Probing green algal hydrogen production

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The recently developed two-stage photosynthesis and H₂-production protocol with green algae is further investigated in this work. The method employs S deprivation as a tool for the metabolic regulation of photosynthesis. In the presence of S, green algae perform normal photosynthesis, carbohydrate accumulation and oxygen production. In the absence of S, normal photosynthesis stops and the algae slip into the H₂-production mode. For the first time, to our knowledge, significant amounts of H₂ gas were generated, essentially from sunlight and water. Rates of H₂ production could be sustained continuously for ca. 80 h in the light, but gradually declined thereafter. This work examines biochemical and physiological aspects of this process in the absence or presence of limiting amounts of S nutrients. Moreover, the effects of salinity and of uncouplers of phosphorylation are investigated. It is shown that limiting levels of S can sustain intermediate levels of oxygenic photosynthesis, in essence raising the prospect of a calibration of the rate of photosynthesis by the S content in the growth medium of the algae. It is concluded that careful titration of the supply of S nutrients in the green alga medium might permit the development of a continuous H₂-production process.

Keywords: *Chlamydomonas reinhardtii*; hydrogen production; hydrogenase; photosynthesis; substrate catabolism; sulphur deprivation

1. INTRODUCTION

Concerns about energy supply and demand, global warming and environmental pollution, combined with projections of potential fossil fuel shortfall toward the middle of the 21st century, make it imperative to develop alternative energy sources that are clean, renewable and environmentally friendly. H₂ is thought to be ideal as an alternative fuel and carrier of energy. A challenging problem in establishing H₂ as a useful energy carrier for the future is the renewable and environmentally friendly generation of large quantities of H₂ gas. Promising in this respect is the process of photosynthetic H₂ production by unicellular green algae.

The process of oxygenic photosynthesis begins with the utilization of sunlight for the oxidation of water molecules and culminates with the conversion of inorganic minerals (e.g. carbon dioxide, nitrate and sulphate) into the major building blocks of life. The absorption of sunlight and the conversion of excitation energy to chemical energy by PSII and PSI in the thylakoid membrane (Hill & Bendall 1960; Duysens *et al.* 1961) provide the driving force of this overall endergonic reaction. Light energy in PSII specifically facilitates the generation of a strong oxidant capable of oxidizing H₂O molecules. As a result of this oxidation, molecular O₂ is released within the chloroplast (Barber 1989) and gradually diffuses into the atmosphere. Electrons extracted upon the oxidation of H₂O are processed through the electron-transport chain in the chloroplast

thylakoids until they reach the electron carrier, ferredoxin. The potential energy of electrons in ferredoxin is far greater than that in H₂O.

A number of unicellular green algae are known to contain genes encoding for an Fe-type 'hydrogenase' (Roessler & Lien 1984; Adams 1990; Happe & Naber 1993; Appel & Schulz 1998). This Fe hydrogenase is coded in the nucleus by the *hydA* gene (Florin *et al.* 2001; Wünschiers *et al.* 2001), synthesized in the cytosol and imported into the chloroplast (Happe *et al.* 1994). The Fe hydrogenase can receive electrons from reduced ferredoxin and efficiently donate them to protons (Greenbaum 1988; Peters *et al.* 1998), thereby generating molecular H₂ (Gaffron & Rubin 1942). In theory, the photosynthetic apparatus of unicellular green algae could photo-oxidize H₂O, elevate the potential energy of the resulting electrons by passing them through the electron-transport chain to ferredoxin, and release H₂ via the Fe hydrogenase. This would constitute an efficient H₂-photoproduction system. In practice, however, the simultaneous H₂O photo-oxidation/O₂ evolution and H₂ photoproduction is not observed because the Fe hydrogenase is extremely sensitive to molecular O₂. O₂ is a positive suppressor of *hydA* gene expression (Florin *et al.* 2001) and a potent inhibitor of the Fe hydrogenase (Ghirardi *et al.* 2000). Anaerobic conditions are required for the hydrogenase gene expression and function (Greenbaum 1982; Happe & Naber 1993), and low concentrations of O₂, as low as 0.2% O₂ partial pressure, are sufficient to bring about a 50% inhibition in H₂ photoproduction in *Chlamydomonas reinhardtii* (Ghirardi *et al.* 2000). In brief, the simultaneous H₂O photo-oxidation–O₂ evolution and H₂ photoproduction reactions in the chloroplast of green algae have appeared to be mutually exclusive. Moreover, given

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the O₂ sensitivity of the [Fe] hydrogenase and the abundance of O₂ in the present-day environment, questions have been asked as to the biological and commercial significance of the [Fe] hydrogenase. Could it be anything more than a relic of the evolutionary past of the chloroplast in green algae, or could this enzyme and the process of photosynthesis be utilized to generate H₂ gas for commercial purposes (Melis & Happe 2001)?

In a recent advance, the mutual incompatibility of O₂ evolution and H₂ production was successfully bypassed via a temporal separation of the two processes upon the S deprivation of the algae (Melis *et al.* 2000). This novel protocol employs a repetitive application and removal of sulphate minerals to the growth medium of the algae (Ghirardi *et al.* 2000). In the presence of sulphates, green algae perform normal photosynthesis, biomass accumulation and O₂ production. In the absence of sulphates, normal photosynthesis and O₂ evolution stop. An alternative form of photosynthesis is established in the chloroplast of the algae, one that utilizes sunlight and the [Fe]-hydrogenase pathway, leading to H₂ production. Inhibition in cell division and a gradual loss in biomass (starch and protein) accompany the latter (Zhang *et al.* 2002). The Fe-hydrogenase pathway may be viewed as a light-dependent bioenergetic process, which employs the photosynthetic electron-transport chain in chloroplasts for the generation of ATP and utilizes protons (H⁺) as the terminal electron acceptors, resulting in the generation of substantial amounts of H₂ gas by the green algae. This process is thought to ensure the survival of the S-deprived cells over prolonged periods of time. The present report investigates further biochemical and physiological aspects of this photosynthetic H₂-production process with an emphasis on the experimental conditions that might improve the continuity and increase the yield of the process.

2. MATERIAL AND METHODS

(a) *Growth of the algae*

Chlamydomonas reinhardtii strain CC124 (mt⁻) was grown photoheterotrophically in a TAP medium, pH 7.0. Liquid cultures were grown at 25 °C in flat Roux-type bottles (*ca.* 4 cm optical path length, 900 ml capacity) with stirring and under continuous cool-white fluorescence illumination of ~200 μmol of photons m⁻² s⁻¹. Culture density was measured by cell counting with the improved Neubauer ultraplane hemacytometer and a BH-2 light microscope (Olympus; Tokyo) operated at a magnification of ×200. Chl was measured according to Arnon (1949). Cells were grown to mid-late logarithmic phase (*ca.* 2–4 × 10⁶ cells ml⁻¹). After they reached this density, cells were suspended in the absence of S and incubated under continuous illumination for up to 120 h. S-free media (TAP-S) were prepared upon replacement of the S salts by their chloride counterparts.

(b) *Oxygen exchange and gas collection measurements*

Oxygen-exchange activity of the cells was monitored at 25 °C with a Clark-type O₂ electrode illuminated with a slide projector lamp. Yellow actinic excitation was provided by a CS 3-69 cut-off filter (Corning, NY). A 5 ml aliquot of the culture was supplemented with 100 μl of 0.5 M NaHCO₃, pH 7.4 (Melis *et al.*

1999). Measurements were taken with the O₂ electrode, beginning with the registration of dark respiration in the cell suspension and followed by measurement of the light-saturated rate of O₂ evolution. The rate of each process was recorded for *ca.* 5 min. Culture bottles were sealed with a size 5 silicon stopper perforated with a gauge 10 Hamilton stainless steel syringe. Teflon tubing (HPLC, Aminco; Lake Forest, CA), attached to the outside-protruding portion of the syringe, was used to conduct the gas evolved by the algae in the culture bottle to an upside-down burette filled with water. The volume of the gas collected in the burette was measured directly from the volume of water that was displaced.

(c) *Spectrophotometric measurements*

The amplitude of the light minus dark absorbance difference measurements at 320 and 700 nm was employed for the direct quantitation of Q_A and P₇₀₀ in the thylakoids of *C. reinhardtii* (Melis 1989). These measurements provided estimates of the concentration of the functional PSII and PSI reaction centres, respectively, in the samples at various times following S deprivation or S limitation. Thylakoid membrane isolation procedures were described earlier (Melis *et al.* 1996).

(d) *SDS-PAGE and Western blot analysis*

Cells were harvested by centrifugation at 3000g for 3 min at 4 °C. For total cell extract analysis, pellets were resuspended in a small volume of solubilization buffer, containing 0.5 M Tris-HCl (pH 6.8), 7% SDS, 20% glycerol and 2 M urea. The mixture was incubated at room temperature for 60 min with frequent vigorous vortexing. At the end of the 60 min incubation, microscopic examination revealed a quantitative disintegration of the cells. The concentration of Chl was determined in this crude extract, β-mercaptoethanol was added to make a 10% final concentration, followed by heating of the sample to 70 °C for 5 min. Prior to loading, the crude extract was centrifuged at 1000g for 1 min to remove cell debris. Protein electrophoresis was carried out according to Laemmli (1970), using a 4.5% acrylamide stacking gel and a 12.5% acrylamide resolving gel. Unless otherwise stated, gel lanes were loaded with an equal amount of Chl (4 nmol). Electrophoresis was carried out at 4 °C and at a constant current of 9 mA for 16 h. Gels were stained with 1% Coomassie Brilliant Blue R for protein visualization. Essentially the same results were obtained upon inclusion or omission of 4 M urea in the gel system. Electrophoretic transfer of the SDS-PAGE-resolved polypeptides to nitrocellulose was followed by incubation of this membrane filter with specific polyclonal antibodies. The cross-reaction between antibodies and protein was detected via a chromogenic reaction with anti immunoglobulin-G secondary antibodies conjugated with alkaline phosphatase (BioRad; Hercules, CA). Immuno-decorated membranes were scanned with a Hewlett Packard Scanjet 5300C optical scanner connected to a Macintosh/G3 computer.

3. RESULTS AND DISCUSSION

(a) *Photosynthesis, respiration and hydrogen production as a function of sulphur deprivation*

The lack of sulphate minerals from the growth medium brings about a gradual but reversible inhibition in the photochemical activity of PSII in chloroplasts, resulting in a gradual slow-down of oxygenic photosynthesis. The reason for such inhibition in the absence of S is the apparent

Figure 1. Light-saturation curves of photosynthesis in *C. reinhardtii*. The rates of O₂ evolution on a per Chl basis were measured as a function of incident light intensity to the cell suspension. Aliquots of cells were taken from a culture at different time points of the S deprivation (0–30 h). Note the declining rate of light-saturated photosynthesis as a function of time in the absence of S. Open circles, 0 h; large filled circles, 5 h; filled squares, 8 h; small filled diamonds, 11 h; small filled circles, 24 h; filled diamonds, 30 h.

inability of chloroplasts to do high rates of *de novo* protein biosynthesis, which are needed for the frequent replacement of the D1/32 kD reaction-centre protein in the H₂O-oxidizing PSII complex (Mattoo & Edelman 1987). In the absence of S, which is an essential component of the amino acids cysteine and methionine, protein biosynthesis is impeded and the PSII repair cycle is blocked (Wykoff *et al.* 1998).

The effect of S deprivation on the characteristic signatures of photosynthesis, i.e. the light-saturation curve of photosynthesis (the so-called 'P versus I' curve) in which the rate of O₂ evolution, or CO₂ assimilation, is measured and plotted as a function of the probing actinic light intensity, is shown in figure 1. In such measurements, the rate of photosynthesis first increases linearly with light intensity and then reaches a plateau ($P_{\max}$) as the saturating light intensity is approached. The light-saturated rate ($P_{\max}$) provides a measure of the capacity of photosynthesis for the algal sample (Powles & Critchley 1980; Neale *et al.* 1993). Figure 1 shows a family of curves measured with *C. reinhardtii* under different stages of S deprivation (0–30 h). It is evident that the light-saturated rate ($P_{\max}$) declines as a function of time in S deprivation. After 24–30 h of S deprivation, the rate of the residual photosynthesis was less than that of respiration, resulting in negative $P_{\max}$ values (figure 1, 24 and 30 h samples).

The relationship between photosynthesis and respiration in the S-deprived green algae was probed in greater detail and over a longer period of time. In the total absence of S (figure 2, 0 μM , open circles), the activity of photosynthetic H₂O oxidation and O₂ evolution ($P_{\max}$) declined with a half-time of *ca.* 13 h (figure 2a, open circles), from *ca.* 40 mmol O₂ (mol Chl)⁻¹ s⁻¹ at $t=0$ h, to *ca.* 4 mmol O₂ (mol Chl)⁻¹ s⁻¹ after *ca.* 40 h of S deprivation. Interestingly, photosynthetic O₂ evolution activity appeared to become stabilized at this low level for times $t > 60$ h (see also Melis *et al.* 2000; Ghirardi *et al.* 2000).

Figure 2. (a) Absolute activity of oxygenic photosynthesis and respiration in *C. reinhardtii* suspended in S-deprived (0 μM , open circles) or S-limited (50 μM , filled circles) media. The rate of cellular respiration (open triangles) was recorded in the dark, followed by measurement of the light-saturated rate of photosynthesis. Cultures at 0 h contained 2.2×10^6 cells ml⁻¹. (b) H₂-gas production and accumulation by *C. reinhardtii* cells suspended in S-deprived (0 μM , open circles) or S-limited (50 μM , filled circles) media. Gases were collected in an inverted burette and measured from the volume of water displacement.

This result indicated that ~10% of the PSII water oxidation activity is retained, even after prolonged S deprivation. When *C. reinhardtii* cells were suspended in media with limited amounts of sulphates (figure 2, 50 μM , filled circles), H₂O-oxidation activity declined with a half-time of 22 h, i.e. longer than that in the absence of S. These results indicate that onset of inhibition in PSII activity is strongly dependent upon residual S in the growth medium, and point to the possibility of regulation of PSII activity by S titration of the growth medium.

The activity of mitochondrial respiration was not significantly affected by the regime of S deprivation (figure 2a, open triangles). It declined only gradually as a function of time in S deprivation from *ca.* 12 mmol O₂ (mol Chl)⁻¹ s⁻¹ at $t=0$ h to *ca.* 6 mmol O₂ (mol Chl)⁻¹ s⁻¹ at $t=120$ h. In summary, over a period of 120 h, S deprivation specifically affects oxygenic photosynthesis but not mitochondrial respiration. As a result of the specific lowering of oxygenic photosynthesis, the rate of O₂ production by the chloroplasts became slower than the rate of O₂ consumption by the mitochondria. This 'crossover' point was reached within *ca.* 22 h in the S-deprived sample (figure 2a, 0 μM , open circles) or

Figure 3. Time-course of the density of cells in *C. reinhardtii* cultures suspended in S-deprived (0 μM) or S-limited (50 μM) media. Cells were first grown on TAP medium. Incubation under S-deprived and S-limited conditions started at 0 h. Open circles, 0 μM S; filled circles, 50 μM S.

within *ca.* 40 h in the S-limited sample (figure 2a, 50 μM , filled circles). Sealed *C. reinhardtii* cultures became anaerobic soon after this crossover point between the activities of photosynthesis and respiration (Ghirardi *et al.* 2000). Bubbles of H₂ gas could be seen soon after this crossover point and the establishment of anaerobiosis in the culture, commencing at *ca.* 24 h in S deprivation (figure 2b, 0 μM). The rate of H₂ production was fairly constant at 2.5 ml h⁻¹ during the 27–60 h period. This is the equivalent of *ca.* 5 mmol H₂ (mol Chl)⁻¹ s⁻¹, which is only *ca.* 15% of the light-saturated rate of O₂ evolution (P_{max}) in the control (figure 1, 0 h). The rate of H₂ production gradually declined at S-deprivation times that were longer than 60–70 h (figure 2b). In the 50 μM S sample, H₂ production commenced later (at *ca.* 40 h) and lasted longer, leading to greater yields of gas. However, the yield of H₂ production was less per cell in the 50 μM S sample than in the 0 μM S sample, apparently due to a greater cell density in the former than in the latter. Indeed, figure 3 shows the cell density of cultures incubated in the absence of S (0 μM), or in the presence of limited amounts of S (50 μM). Following cell suspension in a new medium in the absence of S, cell density increased slightly (0–40 h) and then remained constant (40–120 h) (figure 3, open circles). However, following cell suspension in a new medium in the presence of 50 μM S, cell density increased substantially in the 0–50 h period and remained more-or-less constant thereafter (50–120 h). These results are consistent with the notion of a limitation to growth imposed by this nutrient in the concentration range employed. Qualitatively similar results were obtained when Chl content instead of cell density was measured (Zhang *et al.* 2002).

(b) Photosystem stoichiometry in the sulphur-deprived *C. reinhardtii*

The concentration of the functional photosystems (PSII and PSI) in the thylakoid membrane of S-deprived *C. reinhardtii* was investigated spectrophotometrically (figure 4). PSII was measured from the amplitude of the light-minus-dark absorbance change at 320 nm

Figure 4. Concentration of (a) functional PSII (Q_A) and (b) of PSI (P_{700}) in *C. reinhardtii* as a function of time in the absence (0 μM , open circles) or presence of limited amounts (50 μM , filled circles) of sulphate nutrients.

(measuring the photochemical reduction of Q_A in the reaction centre of PSII). Figure 4a shows that the amount of functional PSII Q_A declined under S deprivation with nearly exponential kinetics (Melis *et al.* 2000). Loss of the D1/32 kD reaction-centre protein of PSII was also noted under these conditions (Zhang *et al.* 2002), indicating degradation and removal of PSII under S deprivation. It is noteworthy that loss in the activity of H₂O oxidation (half-time 13 h, figure 2a) was substantially faster than loss in Q_A photoreduction (half-time 35 h, figure 4a), showing that S deprivation first inhibits H₂O-oxidation activity without adversely affecting the primary charge-separation activity at PSII. Only at later times is the primary charge-separation activity adversely affected.

When the cell suspension was transferred to a new medium in the presence of limiting amounts of 50 μM S, there was a lag in the loss of functional PSII, which lasted for *ca.* 24 h (figure 4a, filled circles). This result reinforces the notion that supply of inorganic S to the chloroplast in the green algae is central in the determination of the PSII functional status (Wykoff *et al.* 1998).

PSI activity was measured from the amplitude of the light-minus-dark absorbance change at 700 nm (measuring the photochemical oxidation of the reaction-centre P_{700} of PSI). Figure 4b shows that PSI primary charge-separation activity declined, albeit slowly, with time in the absence of S. The half-time for the loss of functional PSI was *ca.* 85 h (slower than that for PSII) under S-deprivation conditions. No loss of PSI activity

could be detected when cells were suspended in the presence of limiting amounts of 50 μ M S (figure 4b, filled circles).

(c) Protein substrate catabolism under sulphur-deprivation conditions in *C. reinhardtii*

The operation of the [Fe]-hydrogenase pathway and the ensuing production of molecular H₂, observed in *C. reinhardtii* under conditions of low oxygenic photosynthesis and low oxidative phosphorylation activity, may confer a survival advantage to the organism under S-deprivation stress. The associated electron transport pathway generates a pH gradient across the thylakoid membrane, which is utilized for the synthesis of ATP (photophosphorylation) in the absence of carbon assimilation. The residual H₂O-oxidation activity, observed under prolonged S deprivation (figure 2a, 40–120 h), feeds electrons into the Fe-hydrogenase pathway and helps to enhance the level of photophosphorylation in the chloroplast. Mitochondrial respiration scavenges the small amounts of O₂ that evolve due to this residual activity of photosynthesis. Thus, cellular respiration ensures the maintenance of anaerobiosis in the culture and, simultaneously, the operation of a basal level of oxidative phosphorylation (Melis & Happe 2001), thereby adding to the amount of ATP generated by the cell. Obviously, endogenous substrate mobilization and catabolism under S-deprivation conditions must serve to support this integrated bioenergetic process, the end result of which is H₂ production.

Recent work has shown that both starch and protein catabolism takes place upon S deprivation and H₂ production (Zhang *et al.* 2002). The identity of 'target' proteins and their catabolism as a function of S deprivation or S limitation was investigated. Insight was gained in Western blot experiments with specific polyclonal antibodies against Rubisco and the LHCII: the major protein constituents of the chloroplast. Figure 5a shows a Western blot analysis of Rubisco in *C. reinhardtii* suspended in the absence of S (0 μ M) or in the presence of limited amount of S (50 μ M). The results show a precipitous decline in the amount of this protein as a function of time in S deprivation (Ferreira & Teixeira 1992; Giordano *et al.* 2000; Zhang *et al.* 2002). Only ca. 3% of this important protein were present at ca. 48 h of S deprivation. The level of Rubisco was below the detection limit of this method at times $t > 48$ h. This finding indicates that the reductant generated from the residual photosynthetic H₂O-oxidation activity at times $t > 48$ h (figure 2a) is probably not utilized in the Calvin cycle for CO₂ fixation (lack of Rubisco). Rather, electrons derived upon the residual photosynthetic H₂O-oxidation activity under S deprivation are probably channelled through the [Fe]-hydrogenase pathway and thus quantitatively contribute to the generation of molecular H₂ (figure 2b). In the presence of limited amount of S, loss of Rubisco occurred with a half-time that was considerably longer than that in the absence of S (figure 5a, 50 μ M). These results indicate that the onset of Rubisco catabolism is strongly dependent upon the concentration of S in the growth medium.

Figure 5b shows changes in the composition and amount of the LHCII upon S deprivation. It is of interest to note that, under S-deprivation conditions (0 μ M),

Figure 5. Western blot analysis of (a) Rubisco and (b) the LHCII in *C. reinhardtii*. Cells were incubated over periods of time in the absence of S (0 μ M) or in the presence of limited amounts of this nutrient (50 μ M). Lanes were loaded with 4 nmol Chl of the total cell extract. Nitrocellulose filters were probed with specific polyclonal antibodies that were raised either against the large subunit of Rubisco or the LHCII.

alterations in LHCII were recorded at times $t > 60$ h, which coincides with the levelling-off of the H₂O-oxidation activity in the photosynthetic apparatus (figure 2a, 0 μ M). In the presence of a limited amount of S, alterations in the composition and amount of the LHCII occurred at times $t > 80$ h. These results are consistent with the notion of a hierarchy in the targets and pattern of protein catabolism in the chloroplast, with Rubisco catabolism preceding that of the LHCII.

In conclusion, the combined results (figures 1–5) provide evidence that S availability to the chloroplast acts as a metabolic switch, which regulates the reversible transition of the chloroplast between normal photosynthesis and H₂ production in the green algae. This regulatory mechanism affects not only normal photosynthesis and H₂ production, but also cell growth and multiplication, substrate metabolism and the induction of the [Fe]-hydrogenase pathway. In principle then, it should be possible carefully to titrate the supply of S nutrients to the green algae so as to stabilize the activity of oxygenic photosynthesis to a level that is just lower than that of mitochondrial respiration. Under these conditions, i.e. steady-state rates of respiration slightly greater than those of photosynthesis, sealed green algal cultures will become anaerobic in the light. Anaerobiosis will permit the induction and function of the [Fe] hydrogenase in the cells. Such an experimental process would constitute a continuous H₂-production system.

(d) Effect of salinity on photosynthesis and hydrogen production

The purpose of the [Fe]-hydrogenase pathway, leading to H₂ production in chloroplasts, is to generate ATP for the metabolic needs of the cell (Schlegel & Schneider 1978; Melis & Happe 2001). It may then be assumed that physiological and environmental conditions that require a greater cellular expenditure of ATP may cause a stimulation of the rate of electron transport via the Fe-hydrogenase pathway, thereby resulting in greater yields of H₂-gas production. Salinity is an environmental condition that requires expenditure of cellular ATP to prevent accumulation of unwanted Na⁺ cations in the cytosol of green algae. Accordingly, the research investigated the yield of H₂ production as a function of NaCl concentration in the growth medium.

The Chl content of the cells and the viability of *C. reinhardtii* were measured as a function of time upon S deprivation in cultures that contained different amounts of NaCl. Cells were grown in TAP medium until they reached the mid-late log phase. They were then suspended in TAP-S medium in the presence of varying amounts of NaCl. A concentration range of between 0 and 15 mM NaCl was chosen, as this did not negatively impact the rate of normal photosynthesis or cellular respiration. Figure 6 shows the Chl content, cell density and Chl per cell in the culture as a function of time in S deprivation. It is seen that the typical response of the cells to S deprivation was not altered in the NaCl range between 0 and 15 mM. In all cases, upon S deprivation, the cell density in the cultures increased from *ca.* 4×10^6 cells ml⁻¹ at 0 h to *ca.* 6×10^6 cells ml⁻¹ at *ca.* 24 h. Subsequently, cell density stayed constant or declined slightly during the 24–100 h period. Salinity (up to 15 mM NaCl) did not affect the viability of the cells under S deprivation. Figure 6 also shows that, in all cases, the Chl content of the culture increased transiently during the 0–10 h S-deprivation period but declined steadily thereafter. After 100 h of S deprivation, only *ca.* 60% of the Chl had remained in the cells, indicating regulated catabolism of some of the Chl-protein complexes (Melis *et al.* 2000; Zhang *et al.* 2002). The Chl per cell declined from *ca.* 2×10^{-15} mol Chl cell⁻¹ to *ca.* 1×10^{-15} mol Chl cell⁻¹ after 100 h of S deprivation. These results show that a limited number of cell divisions take place only during the first 24 h of S deprivation. A loss of Chl occurs gradually throughout the 10–100 h S-deprivation period. Salinity, up to 15 mM NaCl, did not measurably affect the time-course or pattern of these phenomena.

Measurements of photosynthesis and respiration as a function of salinity in S-deprived cultures were carried out according to the experimental protocol of figure 2. The capacity of photosynthesis, measured from the light-saturated rate of O₂ evolution $P_{\max}$ in *C. reinhardtii* (figure 7a), declined bi-exponentially from *ca.* 40 mmol O₂ (mol Chl)⁻¹ s⁻¹ at *t* = 0 h to *ca.* 2–4 mmol O₂ (mol Chl)⁻¹ s⁻¹ at *t* = 100 h. Cellular respiration, as measured from the rate of O₂ consumption in the dark (figure 7b), remained fairly constant at *ca.* 10–12 mmol O₂ (mol Chl)⁻¹ s⁻¹ over the 0–100 h period. Thus, irrespective of the NaCl concentration used, the absolute activity of photosynthesis dropped below that of respiration in *C. reinhardtii* after *ca.* 25 h of S deprivation. This pattern

Figure 6. Chl concentration, cell density and Chl per cell as a function of time in S-deprived *C. reinhardtii*. The different panels depict this set of measurements with cells suspended in media of variable salinity: (a) 0 mM, (b) 5 mM, (c) 10 mM and (d) 15 mM NaCl. The initial values, at *t* = 0 h, were Chl = 10.6 μ M, cell ml⁻¹ = 4.2×10^6 , Chl cell⁻¹ = 2.5×10^{-15} mol cell⁻¹. White squares, Chl; white circles, cell ml⁻¹; black triangles, Chl per cell.

did not change as a function of salinity, at least in the 0–15 mM NaCl concentration. At higher salinity, the activity of photosynthesis and the ability of cells to function were adversely affected (Neale & Melis 1989).

At S-deprivation times of *t* > 25 h, owing to the significantly greater rate of respiration than photosynthesis in the cells, sealed *C. reinhardtii* cultures became anaerobic in the light. Anaerobiosis was confirmed by measurements with a Clark-type O₂ electrode (results not shown). Under these conditions, *C. reinhardtii* stops normal photosynthesis and turns to the [Fe]-hydrogenase pathway for electron transport, leading to H₂ production. Figure 8 shows

Figure 7. Absolute activity of (a) oxygenic photosynthesis and (b) oxidative respiration in *C. reinhardtii* cells suspended in a medium devoid of S but containing different concentrations of NaCl (0–15 mM). Incubation under S deprivation and a variety of salinity conditions started at 0 h. Aliquots were harvested at different times during this incubation and the rate of cellular respiration was recorded in the dark, followed by a measurement of the rate of light-saturated photosynthesis. Open circles, 0 mM; filled circles, 5 mM; open triangles, 10 mM; filled triangles, 15 mM.

the results of H₂ accumulation with S-deprived cultures suspended under different salinity conditions. It is seen that cultures under mild salinity (5 and 10 mM) perform slightly better than the control (0 mM NaCl) and also better than the 15 mM NaCl sample. The optimal concentration of NaCl for maximum H₂ production was 10 mM. Under this salinity condition (10 mM NaCl), H₂-production activity by the cells was enhanced by *ca.* 30% in the initial rate and 40% in the final yield, compared with that of the control.

These results are consistent with the hypothesis that the [Fe]-hydrogenase pathway serves to drive the formation of ATP for the metabolic needs of the cells (Melis & Happe 2001). The results also indicate that further refinement in the design of the cell-growth media, including the application of mild stress conditions, may lead to further enhancement in the rate constant and yield of H₂ production by green algae.

(e) Effect of ATP biosynthesis uncouplers

The objective of this set of measurements was to test whether uncoupling of the ATP-generation process from the electron-transport chain might release the 'load' and therefore permit greater rates of H₂ production. Two

Figure 8. H₂ gas accumulated by displacement of water in an inverted burette as a function of S-deprivation time in the green alga *C. reinhardtii*. The culture was sealed at *ca.* 25 h after suspension of the cells in a S-free medium. The salinity of the cultures is indicated as follows: circles, 0 mM; squares, 5 mM; triangles, 10 mM; diamonds, 15 mM.

classes of uncouplers were tested and the results are described in the following paragraphs, for each category.

Methylamine hydrochloride sequesters in the interior (lumen) of the photosynthetic membranes. There, it acts by binding protons as the latter are deposited during electron transport. This binding prevents the accumulation of 'free' protons in the lumen and alleviates the potentially inhibitory effect that a Δ pH may have on the process of electron transport. In these experiments, variable amounts (0–10 mM) of CH₃NH₂·HCl (pH 7.4) were added to the *C. reinhardtii* media 24 h after S deprivation and immediately prior to the sealing of the cultures. Hydrogen accumulation was monitored by the inverted burette method. Figure 9a presents a family of curves showing initiation of H₂ production at *ca.* 27 h. In several such experiments, cultures in the presence of low amounts of methylamine (less than 2 mM) produced H₂ with rates and yields similar to the control (figure 9a, 0 mM). Methylamine concentrations in the 2–7 mM range enhanced the rate and yield, albeit by modest amounts. The initial rate of H₂ production in the presence of 5 mM methylamine was greater than that of the control by 20–25%. Yields at 96 h were 10–20% better in the presence of 5 mM methylamine than in the control. These results with methylamine support the notion of enhancement in the rate of electron transport (by 20–25%) due to the uncoupling of the ATP-generation process.

FCCP represents yet another class of ATP biosynthesis inhibitors acting in a mode that does not necessarily involve dissipation of sequestered protons from the chloroplast lumen. Irrespective of the mode of action of this ATP biosynthesis inhibitor, the results depicted in figure 9b show that FCCP invariably lowers the rate and yield of H₂ production. Even low micromolar quantities of FCCP had a significant inhibitory effect, indicating that the mode of action of this ATP biosynthesis inhibitor is detrimental to the H₂-production process. At a concentration of 5 μ M FCCP, the rate and yield of H₂ production were lowered by *ca.* 80%.

Figure 9. H₂ gas accumulated by displacement of water in an inverted burette as a function of S-deprivation time in the green alga *C. reinhardtii*. Cultures were sealed at ca. 24 h after suspension of the cells in a S-free medium. (a) Immediately prior to the sealing of the cultures, CH₃NH₂-HCl was added to the culture medium (concentration range from 0 to 10 mM). Open circles, 0 mM; filled circles, 2.5 mM; filled diamonds, 5 mM; open squares, 10 mM. (b) FCCP was added to the medium (concentration range from 0 to 5 μM). Open circles, 0 μM; filled circles, 2.5 μM; filled diamonds, 5 μM.

4. CONCLUSIONS

In summary, S deprivation of *C. reinhardtii* causes a selective lowering of the photosynthetic H₂O-oxidation activity by ca. 90% without significantly affecting the activity of cellular respiration. In consequence, the activity of photosynthesis becomes less than that of cellular respiration. The ensuing anaerobiosis is necessary and sufficient for the induction of a light-dependent H₂ production in the cells. H₂ production is accompanied by significant catabolic activity of starch and protein (Gfeller & Gibbs 1984, 1985; Zhang *et al.* 2002), which generate endogenous substrate for the cellular bioenergetic processes (Melis & Happe 2001). Among the cellular proteins examined, of interest is the effect of S deprivation on Rubisco and LHCII. Temporally, the catabolism of Rubisco preceded that of the LHCII (figure 5). Western blot analyses showed a prompt and precipitous decline in the amount of Rubisco upon S deprivation. No Rubisco could be detected at $t > 48$ h in S deprivation (figure 5a), indicating that cells quickly abandon the Calvin cycle of CO₂ fixation under S starvation. Also observed was a regulated

catabolism of the LHCII, which, however, was initiated after the completion of the Rubisco catabolism. A fairly abrupt change in the amount and subunit composition of the LHCII appeared after ca. 60 h of S deprivation. The highest-molecular-weight subunit of the LHCII appeared to become reduced in size by ca. 1 kDa (from ~31 to ~30 kDa, figure 5b). We speculate that a quantitative N-terminal clipping of these large subunits of the LHCII is probably the reason for the observed change in their electrophoretic mobility. Moreover, the relative concentration of one of the smaller LHCII subunits, migrating to ca. 28 kDa, increased at S-deprivation times of $t > 60$ h. It is concluded that S deprivation initiates a highly regulated catabolic activity of starch and protein in *C. reinhardtii*. We propose that this regulated process is designed to support and sustain the [Fe]-hydrogenase pathway for the survival of the cells under a protracted S-deprivation stress.

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Discussion

R. D. Britt (*Department of Chemistry, University of California, Davis, CA, USA*). I wonder how the community in general views this kind of work?

A. Melis. The general response from the press, environmental groups and the public at large has been very positive on the question of utilizing genetically modified organisms to produce clean energy, if that's what you mean? It's still an open one and I think the scientific community, all of us, and the public, will have to sort it out. I think it is a matter of us scientists educating the public. We have not done a very good job on this in the past. I am encouraged by the very positive publicity that we received on this discovery, although the work we have published so far did not involve genetic engineering of the green algae. However, it may be necessary to genetically engineer the algae to increase the yield and improve the continuity of the process. The question of whether we can then deploy in the field such genetically modified organisms to generate needed energy is still an open one.

R. D. Britt. Turning to a more scientific question, I'm a little puzzled. I guess it's not clear from your sulphate deprivation why you wouldn't get the same effect by inhibiting PSII in some direct way, like with herbicides, and I assume that some people have tried that and it does not work.

A. Melis. Thank you. I can tell you that we tried that and it did not work very well. Obviously, use of herbicides is one way to slow down the activity of PSII in the cells, but herbicides will kill the cell, so it is hard to apply the method. Once the herbicide is inside the cell, it is difficult to remove it. Whereas, via a titration, it is much easier to manipulate the sulphate content of a culture. For example, give the cells enough sulphate nutrients to last for a limited period of time, and then it runs out. Once we're on this topic, I should also say that, for a period of time, we experimented with bicarbonate. Many of you know that removal of bicarbonate from the photosynthetic apparatus slows down the activity of PSII. Thus, initially, I thought that removal of bicarbonate could be the 'metabolic switch'. But this idea was not successful.

R. D. Britt. So, is there a burst of hydrogen with the initial addition of herbicides, and then it dies off, or do you get some residual hydrogen production?

A. Melis. There is a small amount of hydrogen produced on illumination in the presence of herbicides, and then the activity ceases. Certainly, it cannot be sustained for 80 h. No way.

A. W. Rutherford (*Service de Bioénergétique, Saclay, France*). As I understand it, maybe I got this wrong, I thought the electrons that were ending up on hydrogen were actually coming from water?

A. Melis. Yes. In this S-deprivation approach, we now know with certainty that the majority of the electrons come from water.

A. W. Rutherford. Which means that all attempts to kill PSII in a direct way would very quickly end up snuffing out the hydrogen as well?

A. Melis. Right. An attempt to inhibit 100% of PSII will snuff out all hydrogen. But apparently low-level activity of PSII and small amounts of oxygen can be dealt with by the cell via the endogenous mitochondrial respiration, so that the anaerobiosis of the medium is maintained.

J. Barber (*Department of Biological Sciences, Imperial College of Science, Technology and Medicine, London, UK*). It is related to what we are talking about. If you do the experiment, you have got sulphate starvation and hydrogen evolution and then you add DCMU, for example, to the culture; does that just snuff it out? What happens?

A. Melis. So, what happens when one drops in this hydrogen-bubbling bottle enough DCMU to make it 5 μ M concentration, which, as you know, is enough to totally stop the activity of PSII? Hydrogen production continues with a substantially reduced rate for maybe a couple of hours, and then it stops. This is the actual observation.

E.-M. Aro (*Department of Biology, Plant Physiology and Molecular Biology, University of Turku, Turku, Finland*). How about the developmental status of the chloroplast in your sulphate permease antisense transformants? Are they as beautiful as you see them in normal electron micrographs?

A. Melis. No, they show symptoms of S starvation. The amount of D1 per chloroplast is less than in the control; the amount of Rubisco is less in the chloroplast. Thylakoids we haven't looked at with electron microscopy, yet. But the chloroplasts contain about the same amount of PSI and cytochrome *b-f* complex. Only the level of PSII seems to be down; all other complexes are at about the same level as in the control.

B. Diner (*Central Research and Development Department, EI Dupont de Nemours & Co., Wilmington, DE, USA*). Do you have an estimate of what the quantum requirement is for hydrogen production?

A. Melis. In control green algae, the quantum requirement for hydrogen production is *ca.* 9–10 quanta per two hydrogen molecules, translating into a very high quantum yield, very close to the theoretical maximum. And this has been known for some time. At present, it is not known what the quantum requirement for hydrogen production is under S deprivation. It may turn out to be lower than that of the control.

J. Allen (*Lund University, Lund, Sweden*). Are you quite sure about the requirement for mitochondrial respiration? You know you could construct, on paper at least, a PSI cycle ATP-generating system, which might also help to titrate the level of oxygen to that needed to keep itself going? Have you tried inhibitors of oxidative phosphorylation, for example?

A. Melis. We haven't done that systematically, John. But, on the basis of what I've seen so far, I'm convinced that this process of hydrogen production is the result of interplay between photosynthesis, respiration and catabolism of endogenous substrate. All three mechanisms work together. And if you interfere with one of them, then you interrupt the process of hydrogen production altogether. Obviously, this is a big question and more work needs to be done in this direction.

J. Allen. If I may follow up on that, it's wonderful to see a eukaryote carry out bacterial physiology and bioenergetics, as the case is with *Chlamydomonas*. I always cheer for the anaerobic metabolism that comes up when you prevent *Chlamydomonas* from working just like a higher plant. You said that Rubisco was switched off. So, are you still fixing CO₂ and behaving autotrophically after that time?

A. Melis. There is no CO₂ fixation once Rubisco has been degraded. However, if sulphate is added to the growth medium, then cells *de novo* synthesize Rubisco and, after a lag period of time, they begin to grow photoautotrophically. They recover. Given time, they recover fully.

J. Allen. I wonder, is there any evidence for (a) the reductive TCA cycle because they have pyruvate-ferredoxin oxidoreductase activity, which is one of the key reactions?

A. Melis. There is no evidence. It's something we would like to look into.

J. Allen. And then (b), there's this primitive 'autotrophic' pathway, which works via acetyl co-enzyme A synthetase, and what you need for that is hydrogen and CO₂, which you fix and you make acetate, so you'd be making TAP medium by a direct route, as well. I mean, all of these possibilities could exist. So I do not suppose that you could rule them out at this stage?

A. Melis. They cannot be ruled out, as they have not been systematically investigated. But it may be worthwhile mentioning that, along with hydrogen and ATP, the process generates carbon dioxide. The ratio of hydrogen to CO₂ is 2 : 1 (mol : mol). And this is consistent with the prediction of oxidative respiration of endogenous substrate. The CO₂, by the way, is not released from the medium because the medium has a pH of 7.8–8, so the CO₂ is trapped in the water phase as bicarbonate. And when one re-supplies the culture with sulphate, this bicarbonate may serve as a source of carbon.

S. Santabarbara (*Biology Department, Queen Mary College, University College, London, UK*). It has been shown in *Chlamydomonas* that, under anaerobic conditions, most of the light-harvesting antenna of PSII, via state transitions, migrates to PSI. Then, light drives mostly cyclic electron transport through PSI, while linear electron transport via PSII is switched off, while in your reports, what you are suggesting is different and your DCMU effect is very surprising data.

A. Melis. Well, I do not want to talk about DCMU because DCMU may have side-effects that are difficult to analyse. But I should say that people often asked me in the past 'Why does the cell undertake this elaborate hydrogenase pathway mechanism, the end result of which is hydrogen, if all it needs is ATP? Why does it not simply perform cyclic photophosphorylation, which is cheap and theoretically could generate all the ATP that is needed for survival under these stressful conditions?' And the answer is, I do not know. The cells obviously prefer to do the more complex hydrogenase pathway. Perhaps green algae cannot do the cyclic electron transport and cyclic photophosphorylation as we mechanistically think about it. Maybe they have no choice but to go through the long route via endogenous substrate catabolism, respiration and hydrogen production in order to generate ATP. Maybe it is linear electron transport and not cyclic that they can do, who knows? But it is an open question and I wanted the audience to be aware of it. I guess, with time, we will sort it out.

P. L. Dutton (*Department of Biochemistry/Biophysics, University of Pennsylvania, PA, USA; visitor at Inorganic Chemistry Laboratory, Oxford University, UK*). If I can just add a question, here. So, back to Gaffron. So, you've got

this thing going, and you've developed it, fell on it, so does the bacteria—say, green bacteria, or purple bacteria—when you look at those and give them the same chance, do they produce the same amount of hydrogen with just as cheap a starting point?

A. Melis. I'll say something about cyanobacteria. We have some surprises, here. We think of the higher-plant chloroplast as having evolved from a primitive cyanobacterium upon endosymbiosis of the latter with a eukaryote. The first thing that one should do is to ask whether cyanobacteria code in their genome for an [Fe] hydrogenase. Guess what the answer is? It is a 'No'! Cyanobacteria whose genome has been sequenced do not code for an [Fe] hydrogenase! It is surprising that the progenitors to the green alga chloroplast do not contain genetic information about any Fe dehydrogenase. This is really a paradox. So, a question is raised as to the evolutionary origin of the green alga Fe dehydrogenase.

J. Durrant (*Department of Chemistry, Imperial College of Science, Technology and Medicine, London, UK*). Once you've finished optimizing the system, I was wondering, in terms of energy efficiency, how efficient do you think you're going to be able to be in terms of conversion of sunlight energy into chemical energy of hydrogen?

A. Melis. In theory, we should be able to convert 85% of the photons (PAR) that are absorbed by the green algae into hydrogen because this is the efficiency of photosynthesis.

J. Durrant. That's quantum efficiency. What about energy conversion?

A. Melis. Well, I do not know. I haven't done the calculations in terms of total energy converted because, in photosynthesis, as you know, we work on the basis of quanta. Take so many quanta, put them in the Chl antenna and see how many electrons (hydrogen) are produced. This is the basis upon which I've done the calculation of an 85% conversion efficiency.

GLOSSARY

ATP: adenosine triphosphate

CH₃NH₂-HCl: methylamine hydrochloride

Chl: chlorophyll

DCMU: 3-(3',4'-dichlorophenyl)-1,1-dimethyl urea

FCCP: carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone

LHC: light harvesting complex

PAGE: polyacrylamide gel electrophoresis

PSI: photosystem I

PSII: photosystem II

Q_A: primary quinone acceptor of PSII

Rubisco: ribulose bis-phosphate carboxylase-oxygenase

S: sulphur

SDS: sodium dodecyl sulphate

TAP: tris-acetate-phosphate