Hydrogen Production. Green Algae as a Source of Energy[1]

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Hydrogen gas is thought to be the ideal fuel for a world in which air pollution has been alleviated, global warming has been arrested, and the environment has been protected in an economically sustainable manner. Hydrogen and electricity could team to provide attractive options in transportation and power generation. Interconversion between these two forms of energy suggests on-site utilization of hydrogen to generate electricity, with the electrical power grid serving in energy transportation, distribution utilization, and hydrogen regeneration as needed. A challenging problem in establishing H_2 as a source of energy for the future is the renewable and environmentally friendly generation of large quantities of H_2 gas. Thus, processes that are presently conceptual in nature, or at a developmental stage in the laboratory, need to be encouraged, tested for feasibility, and otherwise applied toward commercialization.

Since the pioneering discovery by Gaffron and coworkers over 60 years ago (Gaffron, 1939; Gaffron and Rubin, 1942), the ability of unicellular green algae to produce H_2 gas upon illumination has been mostly a biological curiosity. Historically, hydrogen evolution activity in green algae was induced upon a prior anaerobic incubation of the cells in the dark (Greenbaum, 1982; Roessler and Lien, 1984; Happe and Naber, 1993; Schulz, 1996). A hydrogenase enzyme was expressed under such incubation and catalyzed, with high specific activity, a light-mediated H_2 evolution. The monomeric form of the enzyme, reported to belong to the class of Fe hydrogenases (Voordouw et al., 1989; Adams, 1990; Meyer and Gagnon, 1991; Happe et al., 1994), is encoded in the nucleus of the unicellular green algae. However, the mature protein is localized and functions in the chloroplast stroma (Happe et al., 1994). Light absorption by the photosynthetic apparatus is essential for the generation of hydrogen gas because light energy facilitates the oxidation of water molecules, the release of electrons and protons, and the endergonic transport of these electrons to ferredoxin. The photosynthetic ferredoxin (PetF) serves as the physiological electron donor to the Fe-hydrogenase and, thus, links the Fe hydrogenase to the electron transport chain in the chloroplast of the green algae (Florin et al., 2001).

Under these conditions, the activity of the hydrogenase is only transient (it lasts from several seconds to a few minutes) because, in addition to electrons and protons, the light-dependent oxidation of water entails the release of molecular O_2. Oxygen is a powerful inhibitor of the Fe hydrogenase (Ghirardi et al., 2000). Current technological developments in this field have not yet succeeded in overcoming this mutually exclusive nature of the O_2 and H_2 photoproduction reactions. Thus, the physiological significance and role of the Fe hydrogenase in green algae, which normally grow under aerobic photosynthetic conditions, has long been a mystery. Given the O_2 sensitivity of the Fe hydrogenase and the prevailing oxidative environmental conditions on earth, questions have been asked as to whether the hydrogenase is anything more than a relic of the evolutionary past of the chloroplast in green algae, and whether this enzyme and the process of photosynthesis can ever be utilized to generate H_2 gas for commercial purposes (Zhang et al., 2001). Nevertheless, the ability of green algae to photosynthetically generate H_2 gas has captivated the fascination and interest of the scientific community because of the fundamental and practical importance of the process. Below is an itemized list of the properties and promise of photosynthesis in green algal H_2 production, and the problems that are encountered with current technology:

(a) Photosynthesis in green algae can operate with a photon conversion efficiency of ≥80% (Ley and Mauzerall, 1982).

(b) Micro-algae can produce H_2 photosynthetically, with a photon conversion efficiency of ≥80% (Greenbaum, 1988).

(c) Molecular O_2 acts as a powerful and effective switch by which the H_2 production activity is turned off.

(d) This incompatibility in the simultaneous O_2 and H_2 photoproduction could not be overcome in 60 years of related research.

Aside from the above described photosystem II (PSII)-dependent H_2 photo-evolution, which involves water as a source of electrons and produces

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2:1 stoichiometric amounts of H_2:O_2, an alternative mechanism has been described in the literature (Gfeller and Gibbs, 1984). Upon a dark anaerobic incubation of the algae and the ensuing induction of the hydrogenase, electrons for the photosynthetic apparatus are derived upon a catabolism of endogenous substrate and the attendant oxidative carbon metabolism in the green algae. Electrons from such endogenous substrate catabolism feed into the photosynthetic electron transport chain between the two photosystems, and probably at the level of the plastoquinone pool. Light absorption by PSI and the ensuing electron transport elevates the redox potential of these electrons to the redox equivalent of ferredoxin and the hydrogenase, thus permitting the generation of molecular H_2 (Gibbs et al., 1986). In the presence of the PSII inhibitor 3-(3, 4-dichlorophenyl)-1,1-dimethylurea (DCMU), this process generates 2:1 stoichiometric amounts of H_2:CO_2. Thus, following a sufficiently long dark anaerobic incubation of the culture, initially high rates of H_2 production can be detected upon illumination of the algae in the presence of DCMU, a PSII inhibitor (Happe and Naber, 1993; Florin et al., 2001).

TWO-STAGE PHOTOSYNTHESIS AND H_2 PRODUCTION IN GREEN ALGAE

Recent work has shown that lack of sulfur from the growth medium of *Chlamydomonas reinhardtii* causes a specific but reversible decline in the rate of oxygenic photosynthesis (Wykoff et al., 1998) but does not affect the rate of mitochondrial respiration (Melis et al., 2000). In sealed cultures, imbalance in the photosynthesis-respiration relationship by S deprivation resulted in net consumption of oxygen by the cells causing anaerobiosis in the growth medium, a condition that automatically elicited H_2 production by the algae (Melis et al., 2000). In the course of this recent work, it was shown that expression of the Fe hydrogenase can be induced in the light, so long as anaerobiosis is maintained within the culture (Ghirardi et al., 2000; Melis et al., 2000). Under such conditions, it was possible to photoproduce and to accumulate significant volumes of H_2 gas, using the green alga *C. reinhardtii*, in a sustainable process that could be employed continuously for several days. Thus, progress was achieved by circumventing the sensitivity of the Fe hydrogenase to O_2 through a temporal separation of the reactions of O_2 and H_2 photoproduction, i.e. by the so-called “two-stage photosynthesis and H_2 production” process (Melis et al., 2000). The novel application of this two-stage protocol revealed the occurrence of hitherto unknown metabolic, regulatory, and electron transport pathways in the green alga *C. reinhardtii* (Zhang et al., 2001), leading to the significant and sustainable light-dependent release of H_2 gas by the cells. Figure 1 shows a photograph of an S-deprived, sealed, and H_2-producing *C. reinhardtii* culture, in which emanating H_2 bubbles are discerned as they are briefly trapped on the walls and neck of the Roux bottle.

Figure 1. A hydrogen-producing *C. reinhardtii* culture. Hydrogen bubbles emanate toward the surface of the liquid medium. The gas is drained through a syringe (inserted in the middle of the silicone stopper) and, through teflon tubing, is collected in an inverted burette and measured by the method of water displacement. Photograph courtesy of Michael Barnes (Office of the President, University of California, Oakland).

This method may serve as a tool for the elucidation of the green alga hydrogen-related metabolism. Upon further refinement, the method may also serve in the generation of H_2 gas for the fuel and chemical industries. The temporal sequence of events in this two-stage photosynthesis and H_2-production process is given below:

(a) Green algae are grown photosynthetically in the light (normal photosynthesis) until they reach a density of 3 to 6 million cells mL^{-1} in the culture.

(b) Sulfur deprivation is imposed upon the cells in the growth medium, either by carefully limiting sulfur supply in the medium so that it is consumed entirely, or by permitting cells to concentrate in the

growth chamber prior to medium replacement with one that lacks sulfur nutrients. Cells respond to this S deprivation by fundamentally altering photosynthesis and cellular metabolism to survive (Davies et al., 1996; Hell, 1997; Zhang et al., 2001).

(c) S deprivation exerts a distinctly different effect on the cellular activities of photosynthesis and respiration. The activity of oxygenic photosynthesis declines quasi-exponentially with a half-time of 15 to 20 h to a value less than 10% of its original rate (Wykoff et al., 1998). However, the capacity for cellular respiration remains fairly constant over the S deprivation period (Melis et al., 2000). As a consequence, the absolute activity of photosynthesis crosses below the level of respiration after about 24 h of S deprivation. Following this cross point between photosynthesis and respiration, sealed cultures of S-deprived *C. reinhardtii* quickly consume all dissolved oxygen and become anaerobic (Ghirardi et al., 2000), even though they are maintained under continuous illumination.

(d) Under S deprivation conditions, sealed (anaerobic) cultures of *C. reinhardtii* produce H_2 gas in the light but not in the dark. The volume and rate of photosynthetic H_2 production was monitored from the accumulating H_2 gas in an inverted burette, measured from the volume of water displacement. A rate of 2.0 to 2.5 mL, H_2 production L^{-1} culture h^{-1} was sustained in the 24- to 70-h period. The rate gradually declined thereafter.

(e) In the course of such H_2 gas production, cells consumed significant amounts of internal starch and protein (Zhang et al., 2001). Such catabolic reactions apparently sustain, directly or indirectly, the H_2 production process.

(f) Profile analysis of selected photosynthetic proteins showed a precipitous decline in the amount of Rubisco as a function of time in S deprivation, a more gradual decline in the level of PSII and PSI proteins, and change in the composition of the light-harvesting complex.

(e) Microscopic observations showed distinct morphological changes in *C. reinhardtii* during S deprivation and H_2 production. Ellipsoid-shaped cells (normal photosynthesis) gave way to larger and spherical cell shapes in the initial (0–24 h) stages of S deprivation and H_2 production, followed by cell mass reductions at longer (24–120 h) S deprivation and H_2 production times (Zhang et al., 2001).

A summary of the current state-of-the-art in this field is given below:

(a) The absence of sulfur from the growth medium of algae acts as a metabolic switch, one that selectively and reversibly turns off photosynthetic O_2 production.

(b) In the presence of S, green algae do normal photosynthesis (water oxidation, O_2 evolution, and biomass accumulation). In the absence of S and absence of O_2, photosynthesis in *C. reinhardtii* slips into the H_2 production mode.

(c) Reversible application of the switch (presence/absence of S) permits the algae to alternate between O_2 production and H_2 production (cycling of the stages; Ghirardi et al., 2000), thus bypassing the incompatibility and mutually exclusive nature of the O_2- and H_2-producing reactions.

(d) Interplay between oxygenic photosynthesis, mitochondrial respiration, catabolism of endogenous substrate, and electron transport via the hydrogenase pathway is essential for this light-mediated H_2 production process.

(e) The release of H_2 gas serves to sustain baseline levels of chloroplast and mitochondrial electron transport activity for the generation of ATP, which is needed for the survival of the organism under the protracted sulfur deprivation stress conditions (see below).

PHYSIOLOGY OF H_2 PRODUCTION IN GREEN ALGAE

Historically, Hans Gaffron made the first observation of hydrogen metabolism in green algae (Gaffron, 1939, 1944). Upon exposure to hydrogen of anaerobically adapted cells, he observed uptake of molecular H_2 by the algae and a concomitant CO_2 reduction in the dark. The reverse reaction, e.g. hydrogen production in the light, was first reported with the green alga *Scenedesmus obliquus* (Gaffron and Rubin, 1942). High rates of H_2 evolution could be measured in the light for short periods of time (from several seconds to a few minutes). Electrons were generated either upon the photochemical oxidation of water by PSII, which results in the simultaneous production of O_2 and H_2 (Spruit, 1958; Greenbaum et al., 1983), or upon the oxidation of endogenous substrate (Fig. 2), feeding electrons into the thylakoid membrane with the simultaneous release of CO_2 to the medium (Kessler, 1974; Bamberger et al., 1982). It is known that *C. reinhardtii* can photoproduce hydrogen when PSII is blocked by DCMU, but no H_2 evolution occurs after an addition of 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone (Stuart and Gaffron, 1972), which blocks the function of the cytochrome *b-f* complex. Under anaerobic conditions in the presence of DCMU, accumulated reducing equivalents from the fermentative catabolism of the algae cannot be oxidized via respiration because the terminal electron acceptor O_2 is absent. An NAD(P)H reductase protein complex that feeds electrons into the plastoquinone pool recently has been identified in many vascular plant chloroplasts (Shinozaki et al., 1986; Kubicki et al., 1996; Sazanov et al., 1998) but so far only from the green alga *Nephroselmis olivacea* (Turmel et al., 1999). Nevertheless, inhibitor experiments have yielded evidence in support of a thylakoid membrane-localized NAD(P)H reductase in *C. rein-*

Figure 2. Hydrogenase-related electron transport pathways in green algae. Electrons may originate either at PSII upon photooxidation of water, or at the plastoquinone pool upon oxidation of cellular endogenous substrate (e.g. via glycolysis and the tricarboxylic acid cycle). Electrons in the electron transport chain are transported via PSI to ferredoxin, which serves as the physiological electron donor to the Fe hydrogenase. P_{680}, Reaction center of PSII; P_{700}, reaction center of PSI; Q, primary electron acceptor of PS II; A, primary electron acceptor of PSI; PQ, plastoquinone; Cyt, cytochrome; PC, plastocyanin; Fd, ferredoxin; Red, NAD(P)H oxido-reductase; H_2ase, hydrogenase; FNR, ferredoxin-$NADP^+$ reductase.

hardtii (Godde and Trebst, 1980), suggesting that electrons derived upon the oxidation of endogenous substrate may feed into the plastoquinone pool (Fig. 2). Thereafter, electrons are driven upon light absorption by PSI to ferredoxin. The latter is an efficient electron donor to the Fe hydrogenase, which efficiently combines these electrons with protons to generate molecular H_2 (Florin et al., 2001).

The physiology of H_2 production upon S deprivation has many similarities and some distinct differences from the process described above. Sulfur-deprived and sealed cultures of *C. reinhardtii* become anaerobic in the light due to a significant and specific slowdown in the activity of the O_2-evolving PSII, which is followed by automatic induction of the Fe hydrogenase and by photosynthetic H_2 production. Biochemical analyses revealed that, concomitant with the H_2 production process, starch and protein content of the cells gradually declined (Zhang et al., 2001). Such catabolic pathway(s) could be generating reductant that feeds electrons into the thylakoid membrane, perhaps via a chloroplast NAD(P)H-dependent process (Gfeller and Gibbs, 1984). More important, starch catabolism must also generate substrate for the cell's mitochondrial respiration. Mitochondrial respiration scavenges the small amounts of O_2 that evolve due to the residual activity of photosynthesis and thus ensures the maintenance of anaerobiosis in the culture (Fig. 3). Thus, the physiology of H_2 production by S deprivation involves a coordinated interaction between:

(a) Oxygenic photosynthesis, i.e. the residual PSII activity for the generation of electrons upon oxidation of water. These electrons are transported through the photosynthetic electron transport chain and eventually feed into the Fe hydrogenase, thereby contributing to H_2 production.

(b) Mitochondrial respiration scavenges all oxygen generated by the residual photosynthesis and, thus, maintains anaerobiosis in the culture.

(c) Endogenous substrate catabolism, including starch, protein, and probably lipid catabolism, yields substrate suitable for the operation of oxidative phosphorylation in mitochondria, and possibly for an NAD(P)H-dependent electron transport in the chloroplast, both of which contribute to the generation of much-needed ATP.

(d) Electron transport via the hydrogenase pathway and the ensuing release of H_2 gas by the algae sustains a baseline level of photosynthesis and, therefore, of respiratory electron transport for the generation of ATP (Fig. 3) and thus ensures the survival of the organism under protracted stress conditions.

It is clear that more research is needed to dissect the four-way interplay and the intricate relationships between the processes of oxygenic photosynthesis, mitochondrial respiration, catabolism of endogenous substrate, and electron transport via the hydrogenase pathway leading to H_2 production. Nevertheless, the discovery of sustainable H_2 production that bypasses the sensitivity of the reversible hydrogenase to O_2 is a significant development in the field. It may lead to exploitation of green algae for the production of H_2 gas as a clean and renewable fuel. However, the actual rate of H_2 gas accumulation was at best 15% to 20% of the photosynthetic capacity of the cells, when

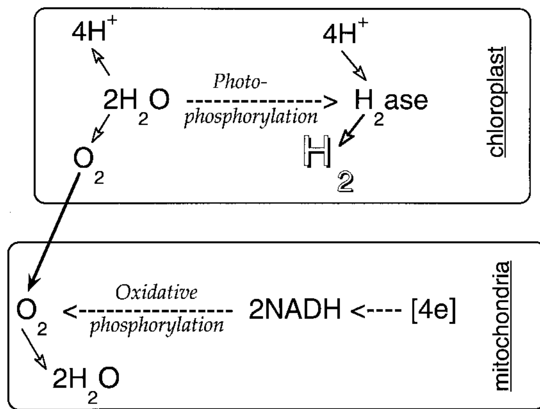

Figure 3. Coordinated photosynthetic and respiratory electron transport and coupled phosphorylation during H_2 production. Photosynthetic electron transport delivers electrons upon photo-oxidation of water to the hydrogenase, leading to photophosphorylation and H_2 production. The oxygen generated by this process serves to drive the coordinate oxidative phosphorylation during mitochondrial respiration. Electrons for the latter ([4e]) are derived upon endogenous substrate catabolism, which yields reductant and CO_2. Release of molecular H_2 by the chloroplast enables the sustained operation of this coordinated photosynthesis-respiration function in green algae and permits the continuous generation of ATP by the two bioenergetic organelles in the cell.

the latter is based on the capacity for O_2 evolution under physiological conditions (Melis et al., 2000). The relatively slow rate of H_2 production suggests that there is room for significant improvement in the yield of the process, by as much as one order of magnitude. Similarly, other improvements must be made to optimize the process under conditions of mass culture of the algae. For example, optical problems associated with the size of the chlorophyll antenna and the light saturation curve of photosynthesis must be addressed (Melis et al., 1999) before green algae can achieve high photosynthetic solar conversion efficiencies in mass culture. Moreover, the continuity of the process needs to be addressed because H_2 production by S deprivation of the algae cannot last forever. The yield begins to level off after about 70 h of S deprivation. After about 100 h of S deprivation, the algae need to go back to normal photosynthesis to be rejuvenated by replenishing endogenous substrate (Ghirardi et al., 2000).

A NOVEL TYPE OF FE-HYDROGENASE IN GREEN ALGAE

Fe hydrogenases originally were cloned from H_2-producing anaerobic microorganisms and protozoa (Meyer and Gagnon, 1991; Bui and Johnson, 1996; Akhmanova et al., 1998; Vignais et al., 2001). These enzymes make it possible to sustain a fermentative metabolism under anaerobic conditions by utilizing protons (instead of O_2) as the terminal electron acceptor and to sustain the process by releasing H_2 gas (Peters, 1999). The Fe hydrogenases are distinguished by their CO sensitivity and a high enzymatic activity that is 100-fold greater than that of the NiFe hydrogenases. The structure of the Fe hydrogenases from *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* were elucidated recently by x-ray crystallography (Peters et al., 1998; Nicolet et al., 1999). These proteins have a multidomain structure with numerous [Fe-S] clusters including a novel type of [Fe-S] cluster (H cluster) within the catalytic site of the enzyme. The H cluster comprises a conventional [4Fe-4S] complex bridged by the sulfur atom of a Cys residue to a unique binuclear iron-sulfur subcluster (Adams and Stiefel, 2000). Highly conserved amino acid residues comprising four Cys ligands and several hydrophobic amino acid residues at the active center are thought to be involved in the formation of H^+ and H_2 channels, thus connecting the catalytic site (located deep within the protein matrix) to the protein surface (Vignais et al., 2001).

Despite the discovery of hydrogen metabolism in green algae over 60 years ago and the great interest in biological H_2 evolution ever since, attempts to clone and characterize the hydrogenase gene from these photosynthetic organisms were unsuccessful. Recently, however, hydrogenase genes have been isolated and reported in the literature from the green algae *S. obliquus* (Florin et al., 2001), *C. reinhardtii* (Happe and Kaminski, 2001), and *Chlorella fusca* (T. Happe, personal communication). All three genes were shown to belong to the class of Fe hydrogenases. However, they showed novel structural properties and suggested a unique biochemical function. It is interesting that Fe hydrogenase genes could not be found in cyanobacteria, the free-living ancestors of plastids, raising the prospect of a non-cyanobacterial origin for the algal hydrogenases.

The Fe hydrogenases from green algae are monomeric proteins of about 45 to 50 kD and have been purified to homogeneity (Roessler and Lien, 1984; Happe and Naber, 1993). The nucleus-encoded polypeptides are synthesized in the cytosol as precursor proteins but the mature protein is localized in the chloroplast stroma (Happe et al., 1994). A transit peptide domain that routes the Fe hydrogenases from the cytoplasm across the chloroplast envelope and into the chloroplast stroma has been identified in the N-terminal region of the enzyme (Florin et al., 2001). The chloroplast-targeting domain of the protein is probably cleaved by a stroma-localized peptidase at a conserved cleavage site. No accessory genes that might be involved in the biosynthesis and/or assembly of Fe hydrogenases have been identified yet, either in green algae or in other microorganisms that contain Fe hydrogenases.

The genetic data on green alga Fe hydrogenases (HydA) reveal unique features in this class of enzymes (Florin et al., 2001). They constitute the smallest known Fe hydrogenase proteins with a significantly shortened N-terminal domain and a conserved

C-terminal domain that contains the catalytic site. The functionally important C terminus of the HydA sequence is very similar to that of other Fe hydrogenases from anaerobic microorganisms. Four highly conserved Cys residues coordinate the special [6Fe-6S] cluster (H cluster) in the catalytic site (Fig. 4). A number of additional amino acid residues define the environment of the active site. It was postulated that 12 mostly hydrophobic amino acid residues might play a role in protecting the H cluster from the surrounding aqueous medium (Peters et al., 1998). Ten residues are strictly conserved, whereas two residues vary within the Fe hydrogenase family (Ser-232 and Ile-268 in *C. pasteurianum*; Ala-109 and Thr-145 in *D. desulfuricans*; Ala-38 and Thr-74 in *C. reinhardtii*; and Ala-44 and Thr-80 in *S. obliquus*). However, the green algal sequences include an insertion of 16 to 45 amino acids that is absent from the bacterial sequences and that forms an external peptide loop in the fully assembled protein. This additional peptide loop in the green alga hydrogenase might be involved in electrostatic binding of the natural electron donor ferredoxin.

In the N-terminal domain of bacterial and other nonalgal Fe-hydrogenases, a number of Cys residues, which are obviously missing from the green algal counterparts, were found to bind accessory iron-sulfur clusters. In all nonalgal Fe hydrogenases, a ferredoxin-like domain (F cluster) coordinates two [4Fe-4S] clusters (Peters et al., 1998; Adams and Stiefel, 2000). Additional iron sulfur clusters were detected within the Fe hydrogenases of *C. pasteurianum* (Fig. 4), *Thermotoga maritima* (Verhagen et al., 1999), and *Nyctotherus ovalis* (Akhmanova et al., 1998). The F cluster in these organisms is responsible for electron transfer from the electron donor (mostly ferredoxin) to the H cluster (Nicolet et al., 2000). These accessory [Fe-S] centers are missing from the algal Fe hydrogenases, indicating a novel electron transport pathway from the donor PetF ferredoxin to the hydrogenase H cluster. The absence of such accessory [Fe-S] centers and the correspondingly

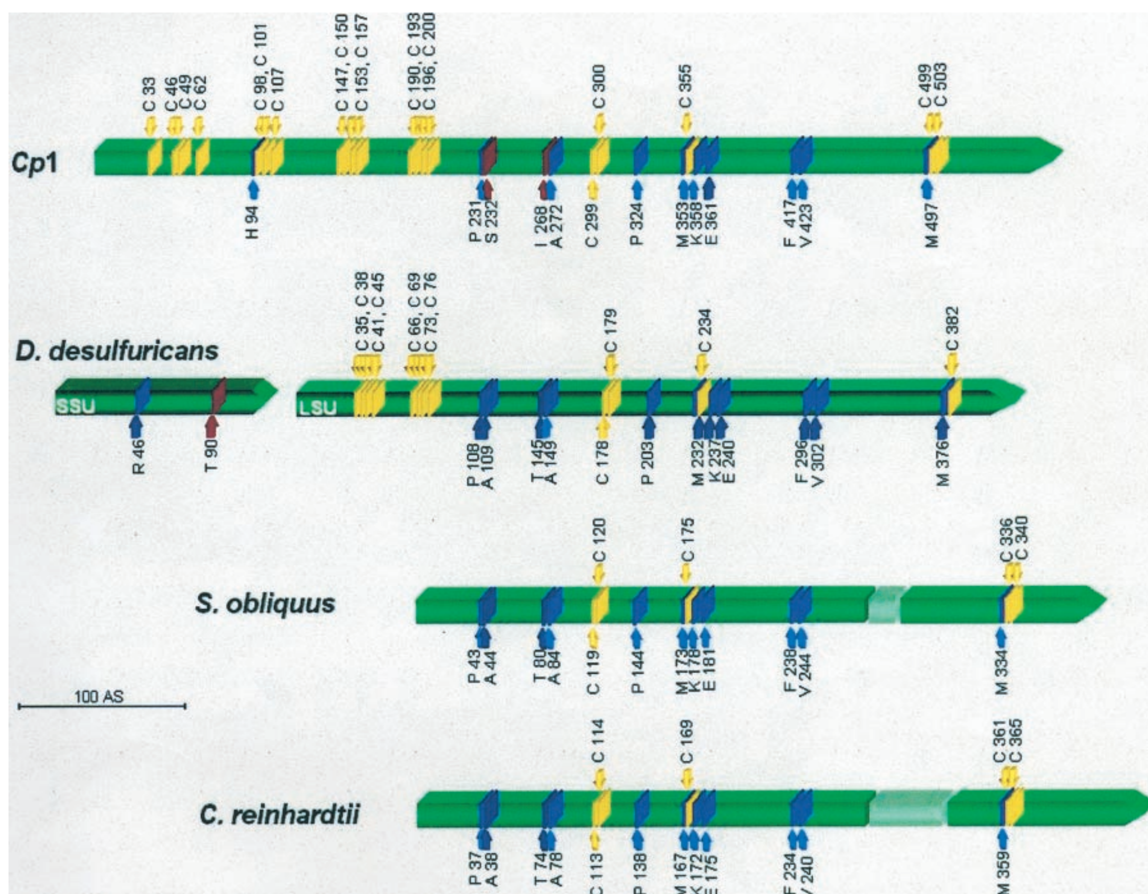

Figure 4. Comparative schematic of the Fe hydrogenase polypeptides from selected H_2-producing organisms. The mature Fe hydrogenase polypeptide is depicted by a green linear rectangle. Vertically pointing arrows and the corresponding parallelograms within the linear rectangle show the position of conserved amino acids in the hydrogenase protein. Cys residues that play a role in the coordination of the [Fe-S] clusters are shown in yellow. Conserved non-Cys amino acids are shown in blue. The insertion of 16 and 45 unique amino acid residues in the Fe hydrogenase of *S. obliquus* and *C. reinhardtii*, respectively, is shown in light-green color.

Table I. *Yield of hydrogen photoproduction by green algae*
Estimates are based on maximum possible daily integrated irradiance and algal photosynthesis characteristics.

Photoproduction Characteristics	Comments on Assumptions Made
Maximum photosynthetically active radiation, 50 mol photons m^{-2} d^{-1} (based on a Gaussian solar intensity profile in which the peak solar irradiance reaches 2,200 μmol photons m^{-2} s^{-1})	Daily irradiance can vary significantly depending on season and cloud cover. It can be greater than 50 mol photons m^{-2} d^{-1} in the summer and much less than that on cloudy days and in the winter (Kirk, 1994).
Theoretical minimum photon requirement for hydrogen production in green algae: 5 mol photons/mol H_2	Based on the requirement of 10 photons for the oxidation of two water molecules and the release of four electrons and four protons in photosynthesis (Ley and Mauzerall, 1982; Greenbaum, 1988).
Theoretical maximum yield of H_2 production by green algae: 10 mol H_2 m^{-2} d^{-1} (20 g H_2 m^{-2} d^{-1}; ~80 kg H_2 $acre^{-1}$ d^{-1})	Assuming that all incoming photosynthetically active radiation will be absorbed by the green algae in the culture and that it will be converted into stable charge separation.

shorter polypeptide of the green algal Fe-hydrogenase significantly reduces the distance from the ferredoxin electron donation site to the H cluster (Florin et al., 2001). In this respect, the external peptide loop of the algal hydrogenases might compensate for the missing domains. The positively charged amino acids in the loop structure may serve as a ferredoxin-docking domain. Thus, it may help to orient the negatively charged ferredoxin to facilitate linkage and efficient electron transfer between ferredoxin and hydrogenase. Such interaction is a prerequisite for the meaningful coupling of the enzyme with the electron transport chain in chloroplasts.

REGULATION OF HYDROGENASE BIOSYNTHESIS IN GREEN ALGAE

Since the discovery of hydrogen metabolism in green algae (Gaffron, 1939), it has been known that the hydrogenase activity can be detected only upon anaerobic incubation of the cells. Recent work (Happe et al., 1994; Happe and Kaminski, 2001) provided molecular insight into this property and showed that the Fe hydrogenase is rapidly induced at the transcriptional level upon anaerobiosis. It is interesting that this induction phenomenon was used as a guiding principle for the cloning of the Fe hydrogenase gene in green algae (Happe and Kaminski, 2001). A special genetic method was employed, the so-called suppressive subtractive hybridization, based on the comparison of mRNA populations from aerobically grown and anaerobically incubated cells. This approach permitted the isolation of a number of cDNA clones that were expressed only under anaerobic conditions. One of these clones encoded the Fe hydrogenase of *C. reinhardtii* (Happe and Kaminski, 2001).

Eukaryotic green algae grown under ambient photo-autotrophic conditions neither consume nor produce molecular hydrogen, suggesting lack of Fe hydrogenase gene expression. It has been shown by western-blot (Happe et al., 1994) and northern-blot analyses (Happe and Kaminski, 2001) that neither protein nor gene transcripts can be detected under ambient photoautotrophic (aerobic) conditions. However, expression of the Fe hydrogenase gene is induced upon incubation of the cells under anaerobic conditions in the dark, e.g. bubbling with argon (Happe and Naber, 1993), or upon incubation under S deprivation conditions, e.g. conditions that selectively diminish the activity of PSII and O_2 evolution (Melis et al., 2000; Zhang et al., 2001). Expression of the Fe hydrogenase gene begins within 10 min after the medium becomes anaerobic and is probably regulated at the transcriptional level. These results suggest that oxygen, directly or indirectly, acts as a positive suppresser of Fe hydrogenase gene expression at the transcriptional level. In the absence of oxygen, suppression of hydrogenase gene expression is alleviated and proceeds unimpeded in the cells. Such triggering is apparently independent of the method employed for the establishment of anaerobiosis, and also independent of illumination and of photosynthesis in the green algae, so long as anaerobiosis is maintained within the cell.

HOW MUCH H_2 CAN ONE EXPECT FROM A MASS CULTURE OF GREEN ALGAE?

Application of the two-stage photosynthesis and H_2 production protocol to a green alga mass culture could provide a commercially viable method of renewable hydrogen generation. Table I provides preliminary estimates of maximum possible yield of H_2 by green algae, based on the luminosity of the sun and the green algal photosynthesis characteristics. Calculations were based on the integrated luminosity of the sun during a cloudless spring day. In mid-latitudes at springtime, this would entail delivery of approximately 50 mol photons m^{-2} d^{-1} (Table I, row 1). It is generally accepted that electron transport by the two photosystems and via the hydrogenase pathway for the production of 1 mol H_2 requires the absorption and utilization of a minimum of 5 mol photons in the photosynthetic apparatus (Table I, row 2). On the basis of these "optimal" assumptions, it can be calculated that green algae could produce a maximum of 10 mol (20 g) H_2 per m^2 culture area per day. If yields of such magnitude could be approached in mass culture, this

would constitute a viable and profitable method of renewable H_2 production.

However, this optimistic scenario cannot be realized with present day know-how. Three biologically "gray areas" directly impact this H_2 production technology. (a) The yield of H_2 production currently achieved in the laboratory corresponds to only 15% to 20% of the measured capacity of the photosynthetic apparatus for electron transport (Melis et al., 2000). (b) The optical properties of light absorption by green algae impose a limitation in terms of solar conversion efficiency in the alga chloroplast. This is because wild-type green algae are equipped with a large light-harvesting chlorophyll antenna size to absorb as much sunlight as they can. Under direct and bright sunlight, they could waste up to 60% of the absorbed irradiance (Neidhardt et al., 1998; Melis et al., 1999). This evolutionary trait may be good for survival of the organism in the wild, where light is often limiting, but it is not good for the photosynthetic productivity of a green algal mass culture. This optical property of the cells could further lower the productivity of a commercial H_2 production farm. (c) The current necessity to cycle a culture between the two stages (normal photosynthesis in the presence of S alternating with H_2 production upon S deprivation) introduces a "down time" as far as H_2 production is concerned. It is inevitable that the "down time" would further erode the yield of the H_2 production process. Thus, with current technology, it is estimated that the actual yield of H_2 production would be lower than that of the theoretical maximum shown in Table I, achieving perhaps a mere 10%, or lower, than the calculated theoretical maximum. It is clear that these three specific biological challenges (a–c) need to be overcome to effect greater actual yields of green alga H_2 production.

CONCLUSIONS

In summary, concerns about global warming and environmental pollution due to the use of fossil fuels, 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. The recently developed single-organism, two-stage photosynthesis and H_2 production protocol with green algae is of interest because significant amounts of H_2 gas were generated for the first time, essentially from sunlight and water (Melis et al., 2000). Further, this method does not entail the generation of any undesirable, harmful, or polluting byproducts and it may even offer the advantage of value-added products as a result of the mass cultivation of green algae. However, several biological and engineering challenges must be overcome before this promising technology becomes a practical reality. Foremost, the cellular metabolism and basic biochemistry that support this process must be well understood and much fundamental research on the mechanism of H_2 production by S deprivation remains to be done.

Ultimately, the advent of hydrogen will bring about technological developments in many fields, including power generation, agriculture, the automotive industry, and other as yet unforeseen applications. It will increase employment, stimulate the economy of all nations on earth, and will have a positive impact on the environment in which atmospheric pollution is all but alleviated and the so-called greenhouse effect is mitigated.

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