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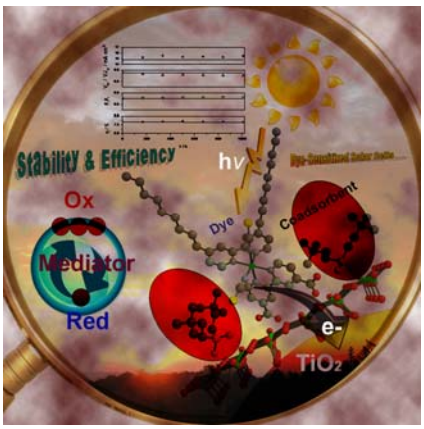

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How algae produce hydrogen—news from the photosynthetic hydrogenase

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Green algae are the only known eukaryotes capable of oxygenic photosynthesis which are equipped with a hydrogen metabolism. Hydrogen production is light-dependent, since the [FeFe] hydrogenases are coupled to the photosynthetic electron transport chain *via* ferredoxin. Algal [FeFe] hydrogenases are one of the most active biocatalysts for the evolution of hydrogen. Therefore, special interest exists in the biophysical characterization and biotechnological usage of these [Fe-S] enzymes. This review traces the discovery of this interesting class of proteins. Recent findings allow insight into the electronic structure and configuration of the [FeFe] hydrogenase active site (H-cluster). Emphasis is placed on novel discoveries of the hydrogenase interaction with its natural electron donor ferredoxin and the mechanism of enzyme inactivation through oxygen.

Introduction

Hydrogenases catalyze a simple reaction, namely the reversible reduction of protons to molecular hydrogen. The discovery of this class of enzymes was made in the 1930s.[1] Years later, Hans Gaffron observed that green algae can either oxidize hydrogen in concert with CO_2 fixation in the "dark reaction"[2,3] or evolve hydrogen gas upon illumination.[4] Since this important finding, the hydrogenase metabolism in photosynthetic algae has been of great scientific interest. Stuart and Gaffron were the first to uncover the direct links between hydrogen evolution and photosynthesis,[5] and in the late 1990s, Melis and co-workers established sulfur deprivation for semi-continuous, photobiological hydrogen production in *Chlamydomonas reinhardtii*.[6]

This breakthrough towards a sustainable hydrogen production was achieved by separating oxygenic photosynthesis and CO_2 fixation from hydrogen evolution in time. Wykoff and Melis could show that a sulfur-deprived culture of *C. reinhardtii* gradually loses its photosynthetic capacity while mitochondrial respiration is left essentially unchanged.[6,7] Photosynthesis is diminished due to the loss of the catalytic active D1 subunit of photosystem II (PSII) which turns over very rapidly.[7] Deprived of sulfur, the amino acids cysteine and methionine run short and D1 can not be replaced at an appropriate rate. Thus, PSII-catalyzed water oxidation and oxygen evolution decline. Once respiration consumes more oxygen than residual photosynthesis can deliver, cells become anaerobic and hydrogen turnover is induced.[8] Under sulfur deprivation, reduction of protons is a sink for (excess) electrons that result from starch breakdown as a product of CO_2 fixation during cell growth under oxygenic conditions.[9,10]

The hydrogenase HydA1 of *C. reinhardtii* receives electrons at the reducing end of the photosynthetic electron transfer chain. The "photosynthetic" ferredoxin PetF shuttles electrons from photosystem I (PSI) to HydA1 which reduces protons to molecular hydrogen.[11] The hydrogenase competes with different electron sinks, in particular ferredoxin-NADP-oxidoreductase as

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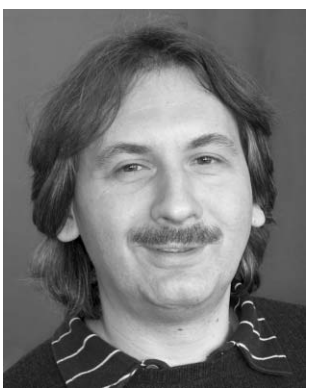

Thomas Happe

Prof. Thomas Happe received his PhD in 1994 from Ruhr-University Bochum. He worked as a postdoc at the University of Bonn in the Department of Molecular Biochemistry and became associate professor in 1998. In the year 2000 he was guest fellow at the University of California in Berkeley. He has been a full professor at the Ruhr-University Bochum since 2003. His research interest focuses on the photobiological hydrogen production in green algae and the characterization of [FeFe] hydrogenases.

an interface with the Calvin cycle.[12-14] Unlike PSII, the PSI complex is essential to hydrogen evolution.[15] "Catabolic" electrons are fed into the photosynthetic electron transfer chain from degradation of starch, glucose or acetate at the level of the plastoquinone pool.[16-18] This PSII-independent hydrogen evolution, which utilizes fermentative oxidation of organic substrates, is referred to as "photofermentation".[14,19]

Hydrogenases are ubiquitous in strict and facultative anaerobes, and the vast majority is found in prokaryotes.[20,21] Hydrogenases are transition metal enzymes, likely to be developed in a pre-photosynthetic, reducing atmosphere.[22,23] In the absence of oxygen, hydrogenases serve as terminal electron acceptors. However, hydrogenases are found in oxidation ("uptake") of molecular hydrogen as well.[24] According to the composition of the bimetallic active site cofactor, [NiFe], [FeFe] and hydrogen-forming methylenetetrahydromethanopterin (Hmd) [Fe] hydrogenases are distinguished.[25-29] Under physiological conditions, [NiFe] hydrogenases generally act as uptake hydrogenases while [FeFe] hydrogenases often catalyze hydrogen evolution.[30,31] Hydrogen release with [FeFe] hydrogenases is fast and in most cases controlled by diffusion of substrates and products.[23,25] [NiFe] hydrogenases were shown to exhibit much higher affinities for hydrogen (as a substrate in uptake) than [FeFe] hydrogenases.[24] [NiFe], [FeFe] and [Fe] hydrogenases (Hmd) are *not* homologs and give a model for convergent mechanistic evolution.[32]

[FeFe] hydrogenases have been described for pro- and eukaryotes, [NiFe] hydrogenases in contrast are solely found in prokaryotes including cyanobacteria. Green algae and cyanobacteria are the only organisms currently known to be capable of both oxygenic photosynthesis and hydrogen production.[33] However, despite the availability of a number of entirely sequenced cyanobacterial genomes, [FeFe] hydrogenases have never been described in cyanobacteria. The photosynthetic cyanophyta ("blue-green algae") are endosymbiotic progenitors of plastids that form chloroplasts in higher plants and algae.[21] These eubacteria possess only [NiFe] hydrogenases and evolve hydrogen by a light-dependent reaction. The cyanobacterial hydrogen metabolism is different to the algal hydrogenase turnover and catalyzed by nitrogenase, the nitrogen fixing enzyme complex.[33] Thus it appears that, in green algae, the hydrogenase has been introduced by a host with a nucleus-encoded [FeFe] hydrogenase of non-cyanobacterial origin.

Besides the natural [FeFe] and [NiFe] catalysts for hydrogen production, chemists have developed several electro- and photochemical hydrogen evolving catalyst systems in the last few years. Based on the [2Fe-2S] cofactor of the H-cluster from [FeFe] hydrogenases, it was shown that these structural and functional [2Fe-2S] mimics can efficiently produce hydrogen.[34-38] Moreover, hydrogen catalysts can also be coupled to photosensitizers and release hydrogen by light-induced water splitting. The progress and the application of the metal-based devices for light driven hydrogen evolution in homogeneous systems was recently summarized by Wang and co-workers.[39]

During the past fifteen years, traditional physiological and biochemical studies have yielded information on photobiological hydrogen evolution in green algae.[6,8] Several review articles summarize the discovery of hydrogen turnover under sulfur deprivation and the isolation of genes encoding for different algal hydrogenases.[11,17,18,31,40,41] The purpose of this article is to highlight the wealth of new results regarding biophysical properties of the [FeFe] hydrogenase HydA1 of *C. reinhardtii*, including the electronic structure of the active site H-cluster, the reaction of this prosthetic group with CO and oxygen, and the interaction of the algal protein with its native donor ferredoxin.

The discovery of hydrogenases in green algae

As already mentioned, the first descriptions of hydrogen evolution by photosynthetic algae were published seventy years ago by Hans Gaffron and co-workers.[4] In 1973, Eric Kessler summarized the relevant information on hydrogen production by photosynthetic algae in a review article, showing that many species of unicellular green algae are equipped for hydrogen metabolism.[42] However, thirty years elapsed between the first observation of a "Cell-free Hydrogenase from *Chlamydomonas*" by Frederick B. Abeles[43] and the purification of the *C. reinhardtii* hydrogenase by Happe and Naber in 1993.[44]

Abeles could show in his pioneering experiments that the cell-free preparations of *Chlamydomonas eugametos* evolve hydrogen when the hydrogenase fraction was incubated with reduced methylviologen as electron mediator.[43] He also analyzed the inactivation of the protein by small amounts of oxygen and carried out his experiments under strict anaerobicity. However, his observation that the hydrogenase is not associated with the chloroplast was incorrect.

Twenty years later, Paul G. Roessler and Stephen Lien developed a method which resulted in a 2000-fold purification of hydrogenase HydA1 of *C. reinhardtii*.[45] The trick was to use an affinity chromatography with immobilised ferredoxin which is the electron donor to the hydrogenase *in vivo*. The preparation was 40% pure and the specific hydrogen evolution capacity of the enzyme was calculated to be 1800 μmol H_2 min^{-1} mg^{-1}.[45] Additional experiments on HydA1 showed that "activation and *de novo* synthesis" of the protein was inhibited by cycloheximide but not chloramphenicol.[46] These results clearly indicated that the hydrogenase gene is nucleus-encoded. Roessler and Lien gave the hydrogenase research in green algae an important impulse leading to the eventual isolation of the hydrogenase from *C. reinhardtii* in the beginning of the 1990s.

To characterize the algal hydrogenase in more detail, the next step was to isolate the protein up to homogeneity. Thomas Happe and Dirk Naber used five column-chromatography steps to purify the enzyme 6100-fold and determined the specific activity for hydrogen evolution as 935 μmol H_2 min^{-1} mg^{-1}.[44] A single band was observed on SDS PAGE gels which had an apparent molecular mass of 48 kDa. The respective protein fraction on non-denaturing gels possessed methylviologen reducing activity. Another study showed that the protein contains iron but no nickel.[47] Therefore, and because of the specific biochemical properties of the enzyme (CO inhibition, extreme oxygen sensitivity, see below), the authors classified the algal hydrogenase as [FeFe] hydrogenase (originally "Hydrogenase of the Fe-only type").

During that time in the 1990s, the results of Happe and Naber were called into question because it was known that cyanobateria, the free-living precursors of plastids, encode exclusively for [NiFe] hydrogenases. Schnackenberg *et al.* published the isolation of an ostensible [NiFe] hydrogenase of the green alga *Scenedesmus obliquus*.[48] While it was not yet established that the *C. reinhardtii*

hydrogenase is encoded in the nucleus,[46] the scientific community knew that the algal chloroplast phylogenetically results from endosymbiosis of cyanobacteria. Hence, doubt was sown that algae can contain any other than [NiFe] hydrogenases.

The conflict was solved when the Happe group isolated the hydrogenase gene from *C. reinhardtii*.[49] The deduced amino acid sequence of HydA1 revealed a conserved C-terminal sequence typical for [FeFe] hydrogenases, including four conserved cysteine residues that coordinate the active site.[41,50] Based on these elementary results, the *hydA* genes of further algal species were isolated in the following years.[51,52] It turned out that the hydrogenase proteins of algae represent a novel class of [FeFe] hydrogenases.[17] The "chlorophyta-type" [FeFe] hydrogenases are smaller (44–48 kDa) because they lack the N-terminal ferredoxin-like domain ("F-domain") present in all [FeFe] hydrogenases isolated back then (see below).[53] Moreover, the reported occurrence of [NiFe] hydrogenases in green algae[48] has never been supported by gene cloning and sequencing and was proven to be erroneous.

Although the genes and the proteins of algal hydrogenases were isolated, another problem had to be overcome to learn more about this class of enzymes. The problem was explained by Roessler and Lien as follows: "More detailed analysis of the active site of *C. reinhardtii* hydrogenase by the use of electron spin resonance (EPR) spectroscopy would be highly desirable for comparative purposes, but the low quantity of hydrogenase present in this organism makes this a difficult task".[45] In the early 1990s, Happe and Naber also reported that they could isolate only 1 μg protein per liter of green algae culture.[44]

To overcome these difficulties, two strategies were used. First, newly established and efficient induction and purification protocols, *e.g.* isolation of the hydrogenase from a sulfur-deprived algal culture, yielded 40 μg HydA1 from one litre green algae corresponding to a 40-fold increase in protein content compared to previous protocols.[54] Second, a heterologous expression system for [FeFe] hydrogenases in the fermentative bacterium *Clostridium acetobutylicum* was established.[55] Using *Escherichia coli* or *Shewanella oneidensis* as hosts, synthesis led to only low amounts of recombinant [FeFe] hydrogenases[56] or high amounts of protein but comparably low specific activities.[57] The heterologous synthesis of [FeFe] hydrogenases with *C. acetobutylicum* in contrast offers the possibility to produce both large amounts of enzyme *and* hydrogenase at high specific activity. After optimizing various parameters, it is possible to isolate about 2 mg of pure and active [FeFe] hydrogenase from one litre of bacterial cell culture.[58] Furthermore, side directed mutagenesis on the plasmid-encoded proteins allows the investigation of structure-function relationships in [FeFe] hydrogenases by analyzing the characteristics of [FeFe] hydrogenase variants.

Basic properties of the [FeFe] hydrogenases from green algae

[FeFe] hydrogenases are small, mono- and dimeric enzymes of 45–65 kDa. The active site cofactor is a unique [Fe-S] compound commonly referred to as "H-cluster".[59] *In vivo*, [FeFe] hydrogenases are usually found in hydrogen evolution.[30] Essayed *in situ*, catalysis is mostly bidirectional. Enzyme activity is easily inactivated by oxygen and CO[22,60] although the characteristics of inactivation differ in the reduction (evolution) and oxidation (uptake) directions.[61] [FeFe] hydrogenases from organisms like *Clostridium pasteurianum* and *acetobutylicum, Desulfovibrio desulfuricans* and *Megasphaera elsdenii* have been described in detail.[25,55,58,62] Table 1 shows a comparison of the basic properties of bacterial and algal hydrogenases, in particular from C. *reinhardtii*, *S. obliquus*, *Chlamydomonas moewusii*, *Chlorococcum submarinum* and *Chlorella fusca*. All [FeFe] hydrogenases are efficient catalysts in hydrogen evolution, but bacterial enzymes like *Cp*I, *Dd*H and HydA of *M. elsdenii* release hydrogen at exceptionally high rates (5000–8000 μmol H_2 min^{-1} mg^{-1}).

Most [FeFe] hydrogenases consist of a single peptide chain. The structures of [FeFe] hydrogenases of *D. desulfuricans* (*Dd*H) and *C. pasteurianum* (*Cp*I) have been resolved by X-ray crystallography.[50,59] *Cp*I represents the typical bacterial-type [FeFe] hydrogenase. Two domains can be distinguished. The C-terminal "H-domain" carries the H-cluster, an electronically coupled [6Fe-6S] cluster described in detail later on. The accessory F-domain holds a set of ferredoxin-type [4Fe-4S] and/or [2Fe-2S] clusters.[20] These [Fe-S] compounds form an electric "wire", shuttling electrons from the protein surface to the H-cluster. The [FeFe] hydrogenases of green algae belong to the smallest hydrogenases known and are about 15 kDa smaller than most bacterial hydrogenase enzymes. The H-cluster is the only catalytically active iron compound in algal hydrogenases. According to sequence alignment, this is true for all [FeFe] hydrogenases found in algae up to now.[52,54,63]

Table 1 Comparison of different prokaryotic and chlorophyta-type [FeFe] hydrogenases

Organism	Name	M_r[a]	V_{max}[b]	Reference
Clostridium pasteurianum	CpI	63.8	5500	Adams 1990 (25)
Clostridium acetobutylicum	HydA	64.3	1750	von Abendroth *et al.* 2008 (58)
Desulfovibrio desulfuricans	DdH	46.1+14.0	8820	Hatchikian *et al.* 1992 (62)
Megasphaera elsdenii	HydA	53.6	7000	Adams 1990 (25)
Chlamydomonas reinhardtii	HydA1	47.5	935	Happe and Naber 1993 (44)
Chlamydomonas reinhardtii	HydA2	47.3	n.d.	Forestier *et al.* 2003 (66)
Scenedesmus obliquus	HydA	44.6	630	Girbal *et al.* 2005 (55)
Chlamydomonas moewusii	HydA1	45.4	1600	Kamp *et al.* 2008 (54)
Chlorococcum submarinum	HydA	45.3	640	Kamp *et al.* 2008 (54)
Chlorella fusca	HydA	45.1	1000	Winkler *et al.* 2002a (17)

[a] M_r Molecular weight in kDa as derived from protein primary structure. [b] V_{max} Specific hydrogen evolution activity expressed as μmol H_2 min^{-1} mg^{-1} with 10 mM methylviologen as electron donor; n.d. not determined.

Interestingly, these proteins are exclusively found in the chloroplast stroma and are not associated with the membrane, which is different to the situation reported for [NiFe] hydrogenases.[64] However, the hydrogenase genes are encoded in the nucleus and a N-terminal transit peptide allows for import of the transcript to the chloroplast where protein biosynthesis is thought to take place.[41,65] While prokaryotic hydrogenases are usually part of the fermentative metabolism, [FeFe] hydrogenases in algae receive reducing equivalents at the end of the photosynthetic electron transfer chain *via* Ferredoxin.[40,41] Therefore, chlorophyta-type [FeFe] hydrogenases have been termed "photosynthetic hydrogenases".[18]

As reported for most bacteria, an isoenzyme HydA2 was found in *C. reinhardtii* and other green algal genomes. The gene *hydA2* possesses all conserved residues and domains identified typical for the active site of this class of [FeFe] hydrogenases.[66] Up to now, the HydA2 protein has not been isolated, and the function and catalytic activity of this isoenzyme remains unclear.

Fig. 1 displays a sequence alignment for different algal hydrogenases with the structurally well-characterized H-domain of prokaryotic enzymes *Cp*I and *Dd*H. The hydrogenase of *M. elsdenii* is plotted because of its minimal set of accessory [Fe-S] clusters (see Fig. 2). For reasons of simplicity, the accessory F-domain is not shown in the alignment. For all [FeFe] hydrogenases, conservation of the active site motif is evident. The position of the H-cluster cysteines (C) is well-preserved and around these residues, sequence similarity is comparably high. However, certain differences set apart bacterial and chlorophyta-type hydrogenases. The N-terminal F-domain is missing (sequence not shown), instead all algal hydrogenases display an "insertion" region (dashed boxes 1 and 2). This insertion (most pronounced in HydA1 of *C. reinhardtii*) is discussed to form a loop responsible for the interaction with the *in vivo* electron donor Ferredoxin.[67] The binding niche for ferredoxin is formed by bulky, basic amino acid residues (K and R in Fig. 1) which are conserved in chlorophyta-type hydrogenases exclusively.

Bacterial-type [FeFe] hydrogenases are very similar to algal [FeFe] hydrogenases in terms of the H-domain primary structure. Variety exists for the accessory F-domain, which structurally differs in prokaryotic [FeFe] hydrogenases and is missing in chlorophyta-type hydrogenases. A relay of [Fe-S] clusters is associated with this domain. However, the amount of bound clusters varies from two (HydA of *M. elsdenii*) to four (*Cp*I).[50,68] Fig. 2 compares the cartoon model crystal structures of *Cp*I (1FEH) and *Dd*H (1HFE) with homology models of HydA of *M. elsdenii* and HydA1 of *C. reinhardtii*.

From Fig. 2, the functional bisection of *Cp*I is easy to see. The upper H-domain holds the H-cluster, the accessory F-domain exhibits three [4Fe-4S] clusters and one [2Fe-2S] compound ("F-clusters"). The overall shape resembles a mushroom.[50] In HydA of *M. elsdenii*, the F-domain is decreased in size. This bacterial [FeFe] hydrogenase holds only two [4Fe-4S] clusters besides the prosthetic group of the H-domain.[68] The periplasmatic *Dd*H differs in structure as the enzyme is a heterodimer and compromises two single-chain subunits, giving the overall molecular weight of approximately 60 kDa. The small 14 kDa chain is discussed to be relevant in translocation to the periplasmatic space.[59] Unlike the F-domain, this subunit does not contain any [Fe-S] clusters or respective binding motifs. However, next to the H-cluster, two [4Fe-4S] clusters are found with the 46 kDa subunit. HydA1 of *C. reinhardtii*, as a representative of chlorophyta-type hydrogenases, lacks the F-domain.[54,69] The putative binding niche of ferredoxin is marked in Fig. 2, as well as the insertion region discussed by Winkler and co-workers.[67]

Electronic structure of the H-cluster

The H-cluster is composed of a ferredoxin-type [4Fe-4S] cluster linked to a [2Fe-2S] moiety commonly known as "$[2Fe]_H$". Each iron atom of the $[2Fe]_H$ cluster is coordinated with one cyanide group (CN^-) and one or two carbon monoxide groups (CO).[59] In respect to the position of the [4Fe-4S] subcluster, the $[2Fe]_H$ iron

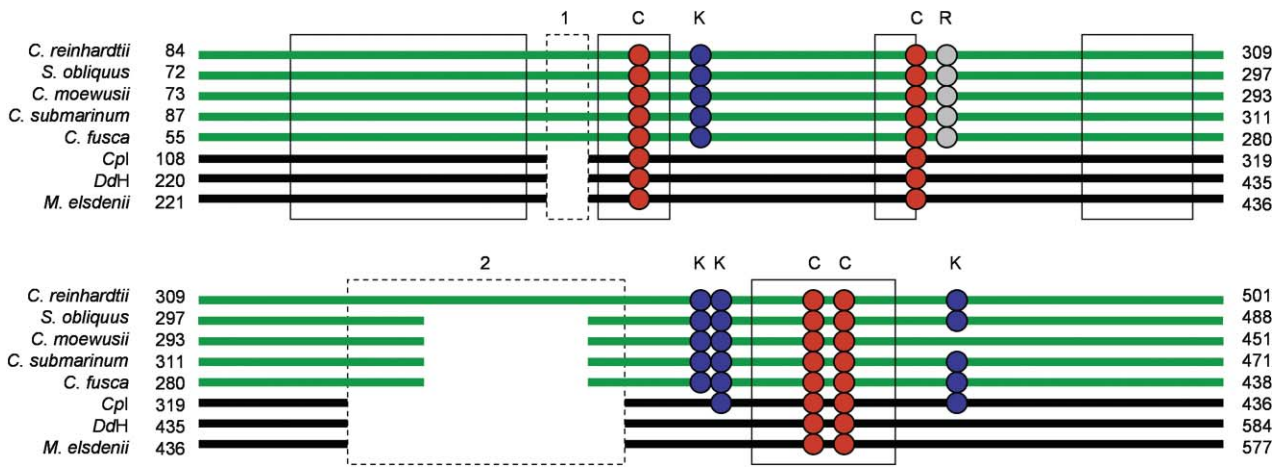

Fig. 1 Sequence alignment of the H-domain primary structure of homolog pro- and eukaryotic [FeFe] hydrogenases (black and green bars, respectively; scoring matrix BLOSUM 62). C- and N-terminal domains are trimmed for optimal fit of the alignment. Areas of high sequence similarity are marked by straight boxes. Dashed boxes show two "insertions" (1, 2) preserved in chlorophyta-type hydrogenases exclusively. According to homology models of HydA1 of *C. reinhardtii*, these sequences form a loop region replacing the F-domain of bacterial [FeFe] hydrogenases.[67] Cysteines coordinating the H-cluster (C, chestnut) are well-preserved in all [FeFe] hydrogenases. Residues K (lysine, blue) and R (arginine, grey) form a positively charged binding niche for the interaction with *in vivo* electron donor ferredoxin. This contact niche is chlorophyta-specific as well.

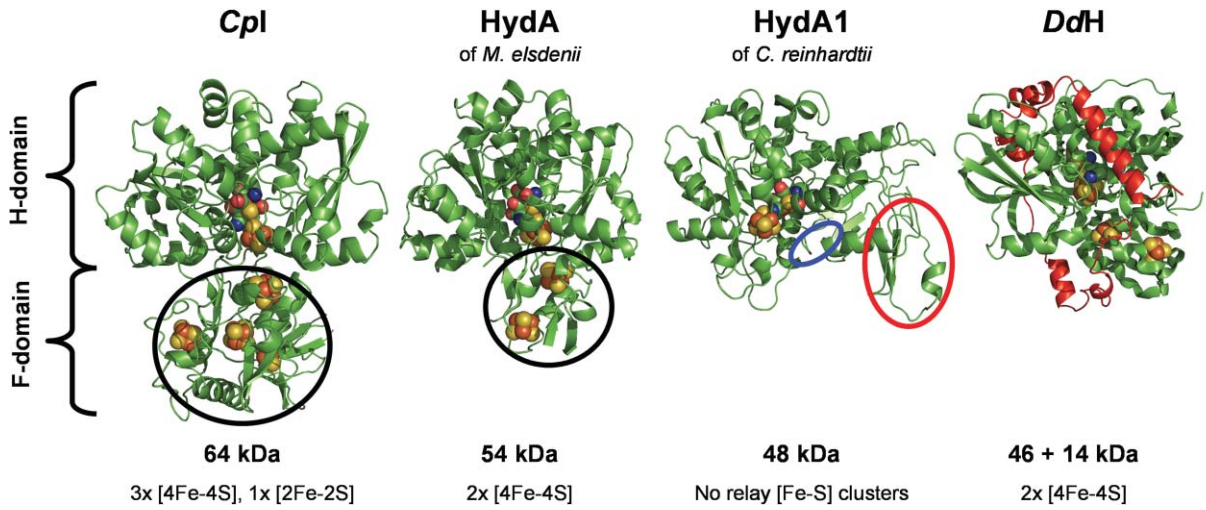

Fig. 2 Comparison of [FeFe] hydrogenases regarding the F-domain and overall structural differences. On the left site, *Cp*I is drawn as a cartoon model from the published structure.[50] Next to *Cp*I, only the structure of *Dd*H (far left) was resolved by X-ray crystallography,[59] HydA and HydA1 of *M. elsdenii* and *C. reinhardtii*, respectively, have been designed by homology modelling. The F-domain and relay clusters of *Cp*I and *M. elsdenii* HydA are marked black. For CpI, four "F-clusters" are annotated. HydA binds two [4Fe-4S] compounds. Instead of the F-domain, HydA1 exhibits an algal-specific "insertion" (red). A positively charged binding niche for interaction with ferredoxin is highlighted (blue).[67] *Dd*H is a heterodimer with a 14 kDa chain folded around the catalytic 46 kDa catalytic subunit like a belt (red cartoon). The large subunit exhibits two [4Fe-4S] clusters wiring the H-cluster to the protein surface.

atoms are labelled "proximal" and "distal". Catalysis is thought to take place at a free binding site of the distal iron atom.[50,59,70]

Different redox states have been described for the H-cluster. The oxidized, catalytically active "H_{ox}" state is paramagnetic and EPR-active. The distal iron atom of the $[2Fe]_H$ moiety Fe_d is less reduced than the proximal iron atom Fe_p, giving the characteristic $[4Fe\text{-}4S]^{2+}$–$Fe_p(I)Fe_d(II)$ assignment.[71] One CO is found in a bridging position as identified by its typical vibrational absorption around 1800 cm^{-1} (see below). H_{ox} can bind a molecule CO at the Fe_d binding site. The paramagnetic state "H_{ox}–CO" is annotated as $[4Fe\text{-}4S]^{2+}$–$Fe_p(I)Fe_d(II)$–CO.[70,72,73] Carbon monoxide is a potent inhibitor of [FeFe] hydrogenase activity.[70,74] Furthermore, all [FeFe] hydrogenases are sensitive to oxygen inactivation, and oxygen competes with CO for the same binding site.[25,75,76] In contrast to oxygen inactivation, inhibition by CO is largely, but not entirely, reversible.[61,70,72,77] Reduction of the distal iron atom gives "H_{red}". This diamagnetic state is assigned as $[4Fe\text{-}4S]^{2+}$–$Fe_p(I)Fe_d(I)$ or hybrido species $[4Fe\text{-}4S]^{2+}$–$Fe_p(II)Fe_d(II)$–H^-, alternatively, and not detectable by EPR spectroscopy.[62,78,79]

The [FeFe] hydrogenases of the *Desulvovibrio* genus *Dd*H and *Dv*H differ from typical bacterial and algal [FeFe] hydrogenases not only in structure but also regarding their insensitivity to oxygen prior a reductive activation treatment.[72,78,80] A novel state "H_{inact}" has been characterized for aerobically isolated *Dd*H and *Dv*H. In this state, the [FeFe] hydrogenases of the *Desulvovibrio*-type are catalytically inactive, EPR-silent and show a typical IR spectrum, including a CO ligand in a bridging position. The Fe_d-binding site is thought to be occupied by either OH^- or H_2O.[50,81] By means of a reductive treatment, H_{inact} is converted to the active form H_{ox} *via* a state "H_{trans}". This state is transient and slightly diamagnetic due to an one-electron reduction of the [4Fe-4S] cluster.[72] It has been characterized by EPR and Fourier-transform infrared (FTIR) spectroscopy. The states H_{inact} and H_{trans} are *not* defined for hydrogenases like HydA1 of *C. reinhardtii* or *Cp*I of *C. pasteurianum* which have to be isolated under strict anaerobic and reducing conditions. Here, hydrogenases (irreversibly) inactivated by oxygen are referred to as "H_{ox}air" to avoid confusion with H_{inact}. The active site composition and precise redox state of H_{ox}air remains a matter of speculation.[75]

Recently, three different algal [FeFe] hydrogenases have been examined by EPR spectroscopy. The hydrogenases from *C. reinhardtii*, *C. moewusii* and *C. submarinum* share similar g-tensors for H_{ox} and H_{ox}–CO.[54] The CO-inhibited form of *C. reinhardtii* HydA1, *e.g.*, shows the characteristic axial EPR signal with g-values of 2.052 and 2.007 (Table 2). Therefore, the electronic configuration of the H-cluster from these algal-type

Table 2 Typical g-tensors for different prokaryotic and chlorophyta-type [FeFe] hydrogenases as determined by EPR spectroscopy. The oxidized states H_{ox} and H_{ox}–CO are EPR-active due to $[4Fe\text{-}4S]^{2+}$–$Fe_p(I)Fe_d(II)$ and $[4Fe\text{-}4S]^{2+}$–$Fe_p(I)Fe_d(II)$–CO, respectively

Organism	H_{ox}	H_{ox}–CO	Reference
Clostridium acetobutylicum	n.d.	2.075, 2.009, 2.009	Von Abendroth 2008 (58)
Clostridium pasteurianum	n.d.	2.072, 2.006, 2.006	Bennet 2000 (117)
Desulfovibrio desulfuricans	2.100, 2.040, 1.999	2.065, 2.007, 2.001	Silakov 2007 (71)
Chlamydomonas reinhardtii	2.102, 2.040, 1.998	2.052, 2.007, 2.007	Kamp 2008 (54)
Chlamydomonas moewusii	2.103, 2.038, 1.998	2.052, 2.008, 2.008	Kamp 2008 (54)
Chlorococcum submarinum	2.100, 2.040, 1.998	2.056, 2.008, 2.008	Kamp 2008 (54)

[FeFe] hydrogenases seems to be similar. Also, it accordingly exhibits similarities to the active sites of the bacterial [FeFe] hydrogenases thus far examined. Still, distinct differences to prokaryotic hydrogenases suggest a slightly different electronic structure of the H-cluster in comparison to *Dd*H which has been characterized by EPR spectroscopy in greater detail before.[24,71,77] Table 2 summarizes the EPR characteristics for some relevant [FeFe] hydrogenases.

Configuration of the H-cluster

The diamagnetic H_{red} state is not accessible by EPR spectroscopy. However, using X-ray absorption spectroscopy (XAS) and FTIR spectroscopy it is possible to get a picture of the H-cluster independent of the redox state. XAS at the K-edge of iron in particular is possible only with chlorophyta-type [FeFe] hydrogenases. The signals from accessory [Fe-S] clusters hamper this iron-specific analysis in bacterial hydrogenases.

By extended X-ray absorption fine structure (EXAFS, a special XAS technique that takes in account the *extended* reach of the absorption edge), the coordination of the iron atoms in $[2Fe]_H$ and [4Fe-4S] cluster could have been distinguished for *C. reinhardtii* HydA1.[69] EXAFS on the H_{red} form of the H-cluster confirmed an overall geometry similar to that of bacterial hydrogenases. The H-cluster remains essentially unperturbed upon hydrogen gas treatment, but oxidation with CO (giving H_{ox}–CO) revealed an increased number of CO ligands at the $[2Fe]_H$ moiety and a ~0.1 Å elongation of the $Fe_p(I)$–$Fe_d(II)$ distance.[69] Although EXAFS can not directly detect electronic states, this elongation is easily attributable to a $Fe_p(I)$–$Fe_d(I)$ attraction in H_{red} lifted upon oxidation.[70] In bacterial [FeFe] hydrogenases, formation of a bridging CO between the $[2Fe]_H$ iron atoms was observed as a consequence of oxidative treatment.[25,70,71,81] The intensified attribution of CO ligands in H_{ox}–CO can be explained alike. Fig. 3 shows the EXAFS analysis in a structural model for the oxidized H_{ox}–CO H-cluster.

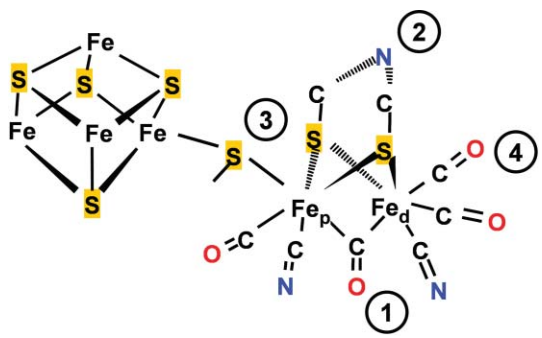

Fig. 3 Structure of the *C. reinhardtii* HydA1 H-cluster in its H_{ox}–CO state as derived from EXAFS. The distance of Fe_p and Fe_d is 2.62 Å, about 0.1 Å longer than reported for H_{red}.[69] One CO is found in a bridging position (1). The heteroatom in the dithiolate ligand (2) was *not* resolved by EXAFS. We follow a recent EPR study[116] and display the ligand as an azadithiolate bridge. A cysteine residue (3) binds the catalytic di-iron unit to the [4Fe-4S] cluster. In H_{ox}–CO, extrinsic CO (4) occupies the Fe_d binding site.

The CO and CN^- ligands of the H-cluster are uncommon in nature, due to the high reactivity of most notably CN^-. Maturation of [FeFe] hydrogenases and the *in vivo* formation of the H-cluster in particular is a field of active research.[57,82,83] Several publications report the *in situ* synthesis is H-cluster analogues, mimicking the unique ligand substitution.[84-87] However, infrared spectroscopy allows for the investigation on these specific groups and the actual situation of the H-cluster in consequence. With an absorption in the range of 2100 to 1800 cm^{-1}, the vibrational modes of CO and CN^- ligands can be analyzed by FTIR spectroscopy without interference from the protein backbone. The typical IR spectrum of the H-cluster can be subdivided into three main regions. Absorption from 2100 to 2050 cm^{-1} is attributable to CN^- stretching vibrations. Within 2050 to 1820 cm^{-1}, the different vibrational modes of the CO ligands absorb incoming IR radiance. This region is the most complex part of the spectrum. From 1820 to 1790 cm^{-1} approximately, the bridging CO can be detected.[72,73,88,89]

The [FeFe] hydrogenase HydA1 from *C. reinhardtii* was subject of a spectro-electrochemical analysis. The enzyme was investigated by FTIR spectroscopy in a "Moss cell".[90] By application of a certain voltage, the redox states of the enzyme can be adjusted without any additional chemical treatment. HydA1 was found to exhibit typical bands in the spectrum from 2100 to 1800 cm^{-1}. The H_{ox} state can be recognized from a prominent absorption band at 1940 cm^{-1} (stretch frequency Fe_d-CO) and a typical 1800 cm^{-1} peak due to the bridging CO stretch frequency.[72,91,92] Lowering the potential, bands at 1935 cm^{-1} and 1891 cm^{-1} (stretch frequency Fe_p-CO) emerge which are attributable to the H_{red} state.[90] Interestingly, a band around 1800 cm^{-1} is observed which might indicate that the CO bridge in HydA1 is not lifted upon reduction of the active site. This observation has important implications as it argues against a catalytic reaction mechanism that involves both $[2Fe]_H$ iron atoms and a bridging hydride.[93] Note that a bridged H_{red} state is in contradiction to what has been reasoned from EXAFS for HydA1 of *C. reinhardtii*.[69] Below −500 mV *vs* SHE, HydA1 was found to adopt a "super reduced" state, comparable to what Albracht *et al.* observed with the bacterial *Dd*H [FeFe] hydrogenase.[72] Potentials more positive than −100 mV gave H_{ox}–CO due to "cannibalization"–an effect indicative of protein degradation and subsequent release of CO which binds H-clusters still intact.[72,77]

Electrochemical analysis of HydA1 of *C. reinhardtii*

In the spectro-electrochemical studies on HydA1 of *C. reinhardtii*, voltage was applied to a solution of protein to adjust for the redox state of the H-cluster.[90] Protein film electrochemistry analyzes protein (mono-) layers in contact with a conductive surface.[23,94,95] Current is recorded as a function of the applied potential and is equivalent to the catalytic redox activity of the protein layer. At potentials more negative than the redox potential of the bound protein, electrons are driven from the (working) electrode to the enzyme, hence reducing immobilized enzyme. Potential values more positive result in an oxidation of the protein layer. Working electrodes are commonly made of gold, platinum and different kinds of graphite. Proteins usually prefer binding to graphite,[23,96] and metal surfaces need to be modified by mercapto-terminated hydrocarbon molecules to circumvent protein degradation and background current due to surface oxidation and absorbed hydrogen layers.[97,98] Modified noble metal electrodes present tailor-made binding surfaces and, in case of gold, provide

the possibility for concerted spectro-electrochemical analyses.[99,100] Graphite electrodes guarantee fast and rather unspecific binding.

Just recently, the [FeFe] hydrogenase HydA1 of *C. reinhardtii* was shown to be catalytically active immobilized on a modified gold electrode.[98] HydA1 was bound to a rough gold surface *via* two different carboxy-terminated self-assembled monolayers (SAM). Current and hydrogen evolution was recorded after immobilization of the hydrogenase and addition of methylviologen as electron shuttle. Whether the SAM was formed from mercaptopropionic acid (3C) or mercaptoundecanoic acid (11C), direct electron transfer (DET) from the electrode surface to the hydrogenase has *not* been observed.[98] By Surface Enhanced Infrared Spectroscopy (SEIRAS), binding kinetics were recorded, and *via* Surface Plasmon Resonance (SPR), the amount of bound protein could have been determined. SEIRAS is an IR spectroscopic technique which enhances the vibrational absorption of adsorbed molecules by more than two orders of magnitude.[101-103] This is due to plasmon excitation in metal surfaces (Au, Pt, Pd) by an incident electric field, an effect utilized in Raman spectroscopy as well. The novel set-up can serve as a device for electrochemical hydrogen production at defined specific activities. Furthermore, IR spectro-electrochemical investigations are possible which bring forth the advantage of full control of the protein layer redox activity *via* potential.

Armstrong *et al.* established protein film electrochemistry on pyrolytic graphite edge for many [FeFe] and [NiFe] hydrogenases.[23] However, immobilization of a chlorophyta-type hydrogenase has not been reported up to now. In recent studies, it was shown that HydA1 of *C. reinhardtii* directly exchanges electrons with the pyrolytic graphite edge electrode.[61,75] This is not trivial as electrons need to tunnel directly into the active site due to the missing [Fe-S] cluster wire in algal hydrogenases. For the first time, the bidirectional character of HydA1 was shown. Fig. 4 displays a cyclic voltammogram of the *C. reinhardtii* hydrogenase. At a given overpotential with regard to the redox potential in either the reduction or oxidation direction, the magnitude of the reduction current is similar to that of the oxidation current. The enzyme exhibits approximately similar activities in reduction (–500 mV *vs* SHE) and oxidation (–200 mV *vs* SHE) at pH 6.0. The infliction marked by the dashed oval reflects the bit of extra driving force necessary due to the lack of accessory clusters in HydA1. Recorded under an atmosphere of 100% hydrogen, it is interesting to note that proton-reducing (hydrogen evolution) activity was not hampered by product (hydrogen) inhibition. A process, commonly referred to as "anaerobic inactivation", occurs at potentials more positive than 0 mV. Current drops and is recovered on the back scan at appropriate rate.

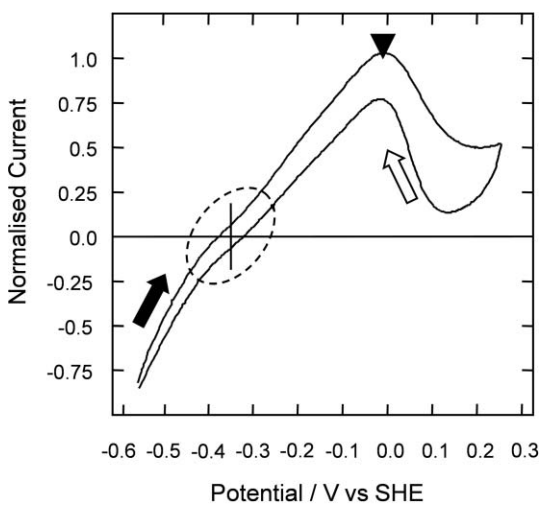

Fig. 4 Cyclic voltammogram of *C. reinhardtii* HydA1 immobilized on a pyrolytic graphite edge rotating disc electrode. At –500 mV *vs* SHE, reductive current is the same as oxidative current recorded at –200 mV. The midpoint redox potential is about –350 mV *vs* SHE. An area of inflection is marked by the dashed oval. At potentials more positive than 0 mV (▼), HydA1 loses activity due to anaerobic inactivation.[60] The arrows give direction of forward (solid) and backward scan (open). Experimental conditions: 100 mM KPi buffer pH 6.0, 20 °C, 1 bar H_2, electrode rotation rate 3000 rpm, scan rate 20 mV/s.

Inactivation at positive potential values is reversible to a different extent depending which enzyme is probed.[60] A new state "H_{ox}inact" is defined for [FeFe] hydrogenases under this conditions,[104,105] setting anaerobic inactivation apart from H_{inact} and H_{ox}air.[72,78,80]

Fully reversible inhibition of hydrogen oxidation by CO was shown for HydA1 of *C. reinhardtii*, alongside protection of the H-cluster by CO against oxygen damage. Surprisingly, reaction with oxygen was found to be ten times slower than that reported for the bacterial-type [FeFe] hydrogenase *Dd*H.[60,75] The irreversible oxygen inactivation of HydA1 is further slowed upon tenfold excess of hydrogen, due to competition for the active site. In summary, experimental evidence is demonstrated that hydrogen, oxygen and CO bind to the H-cluster at the same site. Presumably, this is the distal iron atom of the $[2Fe]_H$ moiety.[50,59,75] Note that a single binding site is not obvious for a [6Fe-6S] compound or di-iron reaction centre, at least.

From a recent EXAFS analysis, it was observed that the [4Fe-4S] part of the H-cluster is disrupted exclusively upon oxygen inactivation. The catalytically active $[2Fe]_H$ unit is initially left intact.[75] As CO is not known to bind to cubane clusters, it must bind to the $[2Fe]_H$ moiety. Thus, the electrochemical demonstration that CO protects the active site from oxygen indicates that oxygen does not *directly* attack the cubane cluster.[70,74,106] Taking these independent observations into account, two ideas of how oxygen inactivates the H-cluster present themselves. Oxygen is either reduced to a reactive oxygen species (*e.g.*, superoxide) or takes one electron from the [4Fe-4S] cluster *via* through-bond oxidation. Reactive oxygen might then be able to attack the cubane subcluster directly.[75,107] Both effects, however, result in oxidation of the [4Fe-4S] cluster and subsequent loss of iron. The Fe K-edge of oxygen-treated samples of *C. reinhardtii* HydA1 displayed a huge peak indicative of ferrous Fe^{2+}.[69] Oxidative disassembly of [Fe-S] clusters is a frequently observed phenomenon (see ref. 103). For the first time, oxygen inactivation was followed by protein film electrochemistry and EXAFS. Due to the relatively slow reaction with oxygen and the absence of any other [Fe-S] compounds than the H-cluster, HydA1 is the only [FeFe] hydrogenase suitable for the set of experiments chosen here.

The interaction of HydA1 with ferredoxin PetF of *C. reinhardtii*

In green algae, hydrogen production is light dependent and coupled to the photosynthetic transport chain *via* ferredoxin

PetF. Although six *fdx* genes were discovered in *C. reinhardtii*, only PetF ("Photosynthetic electron transfer Ferredoxin") is able to reduce the hydrogenase *in vitro*.[108] PetF is discussed to be the central branching point of reducing power in sulfur-deprived algae.[44,108,109] Thus, HydA1 and ferredoxin-NADPH-reductase, which both use ferredoxin as an electron donor, compete for electrons of the photosynthetic transport chain at the level of PetF. It has been shown that this competition determines the hydrogen evolution capacities of the algal cell.[14,110]

A recent study examines the interaction of *C. reinhardtii* proteins HydA1 and PetF with the help of site directed mutagenesis.[67] Several variants were specifically designed on the basis of predicted electrostatic surface distribution and prior *in silico* docking analyses and have been generated using the overexpression system described above.[55,58] Mapping the Michaelis-Menten kinetics of several variants of HydA1 and PetF *via* methylviologen and PetF reduction, a ferredoxin-specific effect was observed for especially two lysine residues. The electron surface potential of HydA1 was simulated to become more negative in these variants. In non-conservative variants, V_{max} is lowered to 60% and 10%, respectively, while hydrogen evolution activity is unchanged for methylviologen as electron donor. These analyses in combination with *in silico* docking studies show that electrostatic interactions between the lysine residues and the C-terminus of PetF play a major role in complex formation and electron transfer.[67] Mapping of significant *C. reinhardtii* HydA1 and PetF residues represents an important method for controlling the physiological photosynthetic electron flow in favour of light-driven hydrogen production.

Outlook

Green algae of the chlorophyta-type encode for [FeFe] hydrogenases smaller and more simple than those known from bacteria. While prokaryotic [FeFe] hydrogenases use a wire of two to four [Fe-S] clusters for translocation of electrons to the active site H-cluster, the algal hydrogenases lacks this accessory subdomain. Therefore, HydA1 of *C. reinhardtii* represents a "minimal catalyst for biological hydrogen production".[69] In this review, we have given a brief overview on the history of an interesting class of [Fe-S] enzymes, the [FeFe] hydrogenases of green algae. We report the most recent biophysical characterizations by electron spin resonance, Fourier-transform infrared spectroscopy, X-ray absorption spectroscopy and protein film electrochemistry. Furthermore, we summarize a work analyzing the specific HydA1–PetF interaction crucial in *C. reinhardtii* photobiological hydrogen production.

Many aspects of the algal hydrogen turnover are still unclear and deserve intensive research. In particular, protein biosynthesis and maturation of the H-cluster is a matter of debate. Organisms encoding for a [FeFe] hydrogenase need at least three maturation enzymes (HydE, HydF and HydG) that catalyze the ligation of the $[2Fe]_H$ moiety and translocation of the prosthetic group onto the hydrogenase apo-protein.

In *C. reinhardtii*, HydE and HydF form a single-chain protein complex.[57,82] HydF is thought to act as the central "scaffold" protein, a sort of construction site from where the H-cluster is transferred to the apoprotein, presumably by the help of HydF GTPase activity.[83,111] Open questions include the specific part of HydE, HydF and HydG in *in vivo* maturation as well as the origins of the CO and CN^- ligands. While there is some data suggesting the origin of the CN^- ligands in [NiFe] hydrogenases,[112,113] the precursors of the ligand groups in [FeFe] hydrogenases have not been identified yet.

The interest in exploitation of algal hydrogenases mainly results from their role in photobiological hydrogen production. Many studies report on the need to produce renewable "biohydrogen" by the use of sunlight and hydrogenases-catalyzed electrolysis.[31,39] One approach is to immobilize both PSI and PSII on electrically linked gold electrodes.[114] On the anodic site, PSII is bound to a special carbohydrate polymer which has been shown to work best for large protein complexes.[115] Water is split when the cell is illuminated, and electrons travel *via* the gold surface to the connected PSI electrode. In analogy to the photosynthetic electron transfer chain, electrons are excited by light at PSI a second and actively transferred from PSI to the hydrogenase.

In this "hydrogen battery", anode and cathode are separated in two gas-sealed cells. Charge exchange is ensured by electron coupling of PSII and PSI electrodes. Protons as the product of water oxidation and the substrate of hydrogen production are free to diffuse from the anode to cathode compartments. The photobiological hydrogen device produces current, oxygen and hydrogen upon illumination. This setup, on the one hand, allows for screening of the optimal components. Each enzyme module can be exchanged by a likely protein—in case of PSII, a stable D1 variant is of interest, *e.g.* from a thermophilic organisms.[114] On the other hand, the battery can directly serve as a fuel cell once all components have been optimized.

All together the new insights into the structural properties of the algal hydrogenases might be used to enhance the photosynthetic hydrogen production process in unicellular green algae and help unravel the molecular principals of hydrogen turnover.

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