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Minireview

Trails of green alga hydrogen research – from Hans Gaffron to new frontiers

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Abstract

This paper summarizes aspects of the history of photosynthetic hydrogen research, from the pioneering discovery of Hans Gaffron over 60 years ago to the potential exploitation of green algae in commercial H_2-production. The trail started as a mere scientific curiosity, but promises to be a most important discovery, one that leads photosynthesis research to important commercial applications. Progress achieved in the field of photosynthetic hydrogen production by green algae includes elucidation of the mechanism, the ability to modify photosynthesis by physiological means and to produce bulk amounts of H_2 gas, and cloning of the [Fe]-hydrogenase genes in several green algal species.

The original discovery

It was Hans Gaffron (1902–1979) who discovered the hydrogen metabolism in unicellular green algae, which are eukaryotic organisms of oxygenic photosynthesis (Gaffron 1939, 1940). Homann (2003), in these history issues, has thoroughly covered the early days of research by Gaffron and his associates. In addition, he has included several photographs of Gaffron and his co-workers. Gaffron observed that, under anaerobic conditions, the green alga *Scenedesmus obliquus* can either use H_2 as electron donor in the CO_2 fixation process in the dark (Gaffron 1942, 1944), or evolve H_2 in the light (Gaffron and Rubin 1942). Over the subsequent three decades, in research that lasted until his retirement in 1973, Gaffron and co-workers contributed greatly to our understanding of the basic mechanisms on photosynthetic hydrogen production (Gaffron and Rubin 1942; Bishop 1966; Kaltwasser et al. 1969; Stuart and Gaffron 1972a, b; Bishop et al. 1977). For example, by using electron-transport inhibitors, Gaffron in the early 1970s arrived at the conclusion that the hydrogenase activity in green algae is linked to the photosynthetic apparatus. Stuart and Gaffron (1972) wrote 'We conclude that all of the algae tested are able to photoproduce H_2 via non-cyclic electron flow through photosystem I to hydrogenase.' Other investigators, many of them associates of Gaffron, contributed with observations on photosynthetic hydrogen production in other unicellular green algae, including *Chlorella* (Spruit 1958; Kessler 1973), *Chlamydomonas* (Frenkel 1952; Frenkel and Lewin 1954; McBride et al. 1977; Greenbaum 1982, 1988; Maione and Gibbs 1986a, b) and other related organisms (Miura et al. 1986, 1992). Eric Kessler (1974) summarized the relevant information in a review article, showing that many but not all green algal species are equipped for molecular H_2 metabolism.

On a more personal note, as a beginning graduate student of Peter Homann, at Florida State University during the early 1970s, the first author (Melis) had the privilege of a guided tour of the Gaffron laboratory in the Institute of Molecular Biophysics, by Hans himself. Images of the 'hydrogen laboratory,' a collection of manometers, water baths, arrays of projection light bulbs, a mass spectrometer etc., are imprinted in Melis' mind, although at the time there was

absolutely no hint about the future course in this field.

The significance

The peculiarity and significance of Gaffron's discovery was that unicellular green algae are organisms of oxygenic photosynthesis, that is, in the light, they produce molecular oxygen. In the presence of O_2, hydrogen metabolism cannot take place. This is because oxygen, whether it is pre-existing O_2 dissolved in the algal growth medium, or photosynthetically generated O_2 from within the chloroplast of the green algae, is a powerful inhibitor of all aspects of cellular H_2 metabolism. Oxygen adversely affects the function of the hydrogenase enzyme (Erbes et al. 1979; Ghirardi et al. 2000) and acts as a positive suppressor of hydrogenase gene expression (Florin et al. 2001; Happe and Kaminski 2002). Historically, this multipoint 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 1984a, b; Miyamoto et al. 1990; Happe and Naber 1993; Schulz 1996), although Gaffron originally termed this process 'adaptation' (Gaffron 1940). Under such imposed anaerobiosis, the hydrogenase enzyme (Adams 1990; Vignais et al. 2001), which is encoded in the nucleus of the unicellular green algae (Florin et al. 2001), was expressed and catalyzed, albeit briefly, a light-mediated photosynthetic H_2-evolution.

The physiological purpose of the [Fe]-hydrogenase pathway is assumed to be an underlying need of the cells to generate ATP (Arnon et al. 1961). Indeed, under imposed anaerobic conditions, oxidative phosphorylation cannot take place in the green algae. Anaerobiosis though is necessary and sufficient for the expression of the [Fe]-hydrogenase pathway and the attendant photosynthetic hydrogen production, leading to generation of ATP, but not NADPH, in the chloroplast thylakoids. Such ATP is needed and probably used by the cell for housekeeping and survival purposes. Thus, the physiological significance of the H_2-production pathway in green algae could simply be the need to generate ATP under anaerobic conditions. However, given the prevailing oxidative environmental conditions on earth and the O_2 sensitivity of the [Fe]-hydrogenase, 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 for commercial purposes (Melis and Happe 2001; Happe et al. 2002). Nevertheless, the ability of green algae to photosynthetically generate molecular H_2 has captivated the fascination and interest of the scientific community because of the many *fundamental* aspects and the *practical* implications of the process (Melis and Happe 2001).

Pathways of electron transport for H_2-production: oxygenic photosynthesis

Interest in green algae emanates from the fact that, in principle, they can employ the highly efficient process of photosynthesis to produce hydrogen (H_2), a valuable fuel, from the most abundant of the natural resources, sunlight and water (H_2O). Under optimal growth conditions, green algae grow with remarkable rates, reaching biomass duplication times of 6 h in the laboratory (Smith et al. 1990) and 24 h under mass culture ambient conditions (Ben-Amotz and Avron 1990). In terms of H_2-production, work by Gaffron and his associates first suggested that H_2O could be the source of electrons for the light-dependent synthesis of molecular H_2 (Gaffron and Rubin 1942; Bishop and Gaffron 1963). This mechanism of photosynthetic H_2-production [also referred to as biophotolysis (Miura 1995; Benemann 1996)] entails H_2O oxidation and a light-dependent transfer of electrons via Photosystem II and Photosystem I to the chloroplast ferredoxin (Randt and Senger 1985) (Figure 1). Ferredoxin efficiently binds to the [Fe]-hydrogenase (Figure 1) and electrons are donated to the catalytic site known as the '*hydrogen cluster*' (HC) of the [Fe]-hydrogenase. The HC cluster utilizes protons as the sink for the photosynthetically generated electrons, leading to the synthesis of molecular H_2 (Peters et al. 1998; Peters 1999; Adams and Stiefel 2000). This process of photosynthetic hydrogen production does not entail CO_2 fixation or energy storage into cellular metabolites (Cinco et al. 1983). The process results in the simultaneous generation of H_2 and O_2 with a H_2:O_2 ratio of 2:1 (Spruit 1958; Greenbaum et al. 1983). In the absence of provision for the active removal of oxygen, this mechanism can operate only transiently (60–90 s) (Ghirardi et al. 1997), as photosynthetically generated O_2 is a powerful inhibitor of the [Fe]-hydrogenase enzymatic reaction and a positive suppressor of [Fe]-hydrogenase gene expression.

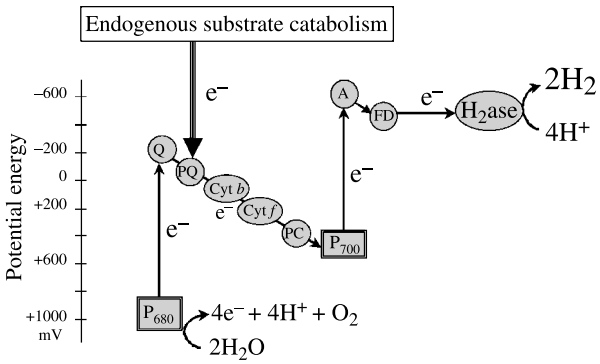

Figure 1. Hydrogenase-related electron transport pathways in green algae. Electrons may originate either at Photosystem (PS) II upon photo-oxidation of H_2O, or at the plastoquinone pool upon oxidation of cellular endogenous substrate (e.g., via glycolysis and the tricarboxylic acid cycle). Electrons in the electron-transport chain are transported via PS I to ferredoxin, which serves as the physiological electron donor to the [Fe]-hydrogenase. Abbreviations: PS = photosystem; P680 = reaction center of PS II; P700 = reaction center of PS I; Q = primary quinone electron acceptor of PS II; A = primary electron acceptor of PS I; PQ = plastoquinone; Cyt = cytochrome; PC = plastocyanin; Fd = ferredoxin; H_2ase = [Fe]-hydrogenase (adapted from Melis and Happe 2001).

Pathways of electron transport for H_2-production: photo-fermentation

Aside from the above-described Photosystem (PS) II-dependent H_2 photoproduction, an alternative source of electrons has been described in the literature. Catabolism of endogenous substrate and the attendant oxidative carbon metabolism in green algae may generate electrons for the photosynthetic apparatus (Gfeller and Gibbs 1984). 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 (Klein and Betz 1978; Godde and Trebst 1980; Gfeller and Gibbs 1985; Ohta et al. 1987; Feild et al. 1998; Bennoun 2001, 2002) (Figure 1). This contention is supported by the observation that *Chlamydomonas reinhardtii* can photoproduce hydrogen when PS II is blocked by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), but no H_2-evolution occurs after an addition of 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) (Stuart and Gaffron 1972), which blocks the function of the cytochrome *b–f* complex. Externally added sources of organic carbon, such as glucose or acetate, substantially enhanced this pathway of electron transport and hydrogen production (Healy 1970; Stuart and Gaffron 1971). Light absorption by PS I and the ensuing electron transport elevates the redox potential of these electrons to the redox equivalent of ferredoxin and the [Fe]-hydrogenase, thus permitting the generation of molecular H_2 (Gibbs et al. 1986). In the presence of DCMU, a PS II inhibitor, this process generates H_2 and CO_2 with a H_2:CO_2 ratio of 2.1 (Bamberger et al. 1982). Thus, following a dark-anaerobic-incubation of the culture (induction of the [Fe]-hydrogenase), initially high rates of H_2 production can be detected upon illumination of the algae in the presence of DCMU (Happe and Naber 1993; Florin et al. 2001).

The two pathways of H_2-production, summarized above, have been shown to operate independently of one another. Elias Greenbaum and co-workers developed conditions for the low-level but continuous operation of an electron transport pathway based on oxygenic photosynthesis (Greenbaum 1982, 1988; Greenbaum et al. 1983). Gaffron and co-workers (Stuart and Gaffron 1971, 1972a) and Gibbs and co-workers (e.g., Gibbs et al. 1986) demonstrated the fermentative pathway of electron transport for H_2-production. However, each of these electron-transport processes has limitations in terms of sustainability and yield. Unless rigorously purged of oxygen (Greenbaum and co-workers), photosynthetic H_2-production with H_2O as the source of electrons cannot be sustained beyond 60–90 s of operation (Ghirardi et al. 1997). This is because molecular O_2, emanating from the activity of photosynthesis, acts as a powerful and effective switch by which the H_2-production activity is turned off. This incompatibility in the simultaneous O_2 and H_2 photo-production remained a problem in 60 years of related research. On the other hand, catabolism of endogenous substrate with the attendant oxidative carbon metabolism in green algae does not have the capacity to sustain high rates of electron transfer to the photosynthetic apparatus. Thus, upon illumination of dark-adapted green algae, initially high rates of PS II-independent H_2-production are observed. These quickly slow-down to negligible levels (Florin et al. 2001; Zhang et al. 2002).

Two-stage photosynthesis and H_2-production in green algae

Progress in the late 1990s contributed to a breakthrough in terms of sustainable hydrogen production. The oxygen sensitivity of the H_2 metabolism reactions was bypassed in a two-stage process in which

O_2-production and H_2-production were temporally separated. In Stage 1, normal photosynthesis, CO_2-fixation and release of O_2 upon oxidation of H_2O enable green algae to accumulate biomass. In Stage 2, the algae are deprived of sulfur-containing nutrients. Sulfur-deprivation acts as a metabolic switch that slows down oxygenic photosynthesis and alters the normal pathways of electron transport in the chloroplast. Specifically, lack of sulfur nutrients from the growth medium of *C. reinhardtii* suppressed the rate of oxygenic photosynthesis (Wykoff et al. 1998) but did 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 (Ghirardi et al. 2000). Under these conditions, it was shown that expression of the [Fe]-hydrogenase is elicited in the light, automatically leading to H_2-production by the algae (Melis et al. 2000). Alteration of the photosynthesis–respiration relationship permitted, for the first time, a continuous H_2 photoproduction process, which could be sustained for days. In the process, electrons for the [Fe]-hydrogenase were contributed both by oxygenic photosynthesis (primary source) and by photo-fermentation (secondary source). The latter was supported by catabolism of endogenous substrate, notably starch and protein. Substantial amounts of H_2 gas, emanating as bubbles from the green algal culture, could be accumulated and measured by simple volumetric methods. In this S-deprivation dependent process, the enzymatic production of H_2 is the last step of a complex cellular metabolism, which entails previously unknown metabolic, regulatory and electron-transport reactions. The underlying biochemistry encompasses a unique four-way interplay between the processes of oxygenic photosynthesis, mitochondrial respiration, catabolism of endogenous substrate, and electron transport via the [Fe]-hydrogenase pathway leading to H_2-production (Melis and Happe 2001).

Figures 2 and 3 schematically show the functional coordination of chloroplast photosynthesis and mitochondrial respiration under S-deprivation. Mitochondrial electron-transport-activity is sufficient to consume photosynthetically generated oxygen, causing anaerobiosis in the culture (Kessler 1966; Francis and Senger 1985; Ghirardi et al. 2000). Anaerobiosis is necessary and sufficient to ensure the continued activity of the H_2-producing reactions. Catabolism of endogenous substrate and cellular morphological alterations take place during Stage 2 (Zhang et al. 2002). 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, that is, by the so-called 'two-stage photosynthesis and H_2-production' process (Melis et al. 2000).

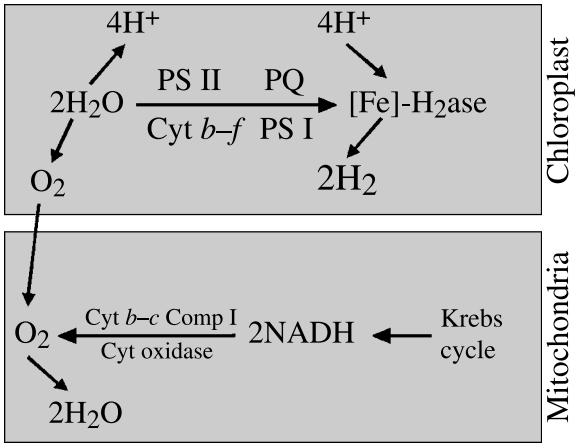

Figure 2. Coordinated photosynthetic and respiratory electron transport and coupled phosphorylation during H_2-production. Photosynthetic electron transport delivers electrons upon photo-oxidation of H_2O to the hydrogenase, leading to H_2-production. The oxygen generated by this process serves to drive the coordinate oxidative phosphorylation during mitochondrial respiration. Electrons for the latter 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 (adapted from Melis and Happe 2001).

The catalyst of H_2-production in green algae

Recent progress contributed to another breakthrough, namely, the first time isolation and characterization of the hydrogenase genes in green algae. The *HydA* gene was characterized in different Chlorophyceae species like *Scenedesmus obliquus* (Florin et al. 2001), *C. reinhardtii* (Happe and Kaminski 2002) and *Chlorella fusca* (Winkler et al. 2002). The gene sequences, and the deduced amino acid sequence of the respective proteins, unequivocally showed that these green algal proteins belong to the class of the [Fe]-hydrogenases (Roessler and Lien 1984a, b; Happe et al. 1994), a group of enzymes with a high specific hydrogen evolution activity. Interestingly, green algae might possess additional gene sequences that encode for

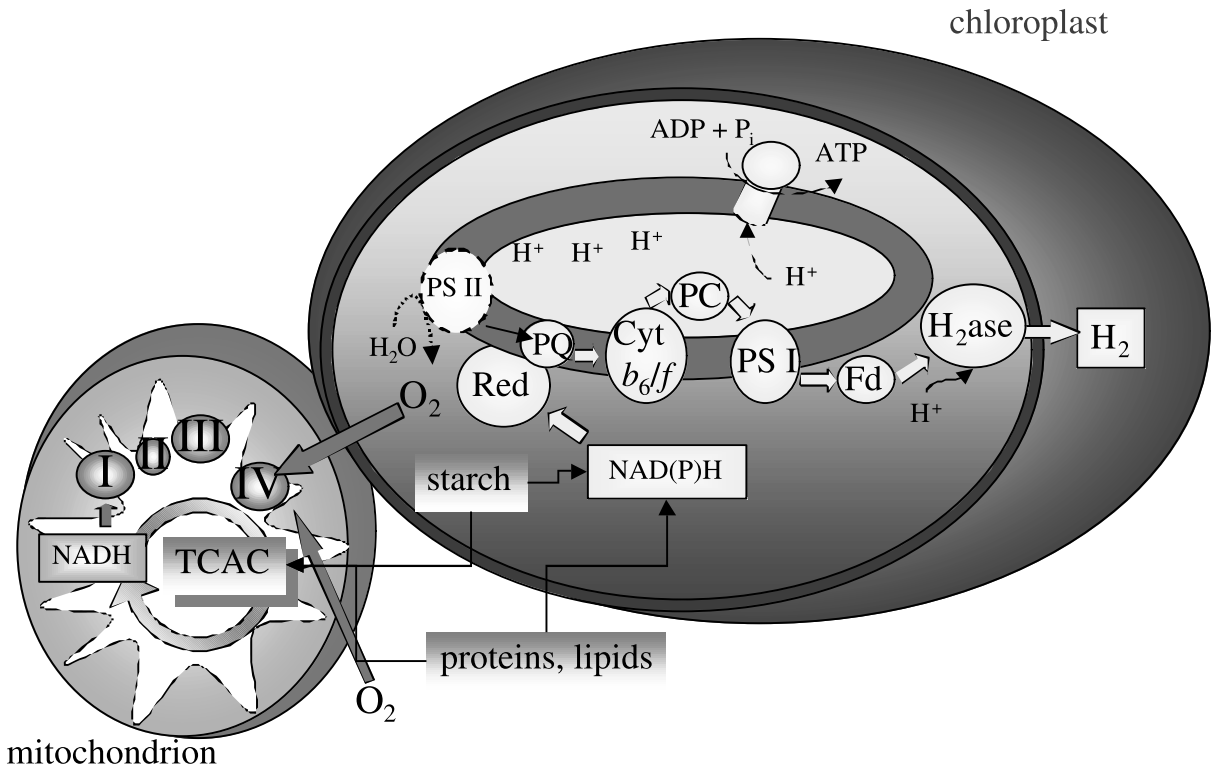

Figure 3. Hydrogen metabolism in *C. reinhardtii* under sulfur-deprivation. Arrows indicate the main electron transport pathway leading to hydrogen production and the oxidation of reducing equivalents during respiration. Abbreviations: PS – photosystem; PQ – plastoquinone; Cyt *b–f* – cytochrome *b–f* complex; PC – plastocyanin; FD – ferredoxin; Red – NAD(P)H plastoquinone-oxidoreductase; H_2ase – hydrogenase; I–IV–complexes of respiratory electron transport chain; TCA – tricarboxylic acid cycle.

hydrogenase-similar polypeptides (Wunschiers et al. 2001). However, it remains unclear whether such '*HydA2*' genes encode for a functional hydrogen-evolving enzyme. Furthermore, from phylogenetic studies, it is evident that the HydA proteins in the green algal plastids share a common origin (Horner et al. 2002). Interestingly, [Fe]-hydrogenase genes have not been found in several genome sequences from cyanobacteria, the presumed endosymbiotic progenitor of the chloroplast in green algae. This raises a question as to the evolutionary origin of the [Fe]-hydrogenases in green algae.

[Fe]-hydrogenases are usually divided in two functional domains (Figure 4) (Peters et al. 1998; Vignais et al. 2001). The well conserved C-terminal part (H-domain) binds the H-cluster, a catalytically active prosthetic group, consisting of a combination of one [4Fe–4S] subcluster and a uniquely structured [2Fe–2S] subcluster (Nicolet et al. 2000, 2001). In the latter, the Fe_{II}-atom represents the effective location of the catalytic activity. The H-cluster is ligated to the polypeptide chain by four highly conserved cysteine residues. The N-terminal part of the [Fe]-hydrogenase is rather variable but, generally, contains additional [4Fe–4S] or [2Fe–2S] clusters (F-clusters) that mediate the electron transfer reactions between the electron donor molecule (ferredoxin) and the H-cluster.

The algal [Fe]-hydrogenases (Figure 4) are unique in this respect as they lack the entire N-terminal domain, while containing only the H-cluster-binding region of the protein. Their truncated N-terminus contains a transit-peptide, which facilitates the import of this nuclear-encoded polypeptide into the chloroplast stroma. An additional unique feature of all green algal [Fe]-hydrogenases is the insertion of an unusual peptide segment in the C-terminus domain of the protein. This appears to be of variable length in the different green algae, but always present in about the same region of the C-terminus domain. The function of this insertion is still unknown, but might

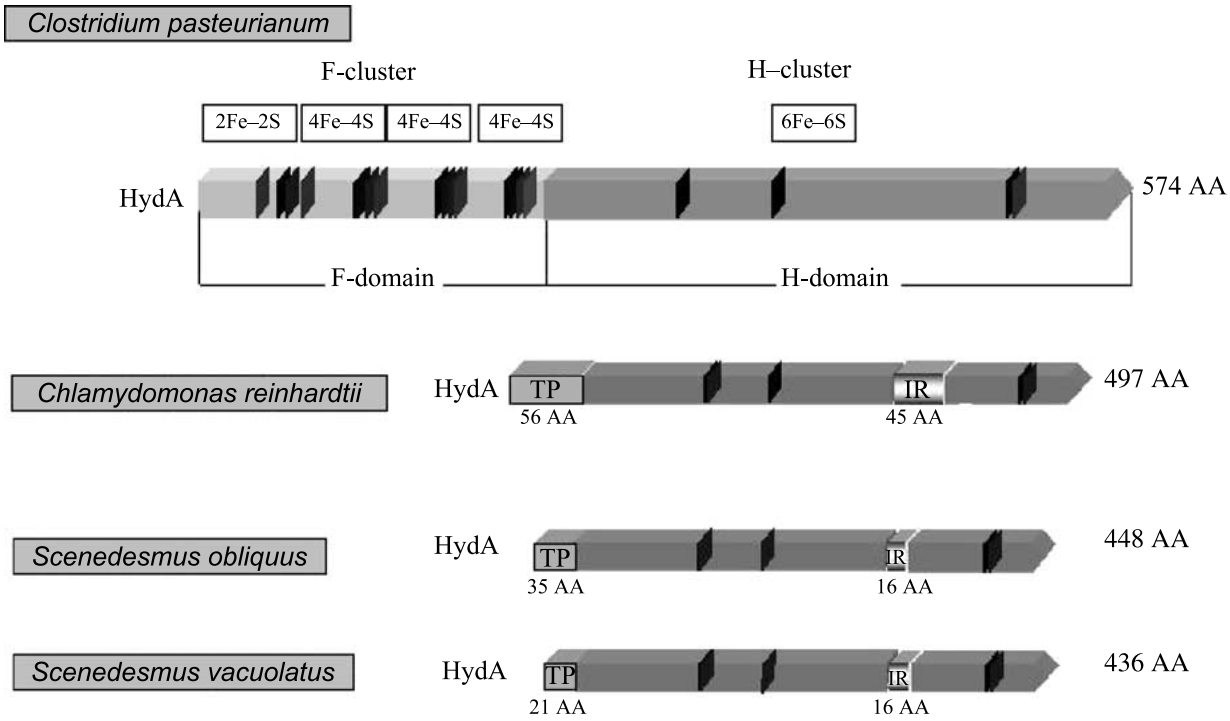

Figure 4. Comparative schematic of the Fe-hydrogenase polypeptides from selected H_2-producing organisms. The mature Fe-hydrogenase polypeptide is depicted linearly from left (N-terminus) to right (C-terminus). Parallelograms within the linear rectangle show the position of conserved cysteine residues that play a role in the coordination of the [Fe–S]-clusters in the hydrogenase protein. The insertion of 45 and 16 unique amino acid sequences in the Fe-hydrogenase of *Chlamydomonas reinhardtii*, *Scenedesmus obliquus* and *Scenedesmus vacuolatus*, respectively, is indicated.

facilitate the proper orientation of the electron donor (ferredoxin) in the binding domain of the green alga [Fe]-hydrogenase.

International conferences on Biohydrogen research

The relevance of H_2 photoproduction has increased substantially in the past several years, evidenced in part by the three international BioHydrogen conferences, recently held in three separate continents. BioHydrogen'97 was organized by Oskar Zaborski in Kona, Hawaii (Zaborsky 1998), where numerous presentations were made on widely divergent aspects of biological hydrogen production, ranging from green alga photoproduction to bacterial dark fermentations and photobioreactor designs. Subsequent international BioHydrogen conferences were held in 1999 in Tsukuba, Japan (Miyaki et al. 2001) and Ede, the Netherlands in 2002 (Van Neil et al. 2002). The reader is referred to the pertinent 'proceedings' for reference as to progress in a variety of BioHydrogen and green algal projects.

Future prospects

The process of hydrogen production by green algae has practical implications because it pertains to the

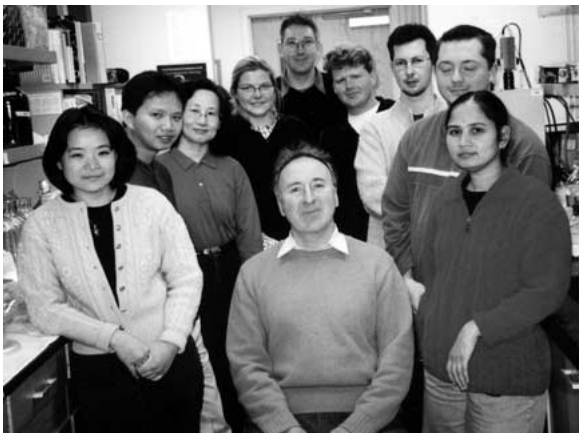

Figure 5. The first author (AM; seated) and his research group at the University of California at Berkeley. *From left to right* Hsu-Ching Chen, Kittisak Yokthongwattana, Yar-Fen Teng, Sabrina Ehnert, Juergen Polle, Eric Johnson, James Kirby, Michael McPhaerson and Sarada Kanakagiri.

Figure 6. The second author (TH; on the far left) and his research group at the University of Bonn. Standing *from left to the right* (after Thomas Happe) Kathrin Happe, Burkhard Heil, Steffi Smolny, Wolfgang Hachtel, Annette Kaminski, Thomas Slabon, Astrid Weber, Wolfgang Schiefer and Martin Winkler. Sitting in the front row are Anja Hemschemeier, Sarah Schwarzer and Annette Zamponi.

question of energy supply and demand for the entire world. Projections of potential fossil fuel shortfall, toward the middle of the 21st century, necessitate the development of alternative energy sources that are clean, renewable and environmentally friendly. In terms of the latter, green alga hydrogen production promises to positively alter the equation on energy supply and demand, alleviate global warming, and mitigate environmental pollution, as hydrogen is generated from water and water is the only byproduct of hydrogen combustion. The advent of photosynthetic hydrogen production will bring about technological developments in many fields and will find many as yet unforeseen applications.

Over 60 years ago, Hans Gaffron embarked on a new trail of scientific research, which entailed the use of sunlight energy to extract hydrogen from water with unicellular green algae serving as the biological catalyst. It is evident from this historical review, that progress along this trail has accelerated substantially in recent years. It is hoped that '*Gaffron's trail*' will soon lead to the renewable production of a valuable fuel, one that will serve our society in many years to come.

Acknowledgments

To recognize all our current co-workers, we show in Figure 5, the research group of the first author (AM) and in Figure 6, the research group of the second author (TH). We are thankful to all our collaborators for their contributions. This manuscript was edited by Govindjee and Andrew Webber.

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