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Review

Genomics of green algal hydrogen research

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Abstract

This article summarizes knowledge on genes and their respective proteins in the field of green algal hydrogen research. Emphasis is placed on recently cloned genes from the unicellular green alga *Chlamydomonas reinhardtii*, including *HydA1* and *HydA2*, which encode homologous [Fe]-hydrogenases, *Tla1*, which encodes a chlorophyll antenna size regulatory gene, *SulP*, which encodes a chloroplast sulfate permease, and *Sta7*, which encodes an isoamylase. Analysis of the structure and function of these genes and of their respective proteins in *C. reinhardtii*, and related unicellular green algae, is presented in light of the role they play in the hydrogen metabolism in these organisms. A discussion is offered as to the potential application of these genes in the field of hydrogen photoproduction.

Introduction

Hans Gaffron discovered hydrogen metabolism in unicellular green algae over 60 years ago (Gaffron 1939). These photosynthetic microorganisms can utilize molecular H_2 in the dark to drive cellular metabolism (Gaffron 1940), or evolve H_2 in the light with electrons derived from the photosynthetic oxidation of H_2O (Gaffron and Rubin 1942). These seminal observations sparked a great deal of interest in photobiological H_2 evolution ever since. Today, it is well established that many, but not all, unicellular green algae, including the model green alga *Chlamydomonas reinhardtii*, are capable of H_2 metabolism (Kessler 1974). Under aerobic conditions, this phenomenon is not manifested since molecular O_2 suppresses expression of the genes associated with H_2 metabolism, undermines the stability of the respective mRNAs, and inhibits the function of the related enzymes (Happe and Naber 1993; Happe and Kaminski 2002). Historically, this multi-point inhibition was alleviated upon a prior anaerobic incubation of the algae in the dark, often referred to as 'induction' (Kessler 1973; Lien and San Pietro 1981; Greenbaum 1982; Roessler and Lien 1984; Miyamoto et al. 1990; Happe and Naber 1993; Schulz 1996). Under such anaerobic conditions, the positive suppression of gene expression by O_2 was lifted, and the unicellular green algae expressed the constituents of cellular H_2 metabolism. A subsequent illumination could drive the hydrogen-evolution process (Stuart and Gaffron 1972a, b). This H_2 photo-evolution lasted for a short period of time as release of photosynthetic O_2 quickly inhibited the process (Ghirardi et al. 1997). The enzymatic generation and release of molecular H_2 under anaerobic conditions, when mitochondrial respiration is inhibited, permits electron flow through the electron transport chain and enables ATP formation (Arnon et al. 1961) without the associated reductant (NADPH) generation or CO_2

fixation. The release of molecular H_2, in addition to ATP energy generation, may also serve as a process by which to remove from the cell accumulated reducing equivalents (Melis and Happe 2001; Happe et al. 2002) and to properly set the redox potential within the chloroplast of the green algae (Appel and Schulz 1998).

This review summarizes recent advances in the genomics of green algal hydrogen research. Specifically, it discusses how recently identified genes and their respective proteins might be used to enhance H_2 metabolism in unicellular green algae, and how the continuity and yield of the photosynthetic H_2-production process might be improved.

[Fe]-hydrogenases

Enzymes that catalyze either the uptake (thereby providing electrons for the reduction of endogenous substrates) or production of H_2 (eliminating excess, possibly damaging cellular reducing equivalents) are termed hydrogenases. They are classified into mainly two groups, according to the cofactor(s) they contain in their catalytic site, as iron [Fe]-only or nickel–iron [NiFe] hydrogenases (Vignais et al. 2001; Boichenko et al. 2004). The [Fe]-only hydrogenases possess a complex and unique electronic lattice of six Fe and six labile S atoms in their catalytic site. Referred to as the 'active-site cluster' (abbreviated as HC), the prosthetic group present in the catalytic site of all [Fe]-hydrogenases, is composed of an electron relay [4Fe4S]-center bridged by cysteine to a unique asymmetrically ligated catalytic [2Fe2S]-center. Three other cysteines coordinate the [4Fe4S]-center to the protein in all known [Fe]-hydrogenases. The [2Fe2S]-center is further coordinated by cyanide and carbon monoxide, while the two sulfurs are bridged by a dithiomethylamine ligand (Nicolet et al. 1998; Peters et al. 1998). Due to its unique chemical nature, this [2Fe2S]-center is highly reactive towards H_2. Accordingly, [Fe]-hydrogenases have much higher turnover numbers when compared to the other class of hydrogenases. On the other hand, they are readily inactivated by molecular O_2.

A monomeric [Fe]-hydrogenase of 48 kDa with high specific activity was first isolated and biochemically characterized from the unicellular green alga *Chlamydomonas reinhardtii* (Roessler and Lein 1984; Happe and Naber 1993; Happe et al. 1994). Despite the early purification to homogeneity of the *C. reinhardtii* [Fe]-hydrogenase (Happe and Naber 1993), initial efforts to isolate hydrogenase gene(s) in unicellular green algae proved unsuccessful. Using the suppression subtractive hybridization approach, a differential expression of genes under anaerobiosis was obtained and analyzed. A PCR fragment with homology to bacterial [Fe]-hydrogenases could be thus isolated (Happe and Kaminski 2002). On the basis of these results, [Fe]-hydrogenase genes (initially termed *HydA*) were identified in different green algae (Florin et al. 2001; Wünschiers et al. 2001; Winkler et al. 2002a; Forestier et al. 2003; Winkler et al. 2004). The cDNA sequence of the *HydA* gene in *C. reinhardtii* contained an ORF of 1494 base pairs, encoding a protein with an apparent molecular mass of 53.1 kDa. The precursor sequence of the [Fe]-hydrogenase includes a transit peptide of 56 amino acids, which targets the cytosolically synthesized protein to the chloroplast stroma. The *HydA* coding sequence showed a typical mosaic structure of a nuclear-encoded gene with seven introns and eight exons.

The product of all *HydA* genes cloned so far belongs to the class of [Fe]-hydrogenases. As a subclass of the [Fe]-hydrogenases, they exhibit unique structural properties, although functionally they catalyze the generation of molecular H_2 in a manner similar to that of their better-known homologues, found in obligate anaerobic bacteria, e.g. *Clostridium pasteurianum* (Peters et al. 1998; see also Nicolet et al. 1998). The C-terminus of the algal enzymes, including the catalytic HC site, has a high degree of identity to that of other [Fe]-hydrogenases; however, a major portion of the N-terminus of the protein in all algae sequenced so far is missing. The green algal [Fe]-hydrogenases lack all auxiliary [FeS] clusters that are found in bacteria. The truncated N-terminus of the protein indicates a direct electrostatic interaction between the natural ferredoxin (PetF) electron donor and the HC catalytic site of the [Fe]-hydrogenase in green algae (Happe and Kaminski 2002). This feature is unique among the hydrogenases and indicates that the presence of an extensive array of auxiliary [FeS] clusters receiving electrons from ferredoxin and transferring them to the HC (Peters et al. 1998) is not an absolute requirement for the efficient function of the enzyme.

As discussed above, hydrogen production is observed in green algae after a period of dark anaerobic incubation of the cells. Northern blot analyses with a probe specific for the *HydA* gene showed that, under such conditions, *HydA* transcription is induced and mRNA accumulation is followed by synthesis of the *HydA* precursor protein. This transcriptional regulation of *HydA* expression was confirmed by promoter analyses. Fusing the promoter region of the *HydA* gene to the arylsulphatase (*Ars*) reporter gene lacking its own promoter, Stirnberg and Happe (2004) showed that the *HydA*/*Ars* construct was transcribed only under anoxic conditions.

Currently, hydrogenase genes have also been cloned and reported in the literature from the unicellular green algae *Scenedesmus obliquus* (Florin et al. 2001; Wünschiers et al. 2001), *Chlorella fusca* (Winkler et al. 2002a) and *Chlamydomonas moewusii* (Winkler et al. 2004). In *Scenedemus obliquus*, the genomic DNA of the *HydA* gene contains five introns and six exons, encoding a protein with a molecular mass of 44.5 kDa (Florin et al. 2001). The complete *HydA* cDNA of 2609 base pairs comprises an open reading frame encoding a polypeptide of 448 amino acids (Florin et al. 2001). The protein also contains a transit peptide that imports the HydA protein to the chloroplast. All four cysteine residues and 12 other amino acids, which are strictly conserved in the domain of the protein that binds the HC active site of the [Fe]-hydrogenases, are present. As the case is with other algal HydA proteins, *S. obliquus* HydA is substantially truncated at the N-terminus compared to that of the bacterial [Fe]-hydrogenases. In this respect, the *S. obliquus* HydA is highly homologous to that of *C. reinhardtii* (Happe and Kaminski 2002, see also Figure 1).

Interestingly, a different [Fe]-hydrogenase gene fragment was also cloned from *S. obliquus* (Wünschiers et al. 2001). Its deduced amino acid sequence contains the conserved HC-binding motif, thought to be unique in the [Fe]-hydrogenases. However, phylogenetically, this enzyme was more closely related to the *Trichomonas vaginalis* [Fe]-hydrogenase. Southern blot analyses revealed that this was a single-copy gene localized in the nucleus. Surprisingly, and unlike the dark anaerobic incubation requirement for the *HydA* gene expression, Northern blot analyses suggested that this [Fe]-hydrogenase is constitutively expressed in *S. obliquus* (Wünschiers et al. 2001). Results from this work raised the prospect of the presence of two slightly different [Fe]-hydrogenase genes in *S. obliquus*. However, neither the protein was isolated nor was the whole cDNA or genomic sequence characterized, so that it is not clear whether this gene encodes for a functional hydrogenase (Wünschiers et al. 2001).

More recent research has revealed the presence and expression of a second [Fe]-hydrogenase in *C. reinhardtii* (Forestier et al. 2003), as was suggested in research with *S. obliquus* (Wünschiers et al. 2001). This second [Fe]-hydrogenase gene encodes a protein that is 74% similar and 68% identical to the previously reported *HydA* hydrogenase from *C. reinhardtii* (Forestier et al. 2003, see also Figure 1). Given the apparent presence of two homologous [Fe]-hydrogenase genes in *C. reinhardtii* and presumably in *S. obliquus*, a proposal was made to change the name of the earlier cloned *C. reinhardtii* gene from *HydA* to *HydA1* (Forestier et al. 2003). Accordingly, the second [Fe]-hydrogenase in *C. reinhardtii* was named *HydA2* (Forestier et al. 2003).

The HydA2 protein contains all the conserved residues and motifs found in the catalytic core of the family of [Fe]-hydrogenases. In contrast to the finding by Wünschiers et al. (2001), both *HydA1* and *HydA2* transcripts were selectively expressed upon dark anaerobic incubation, suggesting regulation of gene expression by oxygen. In Western blot analyses with extracts from anaerobically incubated *C. reinhardtii*, antibodies specific for the HydA2 protein recognized a polypeptide of about 49 kDa, showing that *HydA2* encodes for a translated protein. The nuclear-encoded HydA2, very much like its HydA1 homologue, lacks the N-terminal [Fe–S] centers and suggests a direct interaction between ferredoxin and the catalytic HC of the HydA2 holocomplex.

A comparison of the two *C. reinhardtii* [Fe]-hydrogenase genes revealed that the *HydA1* cDNA contains a 158-nucleotide 5′-UTR and a 747-nucleotide 3′-UTR (excluding the polyadenylated tail) (Happe and Kaminski 2002), while the *HydA2* cDNA contains a 139-nucleotide 5′-UTR and an 873 nucleotide 3′-UTR (Forestier et al. 2003). The ORFs for *HydA1* and *HydA2* encode proteins of, respectively, 497 and 505 amino acid residues. A

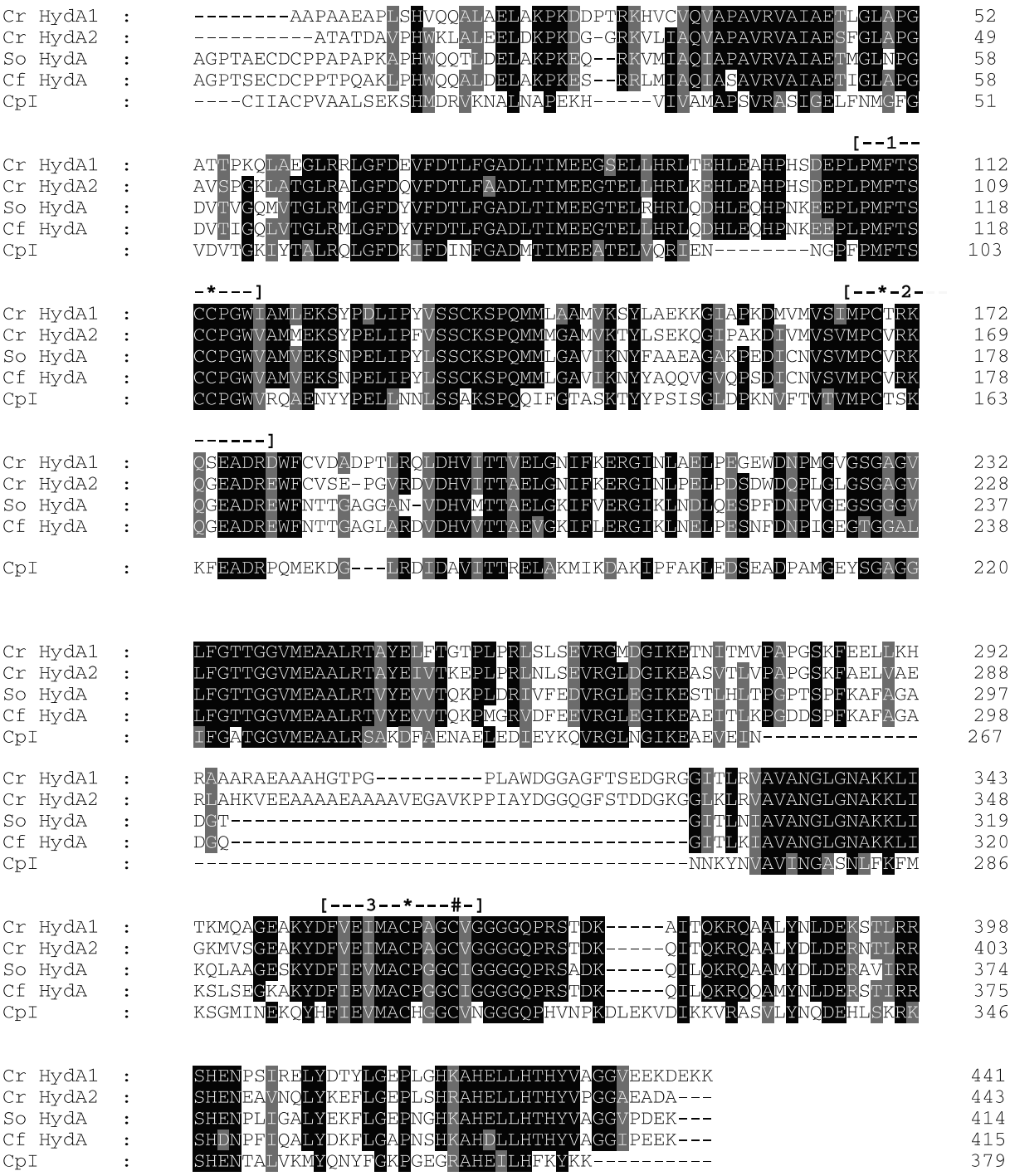

Figure 1. Sequence alignment of [Fe]-hydrogenases. The amino acids highlighted in black represent identity between at least four of the hydrogenases, and those highlighted in gray show similarity between at least four of the sequences. The amino acid alignment of the known and predicted (HydA2) processed forms of algal hydrogenases and of the bacterial [Fe]-hydrogenase from *Clostridium pasteurianum* CpI (shown without the non-conserved N-terminal domain) was done using the CLUSTALW program. Cr, *Chlamydomonas reinhardtii*; So, *Scenedesmus obliquus*; Cf, *Chlorella fusca*. The numbers 1–3 represent the three motifs common to the H-cluster of [Fe]-hydrogenases, the asterisks denote the cysteine residues that ligate the catalytic [4Fe4S] center, and # is the cysteine residue that bridges the [4Fe4S] and [2Fe2S] centers.

polyadenylation signal (TGTAA), which is characteristic of nuclear-encoded genes in *C. reinhardtii* (Silflow et al. 1985), is located 727 and 854 bp downstream from the stop codon in each cDNA, respectively. Furthermore, *HydA1* contains only 8 exons (Happe and Kaminski 2002), while *HydA2* incorporates 10 exons (Forestier et al. 2003). Finally, the two hydrogenase genes are located in different scaffolds (10 and 12, respectively) according to the *C. reinhardtii* genomic DNA sequencing database, which was recently completed and made available by the US Department of Energy's Joint Genome Institute in Walnut Creek, California.

A survey of hydrogen metabolism in unicellular green algae showed that this property is not universally conserved among this group of Chlorophyta (Brand et al. 1989; Horner et al. 2002; Winkler et al. 2002b). Hydrogenase activity was observed in a culture of the unicellular green alga *Chlorella fusca* following dark anaerobic incubation, but not in the related species *Chlorella vulgaris* (Kessler 1974). The cDNA and genomic DNA of an [Fe]-hydrogenase was cloned from *C. fusca* (Winkler et al. 2002a). However, no [Fe]-hydrogenase gene could be isolated from *C. vulgaris* (Happe, unpublished observations). Thus, it is curious that closely related unicellular green algae might be so different in terms of the presence of the *HydA* genes. There are other unicellular green algae that apparently lack the gene of the [Fe]-hydrogenase, e.g. *Dunaliella salina* (Melis and Happe, unpublished observations), raising the question of the evolutionary pressure(s) that enabled some of these unicellular photosynthetic organisms to either acquire or retain an [Fe]-hydrogenase while others did not (Horner et al. 2002). It is also of interest to note that, so far, [Fe]-hydrogenase genes have not been encountered in cyanobacteria, the presumed free-living ancestors of green algal chloroplasts (Tomitani et al. 1999), raising the prospect of a non-cyanobacterial origin for the algal hydrogenases.

Isolation and characterization of the [Fe]-hydrogenase genes from unicellular green algae, and especially *Chlamydomonas reinhardtii*, offers the possibility of manipulating these genes and their respective proteins to enhance gene expression and [Fe]-hydrogenase protein stability. This might result in improved H_2-production characteristics of the unicellular green algae. Notable in this respect is an effort to apply rational design and site-directed mutagenesis (see below) to alleviate, or minimize, the severe O_2-sensitivity of the [Fe]-hydrogenase protein (Ghirardi et al. 1997, 2000). Such performance characteristics of an engineered [Fe]-hydrogenase might permit the occurrence of the H_2-production process under aerobic conditions.

Engineering a solution to the O_2 sensitivity of the [Fe]-hydrogenase

Oxygen, in addition to acting as a powerful positive suppressor of the [Fe]-hydrogenase gene expression, can also adversely affect enzymatic activity by deactivating previously assembled [Fe]-hydrogenases (Bamberger et al. 1982; Roessler and Lein 1984; Happe and Naber 1993; Happe et al. 1994; Ghirardi et al. 1997). Bacterial [Fe]-hydrogenases also showed high O_2 sensitivity, resulting from an irreversible oxidation of the [2Fe2S] center in the catalytic HC site of the enzyme (Adams 1990). Assessment of the bacterial enzyme structure has led to the observation that among several possible routes, O_2 might gain access to the catalytic site through a H_2-channel, which is formed by the folding pattern of the assembled polypeptide and functions in the diffusion of H_2 from the active site to the surface of the protein (Montet et al. 1997; Nicolet et al. 1998). Furthermore, H_2-sensing [NiFe]-hydrogenases have a narrower region between the H_2-channel and the active site, compared to catalytic [NiFe]-hydrogenases, and this has been proposed to explain their greater resistance to O_2 (Bernhard et al. 2001; Volbeda et al. 2002). A similar H_2 gas channel is present in the green algal [Fe]-hydrogenases, as shown by homology structural modeling of the *C. reinhardtii* HydA1 (Figure 2) and HydA2 (Forestier et al. 2003). The high degree of amino acid sequence homology between the algal HydA1 protein and the *Clostridium pasteurianum* CpI [Fe]-hydrogenase (Meyer and Gagnon 1991) are particularly evident within the H_2-channel (62% identity and 92% similarity) and active site (80% identity, 89% similarity) domains. Primarily composed of small hydrophobic residues (i.e. glycine, alanine, valine), the channel environment is characterized by two α-helices and two β-sheets. Homology modeling suggested that the diameter of the channels is indeed larger than the effective diameters of both H_2

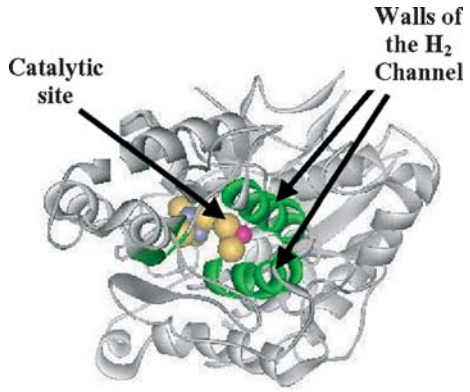

Figure 2. Homology structure model of the *C. reinhardtii* HydA1 hydrogenase. The H-cluster is identified in CPK colors as space-filled atoms. The alpha helices, represented in green, line the hydrophobic H_2 channel, which allows gas diffusion between the active site and the surface of the protein. The viewer is looking straight down the channel. Images of the structures were made with ViewerLite software (Accelrys).

(2.8 Å) and O_2 (3.5 Å) (Nenoff 2000). As a result, the predicted size of the hydrogen channel is sufficiently large to allow for the diffusion of H_2 from the active site to the surface, but it is also big enough to allow for the inward diffusion of O_2, which as noted earlier inhibits hydrogenase function by deactivating the enzyme. Based on these observations and the modeling effort with HydA1 and HydA2, improved tolerance of the algal [Fe]-hydrogenases to O_2 inactivation might be engineered into the protein by narrowing the width of the H_2-channel (King et al. submitted). Physical restriction of access to the site by O_2 (either by reducing the diffusion rate or by shielding the active site) should protect the active site, prevent hydrogenase inactivation, and improve the yield of H_2-production.

Chlorophyll antenna size regulatory genes

Photosynthesis and H_2-production in unicellular green algae can operate with a photon utilization efficiency that is nearly 100% (Ley and Mauzerall 1982; Greenbaum 1988), making these microorganisms an efficient biocatalyst for the generation of H_2 from sunlight and H_2O. However, green microalgal cultures under direct sunlight show rather poor photosynthetic light utilization efficiency. The reason for this shortcoming is that, under bright sunlight, the rate of photon absorption by the chlorophyll (Chl) antenna arrays in Photosystem II (PS II) and Photosystem I (PS I) far exceeds the rate at which photosynthesis can utilize them. Excess photons cannot be stored in the photosynthetic apparatus but are dissipated (lost) as heat or fluorescence. Up to 80% of absorbed photons could thus be wasted (Melis et al. 1999), decreasing light utilization efficiency in the photosynthetic apparatus and compromising both cellular productivity and H_2 generation to unacceptably low levels. Thus, in a high-density mass culture, cells at the surface would over-absorb and waste sunlight; whereas cells deeper in the culture would be deprived of much needed irradiance, as this is strongly attenuated due to filtering by the over layered cells. To attain high performance characteristics in mass culture photosynthesis and H_2 production, it is necessary to minimize the absorption of sunlight by individual cells so as to permit greater transmittance of irradiance through the high-density green alga culture. The advent of molecular genetics in combination with sensitive absorbance-difference kinetic spectrophotometry for the precise measurement of the Chl antenna size in green algae now offer a valid approach by which to pursue a reduction in the number of photosynthetic Chl antenna molecules.

DNA insertional mutagenesis and screening of the green alga, *Chlamydomonas reinhardtii*, was employed to isolate *tla1*, a stable transformant having a *t*runcated *l*ight-harvesting chlorophyll *a*ntenna size (Polle et al. 2003). Molecular analysis showed a single plasmid insertion into an ORF of the nuclear genome corresponding to a novel gene (*Tla1*) that encodes a protein of 213 amino acids. Biochemical analyses showed the *tla1* mutant to be chlorophyll (but not photosystem) deficient, with a functional chlorophyll antenna size of PS II and PS I being about 50% and 65% of the wild type, respectively. It contained a correspondingly lower amount of light-harvesting proteins than the wild type and had lower steady state levels of *Lhcb* mRNA. The *tla1* strain required a higher light intensity for the saturation of photosynthesis and showed greater solar conversion efficiencies and a higher photosynthetic productivity than the wild type under mass culture conditions (Polle et al. 2003). To the best of our knowledge, this is the first gene identified that regulates the Chl antenna size in oxygenic

photosynthesis. The *Tla1* gene (Polle et al. 2003), and functionally similar genes, may find direct application in green algal mass culture for biomass accumulation, carbon sequestration and H_2 production. As such, *Tla1* shows promise in helping to overcome the barrier associated with the low light utilization efficiency of photosynthesis during photobiological H_2 production in green algae.

Sulfate nutrient deprivation as a tool in H_2-production

Sulfur-deprivation in green algae causes reversible inhibition in the activity of photosynthesis (Wykoff et al. 1998). In the absence of sulfur nutrients, rates of photosynthetic O_2 evolution are lowered below those of O_2 consumption by respiration (Melis et al. 2000). As a consequence, sealed cultures of the green alga *Chlamydomonas reinhardtii* become anaerobic in the light (Ghirardi et al. 2000), induce the '[Fe]-hydrogenase' pathway of electron transport and continuously photo-produce H_2 gas in a sustained process that can last for a few days. During the course of such H_2-gas production, cells consume significant amounts of internal starch and protein (Melis et al. 2000; Zhang et al. 2002; Kosourov et al. 2002, 2003). Such catabolic reactions may sustain, directly or indirectly, the H_2-production process. 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 PS II and PS I proteins, and change in the composition of LHC II (Zhang et al. 2002; Zhang and Melis 2002). An increase in the level of the enzyme [Fe]-hydrogenase was noted during the initial stages of S-deprivation (0–72 h), followed by a decline in the level of this enzyme during longer ($t > 72$ h) S-deprivation times. 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, spherical cell shapes in the initial stages of S deprivation and H_2 production, followed by cell mass reductions at longer S-deprivation and H_2-production times (Zhang et al. 2002). This novel approach showed that sulfur nutrient deprivation of green algae serves as a metabolic switch that triggers a reversible change in the metabolic flux within the cell. Compared to metabolism under sulfur-nutrient replete conditions, it entailed a substantially altered interaction between oxygenic photosynthesis, mitochondrial respiration, catabolism of endogenous substrate, and electron transport via the hydrogenase pathway leading to a light-mediated H_2 production (Ghirardi et al. 2000; Melis and Happe 2001; Zhang et al. 2002). Application of the S-deprivation method permitted a temporal separation of oxygenic photosynthesis and biomass accumulation from consumption of endogenous substrate and H_2 production in the light. It was shown that, under S-deprivation conditions, electrons derived mostly from a residual PS II H_2O-oxidation activity (Ghirardi et al. 2000; Antal et al. 2001; Zhang et al. 2002) and in part from endogenous substrate catabolism (Ghirardi et al. 2000; Zhang et al. 2002; Kosourov et al. 2002, 2003; Antal et al. 2003) feed into the hydrogenase pathway, thereby contributing to the H_2-production process in *Chlamydomonas reinhardtii*.

Sulfur-nutrient deprivation proved to be a critically successful tool in the sustained production of H_2 by green algae since, for the first time in 60 years of related research (Gaffron 1939; Melis et al. 2000), substantial amounts of H_2 were produced continuously for 4–5 days in the light and could be accumulated in suitable containers. These results suggested that genes and proteins of the sulfate and starch metabolism are important in the process of H_2 metabolism in unicellular green algae. Research in this direction has indicated a role of a chloroplast sulfate permease and of a chloroplast stroma isoamylase in green algal H_2 metabolism.

Sulfate permease

Genomic, proteomic, phylogenetic and evolutionary aspects of a novel gene encoding a chloroplast-targeted sulfate permease of prokaryotic origin in the green alga *Chlamydomonas reinhardtii* were described (Chen et al. 2003). This nuclear-encoded sulfate permease gene (*SulP*) contained four introns and five exons, whereas all other known chloroplast sulfate permease genes lack introns and are encoded by the chloroplast genome. The deduced amino acid sequence of the protein showed an extended N-terminus, which includes a

putative chloroplast transit peptide. The mature protein contained seven transmembrane domains and two large hydrophilic loops. This novel prokaryotic-origin gene probably migrated from the chloroplast to the nuclear genome during the evolution of *C. reinhardtii*. The *SulP* gene, or any of its homologues, has not been retained in vascular plants, e.g. *Arabidopsis thaliana*, although it is encountered in the chloroplast genome of a liverwort (*Marchantia polymorpha*). A comparative structural analysis and phylogenetic origin of chloroplast sulfate permeases in a variety of species was presented (Chen et al. 2003).

Sulfate permeases may serve as useful tools in H_2 research. For example, this might be achieved upon the bioengineering of the function of the *SulP* gene to limit sulfate uptake and metabolism in the chloroplast of the green algae, thus limiting the rate of photosynthesis to the point where the latter would be more comparable to that of respiration in the cell. Sealed cultures of green algae in which the rate of respiration is equal to or greater than that of photosynthesis would be anaerobic, would constitutively express the [Fe]-hydrogenase pathway and continuously produce H_2 in the light.

Isoamylase

A *C. reinhardtii* library of 6000 colonies, generated by DNA insertional mutagenesis with the *Arg7* gene, was screened with sensitive chemochromic H_2 sensors (Flynn et al. 2002) for clones defective in H_2 production. Screening under defined conditions of dark anaerobic induction and subsequent actinic illumination helped identify a transformant that was deficient in H_2 production. The DNA region flanking the *Arg7* insertion in this mutant indicated plasmid disruption of a gene (*Sta7*), whose putative product showed a high degree of homology with the isoamylase enzyme found in a variety of plants. This enzyme plays a crucial role in starch metabolism (Dauvillée et al. 2001), and the *sta7-10* mutant stores less than 5% of the insoluble starch found in wild type *C. reinhardtii* strains. Starch breakdown has been proposed to generate the endogenous substrate molecules that feed electrons both into the mitochondrial electron transport chain, leading to consumption of O_2 and anaerobiosis, and into the photosynthetic electron chain, thereby contributing to photosynthetic H_2 production (Ghirardi et al. 2000; Melis and Happe 2001; Zhang et al. 2002; Kosourov et al. 2003). Conversely, Posewitz et al. (2004b) demonstrated that lack of accumulated starch in the *sta7-10* mutant has a detrimental effect on photosynthetic hydrogen metabolism and H_2 production in *C. reinhardtii*. An additional regulatory role of the *Sta7* gene was inferred in terms of the expression of the *HydA* genes, since the transcription of the [Fe]-hydrogenase genes is attenuated in *C. reinhardtii* after dark anaerobic induction conditions were applied (Posewitz et al. 2004b).

The *Sta7* gene may also serve as a useful tool in H_2 research. For example, this might be achieved upon bioengineering the *Sta7* gene function to over-express the gene product in the chloroplast of the green algae, thus permitting a greater accumulation of starch during the course of normal photosynthesis. It has been shown that H_2 production upon S deprivation in *C. reinhardtii* would last so long as there are deposits of starch in the green algal chloroplasts (Zhang et al. 2002). Consequently, it is hypothesized that the greater the starch deposits in the cell, the longer the duration and the greater the yield of H_2 production would be in green algae. There is also the possibility that accumulated starch might be mobilized to produce H_2 in the dark, and thus supplement H_2 produced in the light.

Future directions

Photobiological H_2 research has both fundamental and practical value. On the one hand, it addresses the paradox of a light-driven anaerobic H_2 metabolism in oxygenic unicellular green algae (fundamental), while on the other, it may open the possibility of a clean, renewable fuel from nature's most abundant resources, sunlight and water (practical value). Green algal H_2 research is still in its infancy, despite its 60-year history, primarily because earlier research could not overcome the mutually exclusive nature of O_2 and H_2 photoproduction in these microorganisms. Recent advances, including (a) the demonstration of continuous H_2 photoproduction in green algae as the result of imposing a nutritional deprivation, (b) the cloning and sequencing of the first algal [Fe]-hydrogenase genes and the recognition that

these represent a distinct subclass of [Fe]-hydrogenases, (c) the identification of genes that could improve the optical and light absorption properties of the cells, thereby optimizing H_2-production efficiency, and (d) the discovery of additional genes involved in the regulation of cellular metabolism related to H_2 production (Table 1), are evidence of progress and manifestation of the current state-of-the-art. In the future, there will be much to learn at the fundamental level before successful strategies for the commercial production of H_2 are developed. For example, it is clear that the underlying biochemistry as well as the regulation of the H_2 metabolism in the cell must be better understood. Moreover, there is a need to identify all the genes associated with the regulation of hydrogenase gene expression, hydrogenase enzyme assembly, environmental sensing, the partitioning of photosynthetic reductant in algae, and the regulation of metabolic pathways including starch biosynthesis and degradation, both of which affect H_2 production. The development of genomic, proteomic, computational analysis and bioinformatics tools, as well as tools by which to rapidly determine protein structure also offer new opportunities to address long-standing problems in this field.

Among the practical challenges at hand in this field are (a) improving the stability of [Fe]-hydrogenases under aerobic conditions, (b) understanding rate-limiting steps associated with H_2 photoproduction, and (c) addressing the barrier associated with the low light utilization efficiency in photobiological H_2 production. Large photosystem chlorophyll antenna sizes in green algae, were designed to offer a competitive advantage and survival of the organism in the wild rather than to produce H_2 for human consumption. These problems, associated with green algal H_2 research, were recognized long ago. However, efforts based on classical approaches to overcome them have been largely unsuccessful. Nevertheless, photobiological H_2 production by unicellular green algae has received considerable scientific credence following the recent temporal separation of O_2- and H_2-production activities upon sulfate nutrient deprivation of the green algae, the sequencing of specific genes that may directly or indirectly affect the process, and the sequencing of the entire *Chlamydomonas reinhardtii* genome. Finally this review is timely in light of the recent release of the US National Research Council's report on 'The Hydrogen Economy: Opportunities, Costs, Barriers, and R&D Needs' (http://www.nap.edu/books/0309091632/ html/), which suggests that prospects for photo-biological H_2 production in the long term are bright indeed.

Table 1. Summary of genes that are involved in the process of H_2 production or in the cellular metabolism related to H_2 photo-production in unicellular green algae

Gene name	Symbol	Reference
[Fe]-hydrogenase	*HydA1*	Florin et al. (2001), Happe & Kaminski (2002), Winkler et al. (2002a, b)
[Fe]-hydrogenase	*HydA2*	Wünschiers et al. (2001), Forestier et al. (2003)
Chlorophyll antenna size regulatory gene	*Tla1*	Polle et al. (2003)
Sulfate permease	*SulP*	Chen et al. (2003)
Isoamylase	*Sta7*	Posewitz et al. (2004b)

Addendum

Although a lot of information is available concerning the maturation of [NiFe]-hydrogenases, until recently nothing was known about the nature of the accessory genes required for the maturation of an active [Fe]-hydrogenase in any organism. To isolate accessory genes necessary for [Fe]-hydrogenase activity in *C. reinhardtii*, random DNA insertional mutants were screened for clones unable to produce H_2 (Posewitz et al. 2004a). One of the mutants, identified by this procedure and denoted *hydEF-1*, is incapable of assembling active HydA1 or HydA2 [Fe]-hydrogenase. Although the *hydEF-1* mutant transcribes both *C. reinhardtii* hydrogenase genes and accumulates full-length hydrogenase protein, H_2-production activity is not observed. The HydEF protein contains two unique domains, which are homologous to two distinct prokaryotic proteins, HydE and HydF that are found exclusively in organisms containing [Fe]-hydrogenase. Furthermore, in the *C. reinhardtii* genome, the *HydEF* gene is found adjacent to another hydrogenase-related gene, *HydG*, and the *HydE*, *HydF*, and *HydG* genes are found in putative operons with [Fe]-hydrogenase structural genes in several prokaryotic genomes. Posewitz et al. (2004a) demonstrated conclusively that

HydEF and HydG function in the assembly of the algal [Fe]-hydrogenases. Northern blot analyses showed that mRNA transcripts for both the *HydEF* and *HydG* genes were induced anaerobically and concomitantly with the two *C. reinhardtii* [Fe]-hydrogenase genes. In addition, complementation of the *C. reinhardtii hydEF-1* mutant with genomic DNA corresponding to a functional copy of the *HydEF* gene restored hydrogenase activity in the algae. Finally, these investigators showed that the heterologous expression of active algal [Fe]-hydrogenase in the bacterium, *E. coli*, could be accomplished by co-transformating the *HydA1* structural gene along with both the *HydEF* and *HydG* genes.

BLAST searches indicated that both HydEF and HydG belong to the emerging Radical SAM (also known as the AdoMet radical) superfamily of proteins. The HydG protein and the HydE domain of the *C. reinhardtii* HydEF protein both contain the signature Cys-X_3-Cys-X_2-Cys motif that is typically found within the Radical SAM protein superfamily. This motif coordinates a redox active [4Fe4S] cluster under reducing conditions (Frey et al. 2001; Sofia et al. 2001; Ollagnier-de Choudens et al. 2002b; Berkovitch et al. 2004). Radical SAM proteins participate in numerous biochemical reactions including, but not limited to: sulfur insertion, radical formation, organic ring synthesis, and anaerobic oxidation (Frey et al. 2001; Sofia et al. 2001). They are frequently involved in the anaerobic synthesis of complex biomolecules and coordinate unusual [FeS] clusters that are often labile (Krebs et al. 2000; Ugulava et al. 2000; Frey et al. 2001; Sofia et al. 2001; Berkovitch et al. 2004). These characteristics are consistent with the types of chemistries required to synthesize the unique ligands of the H-cluster and to assemble the [Fe]-hydrogenase catalytic domain.

Specifically, the H-cluster requires CN, CO and the putative di(thiomethyl)amine ligands. It is conceivable that the accessory proteins HydEF and/or HydG (Posewitz et al. 2004a) are responsible for the biosynthesis and assembly of these products coordinated to Fe. Since CN and CO are among the most toxic compounds in biology, and likely do not exist freely within the cell, it would be necessary to synthesize these ligands at the site of H-cluster assembly. In the case of the [NiFe]-hydrogenases, strong evidence indicates that CN and CO are synthesized by the HypE and HypF proteins, using carbamoyl phosphate as a precursor to form a thiocarbamate (Paschos et al. 2001; Reissmann et al. 2003). However, no homologues of the HypE and HypF proteins have been observed in *C. reinhardtii*, nor in other organisms containing only [Fe]-hydrogenases (Vignais et al. 2001). This suggests an alternative pathway for CN and CO synthesis or an alternative means to form thiocarbamate. Radical SAM proteins utilize chemistries that include organic radical formation, persulfide formation, pyroxidal phosphate activation, thiocarbonyl formation, and amine migration (Frey et al. 2001; Sofia et al. 2001; Ollagnier-de Choudens et al. 2002a), all or any one of which could be involved in the synthesis of the H-cluster organic ligands. Characterization of these [Fe]-hydrogenase assembly proteins will greatly facilitate additional examination of the mechanism by which [Fe]-hydrogenases are synthesized in nature.

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