CYCLIC PHOTOBIOLOGICAL ALGAL H_2-PRODUCTION

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Abstract

Previous work from our laboratory demonstrated the feasibility of using sulfur-deprived algal cultures to photoproduce H_2 continuously for 3-4 days (Melis et al., 2000; Ghirardi et al., 2000a). The production of H_2 depends mostly on the photosynthetic H_2O-oxidation activity of the cultures (Ghirardi et al., 2000 a and b; Kosourov et al., 2002), and requires concomitant operation of O_2-consumption pathways (Ghirardi et al., 2000a; Kosourov et al., 2002; Antal et al., submitted). Our research has focused on the elucidation of the pathways involved in H_2-production under sulfur-deprivation and on approaches to improve the H_2 production yield of the process.

This year's results show that (a) H_2-production can be optimized by controlling the initial pH of the medium and (b) different maximum amounts of sulfate can be re-added to the system at different points of the sulfur-deprivation process without decreasing H_2 production. Moreover, we present evidence from *in situ* fluorescence measurements, that H_2-production during sulfur-deprivation provides the means to re-activate residual photosynthetic electron transport capacity, which is rapidly down-regulated at the establishment of anaerobiosis. These measurements can be used to determine the transition of the cultures to anaerobic conditions and thus serve as a means to monitor the physiological state of the culture from outside the photobioreactor vessel. Finally, we show that different pathways for photosynthetically-generated O_2 consumption are prevalent during different phases of the process.

We conclude that the rates of H_2-production in the system are limited by the rates of electron transport from the H_2O-oxidizing complex to the hydrogenase under anaerobic conditions, and that these rates can be manipulated, to a certain extent, by changes in pH and addition of sulfate to the medium.

Introduction

Sustained photoproduction of H_2 by green algae can be achieved by the reversible inactivation of photosynthetic H_2O-oxidizing activity, catalyzed by Photosystem II (PSII). Melis et al. (2000) and Ghirardi et al. (2002a) first demonstrated that the removal of sulfate from the growth medium could accomplish this inactivation. Since then, we have refined our understanding of the mechanism of the process. It is clear, now, that, under sulfate-deprivation, the decline in photosynthetic O_2-evolution capacity results from the conversion of PSII centers from a Q_B-reducing to a Q_B-nonreducing form, an intermediate in the PSII repair cycle (Guenther and Melis, 1990; Wykoff et al., 1998). This differential accumulation is believed to occur because photodamaged D1 cannot efficiently be repaired due to the lack of sulfurylated amino acids. As a consequence of the gradual and partial inhibition of O_2-evolving capacity, the rate of O_2 evolution drops below the rate of respiratory O_2 uptake, and *C. reinhardtii* cultures transition

from an aerobic to anaerobic environment (Melis et al., 2000). The establishment of a strong anaerobic environment in the photobioreactor (with a redox potential of about -200 to -300 mV determined against the H_2 electrode [Kosourov et al., 2002]) results in the induction of the [Fe]-hydrogenase, and a sealed *C. reinhardtii* culture starts to produce H_2.

Using a computer-monitored photobioreactor system, Kosourov et al. (2002) showed that sulfur-deprived cultures transition through five consecutive phases: an aerobic phase, an O_2-consumption phase, an anaerobic phase, a H_2-production phase and a termination phase. They also found that re-addition of small quantities of sulfate (12.5 – 50 μM $MgSO_4$, final concentration) back to rigorously sulfur-deprived cell suspensions at the start of the sulfur-deprivation period increases the specific rate of H_2 production and the total yield of H_2 by the system (Kosourov et al., 2002). This was attributed to a positive effect of the added sulfur on the residual PSII activity, suggesting that PSII activity may limit the rates of H_2 production by sulfur-deprived cells. Qualitatively similar results were reported by Zhang et al. (2002).

We also showed that the synchronization of cell growth by alternating light/dark cycles affected the subsequent H_2-production activity of sulfur-deprived cultures (Ghirardi et al., 2001; Kosourov et al., 2002). Maximum H_2-production activity was achieved with cells that had been harvested early in the light phase (Tsygankov et al., 2002), when photosynthetic PSII activity is at its peak. This also corroborates the importance of H_2O-oxidation for H_2 photoproduction.

In attempting to further optimize the rate and duration of H_2 production by sulfur-deprived cultures, we did a pH titration of various metabolic activities during sulfur-deprivation in order to identify those favoring H_2 production. Furthermore, based on encouraging results obtained by addition of sulfate at the start of the sulfur-depletion process (Kosourov et al., 2002), we investigated the addition of limiting amounts of sulfate at other points during the process. To further understand factors that currently limit the rate of H_2 photoproduction, we used PAM fluorometry measurements to externally monitor the *in situ* PSII photochemical activity of the algal culture inside the photobioreactor vessels. Our results demonstrate that, under overreducing conditions, photosynthetic electron transport and H_2 photoproduction influence and regulate each other.

Materials And Methods

Algal Growth and Sulfur Deprivation

Chlamydomonas reinhardtii, strain cc124, was grown photoheterotrophically in 1-L Erlenmeyer flasks containing 800 ml of a standard TRIS-Acetate-Phosphate (TAP) medium, pH 7.3 (Harris, 1989) at 25°C and illuminated continuously with cool-white fluorescent light of about 200 μE • m^{-2} • s^{-1}, PAR. When the cultures reached mid-logarithmic phase (2–5 x 10^6 cells • ml^{-1}), they were harvested by centrifugation at 3000 x g for 5 min and used for the subsequent experiments. For low pH experiments, two additional S-deprived media were used: 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 pH of all media was adjusted with different quantities of 10M NaOH.

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 an automated photobioreactor system (Kosourov et al., 2002) with built-in ports for O_2, E_h, pH, and temperature sensors. The volume of the gas produced by a culture was measured as the volume of water displaced by the gas (the water

was weighed with a digital balance). The data coming from the sensors and balances were continuously recorded by an integrated microprocessor system. The photobioreactor vessels were illuminated continuously with cool-white fluorescent lamps (200-250 μE • m^{-2} • s^{-1}, PAR).

Hydrogenase Activity and H_2 Production

For hydrogenase activity, 1-ml of 10 mM oxidized methyl viologen in 50 mM phosphate buffer and 0.2% Triton X 100 were flushed with argon gas and reduced with 100 mM Na dithionite in 13.5-ml sealed glass vials. Dark H_2 production, mediated by reduced methyl viologen, was initiated by adding 1 ml of anaerobic cell suspension taken directly from the photobioreactor (Kosourov et al., 2002). The rate of H_2 production was measured with a Hewlett-Packard gas chromatograph (Model 3700, Varian, Palo Alto, CA) and expressed on the basis of the sample chlorophyll (Chl) content.

Flash-Probe Chl *a* Fluorescence Measurements

The kinetics of the decay of the fluorescence yield of Chl *a* following a single turnover saturating light flash (3 μs) were measured with a home-built instrument (Ghirardi et al., 1996). Samples used for fluorescence measurements were taken directly from the photobioreactor, concentrated by centrifugation to a final chlorophyll concentration of 40 μg /ml, and adapted in the dark for 5 min under an atmospheric O_2 environment. The samples were then placed in a 4 mm x 10 mm plastic cuvette, and DCMU was added to a final concentration of 30 μM to block any electron transfer between Q_A^- and Q_B. The experiment was initiated by a saturating actinic flash, and the results measured as $(F - F_0)/F_0$ vs. time, where F_0 is the fluorescence yield of the dark-adapted cells measured with the weak probe flashes. From these measurements, F_m (the maximum value of $(F - F_0)/F_0$) was calculated and used as a measure of the photochemical capacity of PSII.

PAM Fluorescence Measurements

In situ Chl fluorescence measurements were obtained with a portable PAM-2000 fluorometer (Walz, Germany), using the so-called Pulse-Amplitude-Modulation (PAM) measuring principle. Fluorescence yield measurements (λ > 710 nm) were obtained with an optical fiber probe affixed closely onto the surface of the glass photobioreactor vessel, which was subjected to continuous illumination as indicated above. The measurements were recorded every 15 min over the total period of sulfur deprivation. An 0.8-s saturating actinic excitation pulse (λ < 710 nm, 1200 μmol • m^{-2} • s^{-1} PAR) from an 8 V/20 W halogen lamp was applied on top of weak modulated probe flashes (3-μs flashes from a 655 nm light-emitting diode at frequencies of 600 Hz or 20 kHz.). The following parameters were recorded: (a) F_t, the fluorescence yield of the cells, exposed to the ambient actinic illumination in the reactor vessels and (b) $F_{m'}$, the fluorescence yield of cells under the same growth illumination, following application of the saturating actinic pulse. The efficiency of photochemical conversion of absorbed light energy (the photochemical activity) in PSII was calculated as $\Delta F/F_m' = (F_m'-F_t)/F_m'$ (Schreiber et al., 1995).

Other Analytical Procedures

The total chlorophyll a + b content was assayed spectrophotometrically in 95% ethanol extracts by the method of Spreitzer (Harris, 1989). The samples for starch, protein, acetate, formate and ethanol analysis were taken directly from a photobioreactor (about 5 ml for each point) and

centrifuged for 5 min at 2000 x g. Starch and protein that accumulated inside the cells were determined in the pellet on a per ml of culture basis according to the method of Gfeller and Gibbs (1984) and Lowry et al. (1951), respectively. Cells were disrupted by sonication for 5 min. The level of acetate and formate in the medium was determined by HPLC (Model 1050, Hewlett-Packard, Palo Alto, CA) using an ion-exchange column (Aminex HPX-87H, Bio-Rad, Hercules CA) and 4 mM H_2SO_4 as the mobile phase. Ethanol was quantified with a Hewlett-Packard gas chromatograph (Model 3700, Varian, Palo Alto, CA). In this case, a Porapak Q column (Supelco) was installed, and argon was used as the carrier gas.

Results

Effect of Initial pH on the Metabolic Activities of Sulfur-Deprived Cultures

As seen previously (Kosourov et al., 2002), the pH of the extracellular medium in sulfur-deprived cultures is not stable and changes with time, increasing by 0.4-0.6 units during the O_2-production and O_2-consumption phases and decreasing gradually during the anaerobic and H_2-production phases by about 0.4 units. The initial increase in the pH is due to the photosynthetic consumption of dissolved CO_2 and uptake of acetate, while the subsequent decrease in the pH is the result of CO_2 evolution and fermentative accumulation of acetate and formate (Kosourov et al., 2002). Due to the co-occurrence of a variety of metabolic pathways during sulfur deprivation, and the different sensitivities of these pathways to pH, we investigated the effect of the initial pH on the rates of H_2 photoproduction. Our aim was to optimize the pH for H_2 photoproduction and to decrease electron drain by alternative pathways. In order to interpret the results below, it is important to remember that the experiments are presented as a function of the pH at *the start of sulfur deprivation*. The actual pH during each measurement depends on the point during the sulfur-deprivation process at which the measurement is taken.

The maximum yield of H_2 per photobioreactor (Fig.1) 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 maximum specific rate of H_2 production (not shown), calculated on a chlorophyll basis, had exactly the same pH dependence as the total yield of H_2 per photobioreactor.

In order to determine how the external pH affects H_2 production, we first 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 here, the pH for these measurements was set *in vitro,* using samples taken from a culture sulfur-deprived at an initial pH of 7.3. The optimum *in vitro* pH for hydrogenase activity was about 7.1 – 7.4 (Fig. 1).

Next, we examined the effect of external pH on the decay of the Chl *a* fluorescence yield in sulfur-deprived cultures. The maximum Chl *a* fluorescence yield (F_m), following a saturating flash and measured in the presence of DCMU, reflects the amount of accumulated reduced Q_A (the primary stable electron acceptor of PSII) and depends on the PSII photochemical capacity. Figure 1 also shows that the residual PSII capacity during H_2 production was highest when the cells were deprived of sulfur at an initial pH of 7.3-7.9. The amount of residual PSII capacity decreased at lower and higher initial pHs, in a manner similar to that of H_2 production.

The changes in the starch and protein concentrations were also measured at different initial pHs. As a rule, algal cells accumulate protein and starch during the aerobic phase of sulfur deprivation and degrade them during the anaerobic and H_2-production phases. We calculated

the quantities of starch and protein degraded during the H_2-production phase. Starch and protein degradation activities peak at initial pHs below 7.0 and decrease at higher initial pHs (Fig. 1). The pH profile of these activities does not correlate with those for either PSII or H_2-production activity.

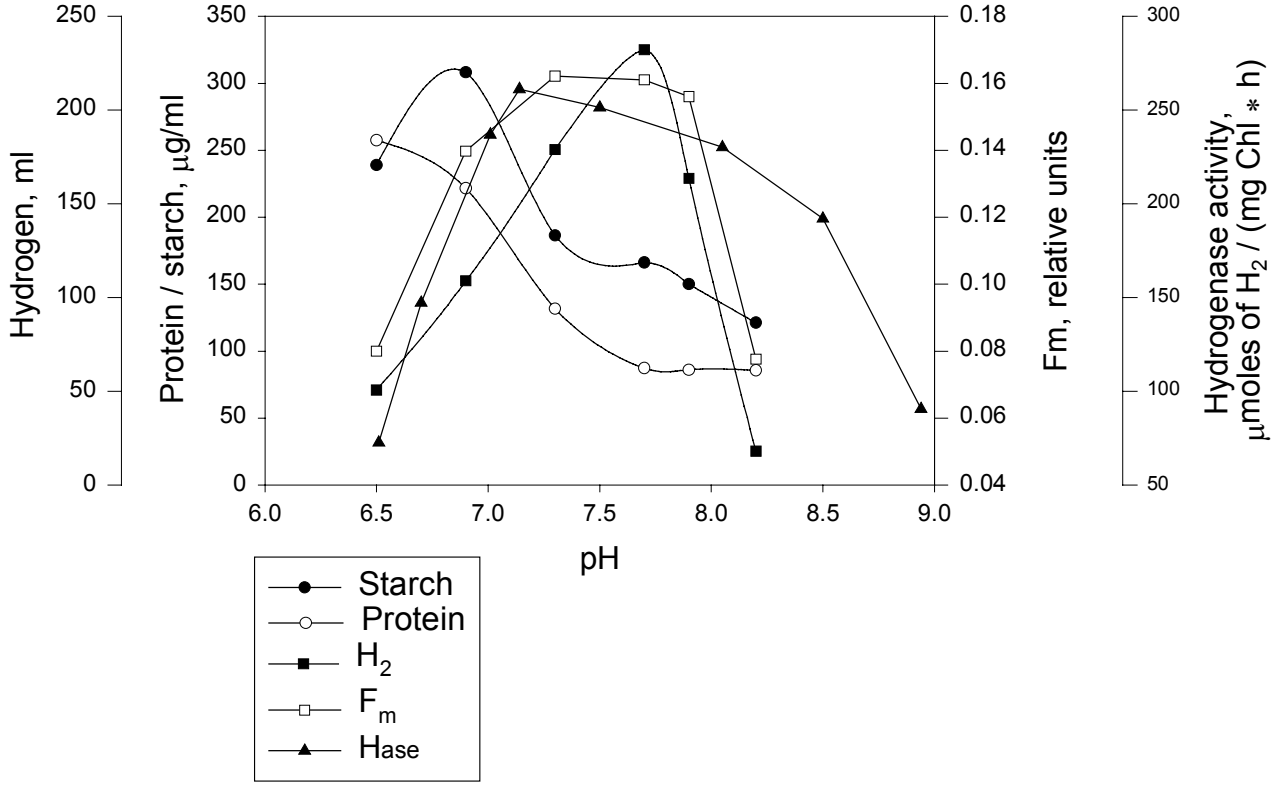

Figure 1. pH titrations of the amounts of starch and protein degraded, the amount of H_2 produced, the maximum Chl *a* fluorescence yield (a measure of PSII capacity) and the *in vitro* hydrogenase activity. As observed in the text, the pHs used in the titration of hydrogenase activity only were set to the actual values measured *in vitro*, and do not represent the initial pH at sulfur deprivation. In order to correlate them to the pH titrations of the other parameters, it is necessary to subtract 0.4 pH units from each value, which corresponds to the change in pH of the cultures from the start of sulfur deprivation to the early H_2 photoproduction phase.

Hydrogen photoproduction in sulfur-deprived algal cultures is accompanied by the accumulation of formate, ethanol and acetate in the medium (Kosourov et al., 2002). The accumulation of fermentative products occurs faster at or below an initial pH of 7.0 (not shown) and does not correlate with H_2 photoproduction. As expected, H_2 production prevented the formation of ethanol by using up the reductants generated during glycolysis. We did not observe any ethanol production at pH 7.3 and 7.7, which were optimal for H_2 photoproduction. In addition, formate and ethanol production was also higher at pH values above 7.7, where only small amounts of H_2 are produced. Finally, due to the fact that acetate is consumed during the initial

phases of sulfur deprivation (Melis et al., 2000; Kosourov et al., 2002) and produced during the later phases, the total amount of acetate required to run the system is a function of the initial pH of the medium. At lower pH, both the consumption and production of acetate are high, and the net change in acetate concentration is about 9 mM. However, as the initial pH increases, acetate consumption and production slow down, and the net change in acetate concentration is only about 5 mM (not shown).

Re-Addition of Sulfate to Sulfur-Deprived Cultures

In previous experiments, we observed that re-adding limiting amounts of sulfate at the start of the sulfur-deprivation period led to higher rates and yields of H_2 photoproduction (Kosourov et al., 2002). We reported that addition of up to 12.5 μM of sulfate to unsynchronized algal cultures resulted in small increases in the specific rate of H_2 photoproduction. We expanded our previous experiments to include sulfate re-addition at two later times during sulfur deprivation: at the beginning of the anaerobic phase and after the start of the H_2 photoproduction phase. Table I shows that it is possible to find a maximum concentration of sulfate that will not inactivate H_2 photoproduction, and that this concentration is different depending on the time at which sulfate is added. When added at the start of anaerobiosis, a concentration of 5 μM sulfate will allow H_2 photoproduction to start at the expected time and to proceed at rates similar to those of samples to which no sulfate was re-added. However, addition of sulfate at concentrations of 1 μM or higher 14 h after the start of H_2 photoproduction will interrupt the process temporarily and result in lower final yields.

Table I. Inhibition of H_2 photoproduction by addition of sulfate at different times during the sulfur-deprivation process.

Time of sulfate addition	Minimum concentration of added sulfate that causes inhibition of the H_2 photoproduction rates
Start of sulfur deprivation	25 μM
Start of anaerobiosis	5 μM
14 h after start of H_2 production	< 1 μM

***In situ* Measurements of Photosystem II Photochemical Efficiency**

We have examined *in situ* changes of PSII photochemical activity by monitoring the PAM chlorophyll fluorescence of *C. reinhardtii* cells during the course of adaptation to sulfur deprivation (Antal et al., submitted). During the O_2-production and consumption phases, neither the Chl concentration nor the *in situ*-monitored fluorescence parameter, F_m', changed significantly (not shown). However since the fluorescence parameter, F_t, increased slowly during this same period, the photochemical activity of PSII ($\Delta F/F_m'$), declined from 0.57 in the middle of the O_2-production phase to 0.44 at the end of the O_2-consumption phase. The observed changes in F_m', F_t and $\Delta F/F_m'$ reflect the existence of long-lived inactive states generated possibly in the Q_B-non-reducing centers of PS II. Thus, during the early stages of sulfur deprivation, PSII photochemical activity gradually declines.

However, an additional, abrupt, and partially reversible down-regulation of PSII photochemical activity begins at the time that anaerobiosis is established. Figure 2 shows that, in this experiment at between 15 and 16 h after the start of sulfur deprivation, there was a rapid (< 5 min) reduction of the *in situ*-measured photochemical yield of PSII ($\Delta F/F_m'$) from 0.4 to 0.1, which reflects the rapid down-regulation of PSII photochemical activity at this time. The loss of photochemical activity was due both to a rapid reduction in F_m' and a rapid increase in F_t. It is very important to note that the observed rapid reduction of $\Delta F/F_m'$ began at the exact time that the O_2 concentration in the culture suspension reached zero. The redox potential of the culture medium also started to decline around this time but not nearly so fast as the PSII photochemical activity. At about one hour of anaerobiosis, the PSII photochemical activity dropped to a minimum value (0.05). After that point, it gradually increased over the next 2 hours and reached a maximum value of 0.085. Hydrogen gas can be detected in the headspace within 5 min of anaerobiosis but the gas cannot be collected for several hours. It takes this amount of time for the rates of electron transport to increase, for the hydrogenase to become fully induced, and for the growth medium to become saturated with hydrogen.

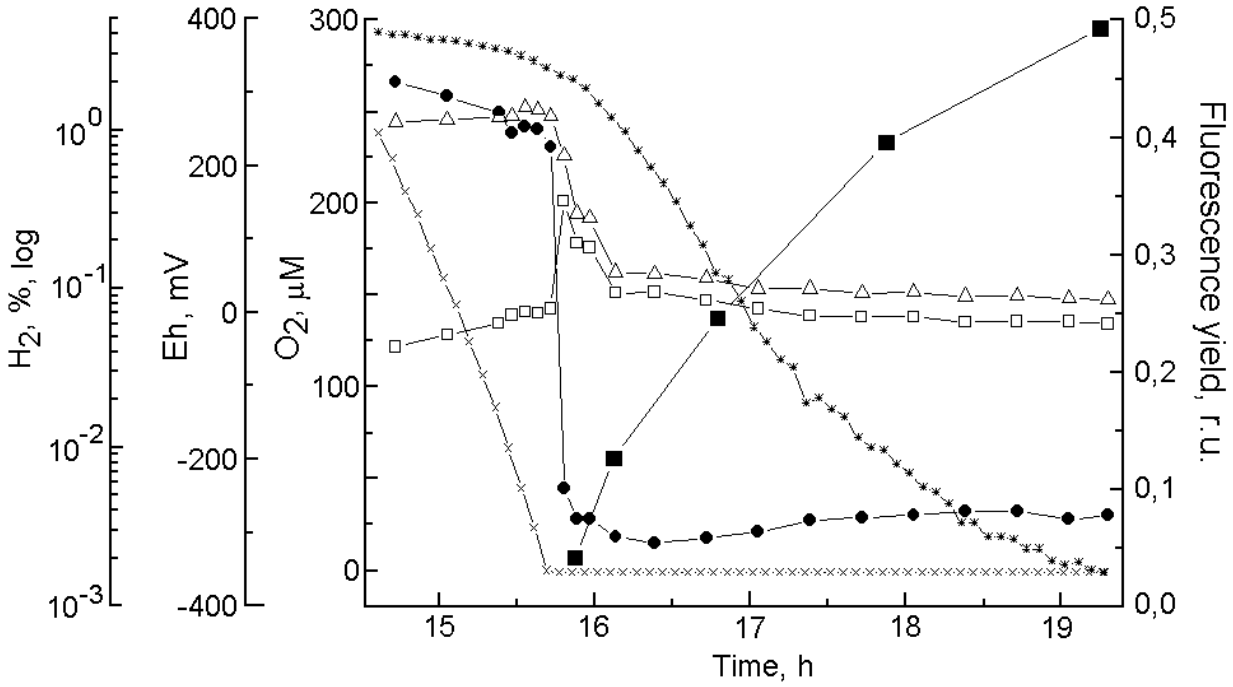

Figure 2. Time course of physiological parameters and H_2 production in *C. reinhardtii* cells during incubation under sulfur-deprived conditions. F_t (open squares), $\Delta F/F_m'$ (solid circles), F_m' (open triangles), dissolved oxygen (pO_2, crosses), redox potential (E_h, asterisks) and H_2 content (%) in the gas phase of the culture vessel (solid squares). Incubation in sulfur-deprived medium started at 0 h.

Discussion

Effects of pH

The maximum yield and initial rates of H_2 photoproduction by sulfur-deprived cultures were observed at pHs that favor the residual activity of PSII (Fig. 1), strengthening our previous

observation that photosynthetic water oxidation is the main source of electrons for H_2 production (Ghirardi et al., 2000a and b; Kosourov et al., 2001; Kosourov et al., 2002). At the equivalent optimal initial pH of 7.7, hydrogenase activity is close to its maximum rate. In contrast, starch and protein degradation peak at acidic pHs, and generate fermentation products (acetate, formate and ethanol). Indeed, a specific inhibitor of PSII activity, DCMU, inhibits 80% of the rate of H_2 production (Ghirardi et al., 2000b; Antal et al., submitted), while DBMIB, an inhibitor of PQ oxidation by the cyt f/b_6 complex, completely inhibits H_2 photoproduction (Kosourov et al., submitted). Based on this observation, the remaining 20% of the reductant necessary for H_2 production must be derived from the anaerobic degradation of starch and/or protein and subsequent operation of the chlororespiratory pathway. Besides providing some reductants for H_2 production, starch and protein degradation must also generate reductants to remove the O_2 generated by the residual activity of PSII. This function is essential for preventing hydrogenase inactivation by O_2 and to sustain hydrogenase activity.

The major anaerobic products of starch and protein degradation in the dark are formate, acetate and ethanol (Gfeller and Gibbs,1984; Kreuzberg, 1984). Minor products, such as glycerol and lactate, are usually detected only at low pH (Kreuzberg, 1984). Anaerobic degradation of starch by *C. reinhardtii* cells in low light (Gfeller and Gibbs,1984) yields formate, CO_2 and H_2, reflecting a partial shift from anaerobic to aerobic respiratory pathways. Ethanol formation under these conditions is minimal since its production competes with the hydrogenase for reductants generated during the earlier steps of glycolysis. The observed shift in metabolic pathways in the light is explained by the photosynthetic production of O_2 in the light, which must be consumed by aerobic respiration. Under the experimental conditions used by Gfeller and Gibbs (1984), electron transport through the photosynthetic pathway is limited, and the amount of photosynthetically-generated O_2 is immediately consumed by cellular respiration, which maintains anaerobiosis in the photobioreactor and allowed for hydrogenase-catalyzed H_2 production to occur.

Similar shifts in the metabolic pathways of sulfur-deprived algal cells were observed as a function of the initial pH of the external medium. At or below pH = 7.0, 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 2:2:1.5:1.5. As the initial pH increases, the amount of H_2 produced reaches a maximum at pH 7.7, the amount of ethanol produced becomes negligible, and the stoichiometric amount of CO_2 increases, signaling a shift to aerobic metabolism. Finally, at pH 8.2, the anaerobic metabolism once again predominates, some ethanol is produced and little CO_2 and H_2 are evolved.

These results mean that, in terms of a future applied system, sulfur-deprived algal cultures can not only produce H_2 but will also produce fermentation products, if desired. We will thus be able to control the product mix simply by controlling the pH inside the photobioreactor. If fermentation products are desired, the algal system is robust enough to shift its metabolism to a fermentative mode upon lowering the pH.

Sulfate Re-addition

The selective inactivation of PSII in algal cultures can be induced by the deprivation of either sulfur or other nutrients from the growth medium (Ghirardi et al., 2000b). We showed that partial recovery of PSII activity occurs when sulfate is re-added in limiting amounts to the growth medium at the start of sulfur deprivation, following intensive sulfur removal by repeated centrifugation and washes (Kosourov et. al., 2002). This work further demonstrates that it is possible to re-add some sulfate to the sulfur-deprived cultures at other points during the process

without inactivating H_2 photoproduction (Table I). Additional experiments will be done to confirm whether this protective effect of sulfate is the result of partial re-activation of PSII as well. These results open up the possibility of maintaining high PSII capacity during the H_2-production phase by continuously supplying the medium with very low amounts of sulfate.

Photosystem II Photochemical Efficiency and O_2 Consumption Pathways

The maximum rate of H_2 production by sulfur-deprived algae is about 4 ml/h per photobioreactor or 9.4 µmoles H_2 • mg Chl^{-1} • h^{-1}. The corresponding PSII capacity for electron transport, measured under aerobic conditions, on the other hand, is about 40 µmoles H_2 • mg Chl^{-1} • h^{-1} (Melis et al., 2000), and the *in vitro* hydrogenase activity is 100 µmoles H_2 • mg Chl^{-1} • h^{-1} or higher (Fig.1; Ghirardi et al., 2000b; Kosourov et al., 2002). It is clear that the latter is not limiting H_2 production. Based on these observations, we would expect the maximum rate of H_2 photoproduction to be four times our current values. The fact that it is not suggests that anaerobiosis must somehow affect the activity of PSII. Indeed, we have shown that, under the anaerobic reducing conditions prevalent in our system, PSII photochemical activity is down-regulated and quickly reaches zero. What are the actual mechanisms responsible for the observed *in situ* changes in PSII activity? In sulfur-deprived cultures, the decreased ability of the cells to replace photodamaged D1 protein (Wykoff et al., 1998) leads to the accumulation of Q_B-non-reducing PSII centers that cannot produce O_2. These changes result in the establishment of anaerobic conditions in the reactor vessel in the light (Melis et al., 2000). Just as anaerobiosis is established, we demonstrated that a new, rapid down-regulation of PSII photochemical activity occurs (Fig. 2). This rapid down-regulation is not accompanied by a proportional loss of PSII capacity as previously measured by the capability of PSII to accumulate Q_A^- or to evolve O_2 when the cells are removed from the culture and exposed to aerobic conditions (Melis et al., 2000; Zhang et al., 2002; Antal et al., submitted). We suggest that the rapid *in situ* PSII down-regulation is a response of the algae to the over-reduction of the PQ pool, which must accompany the establishment of anaerobiosis (Nash et al., 1984). We also proposed that this must occur by the operation of non-photochemical quenching system (Antal et al., submitted). Since there is no O_2 to re-oxidize the PQ pool under anaerobic conditions and since PSI cannot further dispose of electrons from the pool (the hydrogenase is not active yet and there is little Rubisco to utilize photosynthetically-generated electrons (Zhang et al., 2002)), PSII activity is *reversibly* down-regulated. This phenomenon must occur to protect the PSII reaction center from damage by Chl triplet states formed by charge recombination in PSII, as proposed to occur during anaerobic photoinhibition (Andersson et al., 1992). A similar mechanism (PQ pool reduction by chlororespiration but at a slow rate) has been used to explain the irreversible loss of PSII activity in eukaryotic algae following exposure to high temperature (Yu et al., 1996) or when deprived of nutrients other than sulfur (Yu et al, 1996;, Guikema et al., 1983; Yu et al., 1989).

The consumption of photosynthetically produced O_2 during the H_2-production phase is essential for maintaining the culture medium anaerobic for the continued operation of the hydrogenase. Oxygen consumption in *C. reinhardtii* is catalyzed by three main oxidases: (a) the chloroplast PQ-oxidase, (b) the mitochondrial cytochrome c-oxidase, and (c) the mitochondrial alternative oxidase. The mitochondrial cytochrome oxidase in *C. reinhardtii* is inhibited by KCN, CO and sodium azide, while the alternative oxidase is specifically affected by salicyl hydroxamic acid (SHAM). The putative chloroplast PQ oxidase, on the other hand, is not inhibited by SHAM and only slightly affected by KCN (Cournac et al., 2000). By measuring the influence of the two inhibitors, KCN and SHAM, on the rates of dark respiration, we estimated the relative contributions of each of the three oxidases to the consumption of O_2 at different times after sulfur deprivation (not shown; Antal et al., submitted). The actual contributions of the three

oxidases to the scavenging of O_2 *in situ* depend on many factors such as their relative K_M's for O_2 and the rate of diffusion of O_2 from the chloroplast to the mitochondria. At the start of sulfur deprivation, the cytochrome oxidase and the alternative oxidase contribute, respectively, about 60 and 25% to the overall respiratory capacity of the cells (measured in the presence of 0.17 µmoles/ml O_2). At the start of the anaerobic phase, the activities of the two mitochondrial oxidases decrease substantially to 16 and 0% of the total respiration capacity, respectively. This indicates a rapid shift from mitochondrial respiration to chlororespiration. The shift towards chlororespiration was coupled to the down-regulation of electron transport from PSII (Fig. 2), caused by the complete removal of O_2 from the sulfur-deprived culture medium and the over-reduction of the PQ pool. Once H_2 production started and accompanying up-regulation of PSII began, photosynthetically-generated O_2 appeared to be consumed equally by the mitochondrial (49%) and chloroplast (51%) oxidases. During the H_2-production phase, the contribution of the chlororespiratory PQ oxidase remained unchanged. However, over the same period of time, the contribution of the mitochondrial cytochrome oxidase declined dramatically while that of the alternative oxidase increased. At the end of the H_2-production phase, the mitochondrial respiratory activity was due mostly to the alternative oxidase. Thus, the inhibitor studies support the active role of chlororespiration in maintaining the anaerobic conditions in the chloroplast required for H_2 photoproduction, and they are also consistent with the idea that the alternative oxidase is expressed preferentially under stress conditions (Weger and Dasgupta, 1993; Parsons et al., 1999).

In summary, changes of PSII activity in *C. reinhardtii* cells deprived of inorganic sulfur are characterized by complex dynamics during the course of cell adaptation to the nutrient stress. We conclude that the redox state of the PQ pool is the primary factor in regulating the activity of PSII water-splitting capacity during all stages of sulfur deprivation. Thus, the redox potential of the PQ pool depends on the relationship between the rates of photosynthesis, chlororespiration, respiration, and H_2 production. We show that the technique of PAM fluorescence presents us with a practical and inexpensive means to externally monitor anaerobiosis and the start of H_2 photoproduction by the cultures inside the photobioreactor and could be further developed for use with future applied systems.

Acknowledgments

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