *psbA* gene into the CC-741 mutant. Transformants were selected on the basis of spectinomycin and streptomycin resistance conferred by the *aadA* gene (Ruffle et al. 2001). All mutations were confirmed by DNA sequencing.

Culture growth and Chl determination

The D1 mutants, R323H, R323N, R323D, R323E, and R323L, as well as pWt were grown at 25°C either photomixotrophically in TAP medium at pH = 7.2 (Harris 1989) or photoautotrophically in Basal Salts medium (Flynn et al. 2002). The growing cultures were continuously purged with a mixture of air and 3% CO_2 and illuminated with cool-white fluorescence light (60–70 μE m^{-2} s^{-1}, PAR). Chlorophyll $a + b$ content in the samples was assayed spectrophotometrically in 95% ethanol extracts by the method of Spreitzer (Harris 1989). The number of algal cells per unit volume was counted by optical microscopy using a hemocytometer-type counting chamber. The R323N and R323L mutants will be described in the Appendix, but for the sake of simplicity, they will not be discussed in the text except in Fig. 1.

Measurements of PSII activity

The activity of PSII was recorded as the rate of O_2 evolution (Table 1), measured with a Clark electrode (CB1-D, Hansatech Instruments LTD, Kings Lynn, England) at 28°C. Cell samples (4 ml) were taken from growing cultures at mid-logarithmic phase, placed in an O_2-electrode chamber, equilibrated with air, and then dark-adapted for 2 min. After addition of 80 μl of 0.5 M $NaHCO_3$ to a final concentration of 10 mM, the samples were illuminated with saturating light (~1000 μE m^{-2} s^{-1} of heat-filtered incandescent light) for 5 min. The observed rates of O_2 evolution were corrected for the rates of dark respiration, measured for 4 min at the end of the illumination period.

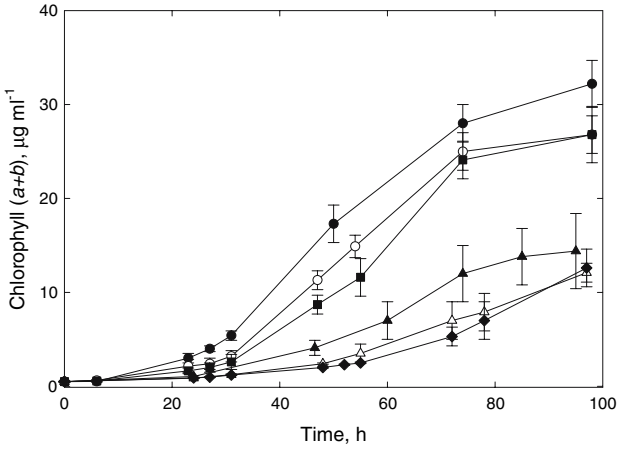

Fig. 1 Chlorophyll accumulation in *C. reinhardtii* pWt (closed circles) and the R323H (open circles), D1-R323N (closed squares), D1-R323D (closed triangles), D1-R323E (open triangles), D1-R323L (closed diamonds) mutants. The cultures were grown photomixotrophically on TAP medium. Each growth curve represents the average of five experiments, and the error bars represent ± one standard deviation

The photochemical capacity of PSII was estimated from flash-probe fluorescence yield measurements in the presence of DCMU with a home-built instrument (Ghirardi et al. 1996). Samples, taken directly from the growth vessel, were concentrated (total chlorophyll concentration, 40 μg/ml), incubated in the dark for 5 min, and then measured in the presence of 30 μM DCMU. Fluorescence was initiated with a saturating, single-turnover actinic flash and probed with low-intensity probe flashes, and the resulting fluorescence yield profiles were measured as $(F-F_0)/F_0$. Data were collected and analyzed using Data Transition Global Lab software.

Measurements of the chlorophyll *a* fluorescence yield (PSII photochemical activity), as well as the fluorescence induction kinetics were obtained with a PAM-2000 fluorometer (Walz, Germany), using the procedures described in Antal et al. (2003) and Kosourov et al. (2005). For recording fluorescence induction kinetics, the samples were removed from the culture vessel aerobically and placed in a transparent plastic cuvette with a closely affixed optical fiber probe. After dark adaptation for 1 min, the cells were illuminated with actinic light ($\lambda < 710$ nm, 450 μE m^{-2} s^{-1}) applied on top of weak measuring modulated light (λ, 655 nm; 0.3 μE m^{-2} s^{-1}). In experiments where the F_v/F_m value was measured on samples withdrawn from the culture aerobically (Table 1), an 0.8-s saturating actinic excitation pulse ($\lambda < 710$ nm, 6000 μE m^{-2} s^{-1} PAR from an 8V/20W halogen lamp) was applied after dark adaptation. The Chl concentration in the samples was 12–13 μg Chl/ml.

In situ measurements of the Chl *a* fluorescence yield (Figs. 2 and 3) were also performed with a PAM-2000 fluorometer, as previously described (Antal et al. 2003; Kosourov at al. 2005). An optical fiber probe was affixed closely to the surface of the illuminated photobioreactor (the ambient actinic illumination was about 250 μE m^{-2} s^{-1}), and an 0.8-s saturating actinic excitation pulse was applied every 15 min. The efficiency of photochemical conversion of absorbed light energy in PSII (the photochemical activity) was calculated as $\Delta F/F'_m = (F'_m - F_t)/F'_m$, where F_t is the fluorescence yield of the cells exposed to the ambient light in the reactor vessels, and F'_m is the fluorescence yield following the application of a saturating actinic pulse to the illuminated cells (Schreiber et al. 1995).

Sulfur deprivation

The D1 pWt and the R323H, R323D, and R323E mutants, chosen for these experiments, were grown photomixotrophically, harvested at mid-logarithmic phase, and washed three times in TAP-minus-sulfur medium (all procedures

Table 1 Characterization of *C. reinhardtii* D1-R323 mutant cultures during the mid-logarithmic phase of growth on TAP medium

Strain	Chlorophyll (a + b) content per cell, μg ($\times 10^{-6}$)	Chl a/b ratio	Oxygen evolution, μmol O_2 (mg Chl)$^{-1}h^{-1}$	F_v/F_m*, rel. units	Relative content of PSII active centers**, % of pWt	Q_B-non-reducing PSII centers***, % of total PSII centers
PWt	5.33	2.6 ± 0.2	148 ± 21	0.70 ± 0.04	100	32 ± 3
R323H	5.11	2.4 ± 0.1	92 ± 18	0.53 ± 0.06	95 ± 3	43 ± 2
R323N	5.80	2.4 ± 0.2	82 ± 12	0.48 ± 0.06	82 ± 5	47 ± 2
R323D	6.12	2.5 ± 0.1	74 ± 15	0.3 ± 0.08	42 ± 4	50 ± 3
R323E	5.17	2.1 ± 0.3	30 ± 15	0.15 ± 0.10	27 ± 6	70 ± 5
R323L	5.00	2.0 ± 0.3	0	–	–	–

* Measurements of F_v/F_m values were obtained with a PAM-2000 on dark adapted samples withdrawn from the aerobic cultures as described in the "Materials and Methods"

** F_{max} values from the flash-probe fluorescence decay kinetics were analyzed (see Appendix, Fig. 3), and the kinetics was recorded in the presence of DCMU as described in the "Materials and Methods"

*** Measurements of the fluorescence induction kinetics were obtained with a PAM-2000 (see Appendix, Fig. 2) as described in the "Materials and Methods". The fluorescence induction kinetics was analyzed by the method of Lazár (1999)

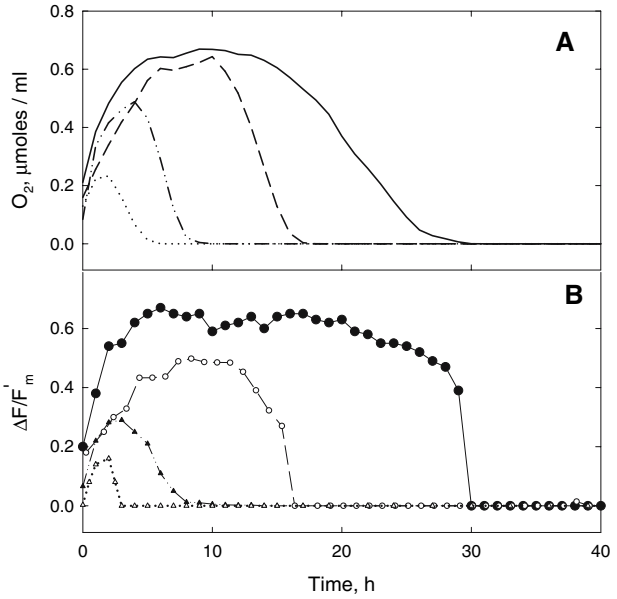

Fig. 2 Changes in dissolved oxygen (**A**) and in situ photochemical activity ($\Delta F/F'_m$) (**B**) during the incubation of pWt (solid line, closed circles) and the D1-R323H (dashed line, open circles), D1-R323D (dashed-dotted line, closed triangles), and D1-R323E (dotted line, open triangles) mutants under sulfur-deprived conditions

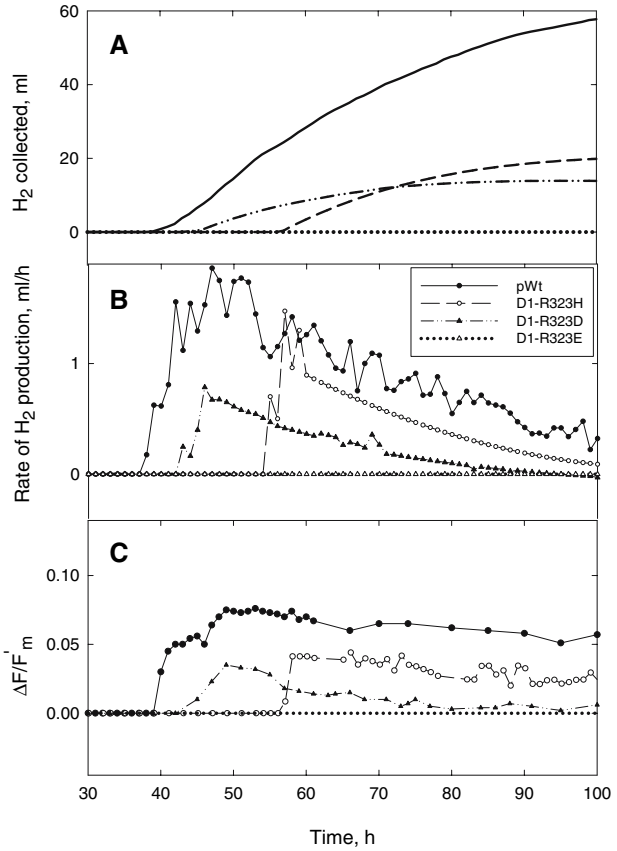

Fig. 3 The total yield of H_2 produced (**A**), the rate of H_2 production (**B**), and the in situ photochemical activity ($\Delta F/F'_m$) (**C**) during the incubation of pWt (solid line, closed circles) and the D1-R323H (dashed line, open circles), D1-R323D (dashed-dotted line, closed triangles), and D1-R323E (dotted line, open triangles) mutants under sulfur-deprived conditions

were described by Kosourov et al. 2003). Sulfur-deprived cell suspensions of pWt and the R323H, D, and E mutants (about 12–14 µg Chl/ml) were placed in four specially fabricated, 1.2 l glass photobioreactors with a built-in port for an O_2 sensor. Dissolved O_2 (Fig. 2) was measured with this sensor, and the volume of H_2 gas produced by each culture (Fig. 3) was measured, as the weight of gas-displaced water on a digital balance (Kosourov et al. 2002). The data from the sensors and balances were continuously recorded with an integrated microprocessor system. The photobioreactors were placed under two-sided illumination (250 µE m^{-2} s^{-1}, PAR) from cool-white fluorescence lamps, and the cultures inside the photobioreactors were mixed using magnetic stirs.

Starch, acetate, and formate determinations

The samples for starch assay were taken directly from the bioreactors and centrifuged for 5 min at 2,000*g*. The pellets were stored frozen at -80°C until all samples were ready for processing. Starch levels inside the cells were determined in the pellet on a per ml of culture basis according to the method of Gfeller and Gibbs (1984). Starch was measured as glucose equivalent (180.2 g mol^{-1}). The levels of acetate and formate in the medium were determined by HPLC (Model 1050, Hewlett-Packard, USA) using an ion-exchange column (Aminex HPX-87H, Bio-Rad, Hercules, USA) and 4 mM H_2SO_4 as the mobile phase.

Results and discussion

Growth characteristics of the D1-R323 mutants

Figure 1 shows that under photomixotrophic conditions all of the R323 mutants tested were affected in growth. Longer doubling times (from 10 h with the pWt to 25 h with the R323L as measured by rates of Chl accumulation) and lower maximal Chl contents at the end of the logarithmic phase (from 33 µg/ml in the pWt to 12 µg/ml in the R323L) were observed as a result of the mutations. While the R323H mutant was able to grow photoautotrophically at rates comparable to those of the pWt, the R323N and R323D mutants grew photoautotrophically only at very low rates (data not shown). The D1-R323E and D1-R323L mutants could not grow photoautotrophically at all.

Despite their lower growth rates, no significant differences were found in the total Chl content per cell in pWt compared to the mutant strains (Table 1). The Chl *a/b* ratio values were similar in the pWt, the R323H, R323D, and

R323D-mutants, but they were slightly lower in the R323E and R323L mutants, suggesting enhanced LHC content per reaction center in the latter two cases. In all other experiments we will report results for only the pWt, and the R323H, R323D, and R323E mutants.

Characterization of PS II activity in the D1-R323 mutants

The activity of PSII was initially determined from the rate of O_2 evolution by each culture. This rate decreased progressively as follows: pWt > R323H > R323D > R323E mutant. However, the decrease in rate was not due to the lack of assembled PSII complexes, as indicated by EPR spectroscopy (see the Appendix, Fig. 5 for details).

In order to evaluate whether the low O_2-evolution rates are due to the loss of PSII photochemical capacity by the D1-R323 mutants, we measured the maximum fluorescence yield (F_{max}) in dark-adapted samples, using the flash-probe fluorescence technique (see Materials and Methods). F_{max} is proportional to the amount of Q_A^-, the primary acceptor, reduced after flash-induced charge separation in PSII when detected in the presence of DCMU (Nixon et al. 1992b). From the F_{max} values, we estimated the number of photochemically active PSII centers (Table 1), which were shown to decrease according to pW > R323H > R323D > R323E.

The presence of photochemical activity does not necessarily imply the ability to convert the initial charge-separated state into useful photosynthetic reductants. The ratio of Q_B-reducing to Q_B-non-reducing centers determines the actual capacity of PSII centers to provide electrons to the photosynthetic electron transport chain (Guenther et al. 1990). Analysis of the fluorescence induction kinetics recorded with dark-adapted cells can give information about this ratio in the mutants (Krause and Weis 1991; Lazar 1999). Notable increases in the number of Q_B-non-reducing PSII centers in the mutants as compared to pWt were also observed (Table 1; see also Appendix, Fig. 6 for details). The deceleration in the rate of PQ-pool reduction (characteristic of an increase in the number of Q_B-non-reducing centers) was probably due to disturbance of electron donation from the OEC to the reaction center, since the R323 residue is located after the last trans-membrane alpha helix near the carboxyl end of the D1 protein (see Appendix, Fig. 7 for more conclusive evidence).

In summary, mutations on the D1-R323 residue affect PSII function by (a) decreasing O_2-evolving activity, (b) decreasing PSII photochemical activity, (c) increasing the relative number of PSII Q_B-non-reducing centers, and (d) impairing electron transfer from the OEC to the PSII reaction center. The observed loss of PSII activity correlates with the charge on the amino acid residue substituting for arginine in the D1-R323 position (Table 1). The replacement of arginine with histidine (positive/neutral) lowered the F_v/F_m ratio by 25%, increased the number of Q_B-non-reducing centers from 32 to 42%, and resulted in a 30–35% lower rate of O_2 evolution in the R323H mutant compared to the pWt. The most dramatic changes in the O_2-evolving activity were found when the positively charged arginine residue was replaced by negatively charged amino acid residues (R323D and R323E). In these mutants, the rate of O_2 evolution dropped to 50% and 20%, respectively, while the remaining photochemical activity decreased to only 40–20% of that found in the pWt (Table 1). The relative amount of Q_B-non-reducing centers in these two mutants comprised 50–70% of the total PSII centers.

Hydrogen production and photochemical activity of the D1-R323 mutants under sulfur-deprivation

Sulfur deprivation of green algae causes the gradual inactivation of PSII function in cells (Wykoff et al., 1989) but a residual level of 5–10 % of the initial PSII O_2-evolving activity persists throughout the H_2-photoproduction phase (Melis et al. 2000). This residual PSII activity plays a central role in H_2 production by providing most of the electrons for the hydrogenase-driven reaction (Ghirardi et al. 2000; Antal et al. 2003). However, maintenance of anaerobiosis during the H_2-photoproduction phase is highly dependent on the accumulation of starch, which occurs mainly during the early stages of sulfur deprivation (Tsygankov et al. 2002; Zhang et al. 2002) and is also dependent on PSII activity. In order to evaluate the correlation between H_2 photoproduction and PSII activity, we sulfur-deprived pWt and the R323H, R323D, and R323E mutants, and then studied subsequent H_2-production activity. Analysis of the photoinduced EPR signal attributed to the cation-radical $P700^+$ demonstrated that none of the mutations localized on D1-R323 affected PSI activity (data not shown).

We previously showed that the process of H_2 production in wild type *C. reinhardtii* cells under sulfur-deprived conditions involves the sequential transition through the following five phases: O_2 evolution, O_2 consumption, anaerobic, H_2 production and termination (Kosourov et al. 2002). As seen in Fig. 2A, the O_2-evolution period varied from <2 to 10 h in the different mutant cultures. In the pWt and R323H cultures, the maximum level of dissolved O_2 was reached after about a 10 h incubation period. However, the O_2-evolution period lasted only about 4 h in the R323D-mutant and <2 h in the R323E-mutant cultures. The duration of the subsequent O_2-consumption period exhibited the same trend, longer in pWt (20 h) and shortest in the R323E mutant (3 h). Table 2 summarizes our data for the duration of the aerobic phase (O_2 evolution + O_2

consumption), which gradually decreased from 30 to 5 h (pWt > R323H > R323D > R323E). The establishment of anaerobic conditions in the photobioreactor is driven by cellular respiration but depends significantly on the loss of O_2-evolving activity in cells, which proceeds gradually after removal of sulfate from the medium (Melis et al. 2000). Therefore, it is not surprising that the cultures with low initial PSII activity transition to anaerobiosis faster when acetate is present in the medium.

The efficiency of photochemical conversion (photochemical activity) of absorbed light energy in PSII (PAM measurement of $\Delta F/F'_m$), measured in situ, increased during the O_2-evolution phase, as described previously by Antal et al. (2003). The maximum $\Delta F/F'_m$ values (Fig. 2B) were lower in the mutants (70% in R323H, 40% in R323D and 20% in R323E) relative to that of the pWt (100% = 0.7 r.u.). As expected, these data are consistent with the F_v/F_m data obtained with dark-adapted samples withdrawn from pWt and mutant cultures during growth on sulfur-replete, TAP-medium (Table 1). A gradual decrease in $\Delta F/F'_m$ that occurs at the beginning of the O_2-consumption phase was observed with both pWt and the R323H mutant. The slow decrease was followed by an additional rapid declined in the $\Delta F/F'_m$ ratio as the O_2 concentration in the culture suspension reached zero (Fig. 2B; see also Antal et al. 2003). The R323D and R323E mutants exhibited only a gradual decrease in the efficiency of photochemistry during the O_2-consumption phase (due to gradual changes in the individual F'_m and F_t values), reaching zero after 8 h and about 3 h of sulfur deprivation, respectively; the sharp drop in efficiency was not observed in these mutants. Fluorescence induction curves measured with samples withdrawn from the photobioreactor during the O_2-evolution phase (data not shown) indicated a gradual over-reduction of the PQ pool over the time course of sulfur deprivation. Similarly, kinetics obtained from flash-probe fluorescence yield measurements in dark-adapted, sulfur-deprived cells confirmed the decreasing number of photochemically active PSII centers in pWt and mutants cultures (data not shown). As expected, mutations that resulted in lower O_2 production also transitioned faster to anaerobiosis in the presence of exogenously-added acetate.

The duration of the H_2-production phase decreased as the duration of the aerobic phase decreased (see mutants R323H and R323D in Table 2). The total yield of H_2 produced by the pWt cells was 60–80 ml (from experimental data), which was 3 to 4 times higher than the yield of H_2 produced by either R323H or R323D (Fig. 3A). The rates of H_2 production were not constant and changed significantly with time (Fig. 3B) as observed previously (Kosourov et al. 2003). The rates rose at the beginning of the H_2-production phase, reaching a maximum after 10–15 h (pWt) and 5 h (R323H and R323D), and then declined gradually thereafter. The maximum rates of H_2 production decreased to 75% and 40% in the R323H and R323D mutants, respectively, as compared to the pWt. When H_2 production began, the recovery of the photochemical activity of PSII was partially restored (Fig. 3C; Antal et al. 2003). A correlation between the H_2-production rates and the $\Delta F/F'_m$ values was observed in pWt and all mutants during the H_2-production phase. The maximum $\Delta F/F'_m$ value of R323H was 65% and that of R323D was 50% compared to the pWt. Finally, the R323E mutant culture achieved an anaerobic state, but H_2 production was not observed.

Starch accumulation and degradation during sulfur deprivation

Starch degradation during the anaerobic stage of sulfur deprivation plays a dual role in H_2 production. First, starch respiration removes O_2 generated photosynthetically and, thus, maintains anaerobic conditions in algal cultures in the light. Second, it can also provide reductants for H_2 photoproduction (Gfeller and Gibbs 1984; Ghirardi et al. 2000).

Figure 4A shows that the highest amount of starch accumulated during the aerobic phase of sulfur deprivation in the R323H and R323D mutants was half that in pWt. This observation could be easily explained by the differences in the photosynthetic activity of each strain during the initial aerobic period of sulfur deprivation, as shown in Fig. 2. The starch accumulated in the pWt and R323H mutant was gradually degraded during the anaerobic and H_2-production phases (compare Fig. 4A with Table 2). In

Table 2 Duration of the different phases of sulfur-deprivation in pWt and the D1-R323(H,D,E) mutant cultures

Strain	Aerobic phase duration (h)	Anaerobic phase before onset of H_2-production duration (h)	H_2-production phase duration (h)
pWt	30–36	6–11	52–93
R323H	20–21	35–36	42–78
R323D	8–10	34–35	39–52
R323E	5–6	Stays anaerobic after transition	0

The data presented are ranges obtained from four independent experiments

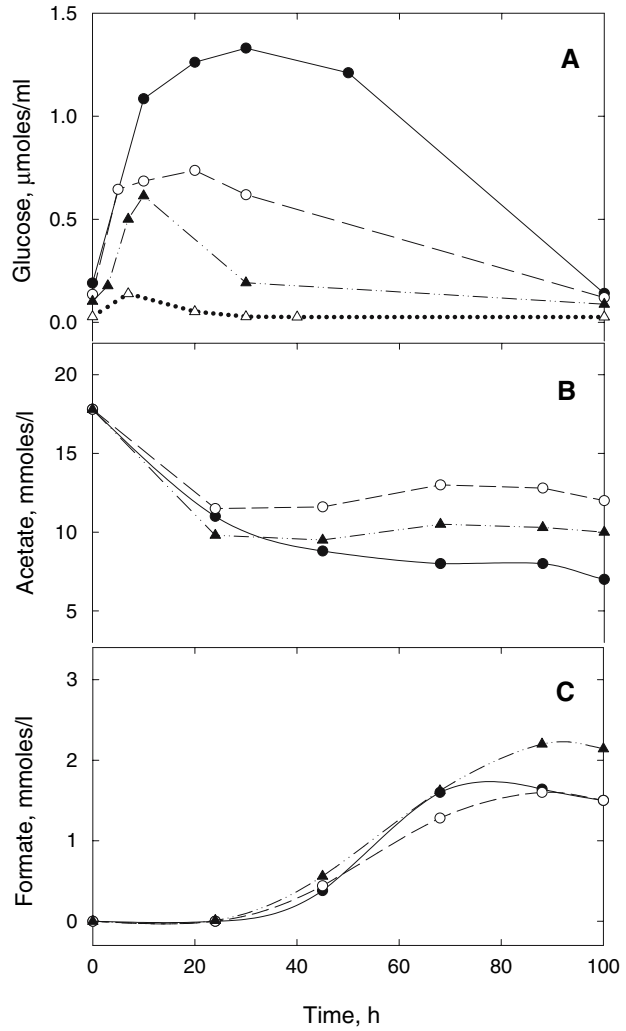

Fig. 4 Accumulation of starch as glucose equivalent (**A**), acetate (**B**) and formate (**C**) content in pWt (solid line, closed circles) and the D1-R323H (dashed line, open circles), D1-R323D (dashed-dotted line, closed triangles), and D1-R323E (dotted line, open triangles) mutants under sulfur-deprived conditions

the R323D mutant, most of the accumulated starch was degraded during the anaerobic phase, before the onset of H_2-production. Very little starch was accumulated during the short aerobic phase in the R323E mutant, and no H_2 was produced as a result. The consumption of acetate by pWt cells gradually decreased before the onset of H_2-production, and then the acetate remained constant (Fig 4B). A similar pattern was detected in both of the H_2-producing R323H and R323D mutants, except that the acetate concentration increased a bit after the end of the anaerobic phase. Acetate consumption during the aerobic phase has been suggested to be important for concomitant accumulation of starch, and its function can be partially replaced by supplementation with extra CO_2 or bicarbonate to heavily-buffered medium (Tsygankov et al. 2006; Kosourov et al. 2007). On the other hand, the amount of acetate produced during the anaerobic phase of sulfur deprivation must reflect the slightly higher rate of starch degradation compared to the residual PSII activity in the mutants. The same rationale applies to the observation that no significant difference in the concentration of formate was observed until about 30–40 h of sulfur deprivation, and that the R323D mutant culture consistently accumulated about 30% more formate after 80 h of sulfur deprivation compared to the pWt and R232H (Fig. 4C).

Finally, it is worth mentioning that the presence of less stored starch in the PSII mutants resulted in a slower transition to H_2 production (Fig. 3B). This effect is due to lower transcription of the hydrogenase genes, when starch is not present (Posewitz et al. 2004). Although the exact signal transduction mechanism is not known, the involvement of the redox state of the plastoquinone pool has been invoked, as a possible intermediate in the process of the activation/inactivation of H_2 production (Ghirardi et al. 2005) and hydrogenase gene expression (Ghirardi et al. 2007).

Conclusions

In this report, mutants with intact PSI function, but decreased PSII photochemical activity, exhibited significant changes in the normal parameters associated with the H_2-photoproduction process under sulfur-deprived conditions. These include a decreased aerobic phase, lower accumulation of starch, a prolonged anaerobic phase before the onset of H_2-production, shorter duration of H_2-production, lower H_2 yield as compared to pWt, and slightly higher production of dark fermentation products, such as acetate and formate. The more compromised the PSII photochemical activity, the more dramatic was the effect on the H_2-production process, which depends both on the presence of residual PSII activity and the amount of stored starch. Thus, the R323D mutant, which exhibits about half the O_2-evolving and photochemical activities of the pWt was able to sustain about 1/3 of the H_2-production activity of the pWt under sulfur-deprived conditions, while the R323E mutant, with only 20% of O_2-evolving activity and photochemical activities of pWt, was unable to photoproduce H_2 at all. Additionally, a delay in H_2 production was observed in the mutants, which correlates with the amount of stored starch.

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Appendix

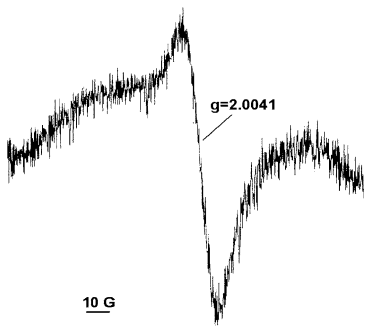

Fig. 5 EPR spectrum of pWt cells sampled during the mid-logarithmic phase of growth. The amount of assembled PSII complex was determined from EPR measurements of the SII signal performed with a Bruker E500 spectrometer at low temperature (77 K). All samples exhibited a narrow (line width, ΔH = 9–12 Gauss) EPR signal at g = 2.0041, attributable to presence of the Y_D^+ paramagnetic species. No significant difference was found in the EPR signal parameters or the concentration of Y_D^+ spins, calculated on a Chl basis, between the pWt and any of the R323 mutants. The EPR signal intensities of the mutants normalized per mg of Chl varied within 20% of the pWt signal, which is shown above. Sample size, 0.3–0.4 ml; total Chl concentration, 1.5–2 mg ml^{-1}. EPR recording conditions: frequency, 9.4 GHz; power, 6 mW; field modulation amplitude 1G; field modulation frequency, 100 kHz; single scan. The spectrum was recorded in dark

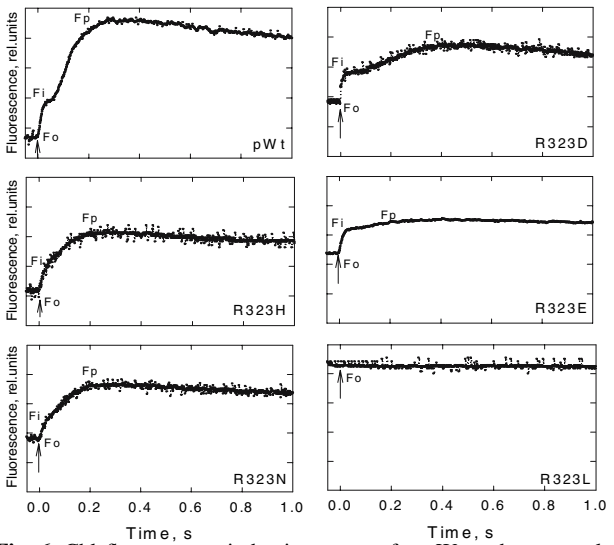

Fig. 6 Chl fluorescence induction curves for pWt and mutant algal culture samples grown in sulfur-replete TAP medium. All curves were measured with a PAM fluorometer. The samples were removed from the bioreactors during the mid-logarithmic phase of growth, adjusted to equal Chl concentration, and dark adapted for 2 min before measurements. The fluorescence induction curve of the control pWt sample exhibited two components. The fast component (from F_0 to F_i) reflects the accumulation of reduced Q_A in Q_B-non-reducing PSII centers and the presence of PSII centers with a doubly-reduced Q_B prior to illumination. The slow component (from F_i to F_p) represents the subsequent accumulation of reductants in the PQ-pool at high light intensity. The induction curves measured with the R323H and R323N mutants had shapes similar to that of pWt. However, the F_0 level was increased, and the maximal fluorescence yield was reduced compared to the pWt. The R323D curve had an intermediate character. The fluorescence induction curve measured with the R323E mutant exhibited a barely-detectable, residual, slow-rise component. The halftime for transition from the F_i to the F_p level (in ms), which is a measure of the time it takes to reduce the PQ pool, also increased progressively in the mutants: pWt (170), R323H (240), R323N (270), and R323D (390)

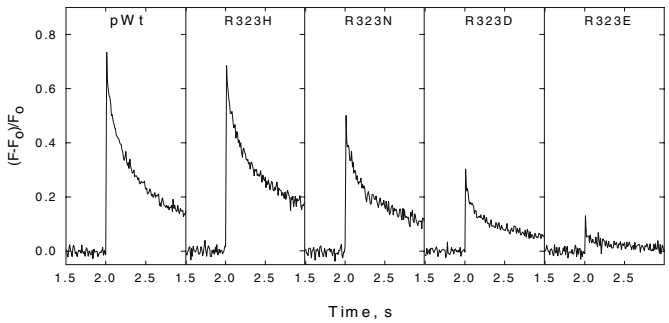

Fig. 7 Flash-probe chlorophyll fluorescence decay kinetics of pWt and R323H,N,D,E cells in the presence of DCMU (which blocks $Q_A \rightarrow Q_B$ electron transfer). Samples were taken from the bioreactors during the mid-logarithmic phase of growth on TAP medium, concentrated by centrifugation to a Chl concentration of 40 μg ml^{-1}, and dark adapted for 5 min before measurement

Table 3 Flash-induced fluorescence decay after a single flash

Strain	Component 1		Component 2	
	$t_{1/2}$ (ms)	(%)	$t_{1/2}$ (ms)	(%)
PWt	35 ± 2	(34 ± 4)	376 ± 19	(66 ± 4)
R323H	35 ± 3	(33 ± 2)	402 ± 10	(67 ± 2)
R323N	34 ± 2	(38 ± 3)	394 ± 25	(62 ± 3)
R323D	26 ± 3	(41 ± 4)	393 ± 18	(59 ± 4)
R323E	10 ± 6	(55 ± 5)	342 ± 25	(45 ± 5)

Multi-exponential, least-squares fluorescence decay curve fitting was done using free running parameters according to: $F = F_0 + A_1e^{-k1t} + A_2e^{-k2t} + ... + A_ne^{-knt}$, where F is the fluorescence level at time t, A_n is the amplitude of the different decay phases, k_n are rate constants, and F_0 is the initial fluorescence level before the actinic flash. The highest fluorescence level, F_{max}, was used as a measure of photochemical capacity of PSII (Ghirardi et al. 1998). These measurements allowed us to monitor the back reaction from Q^-_A to either Y^+_Z or the S_2 state of the OEC. The kinetic curves were analyzed assuming two components, which yielded a satisfactory fit. The fast component, with a halftime of about 10–35 ms is attributed to an accelerated back reaction between Q^-_A and Yz^+ (Nixon and Dinner 1992a, Chu et al. 1994). The halftime of the slow component (around 400 ms) corresponds to the rate of recombination between Q^-_A and the S_2 state of the OEC (Chu et al. 1994, 1995). The increased contribution of the fast component demonstrates that the R323-mutations progressively impair electron transfer from the OEC to the reaction center, and that the mutants lack a fully functioning OEC

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