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# Effects of Extracellular pH on the Metabolic Pathways in Sulfur-Deprived, $H_2$-Producing *Chlamydomonas reinhardtii* Cultures

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**Sustained photoproduction of $H_2$ by the green alga, *Chlamydomonas reinhardtii,* can be obtained by incubating cells in sulfur-deprived medium [Ghirardi et al. (2000b) *Trends Biotechnol.* 18: 506; Melis et al. (2000) *Plant Physiol.* 122: 127]. The current work focuses on (a) the effects of different initial extracellular pHs on the inactivation of photosystem II (PSII) and $O_2$-sensitive $H_2$-production activity in sulfur-deprived algal cells and (b) the relationships among $H_2$-production, photosynthetic, aerobic and anaerobic metabolisms under different pH regimens. The maximum rate and yield of $H_2$ production occur when the pH at the start of the sulfur deprivation period is 7.7 and decrease when the initial pH is lowered to 6.5 or increased to 8.2. The pH profile of hydrogen photoproduction correlates with that of the residual PSII activity (optimum pH 7.3–7.9), but not with the pH profiles of photosynthetic electron transport through photosystem I or of starch and protein degradation. In vitro hydrogenase activity over this pH range is much higher than the actual in situ rates of $H_2$ production, indicating that hydrogenase activity per se is not limiting. Starch and protein catabolisms generate formate, acetate and ethanol; contribute some reductant for $H_2$ photoproduction, as indicated by 3-(3,4-dichlorophenyl)-1,1-dimethylurea and 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone inhibition results; and are the primary sources of reductant for respiratory processes that remove photosynthetically generated $O_2$. Carbon balances demonstrate that alternative metabolic pathways predominate at different pHs, and these depend on whether residual photosynthetic activity is present or not.**

**Keywords**: *Chlamydomonas reinhardtii* — Fermentation — Hydrogen production — pH — Photosynthesis — Sulfur deprivation.

Abbreviations: BTAP, BIS-TRIS-acetate-phosphate medium; Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-6-isopropyl-3-methyl-1,4-benzoquinone; DCIP, 2,6-dichlorophenol-indophenol; MAP, MOPS-acetate-phosphate medium; NHE, standard hydrogen electrode; PAR, photosynthetically active radiation; PhBR, photobioreactor; PQ, plastoquinone; PSII, photosystem II; TAP, TRIS-acetate-phosphate medium.

## Introduction

Sulfur is one of the six macronutrients required by algae, plants, fungi, animals and humans for growth and development (Grossman and Takahashi 2001). Sulfur is necessary for protein biosynthesis and is found in a wide range of secondary cell compounds, including glucosinolates and sulfolipids (Leustek and Saito 1999). In the absence of sulfur, *Chlamydomonas reinhardtii* cells stop dividing (Hase et al. 1958, Hase et al. 1959), accumulate and then degrade endogenous protein and starch (Melis et al. 2000, Kosourov et al. 2001, Kosourov et al. 2002, Tsygankov et al. 2002, Zhang et al. 2002), synthesize sulfatases (de Hostos et al. 1988, de Hostos et al. 1989, Takahashi et al. 2001) and high-affinity sulfate transporters (Yildiz et al. 1994), and degrade some enzymes and polypeptides (Zhang et al. 2002). These responses are of physiological significance for the cells, allowing them to maintain required energy conversion levels and efficiently utilize the limiting nutrient.

One of the most interesting adaptive responses of algae to sulfur-deprivation is their ability to reduce photosystem II (PSII) activity in the light (Davies et al. 1996, Wykoff et al. 1998, Melis et al. 2000). For example, $O_2$-evolution capacity in *C. reinhardtii* cells decreases to approximately 30% of the initial value within 24 h after sulfate is removed from the culture medium (Wykoff et al. 1998, Melis et al. 2000). Wykoff et al. (1998) suggested that the decline in photosynthetic $O_2$ evolution results from the conversion of PSII centers from the $Q_B$-reducing to the $Q_B$-non-reducing form, an intermediate in the PSII repair cycle (Guenther and Melis 1990). Accumulation of the $Q_B$-non-reducing form is believed to occur because photodamaged D1 cannot be repaired efficiently due to the lack of sulfurylated amino acids (Wykoff et al. 1998). As a consequence of the gradual inhibition of the PSII activity, the rate of $O_2$ evolution drops below the rate of respiratory $O_2$ uptake, and the algal culture transitions from an aerobic to anaerobic environment (Melis et al. 2000). This transition is followed by a reduction in the redox potential of the medium from +400 mV to about –300 mV vs. NHE (Kosourov et al. 2002). The establishment of a strong anaerobic environment in the photobioreactor results in the induction of the [Fe]-hydrogenase, and subsequently the algal culture starts to produce $H_2$.

Using a computer-monitored photobioreactor system, Kosourov et al. (2002) showed that sulfur-deprived cultures of

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*C. reinhardtii* transition through five consecutive phases: an aerobic, an $O_2$-consumption, an anaerobic, a $H_2$-production and a termination phase. They found that re-addition of small quantities of sulfate (12.5–50 μM $MgSO_4$, final concentration) back to a rigorously sulfur-deprived cell suspension at the start of the sulfur-deprivation process increases the specific rate of $H_2$ production and the total yield of $H_2$ by the system. This was attributed to the effect of sulfur on residual PSII activity, suggesting that electron transport from PSII may limit the rates of $H_2$ production by sulfur-deprived cells. Qualitatively similar experimental results were reported by Zhang et al. (2002).

The dependence of $H_2$ photoproduction on PSII activity was first reported by Ghirardi et al. (2000a) and confirmed recently by Antal et al. (2001). The latter used pulse-amplitude-modulated (PAM) fluorometry to monitor the photosynthetic activity of PSII in situ during the sulfur-deprivation process, and they observed a sharp decline in the photochemical activity of PSII at about the time that anaerobiosis was established in the culture. This decline in activity was attributed to the down-regulation of photosynthetic electron transport due to the over-reduction of the PQ pool, since at that point no photosystem I (PSI) electron acceptor was available (Zhang et al. 2002, Antal et al. submitted). During the next couple of hours of anaerobiosis, the synthesis of the hydrogenase enzyme was induced. The start of $H_2$ production and recovery of PSII activity occurred over the same period of time (Antal et al. 2001), suggesting that the hydrogenase activity released some of the PQ regulatory control by providing electron acceptors for the PQ pool through PSI.

Besides affecting photosynthesis, sulfur deprivation has significant effects on intracellular protein and starch levels. During the aerobic phase of sulfur-deprivation, the cultures are able to store significant amounts of protein and starch. However, following the transition to the $O_2$-consumption phase, starch and protein contents of the cells decrease (Melis et al. 2000, Kosourov et al. 2001, Kosourov et al. 2002, Zhang et al. 2002). The primary role of endogenous substrate degradation during sulfur deprivation is to remove the $O_2$ generated photosynthetically by the residual PSII activity, but it also contributes minor amounts of reductants to $H_2$ photoproduction (Ghirardi et al. 2000a). Others have previously shown that starch degradation serves as a source of reductants for green algal $H_2$ production under sulfur-replete conditions (Yanyushin 1982, Gfeller and Gibbs 1984, Ohta et al. 1987). However, during the anaerobic and $H_2$-production phases of the sulfur-deprivation process, we have found that large amounts of fermentation products are also co-generated (Tsygankov et al. 2002). These observations lead to the following questions: what are the contributions of different metabolic pathways to the survival of algal cells under these conditions, and how can they be optimized for $H_2$ production?

We investigated the metabolism of sulfur-deprived *C. reinhardtii* by studying the effects of the extracellular pH, set at the time when sulfur is removed from the culture medium (i.e. the initial pH) on $H_2$ production, residual PSII capacity, photosynthetic electron transport through PSI, protein and starch degradation, and the accumulation of fermentation products. We also studied the effect of pH on in vitro hydrogenase activity. We reasoned that, by understanding the relationship between the different metabolic pathways, we might be able to find the conditions that favor photosynthetic $H_2$ production. Our results show that fermentation predominates at an initial pH below 7.3, while PSII capacity and $H_2$ production are maximal at initial pHs around 7.0–7.7. Photosynthetic electron transport through PSI, on the other hand, increases as a function of the initial pH between 6.8 and 8.2. Ethanol production is completely inhibited when $H_2$ production is maximal, perhaps because reductants from endogenous substrate degradation are preferentially utilized by the NAD(P)H-PQ oxidoreductase to fuel extra $H_2$ photoproduction. Consequently, maximum $H_2$ photoproduction activity occurs at an initial pH of 7.7.

## Results and Discussion

The metabolism of anaerobically induced *C. reinhardtii* cells, as related to $H_2$ photoproduction is complex and involves fermentative, photosynthetic and respiratory pathways, depending on whether the cells are illuminated or not (Gfeller and Gibbs 1984). Sulfur-deprivation further complicates the situation by imposing another variable on the system. The present study attempts to examine these pathways and to determine the optimal conditions for $H_2$ photoproduction.

### *Hydrogen production*

The effect of extracellular pH on $H_2$-photoproduction by sulfur-deprived algal cultures is shown in Fig. 1. Each of the indicated pH values was established at the start of the sulfur-deprivation process (see Materials and Methods). It should be noted that the pH of the medium in the batch culture changes with time, increasing by about 0.6 of a pH unit during the $O_2$ production and $O_2$-consumption stages and decreasing gradually by 0.4 of a pH unit during the anaerobic and $H_2$ production stages (Kosourov et al. 2002). The initial increase in the pH is due to photosynthetic consumption of dissolved $CO_2$ and utilization of acetate, while the subsequent decrease in the pH is the result of $CO_2$ release (Kosourov et al. 2002) and fermentative accumulation of acetate and formate (Tsygankov et al. 2002).

The maximum yield of $H_2$ per photobioreactor (Fig. 1A) occurred when the pH at the start of the sulfur deprivation period was 7.7 and decreased when the initial pH was lowered to 6.5 or increased to 8.2. The corresponding rates of $H_2$ photoproduction are shown in Fig. 1B. The rates of $H_2$ production were not constant during the experiments and changed significantly with time. As a rule, the rate rose in the beginning of the $H_2$-production phase, reached a maximum value after 10–20 h of $H_2$ production, and then declined gradually thereafter. The initial increase can be explained by a corresponding increase in

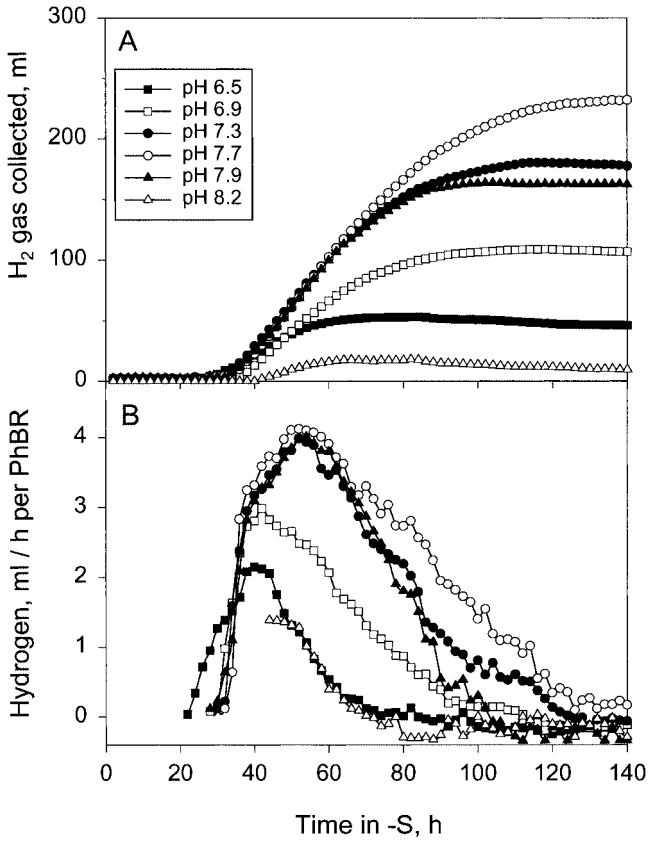


**Fig. 1** Effect of extracellular pH on (A) the total yield of $H_2$ produced and (B) the rate of $H_2$ production per photobioreactor (PhBR) by sulfur-deprived cultures of *C. reinhardtii*. The cultures were harvested at the mid-log phase, washed three times in TAP-minus-sulfur medium and resuspended in MAP- or TAP-minus-sulfur media at different pHs ($t$ = 0). All points are the mean of five to nine independent experiments. PhBR, photobioreactor.

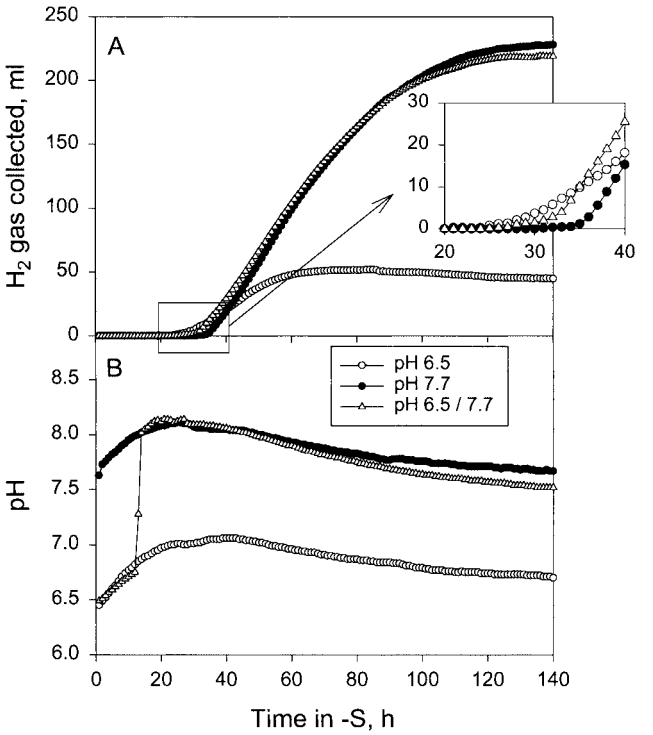


**Fig. 2** (A) Effect of a shift in the extracellular pH (open triangles) on $H_2$ production by a sulfur-deprived culture. The inset shows the changes that occur between 20 and 40 h of sulfur deprivation in more detail. (B) The pH shift was achieved by rapidly titrating the medium with 0.5 N NaOH. Controls: initial pH = 6.5, open circles; initial pH = 7.7, closed circles.

hydrogenase activity as the enzyme is induced by the establishment of anaerobiosis (Forestier et al. 2001, Zhang et al. 2002). The gradual decrease in $H_2$-production activity during the later phases of sulfur deprivation is non-specific and is accompanied by the general deactivation of many other cellular metabolic activities (Wykoff et al. 1998, Melis et al. 2000, Zhang et al. 2002). However, these decreases in rate were more pronounced at the lower and higher initial extracellular pHs. At the optimal pH of 7.7, the rate declined slower than at all other pHs, resulting in the highest total yield of $H_2$.

The maximum *specific* rate of $H_2$ production calculated on a chlorophyll basis (not shown), had exactly the same pH dependence as the total yield of $H_2$ per photobioreactor. The maximum *specific* rate was 9.4 μmol (mg Chl)$^{-1}$ h$^{-1}$ (4.1 ml h$^{-1}$ per photobioreactor) at pH 7.7, and declined to 4.9 and 3.3 μmol (mg Chl)$^{-1}$ h$^{-1}$ (2.2 and 1.4 ml h$^{-1}$ per photobioreactor) at pH 6.5 and 8.2, respectively.

The initial extracellular pH also affects the time at which $H_2$ production starts in the algal cultures (Fig. 1). When the initial pH is between 6.9 and 7.9, $H_2$ production starts at about 30 h under our conditions. Hydrogen production begins early (at about 22 h after sulfur deprivation) in the cultures deprived of sulfur at pH 6.5 and is delayed to 43 h at an initial pH 8.2. The delay observed at alkaline pH can be explained by a delay in the establishment of the anaerobic environment inside the photobioreactor under these conditions (data not shown).

In an effort to capitalize on these two desirable traits (early start of $H_2$ production at low pH and higher yield of $H_2$ photoproduction at pH 7.7), we performed a pH-jump experiment (Fig. 2). The cultures were initially deprived of sulfur at an initial pH of 6.5 and then, right before the start of the transition to anaerobiosis, the pH was rapidly increased to 8.0. This pH corresponds to the pH level observed at the equivalent time in cultures sulfur-deprived at an initial pH of 7.7 (Fig. 2B). This experiment, while succeeding in producing $H_2$ at an early time (see the inset to Fig. 2A), did not increase the final yield of $H_2$ photoproduction compared to cultures incubated at initial pH of 7.7. However, these results do show that it is possible to re-direct the metabolism of *C. reinhardtii* cells towards of $H_2$ production by changing the pH during the sulfur-deprivation process.

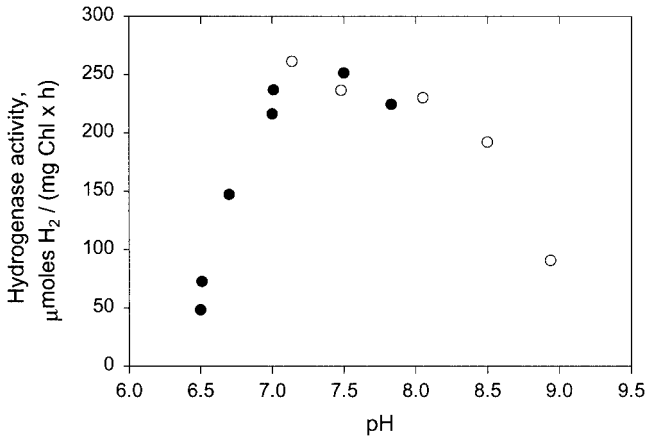


**Fig. 3** Influence of pH on hydrogenase activity by sulfur-deprived algal cells. The activity measurements were done using the dark, methyl viologen assay (Materials and Methods). Samples were taken anaerobically directly from the photobioreactor (initial pH = 7.3) at the start of the $H_2$-production phase, partially solubilized with 0.2% Trixon X100, and set at the indicated pH values before each measurement. Closed circles, 50 mM MOPS buffer (pH 6.5–7.9); open circles, 50 mM TRIS buffer (pH 7.1–9.0).

*Hydrogenase activity*

In order to determine if the observed effects of different initial extracellular pHs on $H_2$ photoproduction rates were related to the activity of the hydrogenase enzyme itself, we measured hydrogenase activity in cells harvested at the beginning of the $H_2$-production phase, using the standard methyl viologen in vitro dark assay (see Materials and Methods). In contrast to the other experiments described in this paper, the pHs used for these measurements were set in vitro, using samples taken from a culture sulfur-deprived at an initial pH of 7.3 and partially solubilized with detergent. Fig. 3 shows that the optimum in vitro pH for hydrogenase activity was about 7.0–7.4. A pH shift either to the alkaline or acidic side decreased this activity, indicating a direct effect of pH on the catalytic function of the enzyme. Roessler and Lien (1982) also observed that the optimum pH for partially purified *C. reinhardtii* hydrogenase was in the pH region close to neutral (pH 6.8–7.0) and that the hydrogenase activity was inactivated at higher and lower pH. Since the pH values shown in Fig. 3 represent the actual pH used in the hydrogenase activity measurements and not the initial extracellular pHs of cultures at the time of sulfur deprivation, as shown in Fig. 1, the two sets of data cannot be directly compared. We observed that the peak of hydrogenase activity occurs somewhat below the optimal initial pH for $H_2$ production but point out that the activity is above 50 μmol $H_2$ (mg Chl)$^{-1}$ h$^{-1}$ at all pHs measured. This in vitro activity is in excess of the highest $H_2$-photoproduction rate observed in sulfur-deprived algal cultures at any pH value (Fig. 1).

*Flash-probe chlorophyll a fluorescence yield*

To study the relationship between $H_2$ production and other cellular activities under sulfur-deprivation conditions, we examined the effect of extracellular pH on the maximum Chl *a* fluorescence yield ($F_{max}$) in sulfur-deprived *C. reinhartii* cultures. Chlorophyll *a* fluorescence yields were measured by the flash-probe method (Ghirardi et al. 1996), following addition of 30 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) to samples that had been removed from the cultures. Since $F_{max}$ in the presence of DCMU is related to the amount of reduced $Q_A$, the primary acceptor formed after flash-induced charge separation in PSII (Nixon et al. 1992), this method can be used to estimate the capacity of intact algal cells to perform PSII charge-separation function.

The top panel in Fig. 4 shows the kinetics of the flash-probe Chl *a* fluorescence yield in control, sulfur-replete samples at pH 7.3. The $F_{max}$ level of 0.6 decays to a $F_{final}$ level of

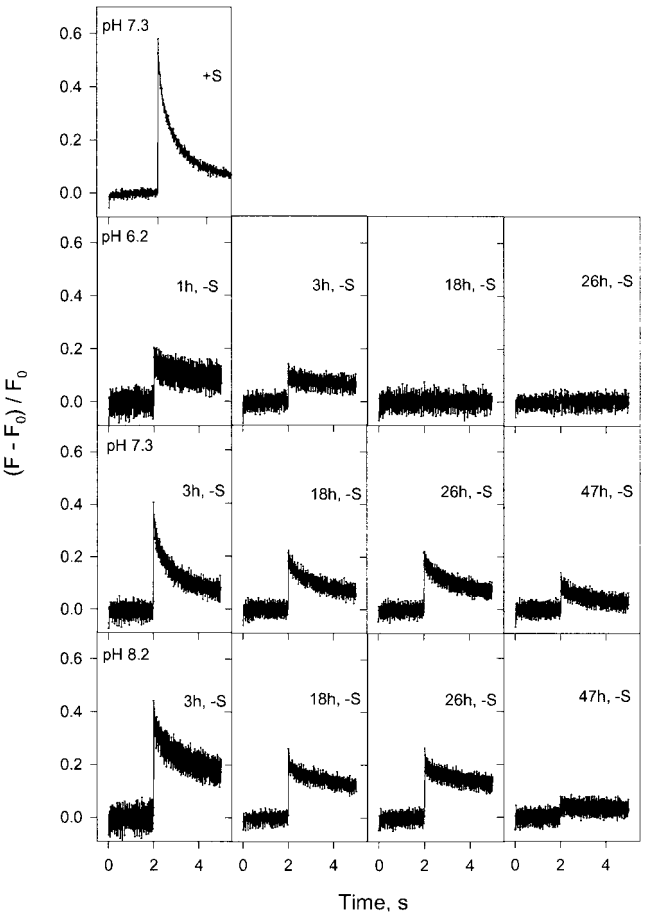


**Fig. 4** Effect of the initial extracellular pH on the flash-probe Chl *a* fluorescence yield in sulfur-replete and sulfur-deprived algal cells. Samples were taken directly from the photobioreactor at different time points following sulfur deprivation, concentrated by centrifugation to a chlorophyll concentration of 40 μg ml$^{-1}$, adapted in the dark for 5 min and placed aerobically in a 4×10 mm plastic cuvette. The fluorescence yield was initiated by a saturating actinic flash, and the variable fluorescence yield was measured as $(F–F_0)/F_0$. DCMU = 30 μM. Each curve is the average of three separate experiments.

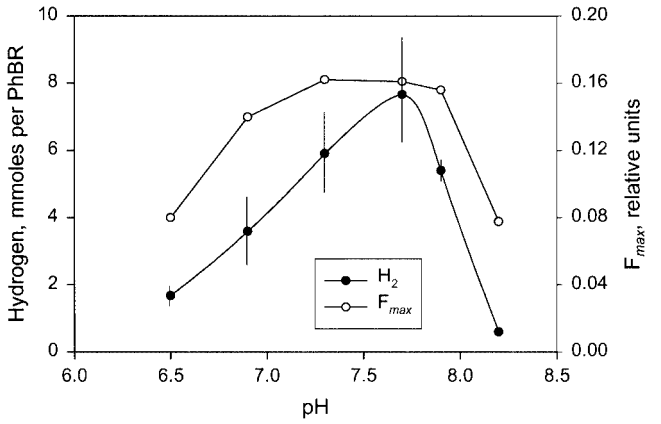


**Fig. 5** Effect of the initial extracellular pH on the amount of $H_2$ gas produced during a 140-h sulfur-deprivation experiment, and the maximum fluorescence yield ($F_{max}$, a measure of PSII capacity) determined at the start of the $H_2$-production phase. The $F_{max}$ points represent the average of three separate experiments, and errors were less than 5% (not shown).

about 0.1 that is stable during the remaining course of the experiment (not shown). The other panels show the effects of sulfur-deprivation (1–47 h) at three different initial extracellular pHs on the $F_{max}$ and $F_{final}$ of the samples. At all pHs examined, sulfur deprivation resulted in a substantial decrease in PSII photochemical capacity over time. The decrease was more pronounced and occurred at earlier times in cultures resuspended at an initial pH of 6.2 and 8.2 than at pH 7.3. For example, at pH 6.2, the $F_{max}$ of a sample incubated in sulfur-depleted medium for 1 h is 1/3 of the control sample before sulfur depletion, and reaches zero after 18 h of incubation. While this pH resulted in a very early transition of the culture to an anaerobic environment, the algal culture did not produce $H_2$ (data not shown). At an initial pH of 7.3, the decline of the PSII activity upon sulfur deprivation was much more gradual ($F_{max}$ decreased to about 75% of the value in control, sulfur-replete samples after 3 h of incubation), and substantial PSII activity was still observed at $t$ = 47 h, close to the peak of the $H_2$-production phase (Fig. 1B). At an initial pH of 8.2, PSII was inactivated by about 50% after 18 h of sulfur-deprivation. Almost complete inactivation was observed at 47 h, and this explains the low $H_2$ yield seen in Fig. 1A at this pH.

Finally, we plotted the amount of residual PSII capacity (measured as $F_{max}$ at the peak of the $H_2$-production phase in Fig. 1B) and the total amount of $H_2$ photoproduced as a function of the initial pH. Fig. 5 shows that both parameters peaked, respectively, at pH 7.3–7.9 and pH 7.7, and decreased at lower and higher pHs.

*Starch and protein breakdown and fermentative metabolism*

Changes in the concentrations of starch and protein upon sulfur deprivation at different initial pHs of the medium are examined in Fig. 6. As a rule, algal cells accumulate protein

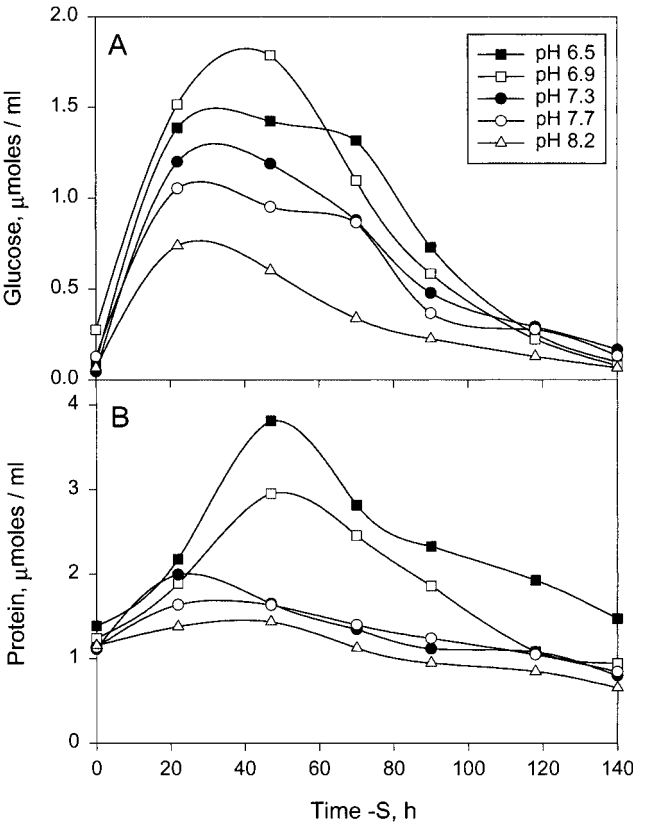


**Fig. 6** Influence of the initial extracellular pH on (A) starch and (B) protein content in sulfur-deprived cultures of *C. reinhardtii* at different times after sulfur deprivation. The figure shows points from one experiment but similar trends were observed in replicate experiments.

and starch during the aerobic phases of sulfur deprivation and degrade them during the anaerobic and $H_2$-production phases (Melis et al. 2000, Kosourov et al. 2001, Kosourov et al. 2002, Zhang et al. 2002, Tsygankov et al. 2002). Based on the data from Fig. 6, we calculated the quantities of starch and protein produced and degraded before and during the $H_2$-production phase, respectively. These data are shown in Fig. 7. Fig. 6 and 7 show data for only one experiment. However, similar trends were observed in a second series of experiments with a different sample (not shown). Starch accumulation during the early stages of sulfur deprivation peaks at pH 6.9 and decreases at lower and higher pHs, while protein accumulation peaks at pH <6.9. The starch and protein degradation activities show similar pH responses, but their pH profiles do not correlate with those for either PSII of $H_2$ photoproduction activities shown in Fig. 5.

We have shown previously (Tsygankov et al. 2002) that $H_2$ production in sulfur-deprived *C. reinhardtii* cultures is accompanied by the accumulation of formate, ethanol and acetate in the extracellular medium. Fig. 8 and 9 show that the accumulation of fermentation products predominates at low pHs, where starch and protein degradation activities are highest (Fig. 7). Ethanol production does not occur at pH 7.3 and 7.7,

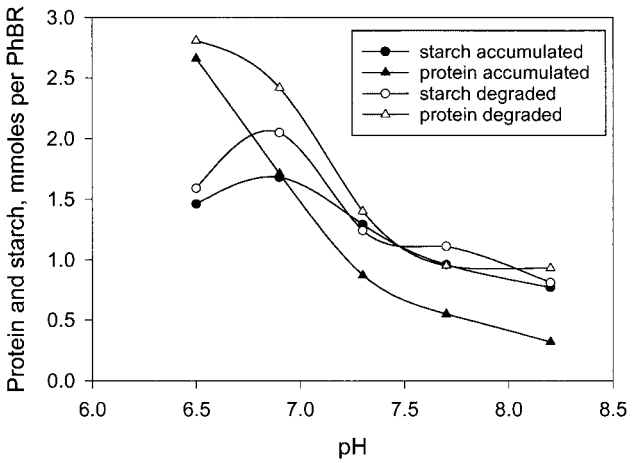


**Fig. 7** Effect of the initial extracellular pH on starch (circles) and protein (triangles) accumulation (closed symbols) and degradation (open symbols) during sulfur deprivation of *C. reinhardtii* cultures. The figure shows points from one experiment (values used in Table 1). Similar trends were observed in replicate experiments.

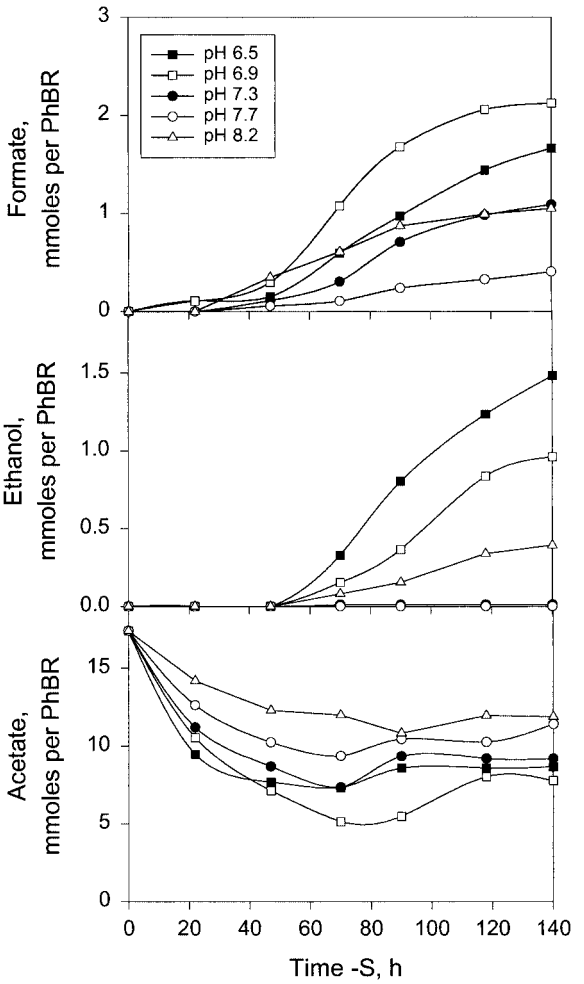


**Fig. 8** Influence of the initial extracellular pH at the start of sulfur deprivation on the fermentative metabolism of sulfur-deprived algal cultures. Formate and acetate contents in the culture media were measured by HPLC and ethanol by gas chromatography. The figure shows points from one experiment but similar trends were observed in replicate experiments.

where $H_2$ photoproduction peaks, but is significant at lower or higher pH values. This observation can be explained by fact that ethanol production consumes the reductants generated during the initial steps of the glycolytic oxidation of starch, preventing their re-direction to NAD(P)H-PQ oxidoreductase and $H_2$ photoproduction (see next section). High $H_2$ photoproduction activity, thus, is accompanied by low ethanol production due to competition for reductants between the two pathways (Happe et al. 2002). Similarly, formate production is lowest at an initial pH of 7.7, probably due to the preponderance of aerobic metabolic activity required to remove the residual amount of photosynthetically generated $O_2$. We currently do not have an explanation for the relatively large amount of extracellular acetate present at the end of the experiment done at initial pH 7.7 (Fig. 8).

*Effect of electron transport inhibitors on $H_2$ production*

The pH titration of PSII capacity during the $H_2$-photoproduction phase shows a pattern similar to that of the $H_2$ photoproduction (Fig. 5), but it is very different from those of starch and/or protein accumulation or degradation (Fig. 7). This suggests a stronger correlation between PSII activity and $H_2$ photoproduction than with substrate metabolism. Indeed, there is much previous evidence linking photosynthetic water oxidation with $H_2$ photoproduction in sulfur-deprived cultures. For example, (a) re-addition of limiting quantities of sulfate at the start of the sulfur-deprivation process increases the rates and yields of $H_2$ photoproduction as well as the residual capacity of PSII but not the in vitro hydrogenase activity (Kosourov et al. 2002); (b) the in situ PSII photochemical activity measured with a PAM fluorometer in sulfur-deprived cultures is down-regulated during the establishment of anaerobiosis and then partially recovers when $H_2$ photoproduction commences (Antal et al. 2001, Antal et al. submitted); and (c) a specific inhibitor of PSII activity, DCMU, inhibits 80% of the rate of $H_2$ photoproduction (Ghirardi et al. 2000a, Antal et al. submitted) and 100% of photosynthetic $O_2$ evolution.

Having established that PSII-catalyzed $H_2O$ oxidation is the main source of electrons for $H_2$ photoproduction in sulfur-deprived cultures, we addressed the question of alternative sources of reductants (i.e. the 20% that is not inhibited by DCMU). The remaining 20% probably depends on the transfer of reductants from endogenous substrate degradation to the photosynthetic electron transport chain, through the operation of the NAD(P)H-PQ oxidoreductase (the electron-accepting side of the chlororespiratory pathway). To prove this, we measured the effects of 2,5-dibromo-6-isopropyl-3-methyl-1,4-

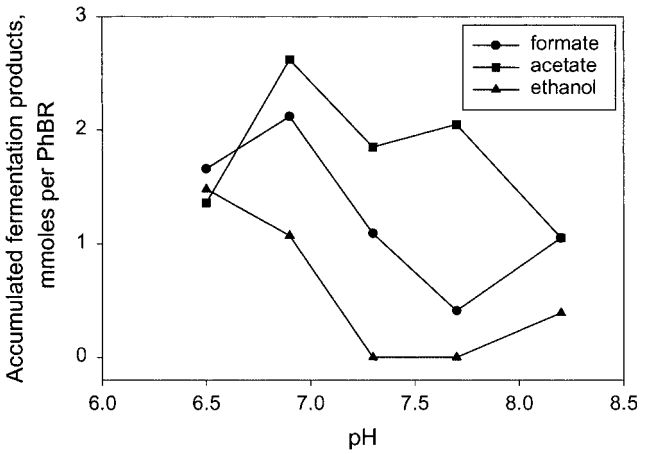


**Fig. 9** Effect of initial extracellular pH on the accumulation of acetate (squares), formate (circles) and ethanol (triangles) during the $H_2$-production phase of sulfur deprivation in *C. reinhardtii* cultures. The figure shows points from one experiment (values used in Table 1). Similar trends were observed in replicate experiments.

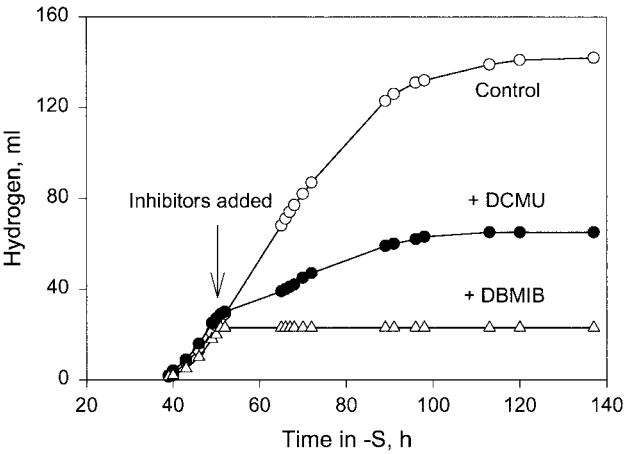


**Fig. 10** Effects of adding 10 μM DCMU (closed circles) or 40 μM DBMIB (open triangles) on $H_2$ photoproduction by sulfur-deprived control (open circles) algal cultures. The inhibitors were added at the point indicated by the arrow. In this experiment, $H_2$ gas was collected by displacing water in an inverted cylinder as described in Melis et al. (2000).

benzoquinone (DBMIB), an inhibitor of PQ oxidation by the $b_6/f$ complex, which prevents electron flow from both PSII and the NAD(P)H-PQ oxidoreductase to PSI. Fig. 10 demonstrates the different effects of DCMU and DBMIB on $H_2$ photoproduction by sulfur-deprived cultures. The addition of 10 μM DCMU inhibits the rates of $H_2$ photoproduction to about 21% of the control (average from four different experiments). The percentage of inhibition in this particular experiment was determined by comparing the rates of $H_2$ production by control (3.2 ml $h^{-1}$) and DCMU-treated samples (0.74 ml $h^{-1}$) right after addition of the inhibitor. Similar results were observed upon addition of a mixture of 10 μM DCMU and 15 μM atrazine (data not shown). Addition of 40 μM DBMIB, on the other hand, completely blocks $H_2$ gas production (results from three separate experiments), strongly indicating that substrate degradation is responsible for about 20% of the overall $H_2$ photoproduction activity of the cultures (Ghirardi et al. 2000a).

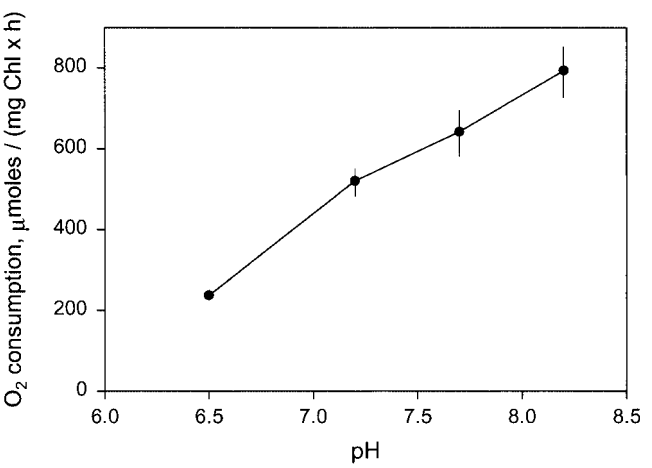


**Fig. 11** Measurements of the Mehler reaction in samples sulfur-deprived at different initial pH values. Three separate measurements were taken with different samples during the initial phases of $H_2$ production.

*Electron transport through PSI*

In order for substrate degradation to potentially contribute to $H_2$ photoproduction, it is necessary that the electron transport pathway through PSI be operational under the different pH conditions. This was examined by measuring the Mehler reaction in $H_2$-producing cultures as a function of the initial pH of the medium. The assay was done using ascorbate-reduced DCIP as an artificial donor. Fig. 11 shows that, as the initial pH increases, the rates of electron transport through PSI also increase. A similar increase in the rate of the Mehler reaction as a function of pH was observed with cultures that had not been sulfur-deprived and that were not producing $H_2$ (data not shown). Three main conclusions resulted from this work: (a) the capacity of the photosynthetic electron transport chain through PSI is not limiting $H_2$ production at any initial pH value, (b) the pH titration of the capacity of electron transport through PSI does not follow that of $H_2$ photoproduction, and (c) the capacity of PSI is high enough to shuttle electrons from starch/protein degradation to $H_2$ photoproduction at any initial pH tested.

*Carbon balance*

That a substantial amount of fermentation occurs at different initial pHs raises questions regarding the relative contributions of these processes to the metabolism of sulfur-deprived cultures. We investigated this question by performing a carbon

**Table 1** Carbon balance in sulfur-deprived cells at different initial pHs

| Row | Parameter examined | pH 6.5 | pH 6.9 | pH 7.3 | pH 7.7 | pH 8.2 |
|---|---|---|---|---|---|---|
| 1 | C consumed: | | | | | |
| | Starch (mmol glucose) | 1.59 | 2.05 | 1.24 | 1.11 | 0.81 |
| | Total (mmol C) | 9.54 | 12.3 | 7.44 | 6.66 | 4.86 |
| 2 | C produced: | | | | | |
| | Formate (mmol) | 1.66 | 2.12 | 1.09 | 0.41 | 1.05 |
| | Acetate (mmol) | 1.36 | 2.62 | 1.85 | 2.05 | 1.05 |
| | Ethanol (mmol) | 1.48 | 1.07 | 0 | 0 | 0.39 |
| | Estimated [a] $CO_2$ (mmol) | 0.84 | 1.80 | 2.96 | 3.83 | 0.30 |
| | Total (mmol C) | 8.18 | 11.3 | 7.75 | 8.34 | 4.23 |
| 3 | C balance (produced/consumed) | 0.86 | 0.92 | 1.04 | 1.25 | 0.87 |
| 4 | Additional C available from protein degradation (mmol amino acid) | 2.81 | 2.42 | 1.4 | 0.95 | 0.93 |
| | Total (mmol C) | 11.24 | 9.68 | 5.6 | 3.8 | 3.72 |
| 5 | $H_2$ produced (mmol) | 1.67 | 3.59 | 5.91 | 7.66 | 0.60 |

[a] Estimated values based on measured $H_2$-production rates shown in row 5 (see text).

balance during the $H_2$-production phase at different initial pHs. The results are summarized in Table 1. Since glycolysis, under these conditions, is dependent on starch degradation, we initially measured the total amount of carbon consumed from starch (row 1) and the total amount of carbon produced as formate, acetate, ethanol and $CO_2$ (row 2) under five different initial pHs. Since the actual amount of $CO_2$ produced was not measured in our current experiments, we estimated these values based on the known stoichiometry between $CO_2$ and $H_2$. We assumed that 80% of the $H_2$ produced is derived from water oxidation (Fig. 10 and Ghirardi et al. 2000a), which results in the release of $O_2$ with a stoichiometry of 2 : 1 ($H_2/O_2$). The consumption of this $O_2$ by mitochondrial respiration, in turn, generates $CO_2$ with a stoichiometry of 1 : 1 ($CO_2/O_2$), yielding a final $H_2/CO_2$ ratio of 2 : 1. This stoichiometry agrees with the values that we measured and reported earlier at an initial pH of 7.0 (Melis et al. 2000). Our results suggest that starch degradation can account for almost all of the degradation products excreted by sulfur-deprived cells (row 3). An additional source of organic material must be available to explain the >1 ratios of carbon produced vs. consumed at the initial pHs of 7.3 and 7.7. Row 4 shows that some of the carbon released by protein degradation during the process of sulfur deprivation could account for the extra carbon. It must be made clear that not all of the carbon generated by protein degradation generates fermentable products. As a matter of fact, different amino acid residues are metabolized by different degradation pathways and, in some cases, generated end-products that cannot be fermented. Moreover, protein extrusion is known to occur concomitantly with $H_2$ photoproduction in sulfur-deprived cultures, and this could account for part of the carbon released by intracellular protein degradation (as high as 50% at an initial pH of 7.3; Kosourov et al. 2001).

Table 1 illustrates a major shift in metabolic pathways in sulfur-deprived cells as a function of the initial pH of the external medium. At low pHs, the residual PSII activity is low, small amounts of $H_2$ are produced, and starch and protein degradation generate a mixture of formate, acetate, ethanol and $CO_2$ with a stoichiometry of, approximately, 2 : 2 : 2 : 1. As the initial pH is increased, the amount of $H_2$ produced reaches a maximum at pH 7.7, the amount of ethanol produced becomes negligible, the amount of $CO_2$ released increases, and the formate : acetate : ethanol : $CO_2$ stoichiometry is approximately 1 : 4 : 0 : 8. Finally, at pH 8.2, anaerobic metabolism once again predominates (because PSII activity is low), some ethanol is produced, and little $CO_2$ or $H_2$ are evolved. The formate : acetate : ethanol : $CO_2$ stoichiometry changes to 3 : 3 : 1 : 1.

The major anaerobic products of starch/protein degradation by sulfur-replete cultures in the dark are formate, acetate and ethanol (Gfeller and Gibbs 1984, Kreuzberg 1984). Minor products, such as glycerol and lactate, are usually only detected at low pH (Kreuzberg 1984). The anaerobic degradation of starch in the light by sulfur-replete *C. reinhardtii* was studied previously in the F60 mutant, which lacks one of the enzymes of the Calvin cycle (phosphoribulokinase). This deficiency accounts for its low rate of photosynthesis and thus the F60 mutant, under sulfur-replete conditions, resembles sulfur-deprived, wild-type algal cultures. In the mutant, photosynthetically generated $O_2$ is immediately consumed by cellular respiration, and this activity maintains an anaerobic environment in the photobioreactor, which in turn allows for hydrogenase-catalyzed $H_2$ production to occur. Light degradation of starch in the F60 mutant yields formate, $CO_2$ and $H_2$ (Gfeller and Gibbs 1984). This reflects a partial shift from an anaerobic to an aerobic, $CO_2$-releasing respiratory pathway upon illumination. Moreover, ethanol formation by the F60 mutant under these conditions is minimal, since, as observed with sulfur-deprived cultures, its production competes with the hydrogenase for reductants generated during the earlier steps of glycolysis.

In conclusion, the optimal pH for $H_2$ production coincides

with the optimal pH for residual PSII activity in sulfur-deprived cells. At more acidic pHs (<7.7), sulfur-deprived cells degrade large amounts of starch and protein and accumulate fermentation products (acetate, formate and ethanol). At the optimal pH for $H_2$ production, the cells shift to an aerobic metabolism in order to remove the $O_2$ produced by residual PSII activity. At this pH, the cultures do not accumulate any ethanol but produce large amounts of $CO_2$. Finally, at higher pHs, the cells shift back to an anaerobic metabolism and stop evolving $H_2$. The shifts in metabolism described above are the result of higher or lower amounts of residual PSII activity remaining under the experimental pH conditions. The crucial role of residual PSII activity in $H_2$ photoproduction is strongly supported by the observation that the specific PSII activity inhibitor, DCMU, inhibits 80% of the rate of $H_2$ production. Besides providing minor amounts of reductants to supplement reductants from water for $H_2$ production, the main role of starch/protein degradation is to generate the reductants required to remove the $O_2$ evolved by the residual PSII function in order to sustain hydrogenase activity, in agreement with Ghirardi et al. (2000a) and proposals by Antal et al. (2001) and Melis and Happe (2001).

Finally, the results of this work may be helpful in the future design of applied algal $H_2$ production systems, which will require high yields of $H_2$ photoproduction and low yields of fermentation. Both of these goals can be achieved by setting the pH of the culture medium at 7.7 when the cells are first exposed to sulfur-deprived conditions.

## Materials and Methods

*Algal growth conditions*

*C. reinhardtii*, strain cc124, was grown photoheterotrophically in Erlenmeyer flasks containing 800 ml of standard TRIS-Acetate-Phosphate (TAP) medium, pH 7.3 (Harris 1989), at 25°C. Algal cultures were sparged with 3% $CO_2$ in air, mixed by magnetic stirring (PC-131, Corning Inc., NY, U.S.A.), and illuminated continuously with cool-white fluorescent light (~200 μE $m^{-2}$ $s^{-1}$ PAR at the outer surface of the culture flasks). After reaching mid-logarithmic phase (2–5×$10^6$ cells $ml^{-1}$), the cell cultures were harvested by centrifugation at 3,000×*g* for 5 min and resuspended in sulfur-depleted media at the indicated pHs.

TAP-minus-sulfur medium (pH 7.3–8.2) is a modification of a standard TAP medium, in which all sulfates were replaced with chloride salts at the same concentrations. For low pH experiments, two additional S-deprived media were devised: MAP-minus-sulfur (pH 6.5–7.0) and BTAP-minus-sulfur (pH 6.2). These media were modified by replacing 20 mM TRIS with 20 mM MOPS or 20 mM BIS-TRIS, respectively. The initial pHs of all media were adjusted as required with 10 N NaOH. Acetate is necessary for fast inactivation of oxygenic photosynthesis under sulfur-deprived conditions and subsequent establishment of the anaerobic environment inside a photobioreactor (Ghirardi et al. 2000a).

*Sulfur-deprivation procedure*

Harvested cells were washed three times in TAP-minus-sulfur medium (pH 7.3), resuspended in BTAP-, MAP- or TAP-minus-sulfur medium to a final concentration of about 9–12 μg Chl $ml^{-1}$ (4–5×$10^6$ cells $ml^{-1}$) and placed in the automated photobioreactor system described in detail by Kosourov et al. (2002). This system employs four specially fabricated, glass photobioreactors (4-cm optical path length, 1.2 liters culture volume) with built-in ports for $O_2$, $E_h$, pH and temperature sensors. Each bioreactor has two additional ports for gas outlet and culture sampling. The volume of the gas produced by a culture was measured as the weight of gas-displaced water on a digital balance. The data produced by the electrodes and balances were continuously recorded with an integrated microprocessor system.

*Flash-probe Chl a fluorescence*

Flash-probe Chl *a* fluorescence yields were measured with a home-built instrument (Ghirardi et al. 1996). Samples used for the fluorescence measurements were taken directly from the photobioreactor, concentrated by centrifugation to increase the total chlorophyll concentration to 40±1 μg $ml^{-1}$ and adapted in the dark for 5 min under atmospheric $O_2$ partial pressure. The samples were then placed in a 4×10 mm plastic cuvette, and DCMU was added to a final concentration of 30 μM to block electron transfer between $Q_A^-$ and $Q_B$, the secondary plastoquinone electron acceptor. Fluorescence was initiated by a saturating, single-turnover actinic flash, and the resulting fluorescence yield profile was measured as $(F–F_0)/F_0$. The highest fluorescence level, $F_{max}$, appears within 20–40 μs following the saturating flash (Nixon et al. 1992) and decays to a low final level, $F_{final}$, within 3 s (Dekker et al. 1984, Metz et al. 1989, Ghirardi et al. 1996, Ghirardi et al. 1998). The $F_{max}$ level was used as a measure of the functional capacity of PSII in our experiments (Ghirardi et al. 1998).

*Mehler reaction*

A 2.1-ml aliquot of the culture was taken directly from the photobioreactor and placed in a vial with 0.4 ml of the reaction solution to a final concentration of 8 mM ascorbate, 80 μM DCIP, 1 mM $NaN_3$, 37.5 μM DCMU, 1 mM methyl viologen and 0.1% w/v Triton X100. The final mixture was shaken for 3 min to equilibrate the liquid with atmospheric $O_2$ and then transferred to the electrode chamber. Light-induced $O_2$ uptake was measured at 25°C with a Clark-type $O_2$ electrode under saturating light of about 700 μE $m^{-2}$ $s^{-1}$ (Fiber-Light High Intensity Illuminator, model 170-D, Dolan-Jenner Industries) filtered through a 1% $CuSO_4$ solution. All experiments were repeated for 4–5 times. The rates were estimated from the maximum slopes of the curves.

*Other analytical procedures*

The samples for starch, protein, acetate, formate and ethanol analysis were taken directly from the photobioreactor (about 5 ml of algal suspension for each point) and centrifuged for 5 min at ~2,000×*g*. The pellets and the media were separated and stored frozen at –80°C until all samples were ready for processing. Starch and protein levels inside the cells were determined in the pellet on a per ml of culture basis according to the methods of Gfeller and Gibbs (1984) and Lowry et al. (1951), respectively. Protein was measured as mmoles of amino acid, and the weight conversion assumed an average amino acid molecular mass of 110 g $mol^{-1}$. Starch was measured as mmoles of glucose equivalent (180.2 g $mol^{-1}$). The levels of acetate and formate in the medium were determined by HPLC (Model 1050, Hewlett-Packard, Palo Alto, CA, U.S.A.) using an ion-exchange column (Aminex HPX-87H, Bio-Rad, Hercules, CA, U.S.A.) and 4 mM $H_2SO_4$ as the mobile phase. Ethanol was quantified with a Varian gas chromatograph (Model 3700, Palo Alto, CA, U.S.A.). In this case, a Porapak Q column (Supelco, Bellefonte, PA, U.S.A.) was installed, and argon was used as a carrier gas.

For in vitro hydrogenase activity measurements, 1-ml cell suspension samples were taken anaerobically from the photobioreactor at the beginning of the $H_2$-production phase and injected into 13.5-ml

sealed and argon-purged glass vials. The vials contained 1 ml of 10 mM oxidized methyl viologen prepared in $O_2$-free 50 mM MOPS (for pH 6.5–7.85) or 50 mM TRIS (for pH 7.1–9.0) buffer and 0.2% w/v Triton X100 (Kosourov et al. 2002). The reaction was started when methyl viologen was reduced by the addition of 100 µl of anaerobic, 100 mM Na dithionite in 0.03 N NaOH. The assay was performed at 37°C in the dark for 20 min. The rates of the resultant $H_2$-production activities were measured using the Varian gas chromatograph outfitted with a 5Å Molecular Sieve (Supelco, Bellefonte, PA, U.S.A.) column. The rates were expressed on the basis of the chlorophyll content of the sample.

The total chlorophyll *a*+*b* content was assayed spectrophotometrically in 95% ethanol extracts by the method of Spreitzer (Harris 1989).

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## References

Antal, T.K., Krendeleva, T.E., Laurinavichene, T.V., Makarova, V.V., Tsygankov, A.A., Seibert, M. and Rubin, A.B. (2001) The relationship between the photosystem 2 activity and hydrogen production in sulfur deprived *Chlamydomonas reinhardtii* cells. *Dokl. Biochem. Biophys.* 381: 371–374.

Davies, J.P., Yildiz, F.H. and Grossman, A. (1996) Sac1, a putative regulator that is critical for survival of *Chlamydomonas reinhardtii* during sulfur deprivation. *EMBO J.* 15: 2150–2159.

de Hostos, E.L., Schilling, J. and Grossman, A.R. (1989) Structure and expression of the gene encoding the periplasmic arylsulfatase in *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* 218: 229–239.

de Hostos, E.L., Togasaki, R.K. and Grossman, A. (1988) Purification and biosynthesis of derepressible periplasmic arylsulfatase from *Chlamydomonas reinhardtii*. *J. Cell Biol.* 106: 29–37.

Dekker, J.P., Van Gorkom, H.J., Wensink, J. and Ouwehand, L. (1984) Absorbance difference spectra of the successive redox states of the oxygen-evolving apparatus of photosynthesis. *Biochim. Biophys. Acta.* 767: 1–9.

Forestier, M., Plummer, S., Ahmann, D., Seibert, M. and Ghirardi, M. (2001) Cloning of two hydrogenase genes from the green alga *Chlamydomonas reinhardtii*. *In* PS2001 Proceedings: 12th International Congress on Photosynthesis. S037–003. CSIRO Publishing, Melbourne. Online: www.publish.csiro.au/ps2001.

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

Ghirardi, M.L., Kosourov, S., Tsygankov, A. and Seibert, M. (2000a) Two-phase photobiological algal $H_2$-production system. *In* Proceedings of the 2000 DOE Hydrogen Program Review, 9–11 May 2000, San Ramon, CA. NREL/CP-570–28890. Online: www.eren.doe.gov/hydrogen/docs/28890toc.html

Ghirardi, M.L., Lutton, T.W. and Seibert, M. (1996) Interactions between diphenylcarbazide, zinc, cobalt, and manganese on the oxidizing side of photosystem II. *Biochemistry* 35: 1820–1828.

Ghirardi, M.L., Lutton, T.W. and Seibert, M. (1998) Effects of carboxyl amino acid modification on the properties of the high-affinity, manganese-binding site in photosystem II. *Biochemistry* 37: 13559–13566.

Ghirardi, M.L., Zhang, L., Lee, J.W., Flynn, T., Seibert, M., Greenbaum, E. and Melis, A. (2000b) Microalgae: a green source of renewable $H_2$. *Trends Biotechnol.* 18: 506–511.

Grossman, A. and Takahashi, H. (2001) Macronutrient utilization by photosynthetic eukaryotes and the fabric of interactions. *Annu. Rev. Plant Physiol.* 52: 163–210.

Guenther, J.E. and Melis, A. (1990) The physiological significance of photosystem II heterogeneity in chloroplasts. *Photosynth. Res.* 23: 105–109.

Happe, T., Hemschemeier, A., Winkler, M. and Kaminski, A. (2002) Hydrogenases in green algae: do they save the algae's life and solve our energy problems? *Trends Plant Sci.* 7: 246–250.

Hase, E., Morimura, Y., Mihara, S. and Tamiya, H. (1958) The role of sulfur in the cell division of *Chlorella*. *Arch. Mikrobiol.* 31: 87–95.

Hase, E., Otsuka, H., Mihara, S. and Tamiya, H. (1959) Role of sulfur in the cell division of *Chlorella*, studied by the technique of synchronous culture. *Biochim. Biophys. Acta* 35: 180–189.

Harris, E.H. (1989) *The Chlamydomonas Sourcebook: A Comprehensive Guide to Biology and Laboratory Use*. p. 780. Academic Press, San Diego.

Kosourov, S., Tsygankov, A., Ghirardi, M.L. and Seibert M. (2001) Sustained hydrogen photoproduction by *Chlamydomonas reinhardtii* – effects of culture parameters. *In* PS2001 Proceedings: 12th International Congress on Photosynthesis. S037–009. CSIRO Publishing, Melbourne. Online: http://www.publish.csiro.au/ps2001.

Kosourov, S., Tsygankov, A., Seibert, M. and Ghirardi, M.L. (2002) Sustained hydrogen photoproduction by *Chlamydomonas reinhardtii*: Effects of culture parameters. *Biotechnol. Bioeng.* 78: 731–740.

Kreuzberg, K. (1984) Starch fermentation via a formate-producing pathway in *Chlamydomonas reinhardtii*, *Chlorogonium elongatum* and *Chlorella fusca*. *Physiol. Plant.* 61: 87–94.

Leustek, T. and Saito, K. (1999) Sulfate transport and assimilation in plants. *Plant Physiol.* 120: 637–643.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.

Melis, A. and Happe, T. (2001) Hydrogen production. Green algae as a source of energy. *Plant Physiol.* 127: 740–748.

Melis, A., Zhang, L., Forestier, M., Ghirardi, M.L. and Seibert, M. (2000) Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. *Plant Physiol.* 122: 127–136.

Metz, J.G., Nixon, P.J., Rögner, M., Brudvig, G.W. and Diner, B.A. (1989) Directed alteration of the D1 polypeptide of photosystem II: evidence that tyrosine-161 is the redox component, Z, connecting the oxygen-evolving complex to the primary electron donor, P680. *Biochemistry* 28: 6960–6969.

Nixon, P.J., Trost, J.T. and Diner, B.A. (1992) Role of the carboxy terminus of polypeptide D1 in the assembly of a functional water-oxidizing manganese cluster in photosystem II of the cyanobacterium *Synechocystis sp*. PCC 6803: assembly requires a free carboxyl group at C-terminal position 344. *Biochemistry* 31: 10859–10871.

Ohta, S., Miyamoto, K. and Miura, Y. (1987) Hydrogen evolution as a consumption mode of reducing equivalents in green algal fermentation. *Plant Physiol.* 83: 1022–1026.

Roessler, P. and Lien, S. (1982) Anionic modulation of the catalytic activity of hydrogenase from *Chlamydomonas reinhardtii*. *Arch. Biochem. Biophys.* 213: 37–44.

Takahashi, H., Braby, C.E. and Grossman, A.R. (2001) Sulfur economy and cell wall biosynthesis during sulfur limitation of *Chlamydomonas reinhardtii*. *Plant Physiol.* 127: 665–673.

Tsygankov, A., Kosourov, S., Seibert, M. and Ghirardi, M.L. (2002) Hydrogen photoproduction under continuous illumination by sulfur-deprived, synchronous *Chlamydomonas reinhardtii* cultures. *Int. J. Hydrogen Energy* 27: 1239–1244.

Wykoff, D.D., Davies, J.P., Melis, A. and Grossman, A.R. (1998) The regulation of photosynthetic electron transport during nutrient deprivation in *Chlamydomonas reinhardtii*. *Plant Physiol.* 117: 129–139.

Yanyushin, M.F. (1982) Hydrogen evolution and hydrogenase activity in synchronous culture of *Chlamydomonas reinhardtii* in connection with anaerobic degradation of starch. *Sov. Plant Physiol.* 29: 104–109.

Yildiz, F.H., Davies, J.P. and Grossman, A.R. (1994) Characterization of sulfate transport in *Chlamydomonas reinhardtii* during sulfur-limited and sulfur-sufficient growth. *Plant Physiol.* 104: 981–987.

Zhang, L., Happe, T. and Melis, A. (2002) Biochemical and morphological characterization of sulfur-deprived and $H_2$-producing *Chlamydomonas reinhardtii* (green alga). *Planta* 214: 552–561.

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