Chlamydomonas: Hydrogenase and Hydrogen Production

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Abstract An important aspect of *Chlamydomonas reinhardtii's* metabolism is its ability to produce molecular hydrogen (H_2) from protons and electrons. Hydrogen production is catalysed by two [FeFe]-hydrogenases, HYDA1 and HYDA2, although HYDA1 is the main isoform, accounting for ~75% of the H_2 produced. Hydrogen production can be light dependent, with the hydrogenase receiving electrons from the photosynthetic electron transport chain via the ferredoxin PETF, or light independent, where H_2 is produced via fermentation in the dark. Hydrogen production was first reported in microalgae in the early 1940s; however, due to *HYDA* gene expression being induced by anaerobiosis and the extreme oxygen sensitivity of the enzyme, this process only occurred transiently at low levels when the algae were subjected to anaerobic or hypoxic conditions. It was thus considered nothing more than a biological curiosity until the early 2000s, when a method temporally separating oxygenic photosynthesis and H_2 production was developed, which allowed sustained H_2 production in the light over the course of a few days. Light-driven H_2 production has the highest theoretical photon conversion efficiency and is thus of considerable biotechnological interest. However, the calculated theoretical efficiencies are still not achievable in practice, despite the implementation of a wide range of engineering strategies. For an improved H_2 production, a better understanding of the underlying biology is needed. *C. reinhardtii* is the ideal organism in which to study H_2 production, due to the many molecular tools available and the simplicity and long history of study of its hydrogenase.

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1 Introduction

Rising global population growth and concomitant increases in demand for food, water and energy, as well as renewed efforts by governments to reduce carbon emissions, have all heightened interest in hydrogen (H_2) as a renewable fuel. The main attraction of H_2 is that the only by-product of its combustion is water, meaning that it can be carbon neutral or even carbon negative when produced renewably. Hydrogen also has an $\sim 3\times$ higher energy density compared to hydrocarbon fuels (Gupta et al. 2013). Currently, H_2 is produced via expensive energy-intensive fossil fuel-based methods such as steam reformation of natural gas, industrial oil and naphtha reforming, coal gasification and fossil fuel-driven water electrolysis (Gupta et al. 2013). However, it can also be produced under less energy-intensive conditions in cyanobacterial or microalgal systems, which use sunlight as their energy source (Oey et al. 2016).

To date, high bio-H_2 production efficiencies have been reported for eukaryotic microalgae. This is partly due to the high efficiency of the algal [FeFe]-hydrogenase, which is 100-fold higher than that of cyanobacterial hydrogenases, having a turnover rate of up to 10^4 H_2 molecules s^{-1} (Lubitz et al. 2014; Volgusheva et al. 2013). *Chlamydomonas reinhardtii* has emerged as the model organism for the study of photobiological H_2 production in green microalgae. This is likely due to the fact that the *C. reinhardtii* [FeFe]-hydrogenase HYDA1 was the first eukaryotic hydrogenase to be isolated (Roessler and Lien 1984; Happe and Naber 1993) but is also due to the large knowledge base and molecular toolset available for this organism. Since the initial purification of HYDA1, much progress has been made characterising the enzyme and elucidating its maturation, as well as in understanding the underlying regulatory pathways and improving H_2 production efficiencies. The structure, catalysis and oxygen (O_2) sensitivity of the enzyme have all been analysed, and many hydrogen mutants and higher H_2-producing algal strains have been created (see reviews Kruse et al. 2005; Ghirardi et al. 2007; Dubini and Ghirardi 2015; Antal et al. 2015; Torzillo et al. 2015; Oey et al. 2016). In this chapter, we review H_2 production in *C. reinhardtii* with a focus on HYDA1. We detail the induction, structure, maturation and catalytic mechanism of this enzyme and summarise strategies that have been used to improve microalgal H_2 production efficiencies.

2 Microalgal Hydrogen Metabolism

Microalgal H_2 production was first reported for *Scenedesmus* sp. in 1942 (Gaffron and Rubin) and later for *C. reinhardtii* and other species (Stuart and Gaffron 1972). Hydrogen evolution was observed at low levels under dark anaerobiosis and at higher levels in the light (Gaffron and Rubin 1942). Photosystem I (PSI) was identified as being essential for light-dependent H_2 production, whereas

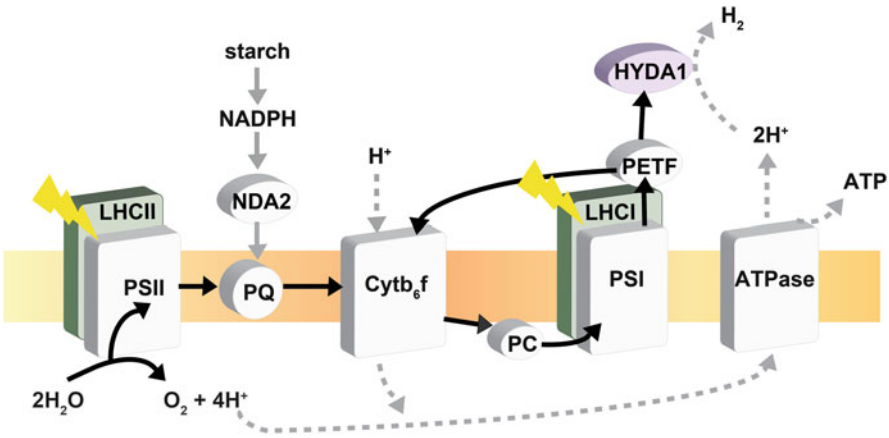

Fig. 1 Overview of the direct and indirect light-dependent hydrogen (H_2) production pathways. In the direct pathway, electrons are derived from the water-splitting reaction at photosystem II (PSII), while in the indirect pathway, electrons are derived from starch catabolism. Both pathways involve the capture of light, either by both light-harvesting complexes I and II (LHCI and LHCII, respectively) or by LHCI, and the transfer of electrons to the hydrogenase HYDA1 through the electron transport chain via the plastoquinone pool (PQ), cytochrome b_6f ($Cytb_6f$), plastocyanin (PC), PSI and the ferredoxin PETF. The indirect pathway additionally involves the NADPH-dehydrogenase NDA2

photosystem II (PSII), although a contributor of electrons, was found to be dispensable (Stuart and Gaffron 1972).

Three H_2 production pathways have since been identified in green microalgae: two that produce H_2 in the light and a third, dark fermentation pathway. The two light-dependent pathways (Fig. 1) include a direct pathway and an indirect pathway. In the direct pathway, HYDA1 receives electrons generated by the splitting of water at PSII via the photosynthetic electron transfer (PET) pathway. This involves the light-dependent excitation of electrons by PSI, the reduction of the ferredoxin PETF and the subsequent transfer of electrons from PETF to the hydrogenase (Melis and Happe 2001; Melis et al. 2000). In the indirect pathway, which is independent of PSII, electrons are also received via PETF but are derived from the catabolism of starch. In this pathway, the plastoquinone pool is reduced by NAD(P)H in a reaction mediated by the type II NADH dehydrogenase NDA2 (Desplats et al. 2009; Fouchard et al. 2005; Godde and Trebst 1980; Maione and Gibbs 1986; Chochois et al. 2009; Baltz et al. 2014; Jans et al. 2008; Mignolet et al. 2012; Mus et al. 2005). However, in this pathway, H_2 production is $\sim 10\times$ lower than in the direct pathway (Chochois et al. 2009). In the dark fermentation metabolism, electrons are also derived from the catabolism of starch but do not enter the PET pathway. Instead, it is thought that they are transferred to pyruvate, which can be oxidised by a pyruvate ferredoxin oxidoreductase and which can then reduce PETF in a process similar to H_2-producing fermentation pathways found in other microorganisms (Grossman et al. 2011; Atteia et al. 2013; van Lis et al. 2013; Noth et al. 2013).

It would appear slightly paradoxical that oxygenic green algae contain fermentative pathways such as the H_2 production pathway, which are more typical of strictly anaerobic microbes. However, green microalgae such as *C. reinhardtii* are in fact frequently exposed to anaerobic or hypoxic conditions and therefore need to be able to readily adapt their metabolisms (Mus et al. 2007; see chapter "Chlamydomonas: Anoxic Acclimation and Signaling" of Volume 1). Although the exact physiological role of H_2 production in microalgae remains unclear, it likely functions as a protection mechanism against the over-reduction of the chloroplast (Hemschemeier et al. 2008a). Under aerobic conditions, photosynthesis produces the carbohydrates that are required for respiration and cell growth. However, under anaerobiosis in the light, oxidative phosphorylation in the mitochondria is largely inhibited by a lack of O_2, leading to the over-reduction of the chloroplast and a reduced electron transfer, which ultimately results in photodamage and decreased levels of ATP. Under these conditions, the protons and electrons derived from water by the remaining PSII in the direct pathway or from starch in the indirect pathway can be fed to the hydrogenase via the PET chain, which recombines the protons and electrons to produce H_2. Therefore the hydrogenase is thought to act as a proton/electron release valve, removing excess protons and electrons by recombining them to produce H_2 gas, which can be easily released from the cells.

3 The *Chlamydomonas reinhardtii* Hydrogenase HYDA1

3.1 Hydrogenases

Three phylogenetically distinct classes of hydrogenases have been identified to date in H_2-consuming and H_2-evolving prokaryotes: [NiFe]-hydrogenases, which contain a nickel (Ni) and an iron (Fe) atom in their active site; [FeFe]-hydrogenases, which contain two Fe atoms; and [Fe]-hydrogenases, which have a mononuclear Fe active site but which are only found in a few methanogenic archaea (Vignais et al. 2001; Meyer 2007). Eukaryotic green microalgae have only been found to contain [FeFe]-hydrogenases, while cyanobacteria only contain [NiFe]-hydrogenases (Ludwig et al. 2006). However, [FeFe]-hydrogenases have also been found in eubacteria such as *Clostridium* sp. and *Desulfovibrio* sp., as well as in anaerobic protozoa such as *Trichomonas* sp. and *Nyctotherus* sp. (Vignais et al. 2001).

3.2 HYDA1 Gene

Two [FeFe]-hydrogenase genes have been identified in *C. reinhardtii: HYDA1* (Happe and Kaminski 2002) and *HYDA2* (Forestier et al. 2003). HYDA1 appears to be the primary isoform as it produces ~75% of the H_2 in the light (Meuser et al. 2012).

Both genes are nuclear encoded; however HYDA1 contains a transit peptide that facilitates its translocation to the chloroplast (Happe and Kaminski 2002). HYDA2 is also predicted to contain a chloroplast transit peptide and is thus also likely to function in the chloroplast (Forestier et al. 2003). Expression of *HYDA1* and *HYDA2*, as well as the hydrogenase specific maturase genes *HYDEF* and *HYDG*, is induced by anaerobiosis (Happe and Kaminski 2002; Happe and Naber 1993; Posewitz et al. 2004; Mus et al. 2007; Hemschemeier et al. 2013). *HYDA1* transcript abundance also increases after copper deprivation (Castruita et al. 2011), and the *HYDA1* promoter has been found to contain two GTAC motifs that are recognised by the *copper response regulator* 1 (CRR1) transcription factor (Pape et al. 2012), which regulates several genes under both copper deprivation and hypoxia/anaerobiosis (Kropat et al. 2005). In fact, anaerobiosis and copper deprivation appear to be linked, with many genes being expressed under both conditions (Castruita et al. 2011; see chapter "Chlamydomonas: Anoxic Acclimation and Signaling" of Volume 1).

3.3 HYDA1 Structure

The *C. reinhardtii* hydrogenase is a small (~48 kDa) monomeric soluble [FeFe]-hydrogenase located in the chloroplast (Happe and Naber 1993; Happe et al. 1994). Its structure consists of two lobes, each containing a beta-sheet surrounded by several alpha-helices, with the active site located between the lobes and interacting with amino acids from seven different protein stretches (Mulder et al. 2010) (Fig. 2b). The structure of HYDA1 and nearly all known [FeFe]-hydrogenases from microalgae features only this one domain, known as the H-domain, which is conserved in all [FeFe]-hydrogenases (Peters et al. 2015). Since additional N-terminal domains with further electron transporting FeS-clusters are present in all non-eukaryotic [FeFe]-hydrogenases, the relative simplicity of HYDA1 contributes to it being one of the model enzymes for hydrogenase research (Lubitz et al. 2014).

Protons can reach the active site of the hydrogenase from the surface by a phylogenetically conserved proton transfer pathway through the H-domain consisting of an arginine, two glutamates, a serine, a water molecule and a cysteine (Peters et al. 1998). Variations of these amino acids were shown to drastically alter both the catalytic competence and the pH optimum of bacterial [FeFe]-hydrogenases (Cornish et al. 2011; Morra et al. 2012). Channels for the diffusion of H_2 and O_2 through the protein towards the active site were discovered in the central domain of the bacterial [FeFe]-hydrogenase CPI from *Clostridium pasteurianum* by *in silico* approaches (Hong and Pachter 2012; Cohen et al. 2005). A complex [6Fe6S]-cluster, known as the H-cluster, forms the active site of [FeFe]-hydrogenases (Fig. 2a). It comprises a standard [4Fe4S]-cluster (the $4Fe_H$-subcluster) coordinated by four cysteine residues, which is linked to a unique [2Fe]-cluster (the $2Fe_H$-subcluster) through one of the cysteines (Peters et al. 1998, 2015; Nicolet et al. 1999). The iron atoms of this $2Fe_H$-subcluster are bridged by both thiolates of a singular aza-dithiolate (adt) ligand with further ligands being one CN^- and one CO molecule per iron, as well as another CO

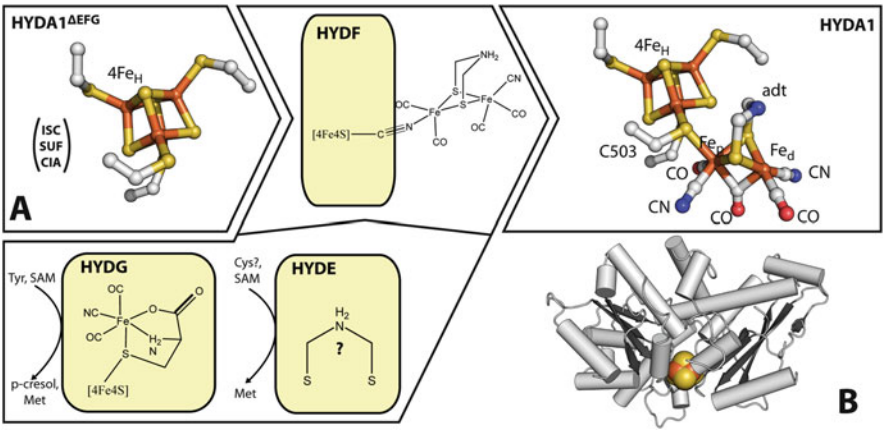

Fig. 2 Structure and maturation of the H-cluster. (**a**) HYDA1 expressed in a background devoid of specific maturases yields protein with the $4Fe_H$-subcluster only, which is supplied by the ISC, SUF or CIA systems. The maturases HYDG and HYDE synthesise parts of the $2Fe_H$-subcluster, which is probably assembled on HYDF. Transfer of the preassembled $2Fe_H$-subcluster from HYDF to HYDA1 completes the H-cluster. (**b**) Structure of HYDA1$^{\Delta EFG}$ in cartoon representation with the beta-sheets in *dark grey* and the $4Fe_H$-subcluster as spheres in *yellow* (S) and *orange* (Fe), respectively (PDB ID 3lx4) (Mulder et al. 2011)

in a bridged coordination between the two Fe atoms (Fig. 2a). While a number of amino acids are well positioned to form stabilising hydrogen bonds with the CN^- ligands in an otherwise hydrophobic active site pocket, the thiolate bond to the cysteine linking the two subsites is the only covalent connection between the $2Fe_H$-subcluster and the protein (Peters et al. 1998) (Fig. 4). In the case of the iron atom located proximal to the $4Fe_H$-subcluster (Fe_p), the linking cysteine is the sixth ligand in an octahedral coordination geometry, while the more distal iron (Fe_d), although restrained by the surrounding amino acids to the same octahedral geometry, is missing one of the axial ligands (Peters et al. 2015). This open coordination site and the amine group of the adt ligand pending above it have been identified as the key reasons for the striking difference in activity between the H-cluster within the protein and chemical compounds of similar composition (Berggren et al. 2013; Simmons et al. 2014; Esselborn et al. 2016).

3.4 Enzyme Maturation

The assembly of the H-cluster of [FeFe]-hydrogenases takes place in two stages. The $4Fe_H$-subcluster is assembled first by the standard FeS-cluster assembly machinery (Broderick et al. 2014), and then the $2Fe_H$-subcluster is added by the specific maturation enzymes HYDEF and HYDG (Posewitz et al. 2004) (Fig. 2). Recently, it became evident that the source of both CO and CN^- is tyrosine, which is

split by radical SAM chemistry in HYDG on a [4Fe4S]-cluster (Peters et al. 2015). The products are then assembled on the other end of a beta-barrel in HYDG to an Fe $(CO)_2(CN)$-cysteine-cluster at a second [4Fe4S]-cluster, turning it transiently into a [5Fe5S]-cluster (Suess et al. 2016; Pagnier et al. 2016). HYDE (in most organisms HYDE and HYDF are separate genes) also relies on radical SAM chemistry and is believed to contribute the aza-dithiolate ligand, as its substrate was shown to be a small sulphur compound (Betz et al. 2015). The product of HYDE appears to come together with two of the mono-iron clusters from HYDG onto HYDF, which serves as a scaffold for the complete [2Fe]-cluster. The interaction between the three maturases is not yet fully understood, but most likely involves GTP hydrolysis, as HYDF has been reported to have GTPase functionality (Peters et al. 2015). Once the [2Fe]-cluster is assembled on HYDF linked to a [4Fe4S]-cluster, the transfer of the [2Fe]-subcluster into the hydrogenase and assembly of the complete H-cluster takes place without additional energy input. However, it requires the $4Fe_H$-subcluster to be present within the hydrogenase (Shepard et al. 2010).

Importantly for hydrogenase research, the specific maturation machinery can be bypassed, as synthetic [2Fe]-clusters can be incorporated spontaneously into [FeFe]-hydrogenases in vitro once the $4Fe_H$-subcluster is formed either by the standard machinery in vivo or by reconstitution in vitro (Berggren et al. 2013; Esselborn et al. 2013). This yields semi-synthetic enzymes, which are indiscernible from their completely in vivo maturated counterparts.

Although much progress has been made using recombinant proteins in in vitro assays (Peters et al. 2015), neither the exact cellular mechanism, nor the cellular compartment in which the incorporation of either subcluster occurs, is known. In eukaryotic microalgae, proteins involved in the assembly of proteins with standard FeS-clusters are located in all cellular compartments, i.e. in the cytosol, plastid stroma and mitochondrial matrix (Balk and Schaedler 2014). Each plant cell compartment is able to synthesise FeS-clusters, but the machineries employed differ: the mitochondria and cytosol contain the FeS-cluster (ISC) and cytosolic FeS-protein assembly (CIA) systems, respectively, while the chloroplast utilises the sulphur mobilisation (SUF)-like machinery (Balk and Schaedler 2014). However, it is likely that the chloroplast-localised HYDA1 enzyme is assembled in the plastid compartment, as current knowledge indicates that only unfolded proteins are transported across the chloroplast outer membranes (Paila et al. 2015). Also, both HYDEF and HYDG contain putative chloroplast transit peptides, and HYDG was detected in the chloroplast in a proteomics study (Terashima et al. 2010), suggesting that at least the addition of the [2Fe]-cluster occurs in the chloroplast.

3.5 HYDA1 Catalysis

A comparative electrochemical analysis of representative [FeFe]- and [NiFe]-hydrogenases concluded that the catalytic competence of the H-cluster is inherently superior to the one from the [NiFe]-cofactor (Hexter et al. 2012). Mössbauer

spectroscopy and pulsed ENDOR/HYSCORE experiments with ^{57}Fe-enriched [FeFe]-hydrogenase showed that both subsites of the H-cluster are electronically coupled and show strong exchange interactions. Substrate binding (H^+/H_2) occurs at the open coordination site at the distal Fe centre (Fe_d) of $2Fe_H$ while being in the most oxidised "active ready" state denoted as H_{ox} (Fig. 3,1). The catalytic cycle, which either oxidatively degrades H_2 to H^+ or reductively converts two protons into H_2, presumably comprises three further catalytic main states (Fig. 3,1–4) while successively taking up or releasing two protons and electrons. The exact sequence of electron and proton transfer steps occurring during catalysis is unknown and has only been speculated to follow an ECEC mechanism in which ***e***lectron- and ***c***hemical proton-transfer steps alternate. However, electron paramagnetic

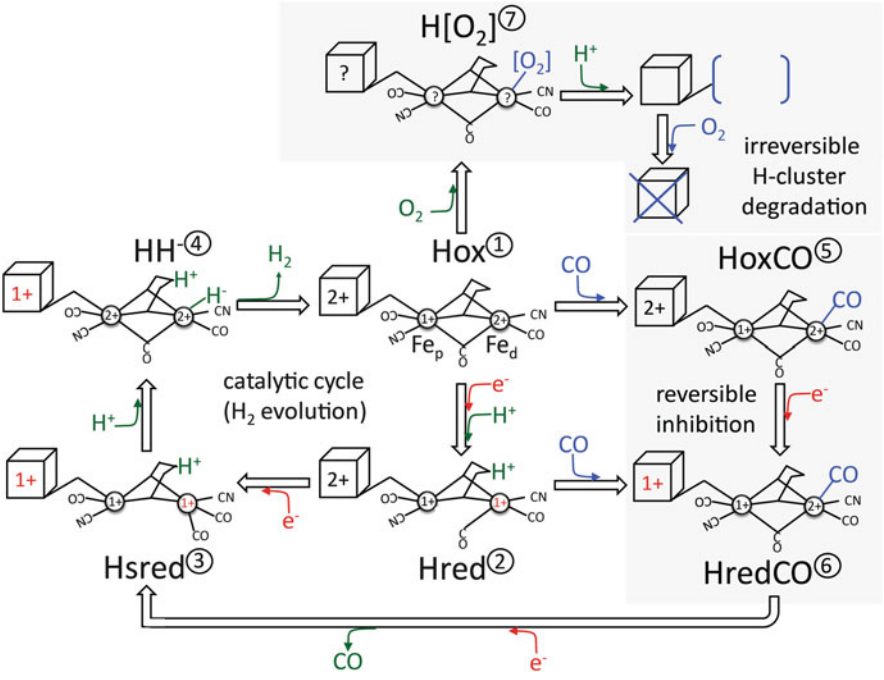

Fig. 3 Catalytic cycle of hydrogen (H_2) evolution and inhibitors of [FeFe]-hydrogenases. **1–4** Schematic working model of the reversible catalytic cycle of H_2 evolution at the H-cluster. Local redox states are indicated in *black* and *red numbers* (transiently lowered redox state). Proton transfer steps are presented according to the favoured ECEC mechanism. The *dotted line* indicates a transition of μCO from the bridging coordination to a terminally bound state. **5–6** Reversible inhibition of the two H-cluster states H_{ox} and H_{red} by CO binding to the open coordination site. Reductive reactivation is achieved via $H_{red}CO$ resulting in H_{sred} after another reduction step. **7** The irreversible process of oxygen (O_2)-induced H-cluster degradation starts with the binding of O_2 to the substrate coordination site, yielding a transient O_2 adduct which presumably requires a protonation step to induce the degradative process. Although the sequence of steps during H-cluster degradation is still under debate, recent experimental data support an initial loss of the $2Fe_H$-site before the oxidative degradation of the remaining $4Fe_H$-cluster occurs.

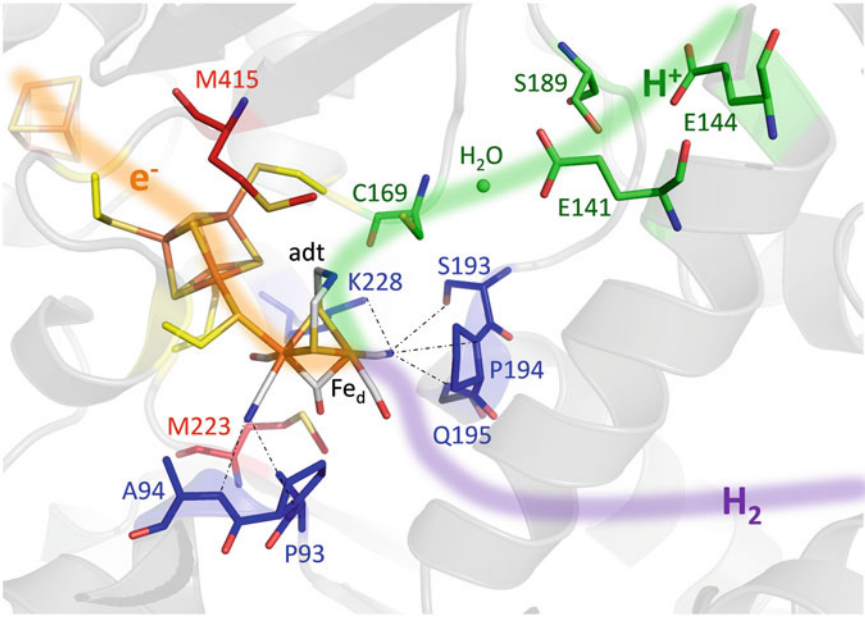

Fig. 4 First and second ligand sphere of the H-cluster in the active site of [FeFe]-hydrogenases. The model was generated using the CPI [FeFe]-hydrogenase holoprotein crystal structure (PDB ID 3C8Y) and depicts the H-cluster, as well as the most important polypeptide positions which provide the necessary environment for catalytic function (stick structures). The labelling corresponds to the homologous positions in HYDA1. Substrate/product pathways for electrons, protons and hydrogen (H_2), leading to or coming from the site of catalytic turnover at Fe_d, are indicated by an *orange*, *green* or *purple glow*, respectively. Amino acid positions contributing to the H-bond networks that stabilise the CN^- ligands are depicted in *blue*; those involved in proton transfer are presented in *green*, while the two methionine residues which allow catalytically relevant ligand movements are shown in *red*.

resonance (EPR) spectroscopy and Fourier transform infrared (FTIR) spectroscopy have enabled the detailed characterisation of the redox and spin state properties for states 1–3 (H_{ox}, H_{red} and H_{sred}). Monitoring H_2 evolution, the first reduction step occurs at the $2Fe_H$-site, thus transforming the paramagnetic H_{ox} state ($4Fe_H$(II)-Fe(I)-Fe(II)) into the EPR silent H_{red} state ($4Fe_H$(II)-Fe(I)-Fe(I)). The second reduction step is restricted to the $4Fe_H$-site, yielding the super-reduced $4Fe_H$(I)-Fe(I)-Fe(I) state (H_{sred}), which accumulates under redox potentials below −500 mV. While H_{sred} has not been found as an active state for the bacterial [FeFe]-hydrogenase DDH and is rather assumed to be an instable artificial product of over-reduction (Roseboom et al. 2006), this state has been demonstrated to be stable and catalytically relevant for the small M1-type enzymes of green algae (Adamska-Venkatesh et al. 2014; Adamska et al. 2012). The final state carrying the hydride intermediate of the heterosynthetic H_2-evolution mechanism (Fig. 3,4) has been presupposed but not yet experimentally verified for any wild-type [FeFe]-hydrogenase. However,

for the virtually inactive HYDA1 variant C169S, which displays a severely compromised proton transfer efficiency, two slightly deviating unknown states have been reported which might correspond to the protonated and unprotonated derivative of the hydride state (HH^-) (Mulder et al. 2014).

The two types of diatomic ligands at the $2Fe_H$-site (CO and CN^-) participate in adjusting its redox chemistry and spin distribution and further provide individual qualities to the H-cluster which are essential for catalytic competence (Winkler et al. 2013).

The CN^- ligands, which are coordinated in a trans orientation to each other, are embedded in strong H-bond networks on either side of the cofactor (Fig. 4, dotted lines), thereby stabilising the orientation and hexacoordinated configuration of the two Fe-sites in the $2Fe_H$-cluster (Winkler et al. 2013).

The latter aspect ensures a terminal coordination of the catalytically generated hydrido species (Fig. 3,4), which would otherwise swap to a kinetically stabilised and thus disadvantageous bridging coordination between the two Fe centres (Winkler et al. 2013). As they do not support H-bond interactions to the environment, CO ligands exhibit a higher degree of configurational flexibility, which is of significance for the role of the third CO ligand (μCO). In the oxidised state, μCO is located in a bridging configuration between both Fe sites (Fig. 3,1), while in the more reduced states (H_{red}; H_{sred}), it stabilises the higher electron density at Fe_d by switching to a terminally bound coordination (Adamska et al. 2012) (Fig. 3,2–3).

The close protein environment of the $2Fe_H$-site provides a second ligand sphere which further participates in the fine-tuning of catalytic features. Apart from the above-mentioned role of the H-bond networks in coordinating the two CN^- ligands, the second ligand sphere provides an efficient coupling to the proton transfer pathway (Fig. 4, green) and with precisely positioned methionine residues (Fig. 4, red), provides a suitable environment for structurally guided and reversible movements of individual ligands such as the proton-shuttling aza-dithiolate ligand or μCO (Knörzer et al. 2012).

Like the active sites of many enzymes, the ligand binding site on Fe_d can also be occupied by small inhibitor molecules such as formaldehyde (FA) or carbon monoxide (CO). While formaldehyde preferably binds to H_{sred} (Bachmeier et al. 2015), CO only interacts with H_{ox} or H_{red}, yielding the catalytically inactive states $H_{ox}CO$ or $H_{red}CO$ (Fig. 3,5–6), which can be reductively reactivated to yield H_{sred} (Fig. 3,3) (Adamska-Venkatesh et al. 2014).

3.6 *Oxygen Sensitivity*

The reversible inhibitory effect of CO and the irreversibly destructive influence of O_2 on the activity of algal hydrogenases were first described for whole cell extracts of *C. reinhardtii* in the late 1970s. It was further demonstrated that the destructive influence of O_2 is antagonised by pre-exposing the cell extract to CO (Erbes et al. 1979). The protective effect of CO gassing prior to O_2 exposure was later

systematically examined by protein film voltammetry, where *C. reinhardtii* HYDA1 was compared to different bacterial-type [FeFe]-hydrogenases (Goldet et al. 2009; Stripp et al. 2009). In contrast to [NiFe]-hydrogenases, all known [FeFe]-hydrogenases exhibit extraordinary high levels of O_2 sensitivity; however different enzyme subtypes show different nuances of sensitivity resulting from differing accessibilities of the gas channel system for O_2 and slight differences in the binding affinities of Fe_d for O_2. The authors further concluded that CO competitively occupies the initial binding site of O_2, thus preventing the following destructive process from being initiated.

Freeze drying renders [FeFe]-hydrogenases surprisingly insensitive towards O_2 (Noth et al. 2015). X-ray absorption spectroscopy demonstrates that exposure of lyophilized [FeFe]-hydrogenase to O_2 results in a stable O_2 adduct which might correspond to a trapped version of the otherwise highly transient initial reaction product between Fe_d and O_2. As proton transfer activity of hydrogenases has been demonstrated to be severely hampered in the freeze dried state, it can be speculated that protonation of the first O_2 adduct is essential for initiating the destructive downstream processes that lead to the irreversible loss of both H-cluster components (Fig. 3,7) (Noth et al. 2015).

While the formation of an initial O_2 adduct is uniformly accepted, the following sequence of events leading to the irreversible degradation of both H-cluster components is still under debate. XAS spectroscopy suggests the production of a reactive oxygen species which detaches from Fe_d and attacks the $4Fe_H$-site before a loss of the $2Fe_H$-cluster can be demonstrated (Stripp et al. 2009; Lambertz et al. 2011). A more recent examination instead favours a loss of the $2Fe_H$-cluster prior to the oxidative degradation of the $4Fe_H$-cluster (Fig. 3,7). The latter model is strongly supported by the fact that a large fraction of the O_2-inactivated enzyme can be reactivated by supplying synthetic [2Fe]-cofactor as a substitute for the lost H-cluster component (Swanson et al. 2015).

4 Photobiological H_2 Production in Practice

As O_2 gradually accumulates during photosynthesis, photobiological H_2 production requires the balancing of oxygenic photosynthesis and cell growth with anaerobic H_2 production. The standard approach for this involves first growing the algae aerobically, to allow for carbon fixation, cell growth and the build-up of carbohydrates, before inducing anaerobiosis and therefore H_2 production via nutrient deprivation. The most common induction method is sulphur deprivation (Melis et al. 2000). Sulphur deprivation results in anaerobiosis due to a reduction in the rate of repair of the PSII reaction centre protein, D1, as a result of a limitation in the sulphur-containing amino acids methionine and cysteine, which are required for D1 protein synthesis. This reduces the level of O_2 production below respiration, resulting in anaerobiosis and *HYDA1* gene expression. Sulphur deprivation also

results in the build-up of starch (Zhang et al. 2002), which is subsequently degraded during H_2 production (Hemschemeier et al. 2008a).

Anaerobiosis can also be achieved through the deprivation of other nutrients. This is possible as the limitation of any macronutrient results in a number of general nutrient deficiency responses in *C. reinhardtii*, many of which are conducive for H_2 production, such as the accumulation of starch and a reduction in photosynthetic O_2 evolution (Ball et al. 1990; Gonzalez-Ballester et al. 2015; Grossman et al. 2010; Zhang et al. 2002; Wykoff et al. 1998). The deprivation of nitrogen (Philipps et al. 2012), phosphorous (Batyrova et al. 2012) and magnesium (Volgusheva et al. 2015) have all been shown to induce H_2 production. Nitrogen and phosphorous deprivation resulted in lower levels of H_2 production compared to sulphur deprivation. In contrast, magnesium deprivation resulted in higher levels of H_2 production (Volgusheva et al. 2015). This was thought to be due to a prolonged H_2 production period of over 7 days, an increased respiration and starch accumulation and a smaller decrease in functional PSII [PSII was only reduced by 20% instead of 80% as in sulphur deprivation (Volgusheva et al. 2013)], resulting in a higher electron availability to the hydrogenase (Volgusheva et al. 2015).

5 Targets for Improved Microalgal H_2 Production

The major problem with nutrient deprivation is that H_2 production cannot be sustained for more than a few days. This is because the algae eventually die due to the lack of the respective nutrient. Therefore, the prolongation of photobiological H_2 production via other strategies has been the focus of much research. Significantly higher H_2 production efficiencies are required in order for biological H_2 production to be commercially viable. Microalgae systems currently only have photon conversion efficiencies of 3%, while the theoretical maximum is ~12–14% (Scoma et al. 2012; Volgusheva et al. 2013). The highest efficiency strains will therefore require a combination of strategies and involve significant re-engineering of the H_2 production process. The ultimate goal is continuous H_2 production where O_2 production and consumption are in balance and the water-splitting reaction is active at the same time as the hydrogenase.

However, there are a few major factors currently limiting sustained H_2 production. These include proton and electron supply to the hydrogenase and the extreme oxygen sensitivity of the hydrogenase enzyme. Factors limiting the growth of the algae and photosynthetic efficiency, such as light capture, also limit H_2 production. In attempts to overcome these bottlenecks, extensive genetic engineering has been performed. A number of high H_2-producing mutants with improvements in one or more of these bottlenecks have been generated and are detailed below. A tabular summary of the various mutants and their H_2 production efficiencies can also be found in the recent review by Dubini and Ghirardi (2015).

5.1 Electron Supply to the Hydrogenase

Electron flow to the hydrogenase is one of the main bottlenecks for sustainable H_2 production. This is due to the large number of other pathways competing for electrons from PETF (Winkler et al. 2011). For example, ferredoxin-$NADP^+$ reductase (FNR), sulphite reductase, nitrate reductase, glutamate synthase and fatty acid desaturases all compete with HYDA1 for electrons (Hemschemeier and Happe 2011). To improve electron flow to the hydrogenase, PETF, FNR and the hydrogenase itself have all been engineered (Long et al. 2009; Yacoby et al. 2011; Wittenberg et al. 2013; Lubner et al. 2011; Rumpel et al. 2014; Sun et al. 2013). For example, a PETF variant with a reduced affinity for FNR has been developed (Rumpel et al. 2014), and PETF and PSI have both been fused to the hydrogenase (Yacoby et al. 2011; Lubner et al. 2011). However, all of the above work has so far only been performed in vitro and remains to be tested in vivo.

Engineering has also focused on various indirect targets. For example, small and large subunit Rubisco mutants (Pinto et al. 2013; Hemschemeier et al. 2008b), cyclic electron flow (CEF) mutants (Kruse et al. 2005; Johnson et al. 2014; Steinbeck et al. 2015; Tolleter et al. 2011), starch degradation mutant strains (Chochois et al. 2009) and respiration mutants (Ruehle et al. 2008) have all been reported to display increased H_2 production levels. In fact, the cyclic electron flow mutants *state transition 6 (Stm6)* (Kruse et al. 2005) and the *proton gradient regulation like 1 (pgrl1)* (Tolleter et al. 2011) and *pgr5* mutants (Steinbeck et al. 2015) have been reported to have the highest H_2 production rates, producing between 540 and 840 mL of H_2 per litre of culture, which is $\sim$10$\times$ that produced by wild-type *C. reinhardtii*. Interestingly, these high H_2-producing mutants all display similar phenotypes: they have an enhanced oxygen consumption capacity and thus increased PSII stability and electron supply to the hydrogenase. The mutant cultures also become anaerobic earlier than the wild type following sulphur deprivation, which is thought to result in an increase in PSII stability due to a reduction in photo-oxidative damage (Steinbeck et al. 2015; Volgusheva et al. 2013). It is important to note that *Stm6* also has increased starch reserves compared to the wild type and thus more available substrate (Kruse et al. 2005).

The effect of adding extra components to the photosynthetic electron transfer pathway has also been tested. The expression of plastid-expressed NAD(P)H dehydrogenase (Baltz et al. 2014) and native and exogenous hydrogenases (Reifschneider-Wegner et al. 2014; Chien et al. 2012) has all been reported.

5.2 Proton Supply to the Hydrogenase

Proton supply to the hydrogenase is another bottleneck for H_2 production. This is because the ATP requirement drops during H_2 production (Das et al. 2014), resulting in a reduced electron transport at cytochrome b_6f (Burgess et al. 2011;

Antal et al. 2009) and an impaired dissipation of the proton gradient and therefore decreased proton availability for the hydrogenase. One strategy to improve H_2 production is to artificially dissipate the proton gradient to increase H_2 production transiently in the presence of the chemical uncoupler carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP), which causes an efflux of protons from the thylakoid lumen into the stroma (Lee 2013; Kruse et al. 2005; Lee and Greenbaum 2003). This suggests that the integration of a proton channel into the thylakoid membrane could more permanently restore proton and electron flow to the hydrogenase. Such a proton channel however would need to be inducibly expressed, as the addition of the uncoupler prior to anaerobiosis was found to abolish hydrogenase activity, suggesting that the proton gradient is important for initial hydrogenase expression (Lee 2013) and aerobic growth. A similar strategy involves the development of a leaky ATPase to increase proton flow and reduce ATP production (Das et al. 2014; Robertson et al. 1990). Reduced ATP production caused by the introduction of a proton channel or mutated ATPase may additionally reduce reactions competing for reducing equivalents and therefore increase electron supply to the hydrogenase (Kumar and Das 2013).

5.3 Oxygen Sensitivity of the Hydrogenase

Sustained H_2 production under standard growth conditions remains a major challenge. The O_2 sensitivity of the hydrogenase is a multifaceted problem due to the fact that O_2 not only inhibits the activity of the hydrogenase enzyme, but also transcription and protein maturation (Cohen et al. 2005). However, to produce H_2 from water using photosynthesis, O_2 will be released; hence O_2 production (photosynthesis) needs to be balanced with O_2 consumption (respiration). Approaches have focused on developing O_2-tolerant hydrogenases or changing the balance between O_2 production and O_2 consumption. As detailed in Sect. 5.1, the highest reported H_2-producing mutants all display increased respiration rates and reach anaerobiosis earlier than the wild type following sulphur deprivation (Kruse et al. 2005; Steinbeck et al. 2015; Schonfeld et al. 2004), thus obtaining the correct balance between oxygen evolution and oxygen consumption appears to be of vital importance for further improvements in H_2 production efficiencies.

Several genetic engineering approaches have been utilised to reduce the O_2 sensitivity of the hydrogenase (reviewed in Ghirardi 2015), including random mutagenesis (Flynn et al. 2002) and targeted mutagenesis of the catalytic site to restrict O_2 access (Stiebritz and Reiher 2012). While engineering approaches have been successful for a bacterial [NiFe]-hydrogenase (Dementin et al. 2009), there has so far been no success for the microalgal [FeFe]-hydrogenase. However, two algae strains were recently reported to be able to express the hydrogenase in the presence of more than 21% O_2 and to produce low levels of H_2 at 15% atmospheric O_2 (Hwang et al. 2014). One problem with an O_2-tolerant hydrogenase however, is

that H_2 would be produced alongside O_2, which would make gas separation difficult and potentially lead to a dangerous gas mixture.

Balancing O_2 production and consumption is perhaps a better, more feasible approach and has already been demonstrated in a number of studies. Photosystem II itself has been the target of engineering. For example, mutant strains with downregulated PSII subunits (Surzycki et al. 2007; Lin et al. 2013) and a mutant containing amino acid substitutions in the D1 protein (Scoma et al. 2012; Torzillo et al. 2009) all displayed increased H_2 production efficiencies. The repression of *psbD* translation (Surzycki et al. 2007) and the downregulation of *psbO* in *Chlorella* sp. *DT* (Lin et al. 2013) both resulted in a lower O_2 evolution. A double amino acid substitution in the D1 protein resulted in an increase in the quantum yield of photosynthesis, a higher respiration rate, a higher carbohydrate accumulation following sulphur deprivation and a higher synthesis of xanthophyll cycle pigments, which was proposed to result in an improved photoprotection (Torzillo et al. 2009, 2015). A mutant with an inactivated Calvin-Benson cycle also displayed a higher respiration rate and higher H_2 evolution compared to the sulphur-deprived wild type (Ruehle et al. 2008).

Alternative approaches have also been tested to control the balance between oxygen evolution and consumption. Leghemoglobins, which are able to sequester O_2, have been expressed in *C. reinhardtii* (Wu et al. 2010, 2011), as has a pyruvate oxidase from *E. coli*, which was found to lower O_2 evolution (Xu et al. 2011). A sulphate permease mutant was also developed, which allowed a greater control over sulphur deprivation (Chen et al. 2005). Another approach targeting controlled sulphur deprivation implemented sulphur microdosing (Kosourov et al. 2005), whereby the sulphur in a sulphur-deprived culture was replaced in small amounts, allowing the repair of PSII and renewed production of protons and electrons to drive H_2 production. Finally, cocultivation of algae and bacteria was reported to result in algal anaerobiosis due to an increased bacterial respiration (Lakatos et al. 2014).

5.4 Indirect Targets

Hydrogen production can be improved further by optimising the general culture conditions. For example, light capture can be optimised so that the photosystems function to their maximal capacities. The LHC antennae systems function to capture photons and dissipate excess light energy to provide photoprotection (Takahashi et al. 2006; Niyogi 1999; Pascal et al. 2005; Oey et al. 2013). Biomass production efficiency, at least in the laboratory, can be improved by reducing LHC antenna size, as this enhances light distribution through the bioreactors and enables the use of increased operational cell concentrations, resulting in improved overall photosynthetic efficiencies (Melis et al. 1999; Mussgnug et al. 2007; Polle et al. 2003; Oey et al. 2013; Beckmann et al. 2009). A number of *C. reinhardtii* antenna mutants have been developed, including *Stm6Glc4T7*, a mutant expressing a permanently active LHC translational repressor NAB1, which resulted in a 10–17%

reduction in LHC antenna size and a ~50% increase in photosynthetic efficiency (Beckmann et al. 2009); *tla1 (truncated light-harvesting Chl antenna)*, an insertional mutant with a reduced LHCI and LHCII antenna complex (50% and 65% of the wild type, respectively) (Polle et al. 2003; Kosourov et al. 2011); and *LO1*, an RNAi knockdown of LHCBM1, 2 and 3 (21%, 81% and 41% expression of *LHCBM1*, *2* and *3*, respectively) (Oey et al. 2013).

6 Conclusion

Microalgal H_2 production is not only a fascinating biological phenomenon but is also of enormous biotechnological significance, as it offers a carbon neutral or even carbon negative approach for renewable energy production. In the more than 20 years since HYDA1 was isolated, remarkable progress has been made in elucidating this protein's structure, catalytic mechanism, induction and regulation. This in vitro groundwork has provided us with a detailed understanding of the enzyme and has paved the way for its in vivo engineering. Considerable work has also been carried out at the physiological level, and a large number of mutants displaying enhanced H_2 productions have now been created. To date, the mutants with the highest reported H_2 production rates have been the CEF mutants, which share the phenotype of an increased oxygen consumption, earlier onset of anaerobiosis and more stable PSII in common. However, further improvements are needed if microalgal H_2 production is to become a commercial reality, particularly in regards to limitations in electron transport, competition for reductants from ferredoxin and the O_2 sensitivity of the enzyme. The most successful approach is likely the combination of a number of different strategies. Solar conversion efficiency also needs to be improved. However, unresolved questions regarding, for example, the underlying regulation of HYDA1 in algae, need to be understood before such improvements can be made. Despite this, we are in a good position to solve these questions, and hopefully a H_2 economy is no longer a distant dream but a future reality.

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