[FeFe]-hydrogenases as biocatalysts in bio-hydrogen production

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Abstract (150-250)

[FeFe]-hydrogenases catalyse H_2 production at exceptionally high turnover numbers (up to 10^4 s^{-1}). They are found in a variety of strict or facultative anaerobic microorganisms, such as bacteria of the genus *Clostridium*, *Desulfovibrio*, *Thermotoga*, and eukaryotes ranging from unicellular and coenobial green algae to anaerobic fungi, ciliates and trichomonads. Key to their activity is an organometallic centre, the H-cluster, that cooperates tightly with the protein framework to reduce two protons into molecular hydrogen. The assembly of the catalytic site requires a specialized cellular mechanism based on the action of three other enzymes called maturases: HydE, HydF and HydG. Recent advancements in the recombinant production of [FeFe]-hydrogenases have provided leaps forward in their exploitation in H_2 production for clean energy storage. [FeFe]-hydrogenases have been used in several fermentative approaches where microorganisms are engineered to overexpress specific [FeFe]-hydrogenases in order to convert low cost materials (*eg* wastes) into H_2. [FeFe]-hydrogenases have also been proven to be excellent catalysts in different *in vitro* devices that can produce hydrogen directly from water, either *via* water electrolysis or *via* light-driven mechanisms, thus allowing the direct storage of solar energy into H_2.

1. Introduction

The issues raised by the massive use of fossil fuels (*i.e.* pollution, climate changes, resource depletion) are promoting the research of new renewable energy technologies (Luque et al., 2008, Jacobson, 2009). The use of hydrogen as an energy carrier would be of great interest because of its high energy content per mass unit, the absence of toxic byproducts and the possibility to be used in highly efficient fuel cells (Dunn 2002, Schultz et al., 2003). Besides the energy sector, hydrogen is an important intermediate in essential industrial processes, such as synthesis of ammonia for the production of fertilizers, various hydrogenation reactions including the petrochemical industry, the food industry and biofuels and methanol production (Ramachandran and Menon, 1998).

Unfortunately, the H_2 production technologies available today still rely mainly on fossil fuels. For this reason, the study of alternative production methods is considered of paramount importance (Holladay et al., 2009, Christopher and Dimitrios, 2012). The production of hydrogen with biological technologies (bio-hydrogen) is of high interest because it is completely renewable and it can be performed under mild ambient conditions, converting water and/or organic waste materials into a valuable fuel (Levin et al., 2004, Kapdan and Kargi, 2006).

Numerous research efforts are focused on [FeFe]-hydrogenases, the enzymes responsible for physiological H_2 production in many microorganisms ranging from the strict anaerobe *Clostridium pasteurianum* to the sulfate-reducer *Desulfovibrio desulfuricans*, to the unicellular green alga *Chlamydomons reinhardtii* to say a few (Vignais and Billoud, 2007). This work will review the catalytic and structural features of these enzymes and the most recent advancements on the recombinant production and protein engineering; the attention will be focused on the exploitation of [FeFe]-hydrogenases for bio-hydrogen production both in microorganisms and bio-hybrid devices.

2. Modular structure, catalytic site and mechanism of [FeFe]-hydrogenases

Hydrogenases are redox enzymes classified on the basis on the metal organization in the catalytic site within three phylogenetically distinct classes: [FeFe]-, [NiFe]- and [Fe]-hydrogenases (Vignais and Billoud, 2007). [FeFe]-hydrogenases are found in many anaerobic prokaryotes, such as clostridia and sulfate reducers, as well as various eukaryotes, such as green algae, anaerobic fungi, trichomonads and ciliates (Vignais and Billoud, 2007).

[FeFe]-hydrogenases are able to catalyze the reversible reaction $2H^+ + 2e^- \leftrightarrow H_2$ in both directions, namely H_2 evolution (direct) and H_2 uptake (reverse) (Fontecilla-Camps et al., 2007, Vignais and

Billoud, 2007). Usually they are physiologically involved in H_2 evolution, and are intrinsically biased towards this direction (Goldet et al., 2009, Hexter et al., 2012). They can use different physiological redox partners such as ferredoxins, flavodoxins and pyridinic cofactors that connect the enzyme activity of [FeFe]-hydrogenases to the energy metabolism of the cell (Demuez et al., 2007, Winkler et al., 2009, Guerrini et al., 2008)

The typical turnover numbers for this class of enzymes are very high, being in the range of 1000-10,000 per second (Woolerton et al., 2012, Peters et al., 2015).

Soluble [FeFe]-hydrogenases are the best characterized members of this class of enzymes, but some periplasmic and membrane bound were also reported (Vignais and Billoud, 2007, Calusinska et al., 2010). They are usually monomeric, though dimeric, trimeric and tetrameric forms also exist. In all cases these enzymes' structure is modular: they are composed by the catalytic domain (H-domain) containing the active site H-cluster, and by some other accessory domains hosting other cofactors, such as iron sulfur centres or NAD(P)H-binding domains. The accessory domains are involved in mediating electron transfer between the redox partners and the H-domain (Meyer 2007, Vignais and Billoud, 2007, Calusinska et al., 2010, Winkler et al., 2013, Peters et al., 2015).

Three conserved signature sequences or motifs have been identified in the H-domain,: L1 (TSC**C**PxW), L2 (MP**C**xxKxxE) and L3 (ExMA**C**xxG**C**xxG) (Meyer 2007, Calusinska et al., 2010). They contain the four cysteines that directly coordinate the active site H-cluster (highlighted in bold).

Only 3 [FeFe]-hydrogenases 3D structures have been solved by x-ray crystallography:

- *Clostridium pasteurianum* CpI. PDB ID: 1FEH (Peters et al., 1998), 1C4A, 1C4C (Lemon and Peters, 1999), 3C8Y (Pandey et al., 2008), 4XDD, 4XDC, 5BYR, 5BYQ and 5BYS (Esselborn et al., 2015).
- *Desulfovibrio desulfuricans* DdH. PDB ID: 1HFE (Nicolet et al., 1999).
- *Chlamydomonas reinhardtii* HydA1. PDB ID: 3LX4 (Mulder et al., 2010) and 4ROV (Swanson et al., 2015).

The catalytic site of [FeFe]-hydrogenases is a unusual organometallic center named "H-cluster" (fig. 1). It is composed of two subclusters: a cubane [4Fe4S] coordinated by the four protein cysteines and bridged to a [2Fe] subcluster via one of these cysteines (Peters et al., 1998, Nicolet et al., 1999). The [2Fe] subcluster is composed by two iron atoms, a proximal Fe_p and a distal Fe_d, coordinated by non-protein ligands that are two terminal CO, two terminal CN, a bridging CO (Pandey et al., 2008) and bridged by an organic ligand, that was recently identified as a di(thiomethyl)amine (Fontecilla-Camps et al., 2007, Vignais and Billoud 2007, Silakov et al., 2009, Berggren et al., 2013, Esselborn et al., 2015). The H-cluster must be considered as an electronically inseparable [6Fe] cluster, due to extensive delocalization of frontier molecular orbitals (Schwab 2006).

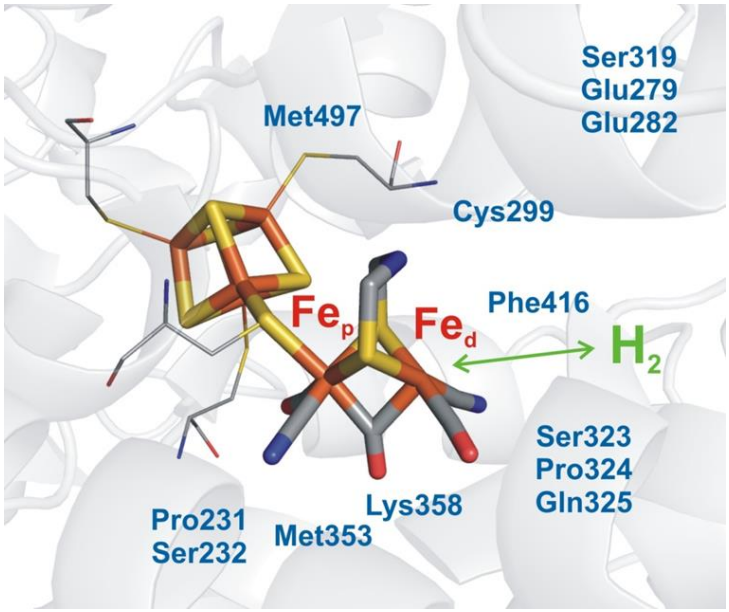

Fig. 1 Structure of the catalytic site of [FeFe]-hydrogenases. The H-cluster is a complex organometallic center hosted in a conserved protein pocket. The H-cluster is represented as thick sticks (Fe = orange, S = yellow, C = grey, N = blue, O = red); four cysteines coordinate the cluster to the protein (represented as thin lines); other residues that are essential for the structure/function of the site are indicated with the position numbers of CpI.

On the basis of the available X-ray crystal structures (Peters et al., 1998, Nicolet et al., 1999) and several spectroscopic studies (De Lacey et al., 2007, Stripp et al., 2009a, Fontecilla-Camps et al., 2009, Lubitz et al., 2014) the H-cluster structure is considered to be essentially the same in all [FeFe]-hydrogenases.

Recently, it is becoming clear that the catalytic features of [FeFe]-hydrogenases are due to the fine interplays occurring between the H-cluster and the protein environment that hosts it (Fig. 1): a hydrophobic pocket, composed by highly conserved residues (Peters et al., 1998, Winkler et al., 2013).

Site directed mutagenesis was used to study the putative role in proton transfer of a chain of conserved residues in CpI (a cysteine, two glutamate and a serine) (Cornish et al., 2011). The mutation of four other conserved residues in CrHydA1 and CpI (two methionines, a lysine and a cysteine) resulted in impairment or loss of activity, demonstrating their importance for H-cluster coordination and functionality (Knörzer et al., 2012). Mutagenesis of a conserved phenylalanine residue in CrHydA1 showed that it is needed to regulate the reversibility of anaerobic inactivation (Fourmond et al., 2014). A site saturation mutagenesis study has shown that replacement of C298 in CaHydA with any other residue strongly affects the enzyme activity, with the only exception of C298D; considering that this mutant showed also a shift in the pH activity profile, these results demonstrated that this cysteine is the key residue in the process of proton transfer to the H-cluster during catalysis (Morra et al., 2012). More detailed spectroscopic studies by EPR and FTIR on this mutant (Morra et al., 2016a) and on a serine mutant at the homologous position in CrHydA1 (Mulder et al., 2014) have further confirmed a relevant role in proton-coupled electron transfer to the H-cluster. The replacement of cysteine with serine, a non-ionisable residue, led to impairment in the proton transfer and severely altered the catalysis by affecting the H-cluster reactivity; on the contrary replacement with aspartic acid, an ionisable residue, sustained catalytic activity at high rates because the proton transfer kinetics are influenced very little and the H-cluster properties are essentially unaffected.

The general framework that is emerging from various mutagenesis studies is that it is important to consider the H-cluster and its protein environment as a dynamic inseparable system that synergistically cooperates for an efficient and fast catalytic mechanism.

H_2 evolution implies several steps: a) intermolecular electron transfer from a low potential electron partner to the accessory FeS clusters; b) intramolecular electron transfer from the accessory FeS clusters to the H-cluster; c) proton transfer from the surface to the active site, through ionisable residues and structural water molecules; d) formation of diatomic hydrogen at the active site; e) diffusion of H_2 out of the protein (Peters 1999, De Lacey et al., 2007, Fontecilla-Camps et al., 2009). H_2 uptake follows the same pathway in the opposite direction.

The reaction site is the distal Fe_d atom of the H-cluster (Peters 1999, De Lacey et al., 2007); the observation that the competitive inhibitor CO binds in this position (Lemon and Peters, 1999) further confirms this hypothesis.

In the very proximity of the active site, the nitrogen atom of the di(thiomethyl)amine bridge coordinating the [2Fe] subcluster plays a key role in proton transfer during the catalytic mechanism (Peters et al., 1998, Cornish et al., 2011, Morra et al., 2012, Morra et al., 2016a).

A number of different redox states of the H-cluster in [FeFe]-hydrogenases were identified by EPR, FTIR and Mössbauer spectroscopy (De Lacey et al., 2007, Lubitz et al., 2007). H_{inact} (also named

H_{ox}^{air}) is an inactive but oxygen-stable state observed only in the hydrogenases from *Desulfovibrio vulgaris* and *D. desulfuricans*, characterized by a diamagnetic $[4Fe4S]^{2+}$ subcluster and a diamagnetic Fe(II)–Fe(II). It can be irreversibly converted in the active H_{ox} form by anaerobic reduction, through the intermediate H_{trans}. The H_{ox} state is characterized by a diamagnetic $[4Fe–4S]^{2+}$ subcluster and a paramagnetic Fe(I)–Fe(II). The H_{red} state is obtained by one electron reduction of H_{ox}, and it is characterized by a diamagnetic $[4Fe4S]^{2+}$ subcluster and a diamagnetic Fe(I)–Fe(I). At lower potentials, the super-reduced state H_{sred} is formed, characterized by a paramagnetic $[4Fe4S]^{1+}$ subcluster and a diamagnetic Fe(I)–Fe(I). The oxidized H-cluster can reversibly bind CO at the Fe_d atom, resulting in the H_{ox}-CO state; only recently, the $H_{'red'}$-CO state has been observed in reduced CrHydA1 bound to CO (Adamska-Venkatesh et al., 2014). The H_{ox}, H_{red}, H_{sred} and H_{ox}-CO states have been described in various [FeFe]-hydrogenases (De Lacey et al., 2007, Lubitz et al., 2014).

The catalytic mechanism was shown to be based on the heterolytic splitting of H_2 (and vice versa) in H^+ and a hydride intermediate that is terminally bound to Fe_d (Vignais and Billoud, 2007;, Lubitz et al., 2007, Mulder et al., 2013, Winkler et al., 2013). Generally, the catalytic cycle is thought to involve H_{ox} and H_{red} but, given the very fast reaction kinetics, the exact involvement of each redox intermediate in the catalytic cycle is not completely clarified; in particular, the exact physiological role of the H_{sred} state is under debate (Adamska et al., 2012, Mulder et al., 2013, Hajj et al., 2014).

[FeFe]-hydrogenases are very sensitive to molecular oxygen, most of them being irreversibly and quickly inactivated by this molecule. Since oxygen sensitivity is a serious limit to their biotechnological use, many studies have been conducted to identify the molecular basis of this feature and how it can eventually be limited.

O_2 inactivation is a complex mechanism, not completely clarified. There is general agreement that it is a multistep process, occurring on different timescales, that starts from O_2 binding at the Fe_d and subsequently producing reactive oxygen species (ROS) that will destroy the [2Fe] subcluster and eventually the [4Fe4S] subcluster of the H-cluster (Goldet et al., 2009, Stripp et al., 2009b, Lambertz et al., 2011, Swanson et al., 2015, Orain et al., 2015). Moreover, very different O_2 inactivation rates are known in different [FeFe]-hydrogenases and this variability has not yet been clarified in details.

3. Maturation, recombinant production and protein engineering

Since the active site of [FeFe]-hydrogenases is a complex organometallic cluster that is not present in any other protein, it requires to be assembled by a specific cellular machinery. This process is a so-

called maturation process that involves at least three maturases: HydE, HydF and HydG (Posewitz et al., 2004, Nicolet and Fontecilla-Camps 2012, Peters et al., 2015).

The maturation process that forms the functional H-cluster consists of two phases: in the first one, the [4Fe4S] subcluster is assembled by the iron sulphur clusters assembly mechanism (ISC); subsequently, the [2Fe] subcluster is assembled by the HydE, HydF and HydG machinery (Nicolet and Fontecilla-Camps 2012, Peters et al., 2015).

HydG is a radical S-adenosyl-methionine (SAM) enzyme, hosting two [4Fe4S] centers, that is responsible for the synthesis of the CO and CN ligands of the H-cluster using tyrosine as a substrate, deriving the two ligands from the COOH and NH_2 groups of the free aminoacid, respectively (Kuchenreuther et al., 2011, Kuchenreuther et al., 2014, Pagnier et al., 2016).

Also HydE is a radical SAM enzyme, but so far it has not been characterized in details. It is essential for the maturation and it has been putatively assigned to a role in the biosynthesis of the di(thiomethyl)amine ligand of the H-cluster (Nicolet et al., 2008, Betz et al., 2015).

HydF has GTPase activity and binds a [4Fe4S] center; it is considered as the scaffold protein that hosts the assembly of the H-cluster, before the final delivery to the functional enzyme. HydF was demonstrated to possess a cluster with CO and CN ligands when coexpressed with HydE and HydG and to be able on its own to activate the hydrogenase (McGlynn et al., 2008, Shepard et al., 2010, Mulder et al., 2010, Czech et al., 2010, Cendron et al., 2011, Nicolet and Fontecilla-Camps 2012, Berto et al., 2012, Albertini et al., 2015).

The discovery of [FeFe]-hydrogenase maturation proteins paved the way for recombinant expression, allowing large scale production of [FeFe]-hydrogenases, that can be exploited for biophysical characterization and biotechnological applications.

The recombinant systems that have been developed are either cell-hosted or cell-free (Tab. 1).

System	**Enzyme**	**Host**	**Maturases**	**Specific activity[1]**	**Yield[2]**	**Ref.**
Cell-hosted	CaHydA	*E. coli*	Ca	75.2	NR	King et al., 2006
		C. acetobutylicum	endog.	1750*	0.8	von Abendroth et al., 2008

		E. coli	Ca	1880±108	1.2	Morra et al., 2015a
	CrHydA1	*E. coli*	Ca	150	0.8-1.0	King et al., 2006
		C. acetobutylicum	endog.	625*	1	von Abendroth et al., 2008
		S. oneidensis	endog.	740±56	0.5	Sybirna et al., 2008
		E. coli	So	641±88	30±11	Kuchenreuther et al., 2010
	Fd-CrHydA1	*E. coli*	Ca	1000	5	Yacoby et al., 2012
	CpI	*E. coli*	So	1087±146	7.9±0.8	Kuchenreuther et al., 2010
Cell-free	CpI	-	-	2037±616	NR	Esselborn et al., 2013
		-	So	~700**	NR	Kuchenreuther et al., 2012
	CrHydA1	-	Ca	700-800	NR	Berggren et al., 2013
	CsHydA	-	Ca	~2.5	NR	McGlynn et al., 2007

Table 1 Recombinant expression systems for [FeFe]-hydrogenases. 1) µmol H_2 mg^{-1} min^{-1}. 2) mg pure protein per litre of culture. Ca) *Clostridium acetobutylicum*. Cr) *Chlamydomonas reinhardtii*. So) *Shewanella oneidensis*. Cs) *Clostridium saccharobutylicum*. endog.) endogenous maturases. NR) not reported. Without other specification, specific activity is reported as H_2 evolution rate. *) V_{max}. **) H_2 oxidation rate.

Three different hosts have been reported for cell-hosted systems: *Escherichia coli* (King et al., 2006, Kuchenreuther et al., 2010, Morra et al., 2015a), *Clostridium acetobutylicum* (von Abendroth et al., 2008) and *Shewanella oneidensis* (Sybirna et al., 2008).

The recombinant expression in *Clostridium acetobutylicum* and in *Shewanella oneidensis* exploits the endogenous maturation system of the host, but usually results in low purification yield. The first attempts to express recombinant active [FeFe]-hydrogenases in *E. coli* were unsuccessful (Voordouw et al., 1987), because this microorganism does not possess endogenous [FeFe]-hydrogenases and their maturation systems. Subsequently, co-expression with the three maturation proteins HydE, HydF and HydG from *Clostridium acetobutylicum* allowed successful expression of various active [FeFe]-hydrogenases. Importantly, this system demonstrated for the first time that it was possible the heterologous maturation of [FeFe]-hydrogenases with maturases from a different organism, suggesting similar assembly mechanism.

The cell-free systems consist of protocols based on the insertion *in vitro* of the [2Fe] subcluster of the H-cluster into an apo-[FeFe]-hydrogenase: in some cases the maturases are added (McGlynn et al., 2007, Kuchenreuther et al., 2012), while in others the cluster is inserted as a chemically synthesized precursor (Berggren et al., 2013, Esselborn et al., 2013, Esselborn et al., 2015).

The possibility to express active [FeFe]-hydrogenases *in vitro* or in recombinant systems also allowed protein engineering of these enzymes. There are several reports on the exploitation by protein engineering means to generate novel artificial variants of [FeFe]-hydrogenases with improved properties.

A fusion protein consisting of CrHydA1 and the PetF ferredoxin has been reported to be able to compete with ferredoxin-NADPH reductase (FNR) and to directly intercept low potential electrons from photosystem I. The redirection of the electron flow resulted in an improved rate of hydrogen photoproduction in isolated plant and algal thylacoids (Yacoby et al., 2011).

The engineering of the putative tunnels that connect the protein surface with the active site was often suggested as a target to limit O_2 inactivation (Cohen et al., 2005, Fontecilla-Camps et al., 2007). Interestingly, even if this approach was effective in [NiFe]-hydrogenases (Liebgott et al., 2010), it was recently demonstrated to be ineffective in [FeFe]-hydrogenases (Lautier et al., 2011). A rational approach using site directed mutagenesis (SDM) allowed to change seven residues in CaHydA where they were putatively involved in the gas tunnel but this demonstrated that increasing the size of the aminoacids do not influence oxygen sensitivity (Lautier et al., 2011).

The use of purely random engineering approaches has been reported in a limited number of works. Gene shuffling was applied to generate a random library from the combination of *C. acetobutylicum* and *C. saccharobutylicum hydA* genes; a clone from this shuffled library showed increased activity,

suggesting that it is possible to improve the catalytic properties of [FeFe]-hydrogenases by mixing enzymes from different sources (Nagy et al., 2007). Despite the encouraging results, unfortunately there is no report in the literature of a follow up of this approach.

More recently, error prone PCR (epPCR) was used to generate random libraries that were then screened with high throughput methods, allowing the identification of a variant of CrHydA1 with higher specific activity (Stapleton and Swartz, 2010) and variants of CpI with decreased oxygen sensitivity (Bingham et al., 2012), that might be very useful for bio-hydrogen production. In the case of improved oxygen stability, it is important to note that, out of the 3 mutations identified, only one was found in the putative gas diffusion tunnel and it does not involve the insertion of a bulky residue, but a fairly conservative substitution of alanine with valine. The other two mutations, including the most influential, are located close to the [4Fe4S] cluster of the ferredoxin-like accessory domain, supporting the hypothesis that O_2 damage involves also other iron sulphur clusters in the protein (Bingham et al., 2012).

4. Bio-hydrogen production

The commercial value in the application of hydrogenases in cell-based, or *in vitro* enzyme-based systems, in combination with photosensitive hybrid materials for the sustainable production of bio-hydrogen cannot be underestimated. When these approaches are combined with the exploitation of waste material and solar energy to drive the reaction the impact on the bioeconomy is even more important.

The hydrogenase reaction can also be combined to a reversible exchange of electrons for the production of other reduced compounds of higher added value, namely NADH and NADPH, that in turn can be used to sustain the biosynthesis of highly stereo- and regio-specific chemicals.

In this respect there is a broad range of strategies that have been tackled in the last years in order to provide the scientific and technological bases for such exploitation.

4.1 Overexpression in microorganisms

The in-cell systems includes few examples of homologous and heterologous overexpression of [FeFe]-hydrogenases in order to enhance hydrogen production from microbial cultures. Heterologous over expression is limited by the availability of the maturation system (as previously described). The homologous overexpression of hydrogenase genes has been performed in *C. tyrobutyricum* (Jo et al, 2010), *C. acetobutylicum* (Von Abendroth et al, 2008) and *C. paraputrificum* (Morimoto et al, 2005). The increase in the yields of hydrogen production starting from glucose are in the range of 1.5 to 1.7 folds, consistent with the overexpression level and activity of the hydrogenase. However, this is still

too limited to suggest that hydrogenase overexpression could overwhelm the limitations imposed by cell metabolism. The overexpression in *C. tyrobutyricum* granted a productivity of 8.67 μmol/mg cell/min (Jo et al, 2010). Nonetheless, the maximum theoretical yield of hydrogen production under these conditions is 4 mol H_2 per mol of glucose. Therefore alternative strategies have been proposed for *in vitro* hydrogen production. The reduction equivalents of NADPH can be exploited *in vitro* entirely for hydrogen production, while *in vivo* the cell metabolism is competing. This includes the *in vivo* processes based on photosynthetic organisms (algae and bacteria) that have also been targeted by *in vivo* metabolic engineering strategies for improving hydrogen production. In this latter cases a yield higher than 4 mol H_2 per mol of glucose can be reached, due to the supplementary energy supplied by light. However, the balance between the photosynthetic activity and the hydrogen production is regulated by a competition between the exploitation of reduced NAD(P)H for sugar synthesis and for hydrogen synthesis. In addition the water splitting activity of photosystem II produces oxygen that is inhibiting the [FeFe]-hydrogenase activity, thus lowering the yields in hydrogen production.

4.2 Hydrogenases coupled to NAD(P)H/glucose *in vitro* and related exploitation for NAD(P)H regeneration

The *in vitro* system proposed by Swartz and co-workers at Stanford (Smith et al., 2012) is based on a NAD(P)H regeneration system previously designed by Adams and co-workers (Zhang et al, 2007) *via* G6P exploitation and already applied to produce hydrogen with [NiFe]-hydrogenases. The system can exploit glucose and xylose sources from ligno-cellulosic material. Reported data of over 90% (11.6 mol of H_2 per mole of glucose) of the theoretical conversion 1 to 12 glucose to hydrogen are available with such approach, though at very low production rates (Woodward et al., 2000). More recently an overall H_2 yield of 96% (9.6 moles of H_2 per mole of xylose) has also been reported with [NiFe]-hydrogenase as final catalyst (Martin Del Campo et al, 2013). In the [FeFe]-hydrogenase supported system reported (Smith et al., 2012) the principle is that the NADPH regenerated by the PPP system can be exploited in an enzyme-based setup, containing the [FeFe]-hydrogenase CpI, FNR (*E. coli* ferredoxin-NADPH-reductase) and Syn Fd ([2Fe2S] single electron-carrying ferredoxin from *Synechocystis*). By varying the relative amount of the three protein catalysts *in vitro* the authors achieved improvement of the turnover frequency (TOF) up to 7 sec^{-1}, and, in a different condition with slightly lower TOF (2-3 sec^{-1}), recorded the highest volumetric productivity at 5.7 mmole H_2 L^{-1} h^{-1}, equivalent to a fuel-value productivity of 1.6 kJ L^{-1} h^{-1}. Further work from the same group (Lu et al., 2015) also validated the use of cell-free unpurified extracts to provide NADPH regeneration

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and subsequent hydrogen production, at similar rates, thus supporting low-cost feasibility of this synthetic system for applicative purposes.

4.3 Electrode immobilisation and electrocatalysis. Towards better catalysts than platinum?

The optimisation of interfacing [FeFe]-hydrogenase with electroactive materials, either carbon-based or metals or semiconductors, is of paramount interest in the perspective of exploiting this biocatalyst for hydrogen production by coupling to other sustainable source of electrons and protons. Therefore several approach have been proposed (Fig. 2).

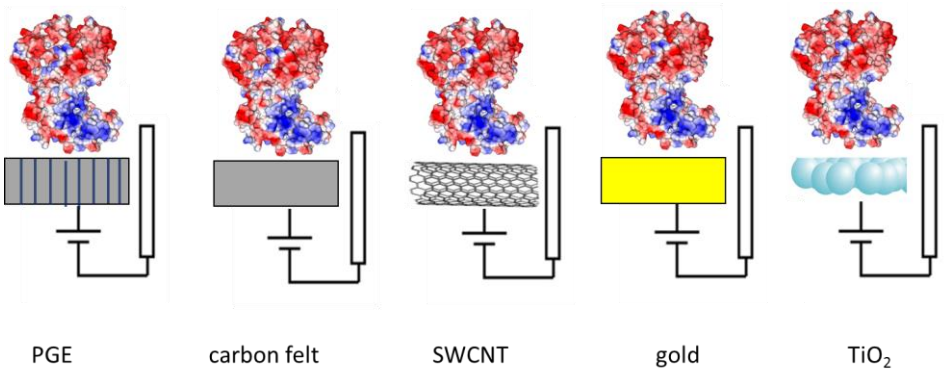

Fig. 2 Scheme showing different approaches in interfacing [FeFe]-hydrogenases to electrode and semiconductor surfaces.

The immobilisation of [FeFe]-hydrogenases has been achieved on several carbon based materials, such as pyrolitic graphite edge (Baffert et al., 2008, Goldet et al., 2009, Baffert et al., 2012), metallic single-wall carbon nanotubes (Svedruzic et al., 2011), glassy carbon and carbon felt (Hambouger et al., 2008). Moreover, to overcome mass diffusion limitations due to the high turnover numbers of [FeFe]-hydrogenases, most studies aiming at calculating rate constants and dissecting the kinetics have been performed on fast rotating disk electrodes. The system of rotating disk is nonetheless limited to theoretical studies and does not allow to reach very high current densities or to propose applicative outcomes directly employing the technique. Recently, the immobilisation of both CrHydA1 and CaHydA on pyrolitic graphite was stabilised by covalent immobilisation, obtaining a longer stability to protein desorption and inactivation (Baffert et al., 2012). Few example of immobilisation on gold electrodes are reported, which allowed for in depth analysis of the

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electrochemical behaviour of the enzyme catalysts but are not feasible to be proposed for exploitation in electrochemical hydrogen-producing devices (Krassen at al., 2009, Krassen at al., 2011). One of the best example of the interaction of these enzymes with carbon based electrodes (Hambourger et al., 2008) employed high surface area carbon felt, a low cost material that proved to be able to interact efficiently with *C. acetobutylicum* [FeFe]-hydrogenase HydA (CaHydA). This approach provided a bio-electrode with cathodic current higher than platinum foil in terms of the nominal two-dimensional area, achieving up to 40% of current densities obtained in platinum based systems in absolute values. These carbon felt/CaHydA electrodes were tested as the cathode in a photoelectrochemical biofuel cell (Hambourger et al., 2008). On the bases of these result (Hambourger et al., 2008), and because of very high turnover numbers and efficient catalysis, [FeFe]-hydrogenases have been proposed to replace the use of rare and expensive noble metals, such as platinum. In this context, the relevance is not only for hydrogen production by also for biohybrid materials to be employed in fuel cells.

TiO_2-based electrodes have also been demonstrated to efficiently adsorb [FeFe]-hydrogenases, including the same specific catalyst (CaHydA) mentioned above, creating stable bio-electrodes possibly by electrostatic interactions. Anatase nanostructured electrodes were tested with different particle size and layer thickness (Morra et al., 2011, Morra et al., 2015b). Coating with a polymer film of Nafion, an efficient proton exchanger, led to an improvement of the protein stability limiting its desorption while maintaining a current density of about 0.1 mA/cm^2. By testing diverse and more efficient [FeFe]-hydrogenases isolated from a pilot plant with high rate of hydrogen production (Morra et al., 2014), the performances in H_2 evolution of TiO_2–enzyme hybrid systems were improved, obtaining bio-electrodes with a very high Faradaic efficiency (98%) and an improved stability over time, even without the Nafion coating (Morra et al., 2015b). The interaction with the nanostructured electrode material, alternatively 20 and 100 nm particle size, allowed for electron densities up to about 1 mA/cm^2 and estimated turnover frequencies of at least 5 sec^{-1}.

4.4 Coupling [FeFe]-hydrogenase to photosynthesis

One of the possible exploitation of bio-hybrid systems based on hydrogenases is the coupling with electron and proton generating setup that exploits the solar harvesting and natural water splitting activity of photosynthesis. Some proof-of-concept setup and the general scheme of the so-called *artificial leaf* have been proposed (Reisner et al., 2011, Mersch et al., 2015), mainly using [NiFe]-hydrogenases, due to their possible stability and slow rate activity even in presence of oxygen. In these systems the natural photosystem II (PSII) was selected as protein catalyst for the bioanode. Serious limitation to these devices is the very low stability of the PSII at the anode and the low energy of the produced electrons that still need an applied potential in the circuit to be able to reduce protons

at the biocathode. As for [FeFe]-hydrogenases, although a similar approach can be proposed, studies have been focusing on improving the efficiency of electron harvesting from Photosystem I (PSI). This has been achieved (Yacobi et al., 2011) by engineering a ferredoxin-hydrogenase (Fd-HydA) fusion protein that can overcome the competition between photosynthesis and hydrogen production over the electrons collected by the ferredoxin:$NADP^+$-oxidoreductase (FNR) bound to PSI and result in more efficient photosynthetic hydrogen production. Also, the direct coupling of [FeFe]-hydrogenases to PSI was proposed as a strategy to increase the rates of hydrogen production (Lubner et al., 2010). By eliminating the diffusion-limited step in electron transfer the efficiency was enhanced to even outperform natural photosynthesis (Lubner et al., 2011, Applegate et al., 2016), as reported on a linked system between PSI from *Synechococcus* sp. PCC 7002, and the distal [4Fe4S] cluster of the [FeFe]-hydrogenase from *Clostridium acetobutylicum* (Figure 3C). This system reached rates of 105 ± 22 e^-PSI^{-1} s^{-1}.

4.5 Light-driven hybrid devices: [FeFe]-hydrogenases and photosentitive nanomaterials

On the basis of the efficient coupling on the electrode and semiconductors materials described above, [FeFe]-hydrogenases have been interfaced to various electroactive materials (King 2013) and exploited as electrocatalysts in several H_2 producing devices. The most interesting are based on photosentitive materials, in order to propose proof-of-concept and strategies (Figure 3) for a solar sustained hydrogen production (Brown et al., 2010, Brown et al., 2012, Vincent et al., 2007, Baffert et al., 2012). As an example CaHydA has been used to develop a photoelectrochemical cell that is able to directly collect the light and to convert it into low potential electrons (using NADH as sacrificial electron donor) to be supplied to the hydrogenase, immobilised on a carbon felt electrode by adsorption (Hambourger et al., 2008). The whole system was demonstrated to produce H_2 under illumination and to be competitive in comparison to bare platinum as cathode.

CaHydA was also exploited in self-sufficient systems (Figure 3B and 3D), in a complex with CdTe nanocrystals (Brown et al., 2010) and CdS nanorods (Brown et al., 2012) achieving turnover frequency numbers (TOF) up to 900 s^{-1} in the latter system. The nanorods are able to capture the light and to promote charge separation (using ascorbic acid as sacrificial electron donor), supplying electrons directly to the enzyme for H_2 production (Brown et al., 2012). Also the site of interaction of nanocrystals and nanorods was suggested to be the same highlighted for the natural and electrostatically stabilised interface of protein-protein interaction between [FeFe]-hydrogenase and ferredoxin.

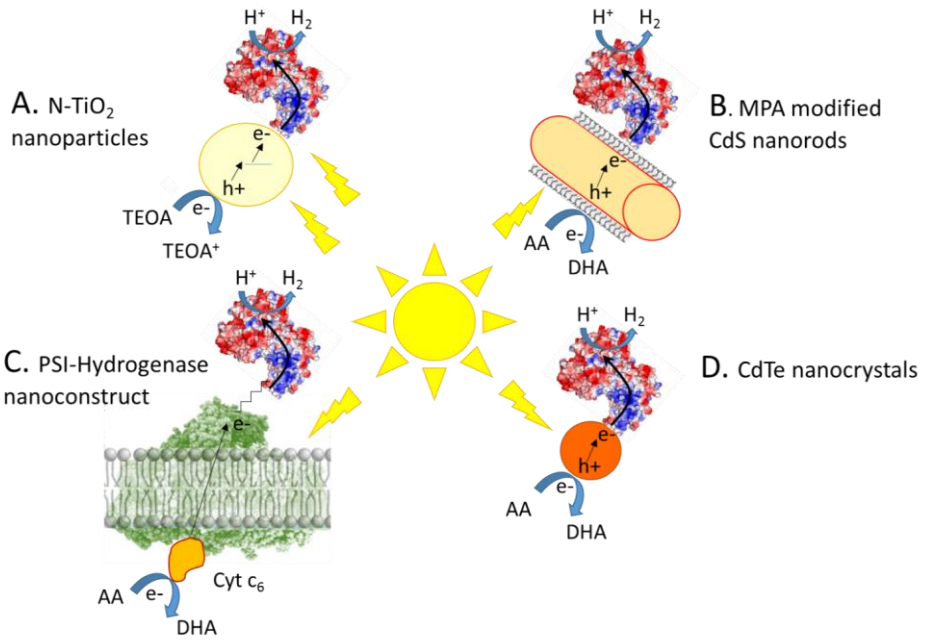

Fig. 3 Overview of the proposed bio-hybrid devices for light-activated hydrogen production based on [FeFe]-hydrogenases directly linked/adsorbed to the photoactive material. A) N-TiO_2-nanoparticles and *Clostridium perfringens* [FeFe]-hydrogenase (Polliotto et al., 2016); B) Mercaptopropionic acid (MPA) modified CdS nanorods and *Clostridium acetobutylicum* [FeFe]-hydrogenase I (Brown et al., 2012); C) PSI–Hydrogenase nanoconstruct based on Photosystem I from *Synechococcus* sp. PCC 7002, and [FeFe]-hydrogenase from *Clostridium acetobutylicum* crosslinked *via* 1,8-octanedithiol. Cytochrome c_6 from *Synechocystis* sp. PCC 6803 was used to transfer electron from the sacrificial donor to the PSI (Lubner et al. 2010, 2011; Applegate et al., 2016); D) CdTe nanocrystals and *Clostridium acetobutylicum* [FeFe]-hydrogenase I (Brown et al., 2010). Sacrificial donors used: AA (ascorbic acid), TEOA (triethanolamine).

Although extremely efficient, these materials suffer from some drawbacks due to the cost and toxicity. As an alternative titanium dioxide was also exploited as photoactive material. The limitation of this very cheap and non toxic semiconductor is the large bandgap, that hinders activation with visible light, limiting to the near UV the solar spectrum that can be harvested if the system is to be exposed directly to sunlight. Previous report of photoactive systems based on titanium oxide with

hydrogenases bypassed the limitation by the use of expensive dye-sensitising with Ru-based systems (Reisner et al., 2009). Alternative materials based on N-doping of titanium oxide has been proven to be more sustainable. The interstitial and substitutional N insertion in the anatase structure can generate intra band-gap levels that are available to excite the electrons of the valence band to the conduction band, even under visible light. This allows to harvest a fraction of photons present in the solar light which, due to lower energies, are not effective in the case of bare TiO_2. In suspension systems based on bare TiO_2 (anatase) and on alternative N-doped (N-TiO2) or reduced TiO_2 (rd-TiO_2), and on novel and highly active *Clostridium perfringens* [FeFe]-hydrogenase CpHydA (Polliotto et al., 2016, Morra et al., 2016b) were tested for hydrogen production under direct solar light using TEOA as a sacrificial donor as well as for buffering the suspension (Figure 3A). These systems showed turnover frequency numbers (TOF) of at least 2.8 ± 0.2 s^{-1} (TiO_2-CpHydA); 4.1 ± 0.1 s^{-1} (N-TiO2-CpHydA) and 0.6 ± 0.1 s^{-1} (rd-TiO_2-CpHydA) in good agreement with values observed for CpHydA immobilized on anatase electrodes in chronoamperometry (Morra et al., 2015b).

5. Conclusions and Future Perspectives

[FeFe]-hydrogenases are excellent natural catalysts that have evolved for efficient H_2 production. The study of the structure/function relationships in this class of enzymes is shedding light on their complex mechanisms. In the future, this will provide information for the production of engineered enzymes with improved features. Their exploitation to produce H_2 at the industrial level has been supported by several studies that suggest a number of possible applications.

Several issues are still being investigated and open to further improvements. The metabolic constraints to in-cell systems due to the fixed availability of NAD(P)H and ATP to supply the electrons and metabolically sustain the hydrogen production explain why there is a limited improvement in anaerobic fermentation and the yields are still too low to provide bio-hydrogen for energetic purposes. Nonetheless, the combination of hydrogen production via dark fermentation with waste treatment can give a relevant benefit in the circular economy perspective by providing a strategy to dispose of waste such as the organic fraction of municipal solid waste and recover a carbon neutral energy storage vector. The fixed maximum theoretical yield that cannot be improved just by *in vivo* strategies is a challenge for developing more advanced *in vitro* or hybrid systems based on engineered hydrogenases.

The quest for oxygen tolerant or stable enzymes is open and engineering the enzyme, in combination with materials protecting from oxidative damage, can provide a biotechnological solution and grant bio-hybrid materials with improved performances for energy-producing devices, also able to harvest solar light for bio-hydrogen production. An engineered and optimized oxygen stability would surmount the hurdles to applications of the isolated enzymes due to the intrinsic oxygen sensitivity of most [FeFe]-hydrogenases.

The widening of researchers interest towards new hydrogenases from still unstudied organisms, as well as deepening the biochemical characterization of enzymes within the scenario of biodiversity (i.e. by exploring the enzyme structure-function fine tuning by evolution in bacteria, archea and eucarya for [FeFe]-hydrogenases) can provide precious information for enzyme engineering and for selection of the most suitable biocatalysts for different exploitation in bio-hybrid materials.

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