Molecular Background of Oxygen Sensitivity in [FeFe] hydrogenases

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aus
Wesel

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What is happening to my skin?
Where is that protection that I needed?
Air can hurt you too
Air can hurt you too
Some people say not to worry about the air
Some people never had experience with...
Air...

_Talking Heads_
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INTRODUCTION

Hydrogen (H\(^1\)) is the most abundant and lightweight element in the universe. The stable form of hydrogen under standard conditions is “dihydrogen” H\(_2\). Dihydrogen is an inert molecule with both nuclei sharing two 1s electrons. The H\(_2\) molecule has a bond enthalpy of 436 kJ mol\(^{-1}\) which reflects its chemical robustness. This extraordinary high bonding energy makes H\(_2\) an excellent energy carrier. Its specific enthalpy is four-fold that of coal, and about 2.5-fold that of diesel or natural gas [1]. With just two electrons shared, the intermolecular attraction of nuclei is weak. Thus, H\(_2\) is highly volatile and only trace levels are left in the lower atmosphere [2]. Dihydrogen escaped the stratosphere due to either diffusion [3] or oxidation in the course of the onset of oxygenic photosynthesis [4, 5]. Hydrogen is scarcely found in the H\(_2\) form but as hydride ion (H\(^-\)) and proton cation (H\(^+\)) in water, Earth’s crust, and all kinds of life.

1.1 Hydrogenases catalyse uptake and release of dihydrogen

Hydrogen in enlivened Nature

In anaerobic segments of deep lakes and hot springs the concentration of H\(_2\) is much higher than in the stratosphere. Strict anaerobe bacteria and archaea make use of protons as terminal electron acceptor in anaerobic respiration and fermentation. Oxidation of organic matter and generation of ATP is coupled to reduction of protons that alternatively stand in for O\(_2\). Anaerobic respiration is not the only metabolic pathway that produces H\(_2\). In carboxytrophic bacteria of the Carboxydothermus genus [6] oxidation of CO to CO\(_2\) is often coupled to proton reduction [7]. Nitrogen-fixing archaea, proteobacteria (e.g., root nodule bacteria Rhizobia spec.), and cyanobacteria (Nostoc, Anabaena) release H\(_2\) as a by-product in the reduction of inorganic N\(_2\) to bio-available NH\(_3\) [8-10]. Nitrogen-fixation is catalysed by the nitrogenase complex and demands an additional energy input of 16 equivalents ATP per N\(_2\) and H\(_2\).

Microbial H\(_2\) release is versatile and typically occurs under anaerobic conditions. However, H\(_2\) uptake is found in many micro organisms as well. Knallgas bacteria and affiliated species of the Desulfovibrio genus use H\(_2\) as a source of electrons to power their metabolism [11, 12]. Interestingly, the notoriety for anaerobiosis is much less pronounced in organisms relying on H\(_2\) uptake than it is found with H\(_2\) release [13]. The non-standard name “Knallgas bacteria” refers to the fact that bacteria like Ralstonia eutropha and Hydrogenobacter spec. can live lithoautotrophically on a mixture of H\(_2\) and O\(_2\) [14].
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Convergent evolution gave three classes of hydrogenases

Hydrogenases are oxido-reductases (EC 1.12) that catalyse uptake and evolution of H\(_2\) with a variety of redox partners. The reaction hydrogenases perform reads \(2 \text{H}^+ + 2 \text{e}^- \leftrightarrow \text{H}_2\). While the nitrogenase-based hydrogen metabolism is a physiological specialisation, hydrogenases are ubiquitous in strict and facultative anaerobes, including some unicellular eukaryotes [15]. Three classes evolved independently: [NiFe], [FeFe] and [Fe] hydrogenases. These types of hydrogenase enzymes do not share common ancestors [16]. The mechanistic similarities, however, are striking and although this work will focus on [FeFe] hydrogenases it is worth learning about hydrogen catalysis in [Fe] and [NiFe] hydrogenases as well.

[Fe] hydrogenases (Hmd) (also known as “[FeS] cluster-free hydrogenases”) have been described for a number of methanogenic archaea. Originally discovered in Methanothermobacter marburgensis [17], the hydrogenase of Methanocaldococcus jannaschii has been crystallized just recently [18, 19]. [Fe] hydrogenases catalyse the uptake of H\(_2\) in a binary reaction.

[Fe] hydrogenases incorporate a low-spin iron atom bound to a cysteine residue. Two intrinsic CO ligands coordinate this central metal ion [20]. A 2-pyridinol compound (“FeGP”) binds the iron atom at two sites presumably: via pyridinol-N and a formyl carbon side chain [21]. Figure 1 shows a schematic drawing of the [Fe] hydrogenase cofactor arrangement. Due to its octahedral geometry, the central iron atom has a vacant binding site. Dihydrogen is dissociated heterolytically: \(\text{H}_2 \rightarrow \text{H}^+ + \text{H}^-\). One proton leaves the active site, the hydride is moved to an associated methenyl-tetrahydromethanopterin substrate [22] (methenyl-H\(_4\)MPT\(^+\), not shown in Figure 1). The iron atom is not catalytically active but serves as a non-redox coordination site, similar to what is found inaconitase (see section 1.2). [Fe] hydrogenases are sensitive to O\(_2\) damage and efficiently inhibited by extrinsic CO [23].
[NiFe] hydrogenases are the most common hydrogenases in bacteria and archaea [15]. Under physiological conditions, these enzymes are mostly found to catalyse H₂ uptake. Contrary to what has been reported for [Fe] hydrogenases, release of H₂ is possible with [NiFe] hydrogenases in vitro as well [24, 25]. A variety of [NiFe] hydrogenase has been crystallized, all of these who stem from sulphate-reducing bacteria [26]. The periplasmatic, membrane-bound hydrogenase of Desulfovibrio vulgaris Miyazaki F. is the best-studied member of the “standard” [NiFe] class [27]. No crystal structure was obtained for [NiFe] hydrogenases of the Ralstonia-type yet.

The active site of [NiFe] hydrogenases consists of a nickel atom, sulphur-coordinated by four cysteine residues. Two of these cysteines bridge to another transition metal compound, an iron atom. Nickel exhibits two free coordination sites, one “terminal” and one “bridging”. The iron atom has been shown to bind three π-accepting ligands, namely two CN⁻ and one CO group. It shares the bridging coordination site with nickel [26, 28, 29]. Figure 2 displays a schematic drawing of the typical [NiFe] prosthetic group. The complex O₂ and CO sensitivity of this class of hydrogenases is reviewed in the Discussion.

[FeFe] hydrogenases attracted a lot of attention in recent years due to their ability of rapid H₂ evolution. Matter of fact, [FeFe] hydrogenases are found in H₂ release in vivo as well as under laboratory conditions with a turnover number about ten times higher than that of [NiFe] hydrogenases [30]. Two [FeFe] hydrogenases have been characterized by crystallography: “Cpl” of Clostridium pasteurianum and “DvH” of Desulfovibrio desulfuricans ATCC 5575 [31-33]. Other [FeFe] hydrogenases have been identified, including “CaHydA” of C. acetobutylicum [34, 35], “DvH” of D. vulgaris [36, 37], and “CrHydA1”, the small algal hydrogenase of Chlamydomonas reinhardtii which represents the main subject of this work.
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The prosthetic group of [FeFe] hydrogenases is built from a [4Fe-4S] cluster (“[4Fe]_H”) and a catalytically active [2Fe-2S] moiety (“[2Fe]_H”). This site is commonly referred to as “H-cluster” [31] (Figure 3). The [4Fe]_H subcluster is bound to the protein by four conserved cysteine residues. One of these connects [4Fe]_H and [2Fe]_H – the catalytic part of the H-cluster is covalently attached to the protein by a single thiolate. Inorganic CN⁻ and CO ligands coordinate the iron atoms of the [2Fe]_H site. Both proximal and distal iron atoms (with respect to the position of the bridging cysteine-S “Fe_p” and “Fe_d”) are substituted with at least one CN⁻ and CO group [31-33]. Depending on the redox state, another CO is found terminally either at the Fe_d or in a Fe_p–Fe_d bridging position [38]. The iron atoms of the [4Fe]_H cluster exhibit a tetrahedral configuration, Fe_d and Fe_p show octahedral geometry. The distal iron atom has a free coordination site that readily attracts hydrogen. A non-protein azadithiolate ligand binds the sulphur atoms of the [2Fe]_H moiety [39].

Different redox states have been described for the H-cluster (Figure 4). The oxidised state “H_ox” (A) is paramagnetic and EPR-active. It is commonly denoted as Fe_p(I)–Fe_d(II) [40]. In the reduced state “H_red” (B), Fe_d is discussed to exhibit a vacant coordination site (Fe_p(I)–Fe_d(I)–X as in (B)) or bind a hydride anion (Fe_p(II)–Fe_d(II)–H⁻, not shown). This state is invisible to EPR. H_red has been characterised by infrared spectroscopy due to the missing 1800 cm⁻¹ band of the CO bridge which is typically found in H_ox [41-43]. External CO binds H_ox to give the paramagnetic state “H_ox-CO” (C) [38].

The O₂ sensitivity of [FeFe] hydrogenases is the main subject of this work. Oxygen irreversibly binds Fe_d and leads to inactivation of the [FeFe] hydrogenase. This state “H_ox-air” is not to confuse with “H_inact” (D). Some Desulfovibrio-type hydrogenases (see next chapter) bind an OH⁻ or H₂O ligand and are stable under air [29]. Catalytic activity is not found in H_inact and it takes a reductive treatment to induce hydrogen turnover. In functional analogy to standard-type [NiFe] hydrogenases [44], activated [FeFe] hydrogenases are re-sensitised to O₂ damage [45].
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The catalytic mechanisms of H₂ turnover remain a matter of debate. [NiFe] hydrogenases were shown to bind a hydride in the Ni–Fe bridging position (“X” in Figure 2) by advanced EPR [46, 47]. From density function theory (DFT) calculations, a similar reaction mechanism was proposed for the Fe–Fe binding site in [FeFe] hydrogenases [48, 49]. The H_red state of most [FeFe] hydrogenases shows a terminal rather than a bridging CO ligand. Thus, a catalytic Fe–H –Fe coordination might be possible. Another theory involves the azadithiolate nitrogen atom (Figure 4D) in catalysis. From EPR [39] and DFT calculations [50-52] it was suggested that the central nitrogen atom accepts a proton (azadithiolate as a base) while Fe₄ binds a hydride (“entatic state” as in Darenbourg et al. [53]). This concerted mechanism is likely to be relevant in H₂ evolution. Protonation of the azadithiolate ligand is further supported by the crystal structures of DdH and Cpl: the gas channel directly points to the azadithiolate nitrogen (see Figure 12 in the Discussion).

Structural differentiation in [FeFe] hydrogenases

Bacterial [FeFe] hydrogenases are either monomeric (Clostridia-type) or dimeric (Desulfovibrio-type). With most Clostrida-type [FeFe] hydrogenases, a functional bisection is observed. The larger part of the enzyme binds the active site H-cluster and is referred to as “H-domain”. The Clostrida-type [FeFe] hydrogenases typically holds three [4Fe-4S] and one [2Fe-2S] cluster in the “F-domain”. These ferredoxin-type [FeS] clusters are in tunnelling distance to each other and “wire” the active site to the protein surface (Figure 5). The mushroom-shaped structure of Cpl (64 kda) clearly illustrates the segmentation of catalytic and accessory domain [31, 33]. In Figure 6B, a cartoon model of the C. pasteurianum enzyme is drawn as crystallized. Among the Clostridia-type [FeFe] hydrogenases, size and [FeS] cluster composition of the accessory domain varies. While Cpl and CaHydA exhibit the maximum number of four clusters, the [FeFe] hydrogenase of Megasphaera elsdenii holds only two [4Fe-4S] clusters [54].

Figure 5 – Iron-sulphur cluster arrangement in Cpl. The F-clusters wire the active site H-cluster to the surface. All clusters are in tunnelling distance (max. ~14 Å). The H-cluster is modelled as H₂ox with a vacant binding site at Fe₄ (X).
The dimeric, Desulvovibrio-type [FeFe] hydrogenases consist of a large and a small peptide chain. The large subunit of \textit{DdH} (46 kDa) not only forms the active site but holds two [4Fe-4S] clusters as well. No [FeS] clusters are found with the small subunit (14 kDa). It folds around the large subunit like a belt. Nicolet and co-workers found structural similarities between bacterial ferredoxins and the small subunit of \textit{DdH} [32]. However, the small subunit is discussed to be relevant in translocation of the protein from the cytoplasm to the periplasmatic space [32, 55]. Figure 6A depicts a cartoon model of the crystal structure of \textit{DdH} in comparison to the structure of \textit{CpI} (6B).

**Figure 6** – Crystal structures of \textit{DdH} and \textit{CpI} including [FeS] cluster equipment. (A) \textit{DdH} (1HFE) of \textit{Desulvovibrio desulfuricans} carries two [4Fe-4S] compounds (yellow spheres) besides the H-cluster (red). These are found with the large subunit (46 kDa). A cluster-free small subunit (14 kDa) folds around the large one like a belt. (B) \textit{CpI} (3C8Y) of \textit{Clostridium pasteurianum} consists of a single 64 kDa peptide chain with three [4Fe-4S] and one [2Fe-2S] clusters. This part of the protein is referred to as ferredoxin- or “F-domain” (F). The H-cluster is buried inside the catalytic part of the protein, the “H-domain” (H).

**The minimal [FeFe] hydrogenases in green algae**

As a representative of Eukaryota, green algae synthesize hydrogenases [56, 57] that have been shown to be of the [FeFe] type and not, as assumed before, [NiFe] hydrogenases [58, 59]. The hydrogenase is usually located in the chloroplast stroma and serves as an “electron valve” under reducing, ATP-deficient conditions – in particular anaerobiosis, which is the key signal for gene expression [59, 60]. Removing electrons from the \textit{photosynthetic electron transport} (PET) chain via proton reduction under conditions where other electron valves like the Calvin Cycle are not available protects the cell from over-reduction. Simultaneously, it allows keeping up the \textit{proton motive force} (PMF) across the thylakoid membrane. Dihydrogen leaves the cell as exhaust. The hydrogenase is coupled to the photosynthetic electron transport chain via photosystem I (PSI) and ferredoxin [60-62]. Numerous authors make use of \textit{C. reinhardtii} in “solar-biological H\textsubscript{2} production” (see Ghiradi et al. [63-66] for review).

The [FeFe] hydrogenases of green algae are known as “\textit{Chlorophyta-type}” hydrogenases [57]. Typically, these enzymes lack the F-domain. No other [FeS] compound than the active site H-cluster is present in \textit{Chlorophyta-type} [FeFe] hydrogenases. Since sequence similarity of the H-domain of \textit{Clostridia} and \textit{Chlorophyta-type} hydrogenases is fairly high, algal [FeFe] hydrogenase can be viewed as “minimal” version of the typical \textit{Clostridia-type} enzyme [57, 67].
The \textit{C. reinhardtii} hydrogenase serves as a role model in [FeFe] hydrogenase research

Isolated from its native host, the [FeFe] hydrogenase of \textit{C. reinhardtii} is laborious to enrich – one litre cell culture gives only about 60 µg protein \cite{60, 68}. Happe and Naber isolated the enzyme with a \( \text{H}_2 \) evolution activity of 935 \( \mu \text{mol \text{H}_2 \, \text{mg}^{-1} \text{min}^{-1}} \). Different systems for the heterologous synthesis emerged since the identity of the “photosynthetic hydrogenase” was resolved \cite{59}. A detailed comparison of the \textit{E. coli}, \textit{C. acetobutylicum}, and \textit{Shewanella oneidensis} systems can be found in the Discussion \cite{35, 69, 70}.

The biophysical characterisation of \textit{Cr}HydA1 holds important advantages. Due to the absence of accessory [FeS] clusters, iron-specific measurements (\textit{X-ray absorption spectroscopy}, XAS) and redox-sensitive methods (\textit{electron spin resonance}, EPR) are facilitated. In the results section, studies on the \textit{Cr}HydA1 H-cluster by XAS and protein film electrochemistry can be found. Furthermore, \( \text{O}_2 \) inactivation and maturation of the H-cluster is best studied with \textit{Cr}HydA1 \cite{71-73}. Regarding the first, a tentative interaction of \( \text{O}_2 \) with F-domain [FeS] clusters does not have to be taken in account. Maturation of \textit{Cr}HydA1 is simplified for the same reason. The catalytic characteristics are solely determined by the H-cluster, accessory clusters do not hamper the analysis.

Although there is no crystal structure of \textit{Cr}HydA1 available yet, theoretical models of the protein structure (Figure 7) help understanding certain aspects of hydrogen turnover. This includes the ongoing issue of gas channels and how these may determine \( \text{O}_2 \) sensitivity \cite{74-76}. Moreover, the protein–protein interaction of \textit{Cr}HydA1 with ferredoxin PetF was discussed in an elaborate study \cite{67}. Reduction by PetF is crucial because the ferredoxin enzyme is thought to be the central branching point of electrons in the chloroplast stroma \cite{61}. Understanding (and facilitating) the hydrogenase–ferredoxin interaction is another approach to enhance \( \text{H}_2 \) production with \textit{C. reinhardtii} \cite{63}.

\textbf{Figure 7} – Homology model of the algal [FeFe] hydrogenase \textit{Cr}HydA1. The structure was modelled after crystal coordinates of \textit{CpI} (3C8Y) using the ExPASy Swiss Prot server. \textit{Cr}HydA1 holds no other [FeS] clusters than the H-cluster (red spheres).
INTRODUCTION

1.2 Iron-sulphur proteins

Iron-sulphur clusters mediate single electron transport reactions

Hydrogenases are [FeS] proteins. This vast and heterogeneous protein class characteristically harbours small inorganic [FeS] compounds bound to the protein scaffold by conserved cysteine motifs. Typical [FeS] compounds are [2Fe-2S] and [4Fe-4S] clusters, with two and four equivalents of free sulphur, respectively. Even an iron atom bound by cysteines can be defined as [FeS] site (“Rubredoxin Fe centre” as in [Fe] hydrogenases (Hmd) [20] and as discussed by Edward et al. [77]). These basic modules can be found combined and twisted in a wide range of different [FeS] cofactors [78], including the hydrogen cycling centres of [NiFe] and [FeFe] hydrogenases.

Typically, [FeS] clusters mediate single electron transport reactions. Iron-sulphur clusters accept and donate reducing equivalents with the overall redox state switching between +1 and +2. Depending on the active site protein environment, the range of reducing potentials is widespread. Iron-sulphur clusters have been described from -0.45 to +0.6 V vs. NHE [79, 80]. Common [FeS] sites serve conductive in low-potential reactions (e.g., in hydrogenases) what sets them apart from high-potential cofactors like heme or NAD(P) [81, 82]. Furthermore, it reflects their origin from a reductive, primordial atmosphere [83]. Exception to the rule is the high-potential [4Fe-4S] ferredoxins (“HiPIPs”) whose cubane cluster redox state intentionally shuttle between +2 and +3 [84-86].

Iron-sulphur proteins are found in a variety of specialisations

It seems likely that [FeS] proteins were the first catalytic enzymes evolved in the heyday of life [87, 88]. Formation of [FeS] clusters requires ferric iron, thiol and sulphide [89]. Moreover, Venkateswara and co-workers could demonstrate that simple [FeS] compounds form spontaneously and remain stable under reducing and O$_2$-deficient conditions [90] – typical characteristics of the atmosphere on early Earth. With subsequent oxidation of the atmosphere, the need for protecting protein scaffold of [FeS] clusters grew. Today, [FeS] clusters are “the most abundant and most diversely employed enzymatic cofactors” [83] and a great variety of proteins has been studied.

Ferredoxins are small and soluble redox proteins that are found in bacteria and photosynthetic eukaryotes [91]. The average redox potential is -420 mV vs. NHE. Plant-type ferredoxins harbour a [2Fe-2S] cluster [92] while bacterial-type ferredoxins are equipped with one or two cubane cluster [93, 94]. PetF, a plant-type ferredoxin, receives electrons at the reducing end of the photosynthetic electron transport chain [61]. Due to their trademark role in electron transport, the accessory [FeS] clusters in [NiFe] and [FeFe] hydrogenases have been termed ferredoxin-type clusters.
**INTRODUCTION**

**Rieske proteins** are high-potential [FeS] proteins that bind a [2Fe-2S] cluster by two cysteine and two histidine residues [95]. Prominently, Rieske proteins are found as part of the cytochrome b_{6f} and cytochrome b_{c1} complexes in the chloroplast and mitochondrial membrane, respectively. The redox midpoint potential of the Rieske protein is about 700 mV more positive than that of plant-type ferredoxins [96]. Like all ferredoxins, the Rieske proteins are solely found in electron transport.

The prokaryotic **high-potential [4Fe-4S] ferredoxins** are another ferredoxin-related class of [FeS] proteins. The active site comprises a bacterial-type [4Fe-4S] cluster completely shut-off from the solvent. Therefore, the cofactor in HiPIP can adopt the +3 redox state without oxidative dismissal of an iron atom [85, 86, 97]. Aromatic residues are discussed to determine the extraordinary redox potential [85, 98, 99]. The maximum midpoint potential is +480 mV vs. NHE, even higher than that of the Rieske protein [79].

**Aconitase** is the best-studied member of the dehydratase family. The enzyme incorporates a [4Fe-4S] cluster that is bound by three cysteine residues (Figure 8A). One iron atom exhibits a free coordination site. Aconitase catalyse the dehydratation of citrate to aconitate by binding two hydroxyl groups of (iso-) citrate to this free iron site [100]. Catalysis is mediated by an interplay of certain residues, not electronic participation of the cubane cluster which nevertheless supports substrate binding. Enzymes of the dehydratase-type are widespread [83, 101, 102].

The **radical-SAM enzymes** employ an analogue coordination mechanism to convert aliphatic molecules [103-105]. S-Adenosylmethionine (SAM) binds the free iron site of a catalytic [Fe-4S] cluster via the carboxy and amino groups of the methinone end (Figure 8B). The cubane cluster donates an electron and an adenosyl-radical is formed [106]. Like in dehydratases, the iron coordination sphere changes from tetrahedral to octahedral geometry. However, the [FeS] moiety does not only bind the substrate but displays redox activity as well. The **pyruvate:formate lyase activating enzyme** (PFL-AE) and **biotin synthase** (BioB) are important examples for the radical-SAM superfamily [107, 108]. Maturation of [FeFe] hydrogenases is supposed to be catalysed by radical-SAM proteins [69].

![Figure 8](image-url) – Iron-sulphur clusters in substrate binding and catalysis. (A) shows the coordination of citrate to the [4Fe-4S] cluster of a dehydratase. In (B) the reaction mechanism of radical-SAM enzymes is displayed. Methionine binds to the free iron site and an electron is passed on to the Adenosyl moiety. The cubane cluster is oxidised 1+ → 2+ in this step. See text for details.
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Next to electron transport and radical synthesis, [FeS] proteins are frequently involved in sensing and regulatory functions. The following chapter will discuss the oxidative disruption of [FeS] clusters. One of the most prominent [FeS] proteins, the fumarate:nitrate reductase, will be introduced subsequently.

Iron-sulphur proteins and the problem with oxygen

Iron-sulphur proteins need to sequester their active site [FeS] clusters from atmospheric exchange if they operate in aerobic environments. Oxygen species like O₂, H₂O₂, and superoxide (O₂⁻) readily bind transition metal compounds: while iron or nickel are good univalent electron donors, oxygen species accept electrons one-by-one [109]. Oxygen sensitivity is not restricted to reactive oxygen species – O₂ is willing redox partner to [FeS] clusters itself [110]. The oxidation state of an oxygen-coordinated [FeS] cluster is +3 (“over-oxidation”). Iron-sulphur clusters as subject to +3 oxidation lose an iron atom due to “solventolysis” as discussed in [97]. The subsequently formed [3Fe-4S]1⁺ compound decomposes if not specifically stabilized by the protein [111-113].

In bacteria and plants, “low-potential” ferredoxins have been reported both sensitive and resistant against oxidative damage [114, 115]. A well-documented example of O₂-stable [FeS] clusters can be found with the chloroplast and mitochondrial inner membranes. The numerous [FeS] proteins of the photosynthetic and mitochondrial electron transport chain (e.g., PSI, NADH dehydrogenase, and the Rieske protein) are insensitive to ambient levels of O₂ [116-118]. The active site in high-potential ferredoxins is lined with aromatic residues. HiPIPs circumvent oxidative damage by excluding any contact of the [4Fe-4S]³⁺ cluster with the solvent. Obviously, O₂ sensitivity of [FeS] proteins is modulated by the peptide scaffold.

Iron-sulphur proteins that mediate electron transport do not necessarily expose their cofactor to the solvent. In [FeS] proteins with substrate binding activity, contact with the solvent is inevitable condition. This selective exposure brings forth the danger of solventolysis [93]. Among the dehydratase and radical-SAM families, different levels of O₂ sensibility have been reported, ranging from insensitive to highly vulnerable [83]. In [NiFe] and [FeFe] hydrogenases, O₂ inactivation is notorious because the enzymes exhibit “tunnels” not only H₂ can travel but O₂ as well [26]. [Fe] hydrogenases display a different strategy. The crystal structure of the O₂-labile [Fe] hydrogenase from M. jannaschii shows that the prosthetic group is liberally exposed to the solvent in a cleft formed by the “open” conformation of the protein [19]. Figure 9 shows a structural comparison of the cofactor environment in CpI and in the [Fe] hydrogenase of M. jannaschii.
There is a wealth of publications reporting the specific reactions of [FeS] proteins with O$_2$ and π-accepting oxidants like CO, H$_2$S, and NO [76, 115, 119-121]. Summed up, [FeS] proteins are generally prone to destructive oxidation. Proteins found under aerobic conditions have been evolutionarily optimized to function at a specific maximum level of O$_2$ – typically by shielding the cofactor from solvent. Iron-sulphur proteins that never had to deal with O$_2$ display pronounced susceptibility. However, with a certain class of oxygen-sensing [FeS] proteins, collapse of a cluster was exploited as regulatory principal [122].

The ubiquitous mammalian [FeS] protein c-aconitase holds a cubane cluster with a single uncoordinated iron atom (as discussed above) [123]. This atom is naturally prone to oxidation and the Achilles’ heel of the cluster. Upon dismissal, c-aconitase acquires the capability to bind RNA. A multifunctional signal cascade is triggered subsequently [124, 125]. The enzyme without its [FeS] core is referred to as “iron regulatory protein” (IRP). The RNA region it binds is specifically termed “iron responsive elements” (IREs). The IRE / IRP system is the typical sensing mechanism for the availability of ferric iron (which directly relies on the O$_2$ content of the cell) [126]. A similar principal is found with the *E. coli* transcriptional regulator SoxP. Here, the oxidative breakdown of a [2Fe-2S]$^2_-$ cluster duplex makes SoxP activate the superoxide response regulon [127, 128]. SoxP senses the concentration of intracellular superoxide and protects the organism by triggering its protection apparatus.
INTRODUCTION

Even more elaborate, the *E. coli* transcription factor **fumarate:nitrate reduction** (FNR) has been discussed to undergo a large structural rearrangement upon O$_2$ sensing [113, 129, 130]. This iron-sulphur protein is essentially responsible for the switch between respiration and fermentation metabolism [131]. The FNR dimer holds a [4Fe-4S] cluster at each N-terminus and binds DNA at the C-terminal regulatory site. Unlike IRP, FNR loses its DNA-binding capacity once the central [4Fe-4S]$^{2+}$ site has been oxidised to a fairly stable [2Fe-2S]$^{2+}$ cluster. Crack et al. traced cluster breakdown via an [3Fe-4S]$^{1+}$ intermediate and reported *in silico* folding studies of a FNR homology model [113, 132]. Two observations are remarkable: (a) cluster dismissal is accompanied by superoxide release, and (b) the final [2Fe2S] cluster on the FNR monomer is apparently not further affected by O$_2$ or superoxide.
RESULTS

Benchmark of contribution

2.1 Optimized over-expression of [FeFe] hydrogenases with high specific activity in *Clostridium acetobutylicum*

P: 20%  E: 30  M: 10%

2.2 Immobilization of the [FeFe] hydrogenase CrHydA1 on a gold electrode: Design of a catalytic surface for the production of molecular hydrogen

P: 50%  E: 30%  M: 40%

2.3 The structure of the active site H-cluster of [FeFe] hydrogenase from green algae *Chlamydomonas reinhardtii* studied by X-ray absorption spectroscopy

P: 50%  E: 30%  M: 60%

2.4 How oxygen attacks [FeFe] hydrogenases from photosynthetic organisms

P: 60%  E: 40%  M: 60%

2.5 Electrochemical kinetic investigations of the reactions of [FeFe] hydrogenase with CO and O₂: Comparing the importance of gas tunnels and active-site electronic/ redox effects

P: 40%  E: 10%  M: 20%

2.6 How algae produce hydrogen – News from the photosynthetic hydrogenase

P: 50%  E: ---  M: 70%
Optimized over-expression of [FeFe] hydrogenases with high specific activity in Clostridium acetobutylicum

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It was previously shown that Clostridium acetobutylicum is capable to over-express various [FeFe] hydrogenases although the protein yield was low. In this study we report on doubling the yield of the clostridial hydrogenase by replacing the native gene hydA1Ca with a recombinant one via homologous recombination. The purified protein HydA1Ca shows an unexpected high specific activity (up to 2257 μmol H2 min⁻¹ mg⁻¹) for hydrogen evolution. Furthermore, the highly active green algal hydrogenase HydA1Cr from Chlamydomonas reinhardtii was heterologously expressed in C. acetobutylicum, and purified with increased yield (1 mg protein per liter of cells) and high activity (625 μmol H2 min⁻¹ mg⁻¹). EPR studies demonstrate intact H-clusters for homologously and heterologously expressed [FeFe] hydrogenases in the CO-inhibited oxidized redox state, and prove the high efficiency of the C. acetobutylicum expression system.

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1. Introduction

The production of the energy carrier hydrogen from renewable resources will be a fundamental prerequisite for a sustainable hydrogen-driven economy. A variety of biological systems serve as a likely base for the development of renewable hydrogen-production technologies [1–4]. Photosynthetic systems in particular can directly link the light energy-driven photosynthetic electron chain to proton reduction, as e.g. the green alga Chlamydomonas reinhardtii [5]. The central enzyme involved in proton reduction in C. reinhardtii is the [FeFe] hydrogenase HydA1Cr catalyzing the reversible reduction of protons to molecular hydrogen [6–8].

Hydrogenases are classified according to the metal content of their catalytic center into [NiFe], [FeFe] and [Fe] hydrogenases [9,10]. The first two classes of hydrogenases are involved in the direct evolution and consumption of hydrogen. [FeFe] hydrogenases show higher catalytic activities for hydrogen evolution than [NiFe] hydrogenases [11]. The active site of all known [FeFe] hydrogenases consists of an unique binuclear iron center which is called the H-cluster. It is of major interest to understand the reaction mechanism of this highly efficient iron-sulfur cluster. HydA1Cr from C. reinhardtii is a promising model for better understanding of [FeFe] hydrogenase structure and function as it is one of the smallest known member (48 kDa) of this enzyme family, due to lack of the FeS-cluster containing F-domain [6,12]. With a better
understanding it could become possible to use [FeFe] hydrogenases in either an artificial hydrogen-production system or in vivo systems including optimized hydrogenases for the renewable production of hydrogen. In this context it will be necessary to over-express highly active [FeFe] hydrogenases in large amounts in order to solve their structure in atomic detail and functionally characterize this important catalyst.

An expression system for [FeFe] hydrogenases had been established in Clostridium acetobutylicum, which achieved high specific activities but rather low protein yield [13]. An alternative system, using Escherichia coli as host, led to high amounts of [FeFe] hydrogenases but comparably low specific activities [14]. In the current report, we present an expression system for C. acetobutylicum that allows for generation of increased amounts of homologously and heterologously synthesized protein with high specific enzyme activities. We demonstrate the high efficiency of this expression system by showing the conservation of the intact H-cluster via EPR-spectroscopy. Based on this work it will now be possible to synthesize large amounts of high quality [FeFe] hydrogenases. This will allow for detailed biophysical analyses of this potent enzyme family leading to an improved description and explanation of their structure and function.

2. Materials and methods

2.1. Growth conditions and maintenance

All C. acetobutylicum strains were grown anaerobically in CGM medium as described previously [15]. For recombinant C. acetobutylicum strains, 40 μg ml⁻¹ erythromycin was added to agar plate and liquid media. C. acetobutylicum strains were stored in spore form at −20 °C.

2.2. Fermentation experiments

Batch experiments with C. acetobutylicum ATCC 824 recombinant strains were performed in a 2.5 l Minifors®-bioreactor (Infors, Augsburg, Germany) with a culture volume of 2.0 l on CGM medium and a glucose concentration of 60 g l⁻¹ as described earlier [13,15].

2.3. DNA isolation, modification and cell transformation

Total genomic DNA from C. acetobutylicum ATCC 824 was isolated as previously described [16]. Plasmids from recombinant strains of C. acetobutylicum were isolated following the protocol of Girbal et al. [17]. All plasmids were constructed initially in E. coli and then transformed into C. acetobutylicum [16].

DNA restriction and cloning were performed according to standard procedures [18]. Restriction enzymes and T4 DNA ligase were obtained from New England Biolabs (Beverly, MA, USA) and Promega (Madison, WI, USA), respectively, and used according to the manufacturer’s instructions. DNA fragments were purified from 2% agarose gels with the GFX™ gel band purification kit (GE Healthcare, Buckinghamshire, Great Britain).

PCR amplifications with Pfu polymerase (Promega, Madison, USA), Pwo polymerase (Roche, Meylan, France) and with the expand long template PCR system (Roche, Meylan, France) were performed as described by the manufacturer in a PCR thermocycler mastercyc personal (Eppendorf, Hamburg, Germany). Oligonucleotide synthesis and de novo-gene synthesis were performed by Eurofins MWG (Ebersberg, Germany) and DNA 2.0 (Palo Alto, CA, USA), respectively.

2.4. Plasmids and genetic construction

Construction and cloning of the expression vectors pTHydA1-C-tag and pTHydA1-C-tag had been described earlier by Girbal et al. [13]. In order to construct and clone the expression vector pTHydA1-C-opt-C-tag, a 5 kb fragment was excised from the plasmid pTHydA1-C-opt using the restriction endonucleases BamHI and Smal. The synthetic gene hydA1-C-opt, containing the green algal hydrogenase gene sequence hydA1-C with optimized codon usage for expression in C. acetobutylicum, was excised from the working plasmid pJ10:hydA1-C-opt using BamHI and EcoRV yielding a 1.3 kb fragment. These two fragments were ligated to construct the expression vector pTHydA1-C-opt-C-tag. Within this vector the gene hydA1-C is under the control of the C. acetobutylicum thiolase promoter and the ribosomal binding site (RBS). The C-terminal Strep tag II sequence was introduced with the 5 kb fragment from the plasmid pTHydA1-C-tag. The expression vector pTHydA1-C-opt-C-tag was excised from the plasmid pJ10:hydA1-C-opt using BamHI and Scal. The linker sequence used to expose the Strep tag II sequence was constructed in a comparable manner, excising the synthetic gene from the working plasmid pJ10:hydA1-C-opt by using BamHI and Scal. The linker sequence used to expose the Strep tag II sequence was constructed in a comparable manner, excising the synthetic gene from the working plasmid pJ10:hydA1-C-opt by using BamHI and Scal.

In order to construct and clone the replicative recombinant plasmid pcons(hydA1-C-opt-C-tag) used for homologous over-expression of hydA1-C with the mutant C. acetobutylicum MGC ΔhydA1-C/hydA1-C-C-tag, two homologous regions (HR) had to be amplified. The homologous region upstream of the native gene hydA1-C was amplified using the primers HydA1 (5’-AAAAAGACCCCTCCTTTTATTGTATTTTTATATTTGCTCAT TGTGC-3’) and HydA2 (5’-GGGGAGGCGCTAAAAGGKGGTCA GTAAAATATTTTCCATCAAGGTAATATTGCTACCAT-3’) yielding a 912 bp fragment, HR1, and introducing a BamHI restriction site at the 5'-end and a Clal-Stul restriction site at the 3'-end. The downstream homologous region was amplified using primers HydA3 (5’-AAATCGATCCCTTTTTT ATGGCCTCCCCCTTCAAGGTGCCCACATTATTATTATTTT TATATTTATATATCTCC-3’) and HydA4 (5’-AAAGAGATCCGTCTCTAAATAAT ATATGAATATATAAAGGTCTTACTACACC-3’) yielding a 1010 bp fragment, HR2, and introducing a Clai-Stul restriction site at the 5’-end and a BamHI restriction site at the 3’-end. Fusion-PCR using both fragments as a template together with the primers HydA1 and HydA4 resulted into a combined fragment HR1:HR2 through the homologous Clai-Stul region with a size of 1922 bp. Digestion with Clai and Stul allowed the directed insertion of a Clai-hydA1-C-opt-C-tag-Stul fragment, which was previously PCR-amplified using the
primers HydA5 (5'-AAAAATGATTCTCTATTTTGGGAGGA-
TAAAATAGG-3') and HydA6 (5'-AAAGCCCTTGACCATGAT-
TAGAATTTCTATGATGC-3'). Subsequent digestion with Stul
allowed the insertion of the Stul–MLSr-cassette, which was
obtained by Stul-digestion of the plasmid puc18-FRT-MLSr-2
[19] and contained the erythromycin resistance gene mlr'.
The final construct BamHI-HR1-hydA1Ca-C-tag-MLSr-HR2-BamHI
was introduced into the BamHI-digested plasmid pcons2-1 [19]
yielding the final recombination vector pcons(hydA1Ca-C-tag).

2.5. Homologous recombination and screening

The replicative recombination plasmid pcons(hydA1Ca-C-tag) was transformed into C. acetobutylicum MGC as described
previously [16] and screened for positive recombinants as
described within the patent of Soucaille et al. [19] for the
positive mutant C. acetobutylicum MGC ΔhydA1Ca/hydA1Ca-C-
tag. Analysis of the positive recombinant was done by
Southern blot analysis [20] using 5μg genomic DNA, digested
with HindIII and separated on a 1% agarose gel. The 517 bp
probe against hydA1Ca was isolated from the plasmid pcon-
s(hydA1Ca-C-tag) by FokI-digestion. The AlkPhos® direct kit (GE
Healthcare, Buckinghamshire, Great Britain) was used for
labeling the probe with alkaline phosphatase. Detection
occurred by chemiluminescence with the CDP-Star-reagent
(Roche, Grenzach-Wyhlen, Germany) and visualization using
a Luminometer model FlurChem 8800 (Alpha Innotech, San
Leandro, CA, USA).

2.6. Purification of Strep tag II – tagged [FeFe] hydrogenases

Protein purification occurred under strictly anoxic conditions
as described by Girbal et al. [13]. An optimized one-step puri-
fication protocol was established for the previously described
[FeFe] hydrogenase HydA1Ca-C-tag[20]. In this case, affinity
chromatography on a 1-mL Strep-Tactin Superflow®
(IBA, Göttingen, Germany) column was carried out using
100 mM Tris–HCl, 0.1 M NaCl, pH 8.0 as buffer, 2 mM dithionite
and 2 mM DTT as reducing agents and 2.5 mM deshiothiobin
for elution of the protein. The presence of Strep tag II-tagged
hydrogenases in the purification fractions was analyzed by
hydrogenase activity assays (see below) and immunoblotting
after 12% SDS/polyacrylamide gel electrophoresis using Strep-
Tactin HRP conjugate (IBA, Göttingen, Germany) at 1:1000
dilution. Low-range standard proteins (GE Healthcare, Buck-
inghamshire, Great Britain) and pre-stained low-range SDS-
PAGE standards (Biorad, Munich, Germany) were used.

2.7. Hydrogenase activity assays

Hydrogenase activity was analyzed by measuring hydrogen
evolution as previously described by Winkler et al. [21], except
for using 100 mM dithionite and 1–50 μL of protein solutions
(max. 50 nM) instead of algal cultures. For determination of
Michaelis Menten kinetics, the concentration of the artificial
electron donor methyl viologen was varied from 0.1 mM to
40 mM. Enzyme kinetics using the natural electron donor-type
protein ferredoxin were performed using 1 mM dithionite and
ferredoxin concentrations ranging from 0.05 μM to 40 μM for
green algal PetF and 10 μM to 100 μM for bacterial ferredoxin
from Clostridium pasteurianum. The values of kinetic constants
were determined from double reciprocal plots.

2.8. EPR spectroscopy

In order to analyze the functionality of the H-cluster of the
purified [FeFe] hydrogenases, pulse EPR-spectroscopy was
applied essentially as described previously [22,23]. The sample
preparations were performed under the previously described
anoxic conditions. Protein samples were concentrated in
Millipore Centricon® Centrifugal Filter Units (molecular
weight cut-off 10 kDa) by centrifugation to a final concentra-
tion of 10–100 μM. Treatment of samples with CO gas was
performed outside the glove box, using gas tight SubaSeal
Tubes. After the treatment samples were transferred into EPR
tubes and frozen in liquid nitrogen. EPR spectra were obtained
at Q-band frequencies, using the 2 pulse electron spin echo
detected EPR technique [24,25]. Electron spin echo (ESE) after
two microwave pulses (π/2 and π) was detected as a function of
the external magnetic field. The delay between the MW
pulses was fixed to τ = 360 ns. The length of the π/2 MW pulse
was set to 36 ns and the π pulse to 68 ns. All pulse Q-band EPR
measurements were performed on a Bruker ELEXSYS E580 Q-
band spectrometer with a SuperQ-FT microwave bridge,
working at 33.88 GHz and a temperature of 20 K.

3. Results

3.1. Homologous over-expression of hydA1Ca in C. acetobutylicum

Previously, homologous expression of the clostridial [FeFe]
hydrogenase HydA1Ca was possible with high specific activi-
ties but protein yield (0.4 mg l⁻¹) was too low for extended
biophysical examinations [13]. The newly available optimized
method for homologous recombination in C. acetobutylicum
[19] gave rise to the approach of engineering an optimal strain
for the homologous over-expression of hydA1Ca. This method
of homologous recombination was applied within the current
study for the first time in the purpose of gene replacement in
C. acetobutylicum. This way it was possible to design the over-
expression mutant C. acetobutylicum MGC ΔhydA1Ca/hydA1Ca-
C-tag. In this mutant the native gene hydA1Ca was replaced via
a double cross-over event with the recombinant gene hydA1Ca-
C-tag, which is equivalent to the native [FeFe] hydrogenase
gene except for a C-terminal Strep tag II sequence. The gene
replacement was proven by Southern blot analysis (Fig. 1).
With this mutant it was possible to obtain a protein yield for
HydA1Ca-C-tag of 0.8 mg l⁻¹. The gene replacement mutant
solely expressed the recombinant hydrogenase hydA1Ca-C-tag
and no native hydA1Ca (data not shown).

Analyzing enzyme kinetics for HydA1ca proton reduction
using the same in vitro-test conditions as for HydA1ca revealed
a high specific enzyme activity for HydA1ca of 1750 μmol
H₂ min⁻¹ mg⁻¹ (using methyl viologen as electron donor) and
2257 μmol H₂ min⁻¹ mg⁻¹ (using [4Fe4S] ferredoxin from Clostrid-
ium pasteurianum as electron donor) (Table 1). EPR-spectro-
trosopic analysis of the CO-inhibited redox state of HydA1ca
revealed the characteristic axial EPR-spectrum and g-values \( g = 2.075, 2.009, 2.009 \) (Fig. 2B).

### 3.2. Heterologous over-expression of the green algal [FeFe] hydrogenase hydA1<sub>Ca</sub> in C. acetobutylicum

Similar to the homologous expression system mentioned above the heterologous expression of green algal [FeFe] hydrogenases in C. acetobutylicum had been shown with high specific activities but protein yield \( 0.1 \text{ mg l}^{-1} \) too low for biophysical examinations [13]. Depending on the age of the mutant spores the protein yield was even further diminished to \( 0.03 \text{ mg l}^{-1} \) of cell culture using this expression system (data not shown). Furthermore, there was significant contamination of purified HydA1<sub>Ca</sub>-C-tag with biotinylated pyruvate carboxylase (PYCA, 127 kD) from C. acetobutylicum. The codon usage of hydA1<sub>Ca</sub> revealed fundamental differences in comparison to C. acetobutylicum resulting in an increased requirement of rare tRNAs and therefore very likely physiological stress for the expression mutant [26]. Adaptation of the codon usage by changing 83% of the codons within the synthetic gene hydA1<sub>Ca</sub>-opt, which was cloned into pThydA1<sub>Ca</sub>-opt-C-tag as previously described for pThydA1<sub>Ca</sub>-C-tag [13], allowed for the reliable expression of significantly increased amounts of algal [FeFe] hydrogenase HydA1<sub>Ca</sub>-C-tag \( 0.3 \text{ mg l}^{-1} \). PYCA contamination was not detected anymore after expression of the optimized gene hydA1<sub>Ca</sub> (Fig. 3A).

Using the strain C. acetobutylicum pThydA1opt-C-tag<sup>exp</sup>, which synthesizes HydA1<sub>Ca</sub>-C-tag<sup>exp</sup> with an exposed C-terminal Strep tag II sequence resulted in further increase of protein yield up to \( 1 \text{ mg l}^{-1} \) (Fig. 3B). Enzyme kinetics for heterologously expressed HydA1<sub>Ca</sub>-C-tag<sup>exp</sup> revealed a specific proton reduction activity of \( 625 \text{ \mu mol H}_2 \text{ min}^{-1} \text{ g}^{-1} \) (Table 1).

In order to prove the functionality of the bimetallic active site of heterologously expressed [FeFe] hydrogenases EPR-spectroscopic analysis of the CO-inhibited enzymes was conducted. The CO-inhibited redox state reveals typical axial spectra for H-cluster containing [FeFe] hydrogenases in general [23,27]. It was possible to assign the axial EPR-spectrum with principal g-values \( g = 2.050, 2.008, 2.008 \) to heterologously synthesized HydA1<sub>Ca</sub>-C-tag<sup>exp</sup> (Fig. 2A).

### 4. Discussion

#### 4.1. Homologously over-expressed [FeFe] hydrogenase from C. acetobutylicum

Gene replacement as a method for creating efficient expression mutants in C. acetobutylicum allowed for deletion of the native hydrogenase gene and incorporation of the recombinant hydrogenase gene within the chromosomal region of hydA1<sub>Ca</sub>. These two events may have caused the twofold increase of purified recombinant hydrogenase in comparison

### Table 1 – Kinetic parameters of HydA1<sub>Ca</sub> and HydA1<sub>Ca</sub> for methyl viologen and ferredoxin as electron donors

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Electron donor</th>
<th>( K_m ) (M)</th>
<th>( V_{max} ) (\text{\mu mol min}^{-1} \text{ mg}^{-1})</th>
<th>( k_{cat} ) (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>( k_{cat}/K_m ) (M&lt;sup&gt;-1&lt;/sup&gt; s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HydA1&lt;sub&gt;Cr&lt;/sub&gt;</td>
<td>Methyl viologen</td>
<td>( 938 \times 10^{-6} )</td>
<td>625</td>
<td>535.4</td>
<td>5.7 \times 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>[2Fe2S] ferredoxin</td>
<td>( 3.4 \times 10^{-6} )</td>
<td>526</td>
<td>450.6</td>
<td>1.3 \times 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>HydA1&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Methyl viologen</td>
<td>( 603 \times 10^{-6} )</td>
<td>1750</td>
<td>1951</td>
<td>3.2 \times 10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>[4Fe4S] ferredoxin</td>
<td>( 3.3 \times 10^{-6} )</td>
<td>2257</td>
<td>2516</td>
<td>7.6 \times 10&lt;sup&gt;5&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
to the plasmid based expression system described by Girbal et al. [13]. Furthermore, the purified enzyme had a specific activity for hydrogen evolution 10- to 175-fold higher than reported earlier for the clostridial hydrogenase HydA1Ca [13,28]. Compared to the E. coli-based heterologous expression of hydA1Ca, the activity described in this paper was more than twenty times higher [14]. The specific activity of HydA1Ca reported in this publication was in the same range as shown for the rather homologous enzyme Cpi from C. pasteurianum or the [FeFe] hydrogenases from Megasphaera elsdenii and Desulfovibrio vulgaris [29].

The EPR-spectroscopic examinations of HydA1Ca revealed a spectrum and g-values (g = 2.075, 2.009, 2.009) different from those of HydA1Cr, but rather similar to the ones found for CO-inhibited Cpi (g = 2.072, 2.006, 2.006) [30]. Compared to HydA1Cr from C. reinhardtii, the left shoulder of the EPR-spectrum for the clostridial hydrogenases was shifted towards lower values, which may indicate differences in structure and functionality between the observed enzymes.

The method of homologous recombination in the context of [FeFe] hydrogenase gene expression opens new perspectives for the use of C. acetobutylicum as a valuable expression host. Subsequent gene deletions, which are possible with the recombination system applied in this work [19], and multiple gene exchanges could allow for the design of further optimized expression mutants. Furthermore, [FeFe] hydrogenases from a wide range of physiological and evolutionary distinct organisms might be synthesized in a stable and highly efficient manner.

4.2. Heterologously over-expressed [FeFe] hydrogenases in C. acetobutylicum

Heterologously expressed [FeFe] hydrogenases from different green algae revealed similar high specific hydrogen evolution activities compared to the native systems [13]. In contrast, heterologous expression of hydA1Cr in E. coli did not lead to any active enzyme, unless the maturation factors hydEF and hydG were co-expressed. Nevertheless, the enzyme synthesized via co-expression of the maturation factors showed a specific hydrogen evolution activity of only 16% and 21% compared to the native [FeFe] hydrogenase HydA1Cr and the heterologously expressed HydA1Cr-C-tag in C. acetobutylicum, respectively [13,14]. For the functional expression of fully active [FeFe] hydrogenases in E. coli additional maturation factors might be necessary, which are presumably naturally available in the expression host C. acetobutylicum. Consequently, the expression of hydA1Cr-C-tagexp in C. acetobutylicum revealed a similarly high specific proton reduction activity of 625 μmol H2 min⁻¹ mg⁻¹ (Table 1), which is about 67% of the native enzyme [6] and 86% of the heterologously expressed enzyme without exposed Strep tag II sequence [13]. The mere insertion of a Strep tag II and a linker sequence had a slightly negative effect on the specific activity of heterologously expressed [FeFe] hydrogenases from C. reinhardtii. Nevertheless, it has to be noted that the specific activity of HydA1Cr-C-tagexp reported in this publication was in the same high range as reported earlier.

Fig. 2 – Q-band 2 pulse ESE detected EPR spectra of the CO-inhibited states of HydA1Cr-C-tagexp (A) and HydA1Ca-C-tag (B) measured at 20 K and 33.88 GHz. Abbreviations: ESE, electron spin echo; EPR, electron para resonance.

Fig. 3 – SDS-PAGE of purified HydA1Cr, coomassie stained, using strain C. acetobutylicum pTHydA1Cr-opt-C-tag (A) and C. acetobutylicum pTHydA1Cr-opt-C-tagexp (B). Abbreviations: E1-E6, eluate fractions; M, marker.
for heterologously expressed HydA1$_{Cr}$ [13] and that to our knowledge in this regard C. acetobutylicum is the most suitable expression host for [FeFe] hydrogenases. The EPR-spectrum of heterologously synthesized HydA1$_{Cr}$-C-tag$^{TM}$ was very similar to natively synthesized and isolated HydA1$_{Cr}$ (g = 2.052, 2.007, 2.007) [23]. This result confirmed that the maturation of the characteristic H-cluster must occur similarly within the expression systems C. acetobutylicum and C. reinhardtii, allowing the heterologous expression of algal [FeFe] hydrogenase with a fully intact H-cluster.

In conclusion, it is now possible to significantly increase the protein yield and maintain a high degree of enzyme functionality within the optimized heterologous expression system for [FeFe] hydrogenases in C. acetobutylicum. This was achieved mainly by adjusting the codon usage and exposing the Strep tag II sequence in the case of green algal hydA1$_{Cr}$. This work has established a reliable method for providing sufficient amounts of enzyme for the biophysical characterization of this smallest known member of the [FeFe] hydrogenase family. This will soon allow a profound insight into the structure and function of this unique class of enzymes.

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References

Immobilization of the [FeFe]-hydrogenase CrHydA1 on a gold electrode: Design of a catalytic surface for the production of molecular hydrogen

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Hydrogenase-modified electrodes are a promising catalytic surface for the electrolysis of water with an overpotential close to zero. The [FeFe]-hydrogenase CrHydA1 from the photosynthetic green alga Chlamydomonas reinhardtii is the smallest [FeFe]-hydrogenase known and exhibits an extraordinary high hydrogen evolution activity. For the first time, we immobilized CrHydA1 on a gold surface which was modified by different carboxy-terminated self-assembled monolayers. The immobilization was in situ monitored by surface-enhanced infrared spectroscopy. In the presence of the electron mediator methyl viologen the electron transfer from the electrode to the hydrogenase was detected by cyclic voltammetry. The hydrogen evolution potential (−290 mV vs NHE, pH 6.8) of this protein modified electrode is close to the value for bare platinum (−270 mV vs NHE).

The surface coverage by CrHydA1 was determined to 2.25 ng mm⁻² by surface plasmon resonance, which is consistent with the formation of a protein monolayer. Hydrogen evolution was quantified by gas chromatography and the specific hydrogen evolution activity of surface-bound CrHydA1 was calculated to 1.3 μmol H₂ min⁻¹ (or 85 mol H₂ min⁻¹ mol⁻¹). In conclusion, a viable hydrogen-evolving surface was developed that may be employed in combination with immobilized photosystems to provide a platform for hydrogen production from water and solar energy with enzymes as catalysts.

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1. Introduction

Hydrogen is one of the most promising energy carriers. Molecular hydrogen is consumed in fuel cells to generate electricity with unrivalled conversion efficiency (Cammack et al., 2001; Karyakin et al., 2005). Nowadays, the major amount of hydrogen is produced by steam methane reforming (Turner, 2004). The electrolysis of water is a costly alternative which allows the incorporation of electricity gained by renewable sources like wind and solar energy (Levene et al., 2007). For this process, rare metals, such as platinum, rhodium, or ruthenium are commonly used as catalysts because of their low overpotentials among the metallic electrocatalysts (Transatti, 1972). The overpotential on other surfaces like Ag, Cu, and Hg can be reduced by the addition of small organic molecules, like 4,4′-bipyridine (Uchida et al., 2008), thiourea (Dayalan and Narayan, 1984; Tian et al., 1990; Bukowska and Jackowska, 1994), pyridine (Hamelin et al., 1990, 1991), or methyl viologen (MV) (Tamamushi and Tanaka, 1987). Still the achieved overpotential is significantly higher than that for a platinum electrode.

Nature has developed highly efficient enzymes to catalyze the reduction of protons to molecular hydrogen. These hydrogenases are able to produce hydrogen with an overpotential close (or equal) to zero (Vignais and Billoud, 2007; Armstrong and Fontecilla-Camps, 2008). Most of these enzymes are iron–sulfur proteins, which contain two metal atoms at their active site, either two iron atoms (as in [FeFe]-hydrogenases) (Peters et al., 1998; Nicolet et al., 1999) or one iron and one nickel (as in [NiFe]-hydrogenases) (Volbeda et al., 1995; Higuchi et al., 1997). [FeFe]-hydrogenases are especially interesting for biotechnological applications because of their high specific activity (Kamp et al., 2008). They are widely distributed among anaerobic living bacteria and some eukaryotic unicellular organisms (Horner et al., 2002).

In particular, hydrogen evolution of photosynthetic green algae has been an active field of basic and applied research during the last 10 years (Melis and Happe, 2001, 2004; Hemmscheimer and Happe, 2005). Recent isolations of the photosynthetic hydrogenase genes (Florin et al., 2001; Winkler et al., 2002) and the discovery of H₂-production under sulfur deprivation in the alga Chlamydomonas reinhardtii (Zhang et al., 2001; Happe et al., 2002) gave an important impulse and opened new ways for biotechnological applications for producing hydrogen.

One approach is to fix the biological catalyst hydrogenase to an appropriate electrode. The modification of an electrode by hydro-
genicase reduces the overpotential to a value close to zero and allows hydrogen production at a higher potential than on a platinum surface. This has been shown for a variety of hydrogenase enzymes including the [NiFe]-hydrogenases of Ralstonia species on a rotating disk graphite electrode (Goldet et al., 2008) and the [FeFe]-hydrogenases of Desulfowlobia desulfuricans (Vincent et al., 2005) and Clostridium acetobutylicum (Baffert et al., 2008). As an advantage, [FeFe]-hydrogenases can have a higher hydrogen production activity (Adams, 1990; Peters et al., 1998; Nicolet et al., 1999) compared to [NiFe]-hydrogenases and suffer less of product inhibition (Léger et al., 2004). As a disadvantage, [FeFe]-hydrogenases are irreversibly inactivated by oxygen, while [NiFe]-hydrogenases can be reactivated by reduction with hydrogen or dithionite (Cammack et al., 2001).

The [FeFe]-hydrogenase of the green alga C. reinhardtii (HydA1) catalyses H₂-evolution with reduced methyl viologen as electron donor with a high specific activity that is comparable to the more complex [FeFe]-hydrogenases of prokaryotes (Happe and Naber, 1993). The monomeric protein of about 48 kDa only consists of the catalytically active H-cluster, but lacks any kind of accessory [FeS]-cluster (Happe and Kaminski, 2002). HydA1 is stable at ambient temperatures and deals with a wide range of buffers and salt concentrations (Forestier et al., 2003; Girbal et al., 2005). Physiologically, the hydrogenase is coupled to the photosynthetic electron transport chain via its natural electron donor, the ferredoxin PetF (Fouchard et al., 2005; Hemschemeier et al., 2008).

In this work, we present the development of an enzyme electrode which is able to catalyze the reduction of protons to molecular hydrogen at minute overpotential. The surface modification and immobilization of the [FeFe]-hydrogenase from C. reinhardtii are probed in situ by surface-enhanced infrared absorption spectroscopy (SEIRAS). Electrochemistry provides evidence for the catalytic activity of surface-bound CrHydA1 in the production of hydrogen. Surface plasmon resonance (SPR) and gas chromatography are used to determine the specific hydrogen evolution activity of the immobilized enzymes.

2. Material and methods

2.1. Purification of Chlamydomonas [FeFe]-hydrogenase

Recombinant [FeFe]-hydrogenase CrHydA1 Strep-tagexp was produced as described before (von Abendroth et al., 2008). Briefly, C. acetobutylicum ATCC 824 recombinant strains were grown in CGM media in a 2.5 L MiniFors®-bioreactor (Infors, Augsburg, Germany) (Girbal et al., 2005; Wiesenborn et al., 1988). An optimized purification protocol was established for the heterologously synthesized CrHydA1 Strep-tag™ enzyme. Ultracentrifugation and affinity chromatography on a 10 mL Strep-Tactin Superflow® column (IBA, Göttingen, Germany) were applied. Cell growth and protein purification were carried out under strict anaerobic conditions. Isolated protein was concentrated to 5 mg mL⁻¹ on Vivaspin 60®-columns (Sartorius Stedim Biotech, Göttingen, Germany) and stored in 10% glycerol and 2 mM sodium dithionite for stabilization. Prior to use in spectroscopic or electrochemical experiments the sample was dialysed for 30 min on 0.025 mM V-series® membranes (Millipore, Schwalbach, Germany) against 10 mM sodium phosphate buffer solution (pH 6.8).

2.2. Surface modification—monitored by surface-enhanced infrared absorption spectroscopy (SEIRAS)

A thin nano-structured gold film was chemically deposited on one side of a triangular silicon prism, as described before (Ataka and Heberle, 2003, 2007). The prism was mounted at the bottom of the spectroscopic glass cell. The infrared beam, provided from the interferometer of the FT-IR spectrometer (Bruker IFS 66 v/s, Bruker Optics, Ettlingen, Germany) was coupled into the single reflection silicon prism at an incident angle of 60°. The beam was totally internally reflected and the intensity was measured by a mercury cadmium telluride (MCT) detector (Oswa et al., 1993; Ataka et al., 1996; Ataka and Oswa, 1998, 1999; Oswa, 2002).

The bare gold surface was incubated in an aqueous solution of 2 mM mercaptopropionic acid (MPA) or an ethanolic solution of 2 mM mercaptoethanodiacacid (MUA) for 60 min (Song et al., 1993; Sun et al., 1993; Chen et al., 2002; Xu and Bowden, 2006; Jiang et al., 2008). A reference IR spectrum of the solvent-covered surface was subtracted from a series of sample spectra, which were recorded after addition of the sample. 789–1578 scans were averaged for each sample and reference spectrum, respectively. After the self-assembled monolayer (SAM) of the heterobifunctional molecules was formed, the surface was first rinsed with the solvent several times and afterwards with 10 mM sodium phosphate buffer solution (pH 6.8). The kinetics of immobilization of 170 μg mL⁻¹ CrHydA1 were monitored by SEIRAS in time intervals of 30 and 60 s.

2.3. Cyclic voltammetry

A gold film or a massive gold electrode was used as a working electrode. Ag/AgCl/3 M KCl and platinum mesh were used as reference and counter electrode, respectively. The cyclic voltammograms were recorded at a sweep rate of 10 mV s⁻¹ with a potentiostat (Autolab PGSTAT 12, Eco Chemie B.V., Utrecht, Netherlands). All potentials are reported versus the normal hydrogen electrode (NHE).

The electrochemical experiments were performed at 20 °C in an anaerobic chamber (Coy Laboratory Products, Grassland, MI, USA) containing 95% nitrogen and 5% hydrogen. Palladium catalysts were used to remove oxygen contaminations by reduction with hydrogen. All solutions were degassed in vacuum for at least 30 min and stored in the anaerobic chamber for at least 2 weeks prior to use. Other equipment, which was transferred to the anaerobic chamber, was evacuated for at least 30 min. In this environment, the recorded cyclic voltammograms were totally stable and not influenced by oxygen during the measurement.

2.4. Amperometric hydrogen production

The electrochemical setup was embedded in a home-made, gastight measuring cell with a total volume of 20 mL. After surface modification the setup was purged with Argon for at least 10 min to remove the atmospheric hydrogen of the anaerobic chamber. 1 mL of the gas phase in the measuring cell was injected into a gas chromatograph (GC–2010, Shimadzu, Kyoto, Japan) equipped with a PLOT fused silica coating molsieve column (5A, 10 m by 0.32 mm; Varian, Palo Alto, CA) to gauge if the hydrogen-containing atmosphere was completely exchanged. Then a potential of −450 mV (vs NHE) was applied for 20 min while the current was monitored. 1 mL of the gas mixture was injected into a gas chromatograph to determine the amount of evolved hydrogen.

2.5. Surface plasmon resonance (SPR)

Surface plasmon resonance experiments were performed on a Biacore 3000 (GE Healthcare, Uppsala, Sweden) with a constant flow rate of 5 μL min⁻¹. Au Sensorchips were used to provide an untreated gold surface for each experiment. To form the SAM on the untreated gold surface, 300 μL of an aqueous 2 mM MPA solution were injected. For the immobilization of hydrogenase, the running buffer was changed from Millipore water to 10 mM
potassium phosphate buffer (pH 6.8) and 300 μL of CrHydA1 with a final concentration of 170 μg mL⁻¹ were injected.

2.6. In vitro activity essay

To probe hydrogen evolution activity under optimal conditions, 1–10 μg CrHydA1 were added to 2 mL of a 100 mM sodium phosphate buffer solution (pH 6.8), containing 1 mM methyl viologen and 100 mM sodium dithionite. This solution was sealed gas-tight in an 8 mL SUBA tube, purged with argon and incubated at 37 °C for 15 min afterwards. The amount of produced hydrogen was measured by gas chromatography (as described above) and the specific hydrogen-evolving activity of the hydrogenase (in μmol H₂ min⁻¹ mg hydrogenase⁻¹) were calculated.

3. Results and discussion

3.1. Immobilization of the hydrogenase

To design a surface which is able to reduce protons to molecular hydrogen, the catalyst, the [FeFe]-hydrogenase from C. reinhardtii CrHydA1 was immobilized on the surface of a solid gold electrode by electrostatic interaction. Immobilization was observed in situ by surface-enhanced IR absorption spectroscopy. Fig. 1 depicts the SEIRA spectra of CrHydA1 during the adsorption to the mercapto- propionic acid-modified surface (A). In Fig. 1(A) bands arise at 1659 and 1550 cm⁻¹. These bands are assigned to the amide I and amide II modes of the protein backbone (Krassen et al., 1986). The band intensities reflect the amount of protein adsorbed to the surface and, therefore, increase during the adsorption process. While the proteins bind to the surface, a negative band arises at frequencies > 1700 cm⁻¹, which overlaps with the amide I band. This band is assigned to water (H₂O-H bending mode), which is displaced from the vicinity of the surface. Signals from the bulk phase are negligible as the surface-enhancement decays exponentially with distance (decay length ~10 nm). The absence of any amide bands

Fig. 2. Adsorption kinetics of 170 μg mL⁻¹ CrHydA1 to an MUA–SAM (▲), an MPA–SAM (○) and a bare gold surface (●) are compared. The peak height of the amide II band at 1550 cm⁻¹ is normalized to 1 for maximum coverage and plotted versus the adsorption time.

upon injecting a similar protein solution to a unmodified gold surface in Fig. 1(B) shows that CrHydA1 does not bind to a bare gold surface.

The gold surface was modified by heterobifunctional molecules to increase the affinity of CrHydA1 to the surface. Mercapto- propionic acid or mercaptopoundecanoic acid was used to form a carboxy-terminated SAM. At the pH of 6.8, the surface is negatively charged and allows electrostatic binding of CrHydA1. The strength of the electrostatic interaction varies with the chosen SAM.

The adsorption kinetics of CrHydA1 to different surfaces are shown in Fig. 2. The peak height of the amide II band is normalized to 1 for maximum coverage and plotted versus the adsorption time. 90% of the maximum coverage with CrHydA1 on an MUA–SAM is reached after 5 min, while it takes 50 min on a MPA–SAM, which is also terminated by carboxy-groups, but less flexible due to the shorter chain length.

In both cases, the immobilized protein film is stable. Rinsing with buffer (10 mM potassium phosphate, pH 6.8) only removes the unspecifically bound protein. The peak height of amides I and II band decreases by less than 5% at the beginning, while further washing does not lead to further decrease in intensity (data not shown).

3.2. Probing the catalytic activity of the surface-bound CrHydA1

The electrochemical activity of CrHydA1-modified gold surfaces is compared among the different adsorption conditions by means of cyclic voltammetry. In the cyclic voltammograms of CrHydA1 on an MUA-modified surface (Fig. 3(A), dotted curve), no increase in the reductive current is observed. This indicates that electrons are not directly transferred from the electrode to the hydrogenase. After addition of the electron mediator methyl viologen the reductive current increases (solid curve) due to electron transfer from the electrode to MV²⁺. When increasing the potential, no oxidation peak appears, indicating that MV is oxidized by transferring electrons to the hydrogenase, where electrons are used to reduce protons to molecular hydrogen.

Fig. 3(B) shows the cyclic voltammogram of CrHydA1 on a bare gold electrode (dotted curve). If electrons would be transferred to the hydrogenase the amplitude of the reductive (“negative”) current would increase below the necessary potential. The absence of this feature proves that there is no direct electron transfer from a
bare gold electrode to CrHydA1 in solution. The addition of MV$^{2+}$ (solid curve) leads to a pair of reduction and oxidation peak with a mid-point potential of $-423\,\text{mV}$, as expected from literature for MV (Stombaugh et al., 1976). The oxidation peak at $-383\,\text{mV}$ is assigned to the re-oxidation of MV at the electrode and its appearance shows, that MV is not (or at a negligible rate) oxidized by the hydrogenase. No (or negligible) mediated electron transfer takes place between a bare gold electrode and CrHydA1, if the protein is not immobilized on the surface.

An increase of the reductive current after addition of CrHydA1 (Fig. 4, solid curve) to a 100 $\mu$M solution of MV$^{2+}$ on an MPA-modified electrode (dotted curve) is observed. Current in the dotted curve is comparable to the cyclic voltammogram of MV as displayed in Fig. 3(B). The reductive current at a potential of $-450\,\text{mV}$ reaches a value of $-47\,\mu\text{A}$, while it is only $-2\,\mu\text{A}$ on a MUA-modified electrode (Fig. 3(A)).

Two factors explain the difference in the catalytic activity of the immobilized hydrogenase on different SAMs: (1) Due to different chain length, the MUA–SAM is more flexible than the MPA–SAM. This results in a different surface structure and probably a different orientation of the hydrogenase. If the electron acceptor site of CrHydA1 gets closer to the surface of the SAM, the accessibility of the acceptor site might be restricted by steric hindrance. This would result in a smaller reductive current. (2) MV$^{2+}$ is not reduced on top of the SAM but partially penetrates the monolayer (see Supplementary informations). The diffusion distance from the place where MV$^{2+}$ is reduced to the electron acceptor site of CrHydA1 is larger for the MUA–SAM. This would result in a smaller reductive current as well.

3.3. Quantification of the hydrogen production

Cyclic voltammetry showed that electrons are transferred to the hydrogenase. However, it is not clear if the transferred electrons are consumed to reduce protons and produce hydrogen. This can be demonstrated by the direct detection of molecular hydrogen by gas chromatography. CrHydA1 is immobilized on a MPA-modified gold film electrode and covered with the MV-containing potassium phosphate buffer ($\text{pH} 6.8$). After a constant potential of $-450\,\text{mV}$ has been applied to this system for 20 min, 1 mL of the gas phase is injected into the gas chromatograph. The area of the hydrogen peak is calculated by integration to an average area of 140, which equates 20 nmol H$_2$ ($n(H_2)$) in the total volume.

While the potential is applied, the current is monitored by amperometry. The transferred charge is calculated to 25 $\mu$C by integration over 20 min, which equals a maximum theoretical H$_2$-production of $n_{\text{max}}(\text{H}_2)=130$ nmol, if all electrons are transferred to the hydrogenase. The catalytic efficiency $n_{\text{cat}}=n(H_2)/n_{\text{max}}(\text{H}_2)$ is calculated to 15% at the applied potential of $-450\,\text{mV}$.

Two factors contribute to the difference between calculated and measured hydrogen: (1) Electrons can be conducted from the gold electrode to the counter electrode without being used by the hydrogenase. (2) A fraction of the surface might be covered with protein which does not produce molecular hydrogen – either denatured during purification/dialysis or bound in an orientation which blocks the electron acceptor site – and transfers the electrons to other acceptors in the solution.

Just recently, Hambourger et al. reported immobilization of the [FeFe]-hydrogenase CoHydA from C. acetobutylicum on glassy carbon and carbon felt (Hambourger et al., 2008). Proteins can bind directly on the surface and receive electrons via direct electron transfer on certain forms of graphite. However, quantification of the bound enzymes is not possible on a graphite electrode, but on a gold electrode as presented here.

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3.4. Specific activity of immobilized hydrogenase

The specific hydrogen-evolving activity of recombinant CrHydA1 in solution is measured by established in vitro tests to 760 μmol H₂ min⁻¹ mg⁻¹ (Girbal et al., 2005). After dialysis, the average specific activity of the CrHydA1 samples used in our experiments drops to 130 μmol H₂ min⁻¹ mg⁻¹ corresponding to 8500 mol H₂ min⁻¹ mol⁻¹. This value defines the upper limit of the specific hydrogen-evolving activity of the immobilized CrHydA1.

Surface plasmon resonance was used to quantify the amount of immobilized CrHydA1. Binding of the protein to the MPA-modified surface and the exchange of the buffer with the protein solution change the refractive index and lead to an increase of the SPR response, respectively (Fig. 5). Before and after the injection, the surface is rinsed with buffer and the difference in the SPR response is solely attributed to the bound protein. These values are noted in Fig. 5 as ΔR₂, for each protein injection. During the first injection, the entire surface of the MPA–SAM is available, while in the following injections only the uncovered parts can bind proteins. Consequently, ΔR₂ and ΔR₁ are much smaller than ΔR₁. The sum of all three ΔR₂ = 2250 R.U. reflects the total amount of specifically bound hydrogenase and is calculated to 2.25 ng CrHydA1 mm⁻² (or 3.42 × 10⁻¹² mol cm⁻²) with the conversion of 1000 R.U. into 1 ng protein mm⁻² (Armstrong et al., 1947; Stenberg et al., 1991). The measured value indicates that a protein monolayer is adsorbed to the surface and the contribution of unspecific multilayer is negligible.

In the amperometric experiment 20 nmol H₂ have been produced in 20 min. The electrode used has a geometrical surface area of 1.45 cm² and a surface roughness of 2.5 (Miyake et al., 2002). From these values, the specific hydrogen-evolving activity of surface-bound CrHydA1 can be calculated to 1.3 μmol H₂ min⁻¹ mg⁻¹ or 85 mol H₂ min⁻¹ mol⁻¹, which is 1% of the activity in the in vitro test.

For the in vitro essay, the strong reductant sodium dithionite is added in excess to immediately reduce oxidized MV²⁺ in the vicinity of the electron acceptor site of CrHydA1 and maintains a constant high concentration of reduced MV. Using a protein monolayer, re-reduction of MV²⁺ takes place close to the electrode surface (tunnelling distance) and the reduced MV diffuses a longer distance to the hydrogenase, which limits the reaction rate. Another explanation for the comparatively low activity of the immobilized CrHydA1 is that the access to the electron acceptor site of CrHydA1 might be hindered by the MPA–SAM, compared to the protein in solution (see above). In addition the temperature is 20 °C for the monolayer experiments and 37 °C for the in vitro tests. This results in a reaction rate lowered by a factor of 3 (from thermodynamics).

3.5. Hydrogen evolution potential

The hydrogen evolution potential is defined as the most positive potential, which allows hydrogen production at the given surface. In Fig. 6 three cyclic voltammograms (pH 6.8) are compared on different electrodes. Each exhibits an increase in the reductive current, at potentials below its hydrogen evolution potential. On a bare gold electrode with methyl viologen as electron mediator, hydrogen evolution takes place below −460 mV (dotted line in Fig. 6). By modifying the gold surface with hydrogenase like CrHydA1, the hydrogen evolution potential is improved to −290 mV (solid line). This characteristic value is close to the potential of −270 mV on platinum surfaces (dashed line), which are used in the industry for electrochemical hydrogen production.

In summary, we demonstrated the successful immobilization of the [FeFe]-hydrogenase CrHydA1 from C. reinhardtii on a carboxy-terminated self-assembled monolayer. For the first time, a "wireless" [FeFe]-hydrogenase (i.e. without electron relay chain) was examined by protein film voltammetry. If methyl viologen is used as a soluble electron carrier, the CrHydA1-modified surface was able to catalyze the reduction of protons to molecular hydrogen at a similar potential as on platinum electrodes. At a potential of −450 mV 15–17% of the provided electrons are used for hydrogen production.

The possibility to investigate the bound hydrogenase by surface-enhanced infrared spectroscopy and to quantify the amount of bound protein via SPR makes the system a promising approach for in-depth analysis of the specific activity per molecule. This will make it possible to distinguish between different surface populations of the hydrogenase and optimize the electrode to highest activity. The system can be used to probe the specific redox activity and efficiency of variable hydrogenase and protein films in general – as a platform technology for further investigations.

Fig. 5. Surface plasmon resonance signal of the binding of CrHydA1 on a MPA-modified gold surface. The surface is continuously rinsed with buffer at a flow rate of 5 μL min⁻¹. At t = 0, 77, and 146 min CrHydA1 is injected for 1 h, respectively. The increase in the SPR response is indicated as ΔR₁ for each injection (ΔR₁ = 2014 R.U., ΔR₂ = 162 R.U., ΔR₃ = 78 R.U.).

Fig. 6. Cyclic voltammograms of hydrogen evolution in sodium phosphate buffer (pH 6.8) on the bare platinum electrode (dashed curve), on the bare gold electrode with MV in solution (dotted curve), and on the CrHydA1-modified electrode (solid curve). In the latter case, methyl viologen is used as soluble electron carrier. The measured current for the platinum electrode is divided by 20 for better comparison.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.jbiotec.2009.01.018.

References

Chlamydomonas reinhardtii


The Structure of the Active Site H-Cluster of [FeFe] Hydrogenase from the Green Alga* Chlamydomonas reinhardtii Studied by X-Absorption Spectroscopy†

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ABSTRACT: The [FeFe] hydrogenase (CrHydA1) of the green alga Chlamydomonas reinhardtii is the smallest hydrogenase known and can be considered as a “minimal unit” for biological H2 production. Due to the absence of additional FeS clusters as found in bacterial [FeFe] hydrogenases, it was possible to specifically study its catalytic iron–sulfur cluster (H-cluster) by X-ray absorption spectroscopy (XAS) at the Fe K-edge. The XAS analysis revealed that the CrHydA1 H-cluster consists of a [4Fe4S] cluster and a diiron site, 2Fe11, which both are similar to their crystallographically characterized bacterial counterparts. Determination of the individual Fe–Fe distances in the [4Fe4S] cluster (∼2.7 Å) and in the 2Fe11 unit (∼2.5 Å) was achieved. Fe–C (═O/N) and Fe–S bond lengths were in good agreement with crystallographic data on bacterial enzymes. The loss of Fe–Fe distances in protein purified under mildly oxidizing conditions indicated partial degradation of the H-cluster. Bond length alterations detected after incubation of CrHydA1 with CO and H2 were related to structural and oxidation state changes at the catalytic Fe atoms, e.g., to the binding of an exogenous CO at 2Fe11 in CO-inhibited enzyme. Our XAS studies pave the way for the monitoring of atomic level structural changes at the H-cluster during H2 catalysis.

Hydrogenases are efficient catalysts for the production and cleavage of molecular hydrogen (H2) (1, 2). Their use in biotechnological applications is expected to significantly contribute to future renewable fuel production (3, 4). The molecular principles of H2 turnover in these enzymes may lead to novel non-platinum catalysts (5). It is thus an important challenge to unravel the reaction mechanism of H2 catalysis in hydrogenases.

In biocatalysis the [FeFe] hydrogenases are of particular interest as they show extremely high rates of H2 production (6, 7). The structures of [FeFe] hydrogenases from two bacterial species, namely, Clostridium pasteurianum and Desulfovibrio desulfuricans, have been resolved by protein crystallography (8, 9). Their active site is a unique iron–sulfur cluster, comprising six Fe atoms commonly known as the H-cluster (6, 10). It can be divided into a [4Fe4S] cluster and a binuclear iron unit, 2Fe11, cysteine-linked to the cubane. This diiron moiety is probably the active site of H2 catalysis (8, 11, 12). This situation differs from that in the [NiFe] hydrogenases where a heterobimetallic complex forms the active site (13). Depending on the redox conditions, the two Fe atoms of 2Fe11 bind three to four CO and two CN ligands. One CO may be found in an Fe–Fe bridging position (14–17). An azadithiolate (adt) moiety has been proposed, but the precise chemical identity of this ligand on top of the 2Fe11 site is not yet settled (12, 18). Besides the H-cluster, bacterial [FeFe] hydrogenases harbor up to four additional FeS clusters, serving as electron transfer relays (19). Signal overlay from these clusters complicates investigations on the reaction cycle of the bacterial H-cluster by Fe-specific techniques, i.e., Mössbauer (20) and X-ray absorption spectroscopy (XAS) (18, 21, 22).

[FeFe] hydrogenases exist not only in anaerobic bacteria but also in photosynthetic eukaryotes. For several green algae, the hydA genes and respective proteins have been identified (23–26). These extraordinarily small proteins may be regarded as a “minimal unit” of biological H2 conversion. Sequence alignments show that the small F-domain carrying the FeS relay clusters in bacteria is missing (24, 25). However, four cysteine residues necessary for H-cluster accommodation in the H-domain are well conserved in all hydrogenase-coding hydA genes (23).

The biophysical characterization of algal [FeFe] hydrogenases is hampered by the fact that these enzymes are extremely O2 sensitive and synthesized in vivo only in small amounts (23, 28). To overcome low protein yields, we have established a general

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§Abbreviations: 2Fe11, binuclear Fe unit of the H-cluster; adt, azadithiolate; A1, as-isolated state; C-Pi, Clostridium pasteurianum [FeFe] hydrogenase; DdH, Desulfovibrio desulfuricans [FeFe] hydrogenase; EPR, electron paramagnetic resonance spectroscopy; EXAFS, extended X-ray absorption fine structure; FTIR, Fourier-transform infrared spectroscopy; CrHydA1, [FeFe] hydrogenase protein of C. reinhardtii; MS, multiple scattering; XAS, X-ray absorption spectroscopy.
system for heterologous expression and synthesis of [FeFe] hydrogenases in *Clostridium acetobutylicum*. The [FeFe] hydrogenase from *Chlamydomonas reinhardtii*, CrHydA1, is efficiently assembled due to the maturation apparatus of the host organism, yielding 2 mg of protein/L of cell culture (28). Hence, a sufficient amount of CrHydA1 [FeFe] hydrogenase is available for in-depth spectroscopic explorations.

CrHydA1 is an ideal candidate to study the assembly, structure, and catalytic mechanism of [FeFe] hydrogenases. First, EPR studies on CrHydA1 and two further algal [FeFe] hydrogenases revealed similar signals of their CO-inhibited state of the active site as found in bacterial enzymes. However, deviations in the EPR g-tensors suggested distinct differences in the electronic structure of the H-cluster (27). Direct information on the geometric structure of the CrHydA1 H-cluster is required to compare it to the situation in bacteria.

In the present study X-ray absorption spectroscopy (XAS) (29) at the Fe K-edge was performed to obtain atomic level structural and electronic information on the H-cluster of CrHydA1. We show that the structural parameters imply an overall organization of the algal H-cluster similar to that in bacterial enzymes. Structural alterations induced by the inhibitor CO and the substrate H$_2$ are addressed.

**MATERIALS AND METHODS**

**Protein Sample Preparation.** *C. reinhardtii* [FeFe] hydrogenase CrHydA1 was heterologously synthesized and isolated as previously described (30). *C. acetobutylicum* ATCC 824 recombinant strains were grown in CGM media containing up to 60 g/L glucose in a 2.5 L MiniFors bioreactor (Infors, Augsburg, Germany) (28, 31). Both cell growth and protein purification were carried out under strictly anaerobic conditions. Isolated protein was concentrated up to 1 mM (about 48 mg/mL) on Vivaspin 6 and Vivaspin 500 columns (Sartorius Stedim Biotech, Göttingen, Germany) and stored in 10% glycerol and 2 mM sodium dithionite (NaDT) for stabilization.

The specific H$_2$-evolving activity was determined by an *in vitro* assay as described before (29). The gas mixture was injected into a gas chromatograph (GC-2010; Shimadzu, Kyoto, Japan) equipped with a PLOT fused silica coating Molsieve column (5 Å, 10 m by 0.32 mm) from Varian (Palo Alto, CA). The specific activity of the hydrogenase was calculated from the detected amount of produced H$_2$.

For gas treatments, protein samples (~1 mM) were filled into open 200 µL PCR tubes and placed in 8 mL Suba tubes, which were rubber sealed under the N$_2$/H$_2$ atmosphere of the anaerobic tent. To humidify the working gases (CO, H$_2$), degassed water was bubbled with each of the two gases for 30 min, respectively. The headspace of the hermetically sealed Suba tubes was flushed with gas for 15–30 min. The final protein concentration of the samples used at the DESY Hamburg was adjusted right after the gas treatments; hence, more diluted protein was flushed. During the treatments, protein was kept on ice and protected against light. For the “as-isolated” samples (AI), no gas treatment was applied. Here, A${}^\text{Red}$ denotes an (optimal) enzyme isolation procedure in the presence of 2 mM NaDT, whereas A${}^\text{Sim}$ samples are preparations without any reducing agents (NaCl was used instead of NaDT to adjust the ionic strength of the buffers). Samples were filled into Kapton-covered acrylic-glass sample holders for XAS and frozen in liquid nitrogen. EPR measurements on the XAS samples (not shown) revealed no indications for significant unspecific iron in the as-isolated reduced CrHydA1 samples as the “rhombic iron” signal at a g-value of ~4 was practically absent. The absence of water-dissolved Fe ions in as-isolated reduced CrHydA1 samples was also likely according to the K-edge spectra, which showed no evidence for Fe–O bonds (see Results).

**X-ray Absorption Spectroscopy (XAS).** Ka fluorescence-detected XAS spectra at the Fe K-edge were collected at 20 K using energy-resolving Ge detectors and helium cryostats as previously described (22, 32) at beamline D2 of the EMBL outstation (HASYLAB, DESY, Hamburg, Germany) and at beamline KMC-1 of BESSY (Berlin, Germany). Harmonic rejection was achieved by detuning of the Si(111) double-crystal monochromators to 50% of their peak intensities. The energy resolution of the used monochromators was ~3 eV at DESY and optimized by appropriate setting of aperture slits and slightly lower, ~4 eV, at BESSY due to the beamline specifics (33). The slightly different energy resolution of the XAS spectra does not affect the conclusions drawn from the data analysis. Spectra were collected maximally for a scan range of 6950–8450 eV, i.e., up to $k = 19.5 \text{ Å}^{-1}$, within ~1 h each. Dead time-corrected XAS spectra were averaged after energy calibration of each scan using the peak at 7112 eV in the first derivative of the absorption spectrum of an Fe foil as an energy standard (estimated accuracy ±0.1 eV) (18). EXAFS oscillations were normalized and extracted as described in refs 29 and 34. The energy scale of EXAFS spectra was converted to the wave vector scale (k-scale) using an $E_0$ value of 7112 eV. Unfiltered $k^2$-weighted spectra were used for least-squares curve fitting employing a curved-wave multiple-scattering (MS) approach with the program EXCURV (35). In our hands, the EXCURV program is able to reproduce the expected coordination numbers of Fe–ligand interactions in Fe model compounds with known structure from fitting of respective EXAFS oscillations ranging up to k-values of 20 Å$^{-1}$ (18). An amplitude reduction factor, S$_0^2$, of 0.9 was used in the EXAFS fits. EXAFS fits initially were based on a model of the H-cluster derived from the crystal structures (8, 9). The CO and CN ligands were classified as units, with almost linear arrangement of the Fe–C=O/N atoms (bond angles close to 180°). For the units, correlation was enabled. A maximal path length in MS calculations of 10 Å and extending over a maximal scattering of 4 of employed. Debye–Waller factors (2σ$^2$) were either fixed at physically reasonable values (see Table 2) or varied in the simulations. Fourier transforms (FTs) were calculated from $k^2$-weighted EXAFS data using the program SimX (29) and employing cos$^2$ windows ranging over 10% at both ends of the k-range. From experimental K-edge spectra the preedge peak region was extracted by subtraction of a polynomial spline through the main edge rise using the program XANDA (36). K-edge energies were determined by the “integral method” (29), using integration limits of 15% and 90% of normalized intensity.

**RESULTS**

**As-Isolated Reduced State of CrHydA1.** Purification of the protein under strictly anaerobic and reducing conditions (30) to obtain the as-isolated state of CrHydA1 (AI$^\text{Red}$) yielded the most active protein (Table 1). Iron XAS spectra of A${}^\text{Red}$ were collected up to a k-value of 19.5 Å$^{-1}$, i.e., to ~1450 eV above the Fe K-edge at ~7120 eV (Figure 1). This very long k-range for XAS on proteins allows for resolution even of closely spaced interatomic distances (34, 37).
Table 1: Specific H₂-Evolving Activities of CrHydA1 Preparations

<table>
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<th>sample</th>
<th>specific H₂ evolving activity (μmol of H₂ mg⁻¹ min⁻¹) ± 20</th>
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The K-edge spectrum of AIred (Figure 1C) was perfectly reproducible in independent protein samples, indicating the high quality of the CrHydA1 preparation. Its small primary maximum at ∼7130 eV revealed the predominant coordination of Fe by sulfur ligands (22, 38) as expected due to the presence of the respective conserved cysteines in CrHydA1. Indications for oxygen ligation of Fe, as expected for oxidatively modified (or damaged) FeS species (22), were absent.

The Fourier transform (FT) of the AIred EXAFS spectrum shows three major peaks (Figure 1A), at reduced distances between ∼1.5 and ∼2.5 Å (the reduced distance is the true interatomic distance minus ∼0.4 Å due to a phase shift). This immediately suggests at least three Fe↔backscatterer shells, i.e., of Fe−C(≡O/N), Fe−S, and Fe−Fe vectors. A shoulder on the high-distance side of the FT peak due to Fe−Fe interactions likely accounted for the expected Fe(C)≡O/N interactions, the contributions of which to the EXAFS spectrum usually are enhanced by multiple scattering (MS) effects due to the almost linear Fe−C≡O/N arrangement (see below). The FT peaks due to Fe−C(≡O/N) and Fe−(C)=O/N interactions may be expected to be similarly large (18). Here, the magnitude of the peak due to Fe−(C)=O/N appears to be diminished due to partial cancelation of respective EXAFS oscillations by interference with EXAFS contributions from the Fe−Fe interactions. A fourth peak at ∼2.2 Å reduced distance became more prominent when the FT was calculated starting at higher k-values (Figure 1A, inset) where metal→metal interactions dominate the EXAFS oscillations (shown in Figure 1B). This result was suggestive of a Fe−Fe distance of 2.5−2.6 Å in the H-cluster. The Fe−Fe distance of the 2Fe₅₂ unit in crystal structures of bacterial [FeFe] hydrogenases is about 2.5 Å, whereas the Fe−Fe distances in the [4Fe₄S] cluster are ∼2.7 Å (8, 9). Thus, the EXAFS data apparently allow to discriminate between the Fe−Fe distances in the [4Fe₄S] and the 2Fe₅₂ subgroups of the C. reinhardtii H-cluster.

Figure 1: XAS analysis of as-isolated reduced CrHydA1 (AIred) at the Fe K-edge. (A) Fourier transform of the EXAFS spectrum in (B). The FT was calculated from k-values ranging over 4.7−19.5 Å⁻¹. Inset: FTs calculated from k-ranges starting at 7.4, 4.9, and 1.6 Å⁻¹ (top to bottom); vertical lines mark contributions from Fe−C(≡O/N) vectors (dots) and from the Fe−Fe vector in 2Fe₅₂ (dashed). (A) and (B): thin lines, experimental data; thick lines, simulations (Table 2C); the dotted line in (A) is a simulation with parameters in Table 2A. (C) Fe K-edge spectra of two AIred preparations measured at DESY (line) and BESSY (dots).
emergence of CO-inhibited species by cannibalization of free CO from denatured protein (17). However, these impurities were not detected in Al\text{red}. A larger apparent coordination number than the expected one of 0.33 (due to two Fe–Fe distances of ~2.5 Å per six Fe ions) of the Fe–Fe distance attributed to 2Fe\text{ox} may suggest contributions from similarly long Fe–S vectors. In the structure of reduced [FeFe] hydrogenase from C. pasteurianum the distance of FeS, at the 2Fe\text{ox} site to the cysteine sulfur atom which links it to the [4Fe4S] cluster is ~2.50 Å, and the Fe–Fe distance in 2Fe\text{ox} is only slightly longer, ~2.55 Å. Thus, such a long Fe–S distance may contribute to the apparent coordination number of the Fe–Fe interaction. We note that in the crystal structures all further Fe–S bonds are shorter than about 2.4 Å, and such distances would not interfere with the 2.5 Å feature.

The coordination number of the Fe–Fe distance of ~2.7 Å (N\text{2,7}) was very close to 2 (2.02) if it was allowed to vary in the fit (Table 2). This value is similar to the one of 2.0 that is calculated for the presence of only the H-cluster in CrHydA1 (i.e., for 12 Fe–Fe distances of ~2.7 Å in the cubane moiety per 6 Fe ions). In the [FeFe] hydrogenases of \textit{C. pasteurianum} and \textit{D. gigas} (CpI and \textit{CpII}, respectively), four and two additional FeS clusters are found so that the N\text{2,7} values would be expected to be significantly larger, ~2.27 and ~2.52, respectively. Thus, our XAS data suggest, in concert with previously performed EPR experiments (27, 30) and multiple sequence alignments (23–25), that only the H-cluster is present in CrHydA1 and further FeS clusters are absent.

A significant further improvement of the fit quality (R\text{F} less than 10%) was achieved by including separate shells for the Fe–C(=O) and Fe–C(=N) interactions and further slight parameter adjustments (Table 2C; Figure 1A,B, thick lines). Now, individual distances for Fe–C(=O) of 1.77 Å and Fe–C(=N) of 1.98 Å were obtained. These distances are close to those of the respective ligands in the crystal structures, where the Fe–C(=O) distance usually is shorter than Fe–C(=N) (8, 9).

We note that the above used “top-down” simulation approach, i.e., starting the EXAFS simulations with a model based on the crystal structures of the H-cluster in bacteria and then refining the structural parameters by additional degrees of freedom in the fit, leads to the same simulation results that were obtained by an inverse (“bottom-up”) procedure where the structural model was developed by the stepwise inclusion of increasing numbers of coordination shells in the fit (not documented).

An unusual bridging ligand (which perhaps is an azadithiolate (adt) ligand) has been proposed to be present in the 2Fe\text{ox} part of the bacterial H-cluster and may serve crucial functions in H\textsubscript{2} production (9, 14, 44–47). The respective carbon and nitrogen or oxygen atoms would be within a range of about 3–4 Å to the Fe atoms of 2Fe\text{ox}. As such distances overlap with those from Fe–C(=O)/N interactions and only weak contributions from these atoms to the EXAFS spectra were expected, the nature of the bridging species in the \textit{C. reinhardtii} H-cluster cannot be deduced from the XAS data.

### **H-Cluster Integrity in Protein from Two Purification Conditions**

The integrity of the active site was compared in as-isolated CrHydA1 samples purified under reducing (Al\text{red}) and mildly oxidizing (Al\text{ox}) conditions (Figure 2). The specific H\textsubscript{2}-evolving activity of Al\text{ox} samples, as determined by the \textit{in vitro} assay, was only slightly lower than in Al\text{red} (Table 1). The Fe K-edge was at ~0.5 eV higher energies in Al\text{ox} compared to Al\text{red} (Figure 2A), indicating that overall more reduced Fe was present in Al\text{red}. Both K-edges were at higher energies than that for Fe(II) species due to the presence of Fe(III) ions in the cubane cluster (48, 49). The shift of the edge to lower energies in Al\text{red} was compatible with the reduction of at least one Fe(III) ion to the Fe(II) state. Furthermore, the preedge peak, due to formally doped-forbidden 1s –> 3d electronic transitions, was increased in Al\text{ox} (Figure 2A, inset). This suggests a less symmetric overall Fe coordination (40), presumably due to the binding of oxygen species to some Fe atoms in Al\text{ox} (see below). Lowering of the site symmetry is expected to cause increased admixtures of metal p levels to the 3d electronic orbitals, increasing the probability of electronic transitions into unoccupied 3d levels and thus the intensity of the respective preedge feature. Due to the limited energy resolution of the monochromator at BESSY, possible spectral substructure on the preedge peaks, as related, i.e., to electronic multiplet interactions (40, 50), remained invisible.

The FTs of EXAFS spectra revealed a pronounced decrease of the peak due to Fe–Fe interactions in Al\text{ox} (Figure 2B, arrow). EXAFS simulations (using a simplified approach) revealed a

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<th>2\sigma (Å)</th>
<th>R\text{F} (%)</th>
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\textsuperscript{a}N\textsubscript{C} coordination number; R\text{F}, Fe–ligand distance; 2\sigma, Debye–Wallen factor. \textsuperscript{b}Fixed values. \textsuperscript{c}0.2\% restricted to values >0. \textsuperscript{d}The sum of N\textsubscript{C}=0.00 values was 1. N\textsubscript{C}=0.00 was coupled to yield equal values for the three spectra. R\text{F}(29) was calculated over reduced distances of 1.3–2.8 Å. All simulations comprise a further multiple-scattering Fe(–C)=O/N shell (with the same N value as for the Fe–C(=O)/N shell); respective distances were in the range of 2.95–3.02 Å; 2\sigma was set to 0.01 Å. |
The protein to the medium, forming hexaquo such an effect was expected if Fe ions had become released from suggested overall more symmetric coordination of the Fe atoms. Possibly due to reduction of H-species (at 0.1 Å). A similar increase of Fe–Fe distance was observed in 2Fe₄H when Fe distance of 2Fe₄H. The respective EXAFS spectrum (Figure 3B) showed only minor changes compared to AI°red. The main difference in the structure of the H-cluster, according to EXAFS simulations (Table 2E), was a decreased apparent Fe–C(=O/N) distance and a slightly decreased Fe–Fe distance of 2Fe₄H in H₂-treated enzyme. Although this small decrease in the Fe–Fe distance may not be significant, it could be caused by the presence of one more oxidized Fe atom (likely Fe₄ of 2Fe₄H; d = distal) due to the binding and reduction of H-species. Notably, the Fe–Fe distance of the [4Fe4S] cluster was not affected by the H₂ treatment (Table 2E).

Carbon monoxide is an effective inhibitor of [FeFe] hydrogenases. In a crystallographic structure for H₄ox–CO of C₃I, electron density at Fe₄ was discussed to be exogenous CO (4I). FTIR and EPR analyses support this notion (4I, 5I). Under certain redox conditions, an endogenous CO is believed to form a “bridge” between the Fe atoms of the 2Fe₄H moiety (6, 4I). A decreased preedge peak in CO-treated CrHydA1 (Figure 3A) was in agreement with an overall more symmetric Fe coordination upon binding of additional CO to 2Fe₄H. The EXAFS data (Figure 3B) revealed an increased coordination number of the Fe–C(=O/N) interactions accompanied by enhanced respective multiple scattering contributions to the spectrum and an overall reduced mean FeC(=O/N) distance (Table 2E). These findings indicate the presence of one to two surplus short Fe–C(=O) interactions. In comparison to AI°red, the Fe–Fe distance of 2Fe₄H was increased significantly by ~0.1 Å. A similar increase of the Fe–Fe distance has been observed in crystal structures of CO-treated bacterial [FeFe] hydrogenase, where one extra CO was found at Fe₄ (4I). Presumably, in CrHydA1 an extra CO also was bound to Fe₄, causing more symmetric (near-octahedral) coordination of this ion.

**Figure 2:** XAS comparison of as-isolated reduced (AI°red) and oxidized (AI°ox) CrHydA1. (A) K-edge spectra (inset, isolated preedge features). (B) FTs of EXAFS spectra (dotted line, AI°ox; solid line, AI°red). FTs were calculated over a k-range of 1.6–12.5 Å⁻¹; the arrow marks the contributions mainly from Fe–Fe interactions. Inset: Fourier isolates over reduced distances of 0.5–3.0 Å (thin lines, experimental data) and simulations according to Table 2D (thick lines).

**Figure 3:** Effects of gas treatment on CrHydA1 CO binding and H₂ reduction. (A) K-edges of indicated CrHydA1 samples. (B) FTs of EXAFS spectra (thin lines, calculated as in Figure 2) and simulations (thick lines, Table 2E). H₂ and CO treatments in (B) were carried out on concentrated protein. Vertical dashed and dotted lines mark shifts in the respective FT peaks due to Fe–S and Fe–Fe interactions.
DISCUSSION

XAS on biological metal centers (BioXAS) is a powerful tool to derive atomic resolution structural information, to determine the electronic configuration, especially in those states which are not accessible by EPR spectroscopy, and to monitor their dynamics during the catalytic reactions (29, 32–55). Here, we used XAS at the Fe K-edge to specifically investigate the catalytic cofactor of biological H₂ turnover in an [FeFe] hydrogenase protein.

The structural parameters from the present XAS study strongly suggest that the overall atomic organization of the H-cluster in the [FeFe] hydrogenase of the green alga C. reinhardtii is very similar to that found in crystallographically characterized enzymes from bacteria. Recent EPR investigations performed on CrHydA1 support this notion (27, 30). Furthermore, the XAS data strongly suggest that in CrHydA1 no additional Fe₅S clusters are found, as opposed to bacterial hydrogenases (2, 8, 9). EXAFS analysis allowed precise determination of interatomic distances in the H-cluster. The individual Fe—Fe distances in the [4Fe4S] part and 2Fe₄ motif of the H-cluster were measured (precision on the order of ~0.02 Å). Changes of the structural parameters upon treatments of CrHydA1 with the inhibitor CO and the substrate H₂ were detected, allowing to address changes, e.g., in catalytic intermediates. The XAS-derived interatomic distances, because of their higher resolution compared to protein crystallography (56), may help to optimize in silico structures of the H-cluster in DFT calculations (57–59).

There are also limitations of the XAS analysis. By the applied methods, predominantly average coordination environments of the six Fe atoms in the cluster were obtained. Characterization of the individual structure and oxidation state of the Fe atoms, in particular in the 2Fe₄ unit, and of the binding sites of substrate and inhibitors is highly desirable. It may be facilitated by future investigations employing site-selective XAS techniques (60). It has been proposed that an azadithiolate is bridging the Fe atoms of the 2Fe₄ site and is essential in H₂ turnover (12, 18, 45–47, 61). The respective C, N, and O atoms are almost impossible to detect by XAS because their distances to Fe are relatively large and overlap with the Fe(C)≡O/N distances and with contributions of atoms from the protein backbone. Thus, discrimination between different bridging species likely cannot be obtained by XAS.

The integrity of the algal H-cluster is easily perturbed, as observed for CrHydA1 purified under mildly oxidizing conditions and upon incubation of dilute protein with H₂ and CO. Such conditions seem to cause the release of Fe ions from the protein into the medium and, hence, at least partial degradation of the H-cluster. Preliminary evidence was obtained that the [4Fe4S] cluster is the primary target of oxidative modification, whereas the 2Fe₄ motif may be more robust. Oxidation of Fe₅S clusters is well-known (62, 63). Purification of CrHydA1 under reducing conditions prevents such deleterious effects and stabilizes an intact H-cluster. In bacterial [FeFe] hydrogenases, the H-cluster is deeply buried in the protein. Induced-fit folding models of CrHydA1 (M. Winkler and T. Happe, unpublished results) suggest that its [4Fe4S] cluster is located just beneath the surface whereas the 2Fe₄ motif lies inside the side of the protein. This arrangement is likely to allow for easy access of gas molecules and redox partners from the bulk to the [4Fe4S] unit and for its rapid modification by O₂.

Treatments of CrHydA1 with H₂ and CO revealed relatively subtle but discernible structural changes of the H-cluster. Inter-


Green algae such as *Chlamydomonas reinhardtii* synthesize an [FeFe] hydrogenase that is highly active in hydrogen evolution. However, the extreme sensitivity of [FeFe] hydrogenases to oxygen presents a major challenge for exploiting these organisms to achieve sustainable photosynthetic hydrogen production. In this study, the mechanism of oxygen inactivation of the [FeFe] hydrogenase CrHydA1 from *C. reinhardtii* has been investigated. X-ray absorption spectroscopy shows that reaction with oxygen results in destruction of the [4Fe-4S] domain of the active site H-cluster while leaving the di-iron domain (2FeH) essentially intact. By protein film electrochemistry we were able to determine the order of events leading up to this destruction. Carbon monoxide, a competitive inhibitor of CrHydA1 which binds to an Fe atom of the 2FeH domain and is otherwise not known to attack FeS clusters in proteins, reacts nearly two orders of magnitude faster than oxygen and protects the enzyme against oxygen damage. These results therefore show that destruction of the [4Fe-4S] cluster is initiated by binding and reduction of oxygen at the di-iron domain—a key step that is blocked by carbon monoxide. The relatively slow attack by oxygen compared to carbon monoxide suggests that a very high level of discrimination can be achieved by subtle factors such as electronic effects (specific orbital overlap requirements) and steric constraints at the active site.

EXAFS | H-cluster | protein film electrochemistry | biological hydrogen production | green algae

Hydrogenases are ubiquitous in bacteria and archaea but are also found in some eukaryotes, particularly green algae (1). There are three distinct classes, known as [NiFe]-, [FeFe]-, and [Fe] hydrogenases, based on the metal components of the active site that binds or releases H2 (2).

Many hydrogenases have extremely high activities (3), a fact that has been emphasized most recently in studies by protein film electrochemistry (4–6). Hydrogenases are able to catalyze both H2 oxidation and H2 evolution with minimal electrochemical overpotential (driving force) (4, 7), comparable to the 2H+/H2 equilibrium established on platinum (5). The [FeFe] hydrogenases are of particular interest as they tend to be more biased toward H2 evolution than [NiFe] hydrogenases (8). The active site of [FeFe] hydrogenases, a complex structure known as the “H-cluster,” consists of a binuclear Fe center (2FeH) linked to a [4Fe-4S] cluster (9). Numerous publications report the chemical synthesis of analogues for the 2FeH domain and even the entire H-cluster (10)—such is the interest displayed not only in understanding the enzymes, but also in finding cheap alternatives to Pt catalysts. However, [FeFe] hydrogenases are extremely prone to irreversible inactivation by O2, and this sensitivity is a key challenge for both the biotechnological and the synthetic chemistry approaches (8).

Viewed in detail, the 2FeH domain consists of iron atoms Fe9 and Fe10 that are, respectively, proximal and distal relative to the [4Fe-4S] domain that is connected to Fe9 by a bridging cysteine sulfur (9). An unusual dithiolate ligand, originally modeled as a 1,3 propane dithiolate, forms a bridge between Fe9 and Fe10. In the oxidized state H+Fe9 as determined from the structure of the CpI enzyme from *Clostridium pasteurianum*, Fe9 is also coordinated by one CO and one CN− ligand and shares a bridging CO with Fe9 (11, 12). The distal Fe is also coordinated by one CO and one CN− ligand, and an additional binding site is vacant (9) or occupied by an exchangeable O ligand, most likely a water molecule (11). In the structure of the [FeFe] hydrogenase from *Desulfovibrio desulfuricans*, which is believed to be crystallized in the HFe9 form, the bridging CO is replaced by a terminal CO on Fe9 (13). A recent EPR analysis favors an oxidation state assignment of [4Fe-4S]<sup>I</sup>−Fe9(1)Fe9(2) for HFe9, with some spin density delocalized onto the [4Fe-4S] domain (14). The EPR-silent HFe9 state is assigned as [4Fe-4S]<sup>I</sup>−Fe9(1)Fe9(2) or a hydrido species [4Fe-4S]<sup>I</sup>−Fe9(2)Fe9(2)H<sup>+</sup> and it appears that the [4Fe-4S] cluster may not access the 1+ level in anything other than a transient manner (15). Crystallography and infrared spectroscopy has shown that exogenous CO, a competitive inhibitor, attacks 2FeH at the vacant/exchangeable binding site of Fe9 (16–18). Carbon monoxide is a σ-acceptor ligand and binds to electron-rich transition metals (19). From a molecular orbital perspective, H2 resembles CO because, by analogy with the Dewar-Chatt-Duncanson model for binding of alkenes to metals (20), back donation of electron density into the antibonding σ-orbital of molecular H2 is important for its binding and activation (20). These facts are highly relevant because they form the basis for CO being competitive with H2 (21) during H2 oxidation, and therefore, Fe9 is likely to be the site for H2 binding (17). Like CO, O2 is also a σ-acceptor ligand and likely to bind to the same site(s); we can anticipate that any such binding could result in the generation of highly reactive oxygen intermediates (22, 23).

Here, we present a study of the mechanism by which O2 irreversibly attacks the H-cluster, by using electrochemical kinetics with the reversible inhibitor CO as a complementary probe. By using X-ray absorption spectroscopy (XAS) at the Fe K-edge, we examine, at atomic level resolution, the nature of the product obtained. Our subject is the [FeFe] hydrogenase CrHydA1 from the photosynthetic green alga *Chlamydomonas reinhardtii*. The [FeFe] hydrogenases of green algae are of particular interest for photosynthetic H2 production (8) and because this class of hydrogenase enzymes contain only the H-cluster (24–26) it is possible to interpret the XAS data without interference from additional electron-donating FeS clusters that are present in bacterial hydrogenases (9, 11). The results


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lead to a mechanistic model of how [FeFe] hydrogenases are inactivated by O₂.

Results

Protein Film Electrochemistry. Cyclic voltammograms recorded for C. reinhardtii [FeFe] hydrogenase CrHydA1 at 1 bar H₂ show the relative activities for H⁺ reduction and H₂ oxidation (Fig. 1). The graph displays two distinct scans obtained at pH 6.0 and pH 8.0 at 20 °C. The potential is swept from −0.55 V to +0.24 V vs. SHE and then scanned back to −0.55 V. The enzyme is clearly a bidirectional hydrogenase; at pH 6.0 (solid line), the catalytic activities for H₂ reduction compared to H₂ oxidation are approximately comparable, whereas at pH 8.0 (dashed line), the activity for H⁺ reduction is much lower, most obviously because the H⁺ concentration is two orders of magnitude lower. In either case the voltammograms cut through the zero-current line (potential axis) at the potential expected for the 2H⁺/H₂ redox couple under these conditions. The slight inflection detectable in this region (dashed oval in Fig. 1) suggests that a small overpotential is required for efficient electron transport to and from the active site. At high potentials (> 0 V vs. SHE, see circles) the enzyme inactivates, giving rise to the anaerobically oxidized state [H₂-inact (7)]. This is apparent from the decrease in H₂ oxidation current, which recovers at least partially on the return scan. The reversibility of this inactivation depends on pH, and it is much more reversible at pH 6.

The kinetics of O₂ inactivation of H₂ oxidation were investigated by chronoamperometric experiments in which the current was monitored following changes in gas composition. The catalytic current is a direct measure of enzymatic turnover rate. Fig. 2 shows the dependence of inactivation rate on O₂ level (Fig. 2A) and H₂ level (Fig. 2B). In all experiments the cell potential was set to −0.05 V vs. SHE to optimize the H₂ oxidation rate while avoiding anaerobic inactivation (see circles in Fig. 1). Experiments were performed at pH 6.0, 20 °C. Reactions with O₂ were initiated by changing the gas composition flowing in the headspace of the cell and simultaneously injecting a solution of buffer preequilibrated with the desired gas composition. This combination of operations ensured rapid initiation and a constant O₂ level throughout the reaction. An important feature of these experiments was that the H₂ concentration in solution always remained constant. Control experiments were performed to assess the current contribution because of direct reduction of O₂ at the graphite electrode, although this was small at −0.05 V (see SI Text and Fig. S1). In all cases O₂ caused almost complete inactivation (>95%).

Fig. 2. Inactivation of CrHydA1 by O₂ by simultaneous gas exchange and injection of O₂-saturated buffers. Experiments were carried out under (A) different concentrations of O₂ and (B) different concentrations of H₂. The H₂ concentration in solution always remained constant (potential axis) at the potential expected for the 2H⁺/H₂ redox couple under these conditions. The slight inflection detectable in this region (dashed oval in Fig. 1) suggests that a small overpotential is required for efficient electron transport to and from the active site. At high potentials (> 0 V vs. SHE, see circles) the enzyme inactivates, giving rise to the anaerobically oxidized state [H₂-inact (7)]. This is apparent from the decrease in H₂ oxidation current, which recovers at least partially on the return scan. The reversibility of this inactivation depends on pH, and it is much more reversible at pH 6.

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10% CO (blue trace). In the third experiment (position in the gas stream throughout all experiments. In the first competitive inhibitor of H

composition (∼0.2 mM in solution) as displayed in Fig. 3B (see below). Fig. 2B shows that the rate of inactivation of H2 oxidation by O2 depends on the H2 concentration (80% H2 compared to 8% H2).

Fig. 3 shows experiments in which CO, known to be a competitive inhibitor of H2 oxidation (21), is used to probe the course of O2 attack. Fig. 3A shows three experiments conducted at pH 6.0, 20 °C, and −0.05 V vs. SHE. The timeline shown in the lower panel provides a guide for the sequence of gas changes. (b) Dependence of rate of inactivation on concentration of O2 (diamonds, red) and CO (squares, blue). Note the rates of inactivation by CO were calculated by performing experiments such as those shown in Fig. 2, that is, by simultaneous injection of CO-saturated buffer and gas exchange.

The decrease in current because of inhibition by CO (ii) is much more rapid than it is for O2 (i), and the comparative rates of inhibition at different inhibitor concentrations are shown in Fig. 3B. For comparison the second-order rate constants for inhibition by CO and inactivation by O2 (obtained from the slopes of the lines in Fig. 3B) are 50 mM−1 s−1 (46 bar−1 s−1) and 0.43 mM−1 s−1 (0.47 bar−1 s−1), respectively. Also, it is clear from Fig. 3A that, when the CO is removed from the gas stream, the current increases to a level consistent with that expected based on natural protein film loss from the electrode measured in control experiments over the same period, showing that CO inhibition of H2 oxidation is reversible. However, removal of O2 from the cell only results in a tiny increase in current, that is, O2 inactivation is almost (but not entirely) irreversible. Experiment (iii) in Fig. 3A demonstrates that CO protects the enzyme against inactivation by O2 as 80% of the initial activity is recovered on removal of CO from the gas stream. This result agrees with the observation reported recently for another [FeFe] hydrogenase CrHydA from Clostridium acetobutylicum (27).

**X-Ray Absorption Measurements.** Fig. 4 depicts XAS spectra of the as-isolated reduced form (Hred) and after O2 incubation (Hredox). The reduced distance is the true metal-backscatterer distance minus approximately 0.4 Å because of a phase shift. (A) Fe K-edge spectra. Inset: isolated preedge features due to 1 s→3d electronic transitions. The shown preedge features were derived by subtraction of a polynomial spline from the main edge rise by using the program Xanda. (B) FTs of EXAFS spectra (see Fig. S2) of Hred (solid line) and Hredox (open circles). The FTs were calculated for k values of 1.6–16.5 Å−1 (7.4–19.5 Å−1 in the inset). Numbers on the FT peaks denote specific Fe-ligand interactions as discussed in the text. The Fe-Fe distance of 2Fe0 of 2.52 Å (FT peak III) is discernable under both conditions; the Fe-Fe distances of approximately 2.7 Å (FT peak IV) from the [4Fe-4S] cluster are largely diminished in Hredox.
at approximately 7,125 eV and a decreased preedge amplitude (# at ~7,111 eV, inset) in H_{red} clearly suggest the binding of O-atoms ligands to Fe ions that subsequently became more symmetrically coordinated (25). This result may be explained by binding of additional O-species to the H-cluster and partial release of Fe from the protein in the form of hexaquo-Fe(II) ions.

Fourier transforms (FTs) of EXAFS spectra for H_{red} and H_{air} are shown in Fig. 4B. The FT of the H_{red} spectrum (solid line and inset) shows four main peaks I - IV. The reduced distances given in the figure are approximately 0.4 Å smaller than the true metal-ligand distances because of a phase shift. These peaks reflect different Fe interactions of the H-cluster, namely Fe-C (= O/N) (I), Fe-S (II), and Fe-Fe (IV) (25). An additional peak III became visible in H_{air} when the FT was calculated from the corresponding EXAFS oscillations starting at higher k-values and extending over a longer k-range (inset, see Fig. S2). This feature represents the Fe-Fe interaction in the 2Fe_{H} moiety (25). Peak III in the spectrum of H_{air} is also attributable to this Fe-Fe interaction. Diminished Fe-Fe interactions from the cubane cluster in the H_{air} spectrum allow for discrimination of peak III even in FT spectra calculated from a k-range starting at lower values. Contributions to the EXAFS spectra from multiple-scattering (MS) effects of the near-linear Fe-C = O/N arrangements are small (25) because of their relatively low coordination number and interference with the Fe-Fe interactions of the [4Fe-4S] cluster.

Precise Fe-ligand distances were determined by simulations of the EXAFS spectra. The coordination numbers per Fe ion (N_{i}) and the Fe-ligand and Fe-Fe distances (R_{i}) for H_{red} (Table 1) are compatible with the expected structure of the reduced H-cluster H_{red} in CrHydA1. Whereas H_{ox} is reported to be in the Fe_{ox}(I)-Fe_{ox}(II) state and has a ‘bridging’ CO between Fe_{p} and Fe_{d}, H_{red} is assigned as Fe_{p}(I)-Fe_{p}(I) and lacks the bridging CO that has become a terminal ligand to Fe_{p} (14, 17, 24, 25). In particular, the Fe-Fe distances of 2.52 Å for the 2Fe_{H} moiety (III, inset in Fig. 4B) and 2.71 Å for the [4Fe-4S] moiety (IV) can clearly be distinguished by EXAFS.

The FT of the EXAFS spectrum of the O_{2}-treated enzyme (H_{air} in Fig. 4B, open circles) reveals a missing contribution from Fe-Fe interactions of the [4Fe-4S] cluster (IV in H_{red}). In addition, a shift of the main FT peak (II) to shorter distances is observed. This is because of contributions from Fe-O bonds, which are shorter than Fe-S bonds. Peak I (representing Fe-C (= O/N) interactions in H_{red}) appears decreased in size in H_{air}. This attributable to interference between Fe-O and Fe-C (= O/N) contributions that cannot easily be discriminated. For H_{air} the 2Fe_{H}-specific FT feature III is even more distinct than in H_{red} (inset). Peaks exclusively appearing in the H_{air} spectrum are marked with asterisks. Peak IV*, which is indicative of MS contributions from the C (= O/N) ligands is resolved more clearly because peak IV is essentially diminished in H_{air}. That the Fe atoms of the [4Fe-4S] cluster may remain bound to the protein but coordinate additional O-ligands is suggested by the observed FT peak V*, which may correspond to long Fe-O-Fe binding motifs (28) prominent in H_{oxair} only.

Simulations of the H_{air} EXAFS spectrum (Table 1) revealed that in H_{air} the Fe-Fe distance in 2Fe_{H} is 0.04 Å longer than the Fe-Fe distance in H_{red} (2.56 and 2.52 Å, respectively), with a coordination number lowered by only ~20%. This is compatible with a Fe-Fe distance elongation upon oxidation from H_{red} to H_{air}CO which we reported recently (25). Accordingly, a fraction of approximately 80% of protein may be calculated to retain a normal 2Fe_{H} unit in which the overall structure is preserved upon O_{2} treatment. Distance elongation may be caused by binding of O-species to 2Fe_{H}. In contrast, there was a 2-fold decrease of the approximately 2.7 Å Fe-Fe interactions (from N_{i} 2.06 to 0.97 in H_{air}) per Fe ion, because of the loss of respective motifs in the [4Fe-4S] cluster. Therefore, in more than half of the protein molecules the native structure of the [4Fe-4S] cluster is degraded.

In summary, the XAS results show that exposure of CrHydA1 to O_{2} causes modification or destruction of the [4Fe-4S] domain of the H-cluster, with the coordination shell of the 2Fe_{H} domain remaining relatively unchanged.

**Discussion**

The electrocatalytic response of CrHydA1 shows that the enzyme exhibits very similar characteristics to other hydrogenases (30). In electrochemical experiments, the enzyme’s activity is directly measured through the catalytic current. The inflection point at the zero-current potential observed for CrHydA1 contrasts with the sharp intersection with the zero-current axis exhibited by other (bacterial) hydrogenases (6, 7, 27). This may be because of the absence of an electron-transfer relay in CrHydA1 (26) and reflects a small overpotential requirement to drive electrons in either direction. Catalytically, the enzyme is bidirectional and its proficiency in H_{red} air is also precedent for CO directly attacking [4Fe-4S] clusters in proteins.

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<th>Table 1. EXAFS fit results</th>
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R_{i} (39) was calculated over reduced distances of 1.3–2.8 Å. Both simulations comprise a further multiple-scattering Fe-C(=O/N) shell (with the same N-value as for the Fe-C(=O/N) shell; average respective distances of 2.98 Å; 2σ was set to 0.01 Å²), N_{i} coordination number; R_{i} Fe-ligand distance; 2πR_{i}, Debye-Waller factor.

*Fixed values in the fit procedure.
The observation from EXAFS, that the fate of the H-cluster in its reaction with O$_2$ is a modification of the [4Fe-4S] domain rather than of the 2Fe$_{Cyt}$ domain, in conjunction with the protective effect of CO, together mean that the reaction of O$_2$ with the 2Fe$_{Cyt}$ domain (as argued above) generates a species that subsequently attacks the [4Fe-4S] domain. In Fig. 5, we consider two mechanisms by which this could occur. The first option involves O$_2$ reacting at the 2Fe$_{Cyt}$ domain to form a ROS, particularly superoxide, that migrates the short distance to attack the [4Fe-4S] domain. The second option involves superoxide being formed by reaction with the 2Fe$_{Cyt}$ domain but remaining bound and exert its destructive effect by causing a through-bond electron transfer from the [4Fe-4S] cluster (B).

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mixture reaching the cell headspace, or (ii) by a simultaneous change in gas mixture in the headspace and injection of an aliquot of solution equilibrated with high gas. Efficient mixing and gas-solution equilibration were achieved through rapid electrode rotation (3,000 rpm).

**X-Ray Absorption Measurements.** Kα-fluorescence-detected XAS spectra at the Fe K-edge were collected at T = 20 K using an energy-resolving 13-element Ge detector and a helium cryostat as previously described (28,29) at beamline D2 of the EMBL outstation (at HASYLAB, DESY). Harmonic rejection was achieved by detuning of the Si (111) double-crystal monochromator to 50% of its peak intensity. Spectra were collected maximally for a scan range of 6,950–8,450 eV. Deadtime-corrected XAS spectra were averaged after energy calibration of each scan by using the peak at 7,112 eV in the 1st derivative of the absorption spectrum of an Fe-foil as an energy standard (estimated accuracy = 0.1 eV) (28,29). Data were then normalized, and extended EXAFS oscillations were extracted (39). The energy scale of EXAFS spectra was converted to the wavevector scale (k-scale) by using an E0 value of 7,112 eV. Unfiltered k2-weighted spectra were used for least-squares curve-fitting employing a multiple-scattering approach with the program EXCURV (40). Fourier transforms were calculated from k2-weighted EXAFS data by using the program SimX (39) and employing cos2 windows ranging >10% at both ends of the k-range. From experimental K-edge spectra the preedge peak region was extracted by using the program Xanda (www.bit.ly/1V1zkE).

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Electrochemical Kinetic Investigations of the Reactions of [FeFe]-Hydrogenases with Carbon Monoxide and Oxygen: Comparing the Importance of Gas Tunnels and Active-Site Electronic/Redox Effects

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Abstract: A major obstacle for future biohydrogen production is the oxygen sensitivity of [FeFe]-hydrogenases, the highly active catalysts produced by bacteria and green algae. The reactions of three representative [FeFe]-hydrogenases with O2 have been studied by protein film electrochemistry under conditions of both H2 oxidation and H2 production, using CO as a complementary probe. The hydrogenases are DdHydAB and CaHydA from the bacteria Desulfovibrio desulfuricans and Clostridium acetobutylicum, and CrHydA1 from the green alga Chlamydomonas reinhardtii. Rates of inactivation depend on the redox state of the active site ‘H-cluster’ and on transport through the protein to reach the pocket in which the H-cluster is housed. In all cases CO reacts much faster than O2. In the model proposed, CaHydA shows the most sluggish gas transport and hence little dependence of inactivation rate on H-cluster state, whereas DdHydAB shows a large dependence on H-cluster state and the least effective barrier to gas transport. All three enzymes show a similar rate of reactivation from CO inhibition, which increases upon illumination: the rate-determining step is thus assigned to cleavage of the labile Fe-CO bond, a reaction likely to be intrinsic to the atomic and electronic state of the H-cluster and less sensitive to the surrounding protein.

Introduction

The increasing need for clean, renewable fuels is stimulating new research on hydrogen (H2) production,1−5 and one promising solution is to exploit microorganisms in ‘H2 farms’. In biology, H2 is evolved by metalloenzymes called hydrogenases, in processes ranging from fermentation to photosynthesis. Hydrogenases are highly efficient enzymes—so much so that when attached to an electrode, they are, like platinum, superb electrocatalysts of both H2 oxidation and H2 production, at or close to the reversible potential for the 2H+/H2 couple.6−8

Of the two main classes, [NiFe]- and [FeFe]-hydrogenases, named according to the metals present in the center at which H2 is activated, the [FeFe]-hydrogenases are considered to be more active in H2 production.9 However, a perceived major disadvantage of [FeFe]-hydrogenases (with respect to [NiFe]-hydrogenases) is their higher O2 sensitivity.10 The [NiFe]-hydrogenases react rapidly with O2 to give inactive, EPR-characterized, Ni(III) forms that can be reactivated by reduction: Ni-A (‘unready’) is reactivated very slowly whereas Ni-B (‘ready’) can be reactivated within seconds, hence there is a rapid repair mechanism for hydrogenases that produce only Ni-B.5,6,11 In contrast, the [FeFe]-hydrogenases appear to undergo irreparable damage when exposed to O2 while in their active state (after reduction).12–14 The incompatibility of O2 with [FeFe]-hydrogenases poses a major limitation to progress in ‘biohydrogen’ production, in particular by modified photosyn-
thesis.\textsuperscript{15} In green algae the O\textsubscript{2}-sensitivity of the [FeFe]-hydrogenase is the bottleneck for producing H\textsubscript{2} from sunlight. Production of H\textsubscript{2} is stimulated during sulfur deprivation, conditions under which only 10\% of the photosystem II remains active and the system effectively becomes anaerobic.\textsuperscript{16} Green algae that could express an O\textsubscript{2}-tolerant [FeFe]-hydrogenase would therefore provide much increased levels of H\textsubscript{2} production.\textsuperscript{15}

The buried active site of [FeFe]-hydrogenases is actually a complex 6Fe unit known as the ‘H-cluster’ which contains a [4Fe-4S] subcluster (generally referred to as [4Fe-4S]\textsubscript{H}) in addition to the di-iron subcluster (2Fe\textsubscript{H}).\textsuperscript{17} The two independent representations of the H-cluster shown in Figure 1 are directly relevant to the catalytically active states known as H\textsubscript{ox} and H\textsubscript{red} that have been extensively characterized.\textsuperscript{18,19} General features of the structure are as follows: a) the [4Fe-4S]\textsubscript{H} subcluster is linked to one of the Fe atoms of the 2Fe\textsubscript{H} subcluster by a bridging cysteine sulfur (the Fe atoms of 2Fe\textsubscript{H} are thus known as ‘proximal’ (Fe\textsubscript{p}) and ‘distal’ (Fe\textsubscript{d}) with respect to the [4Fe-4S]\textsubscript{H} subcluster);\textsuperscript{20} b) both Fe\textsubscript{p} and Fe\textsubscript{d} are coordinated by CO and CN\textsuperscript{−} ligands;\textsuperscript{17} c) an unusual SCH\textsubscript{2}CH\textsubscript{2}S dithiolate ligand forms a di-µ-thiolato bridge between Fe\textsubscript{p} and Fe\textsubscript{d}, and although opinions differ as to whether the bridgehead atom X is an O or N atom,\textsuperscript{21–25} recent investigations with \textsuperscript{15}N HYSCORE have provided direct evidence that X = N.\textsuperscript{21}

In the structure of H\textsubscript{ox}, as determined with the Cpl enzyme from Clostridium pasteurianum, Fe\textsubscript{p} is coordinated by one CO and one CN\textsuperscript{−} ligand and shares a bridging CO with Fe\textsubscript{d}.\textsuperscript{20} In turn, Fe\textsubscript{d} is also coordinated by one CO and one CN\textsuperscript{−} ligand, and an additional binding site is vacant or occupied by an exchangeable O-ligand, most likely a water molecule (Figure 1A). In the structure of the [FeFe]-hydrogenase from Desulfovibrio desulfuricans, which should be in the H\textsubscript{red} form, the bridging CO is replaced by a terminal CO on Fe\textsubscript{d} (Figure 1B).\textsuperscript{26} Recent EPR spectroscopic investigations favor an oxidation state assignment of [4Fe-4S]\textsuperscript{2+}Fe(II)Fe(II) for H\textsubscript{ox}, with some spin density delocalized onto the [4Fe-4S]\textsubscript{H} subcluster,\textsuperscript{27} although H-clusters from different enzymes show minor variations in electronic structure.\textsuperscript{28} The EPR-silent H\textsubscript{red} state is assigned as [4Fe-4S]\textsuperscript{2+}Fe(II)Fe(II) which, if protonated, is formally equivalent to the hydrido species [4Fe-4S]\textsuperscript{2+}Fe(II)Fe(II)H\textsuperscript{−}\textsuperscript{17} As also determined by EPR spectroscopy, exogenous CO, a competitive inhibitor of H\textsubscript{2} oxidation, reacts with H\textsubscript{ox}\textsuperscript{29} Crystallographic and infrared spectroscopic studies of H\textsubscript{ox}-CO further show that binding of CO (which is photolabile) occurs at Fe\textsubscript{p}.\textsuperscript{30–33} Inactivation by anaerobic oxidants gives rise to a form known as H\textsubscript{max}; usually formulated as [4Fe-4S]\textsuperscript{2+}Fe(II)Fe(II), which can be reactivated upon reduction—a process occurring via an intermediate known as H\textsubscript{max} which has been formulated as [4Fe-4S]\textsuperscript{2+}Fe(II)Fe(II), i.e. with the [4Fe-4S]\textsubscript{H} subcluster reduced.\textsuperscript{33} The H-cluster is remarkable among non-macrocycle cofactors because the 2Fe\textsubscript{H} subcluster at which H\textsubscript{2} is produced is connected to the protein through just a half-share of a cysteine sulfur; it is very much an organometallic-like compound physically enclosed in protein.

Despite these intense studies by crystallography and spectroscopy, numerous aspects of the reactions of [FeFe]-hydrogenases remain unresolved. These aspects include the activation process (there is evidence from electrochemical titrations that a two-electron process is also involved)\textsuperscript{33} and many details of the mechanism of catalysis in either direction, including the exact function of the [4Fe-4S]\textsubscript{H} subcluster. The nature and products of the degradation by O\textsubscript{2} are only now coming to light,\textsuperscript{34} and a major issue is whether and how H\textsubscript{2} production could be sustainable at all in the presence of O\textsubscript{2}.

This article describes mechanistic investigations, by protein film electrochemistry, of the O\textsubscript{2} inactivation kinetics of three representative [FeFe]-hydrogenases. These are: the hydrogenase...
from a sulfate-reducing bacterium, Desulfovibrio desulfuricans, 
abbreviated as DdHydAB which has been crystallographically 
characterized;\textsuperscript{28} the hydrogenase from Clostridium acetobutylicum, 
abbreviated as CaHydA, which is potentially of importance 
for \( \text{H}_2 \) production by anaerobic fermentation and has high 
sequence similarity with the crystallographically characterized 
CpI hydrogenase from C. pasteurianum,\textsuperscript{25} and the hydrogenase 
known as CrHydA1 from the green alga Chlamydomonas 
reinhardtii, which is of interest for photosynthetic \( \text{H}_2 \) production.
Both DdHydAB and CaHydA contain a series of Fe–S 
clusters\textsuperscript{25} to relay electrons within the protein for transfer to 
and from the redox partner (in our case the electrode); in contrast 
CrHydA1 possesses no Fe–S clusters apart from the \([4\text{Fe}-4\text{S}]_0 \) 
subcluster,\textsuperscript{35,36} but it is nonetheless electrocatalytically active 
when adsorbed on an electrode.\textsuperscript{34} Although the three enzymes 
differ in their overall tertiary and quaternary structures, their 
H-domains that house the H-cluster are very similar.\textsuperscript{17}

In protein film electrochemistry, an enzyme is immobilized 
on the surface of an electrode such that its properties are 
controlled directly by the electrode potential.\textsuperscript{6,38} Catalytic 
activity in either direction, oxidation or reduction, can be driven 
and recorded at any particular potential value and the catalytic 
rate is directly proportional to the current that flows. Various 
gas mixtures (produced by mass-flow controllers) can be 
introduced and flushed from the sealed electrochemical cell in 
which the electrode is rotated rapidly to provide precise 
hydrodynamic control (supply of reactants and removal of 
products). A particular advantage of this approach is that 
turnover activity in either direction is immediately and directly 
observed from the catalytic current; thus, \textit{rates of change} 
of activity, such as those induced by \( \text{CO} \) or \( \text{O}_2 \), are extracted 
directly from the variation of current with time, all as a precise 
function of the electrode potential. In this way, extremely 
complex reactivities become resolvable; therefore, this technique 
both complements and instigates structural and spectroscopic 
investigations.

We first establish, for each enzyme, how \( \text{H}_2 \) oxidation and 
\( \text{H}_2 \) production are affected by the concentrations of \( \text{H}_2 \) and \( \text{CO} \); next we examine the kinetics of CO binding and release and 
correlate these data with equilibrium values; we then examine the 
kinetides of \( \text{H}_2 \) inactivation of \( \text{H}_2 \) oxidation activity; finally, 
we exploit CO inhibition as a tool to investigate \( \text{H}_2 \) production 
in the presence of \( \text{O}_2 \). Recent studies have established that CO 
is able to protect \([\text{FeFe}]\)-hydrogenases against inactivation by \( \text{O}_2 \).\textsuperscript{34,35} an observation suggesting that the sequence of 
destruction is initiated by \( \text{O}_2 \) coordinating to the same site at which 
exogenous CO binds, i.e. \( \text{Fe}_{\text{ex}} \).\textsuperscript{30–33} Thereafter, the mechanism 
remains less clear, but recent EXAFS evidence obtained with 
CrHydA1 shows that the \([4\text{Fe}-4\text{S}]_0 \) subcluster is altered more than the \( 2\text{FeS} \) subcluster.\textsuperscript{35} Our experiments show clearly how 
the destructive power of \( \text{O}_2 \) varies among the hydrogenases with 
an interesting dependence on catalytic direction (\( \text{H}_2 \) oxidation 
compared to \( \text{H}_2 \) production)—thus implicating sensitivity to the 
level of oxidation of the active site. The results provide insight 
for the quest for solutions to the oxygen problem in bioglycerin

Results

Measurements of the H₂ Concentration Dependencies for H₂ Oxidation and Production. We first evaluated the affinity of the enzymes for H₂ both in terms of Kₐ for H₂ as the substrate in H₂ oxidation (KₐH₂) and the apparent inhibition constant KᵢH₂ for H₂ as the product inhibitor of H² reduction. Figure 2 shows experiments carried out for CaHydA: analogous experiments were carried out for CrHydA1, but films of DdHydAB were not sufficiently stable to obtain accurate measurements over the period of time required (30–60 min.). Experiments to determine KₐH₂ were conducted by varying the ratio of H₂ to N₂ in the headgas and measuring the oxidation current after allowing time for the gas mixture to equilibrate with the cell solution at each concentration of H₂ (Figure 2A). The experiments were performed at an electrode potential of −0.05 V to avoid the anaerobic inactivation that occurs at higher potential (see Figure 3). We and others, have noted that Kₐ and Kᵢ are potential-dependent quantities,⁴⁴,⁴⁵ and the potential must therefore be specified. It was also important to make measurements under conditions where the current was limited by the catalytic rate of the enzyme rather than by mass transport of substrate to (or product from) the electrode. The experiments were therefore carried out at low temperature (10 °C) to decrease the rate of catalysis. The low temperature also minimized the rate of film loss. At each H₂ concentration the electrode rotation rate was stepped between 3000 and 5000 rpm to ensure that the current (and thus the rate of reaction) was independent of rotation rate. Values of KᵢH₂ were calculated from the x-intercept of the Lineweaver–Burk plot shown in Figure 2B and are summarized in Table 1. Experiments to measure the apparent inhibition constant (KᵢH₂), defined in eq 1 where KₛH₂ is the Kₛ

Table 1. Values of KₛH₂ for CrHydA1 and CaHydA

<table>
<thead>
<tr>
<th>enzyme</th>
<th>KₛH₂/µM</th>
<th>KᵢH₂/µM</th>
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<tbody>
<tr>
<td>CrHydA1</td>
<td>0.19 ± 0.03</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>CaHydA</td>
<td>0.46 ± 0.06</td>
<td>6.2 ± 1.1</td>
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</table>

* KₛH₂ and KᵢH₂ were calculated at −0.05 and −0.4 V, respectively.

Figure 2. Determination of KₛH₂ (A and B) and KᵢH₂ (C and D) for CaHydA. (Panel A) chronoamperometric experiment performed at −0.05 V, at pH 6, 10 °C. The broken line traces the progression of film loss throughout the experiment. (Panel B) Lineweaver–Burk plot from experiment shown in A fitted to a straight line. (Panel C) chronoamperometric experiment performed to determine KᵢH₂ at −0.4 V, at pH 6.0, 10 °C. The concentration of H₂ in N₂ (%) in the headspace of the cell is indicated. The rotation rate was varied from 3000 to 4000 and 5000 rpm at each concentration of H₂ to ensure that inhibition was not mass-transport limited. (Panel D) Plot according to the procedure described by Léger et al.⁴⁴ from which KᵢH₂ is determined, showing the line of best fit.

Figure 3. Cyclic voltammograms showing bidirectional electrocatalytic H² reduction and H₂ oxidation by CrHydA1 (A), CaHydA (C) and DdHydAB (E) at pH 6.0 under 1 bar H₂. Panels B, D, F show the time-dependent voltammograms recorded following introduction of CO, introduced prior to the scans shown here, as the potential was being cycled between −0.55 V and +0.3 V, as it is removed from the cell: these reveal different reactions as CO dissociates and rebinds as a function of potential. Experimental conditions for CrHydA1: 10 °C, scan rate 20 mV/s, inhibition was achieved by injection of CO-saturated buffer to give an immediate concentration of 100 µM CO in solution 5 min prior to the scan shown in panel B. Experimental conditions for CaHydA: 32 °C, electrode rotation 3000 rpm, scan rate 20 mV/s, inhibition was achieved by flushing 100% CO through the cell 5 min prior to the scan shown in panel D. Experimental conditions for DdHydAB: 10 °C, electrode rotation 3000 rpm, scan rate 10 mV/s, inhibition was achieved by injection of CO-saturated buffer to give an instant concentration of 30 µM CO in solution 5 min prior to the scan shown in panel F. The dashed arrows indicate the direction of scanning. The asterisks (*) indicate the potential above which anaerobic inactivation takes place for CrHydA1 and DdHydAB.
for binding of the substrate \( \text{H}^+ \) and \( K_i \) is the real inhibition constant) were conducted at \(-0.4 \) V vs SHE using a similar experimental method (Figure 2C). Values of \( K_i^{app} \) were calculated by adapting the method based on that reported by Léger et al.\(^{45} \) which requires plotting the data according to eq 2 (in which \( i_{\text{S}} \) is the current recorded under 100% \( \text{N}_2 \) and \( i_{\text{H}_2} \) is the current recorded at each concentration of \( \text{H}_2 \)) as shown in Figure 2D. The \( K_i^{app} \) values are included in Table 1.

\[
K_i^{app} = \frac{K_i[\text{H}^+]}{K_M^M} \left( 1 + \frac{K_M^M}{[\text{H}^+]^2} \right)
\]

\[
(i_{\text{S}}/i_{\text{H}_2}) - 1 = \frac{[\text{H}_2]}{K_i^{app}}
\]

The \( K_M^{M} \) values are much higher than those we have measured recently for some \( \text{O}_2 \)-tolerant [NiFe]-hydrogenases\(^{46} \) and they suggest that the catalytic current for \( \text{H}_2 \) oxidation will be sensitive to changes in \( \text{H}_2 \) levels even close to 1 bar partial pressure (this is particularly so for \( \text{CaHydA} \)). The results showed that headspace \( \text{H}_2 \) levels should be maintained constant in quantitative \( \text{H}_2 \) oxidation experiments. In contrast, the \( K_i^{app} \) values are so high that the presence of \( \text{H}_2 \) is not expected to pose a problem in studies of \( \text{H}_2 \) production. The order of magnitude difference between \( K_i^{app} \) and \( K_M^{M} \) suggests immediately that the electronic/catalytic state of the \( \text{H} \)-cluster exerts a strong effect on binding affinity, with \( \text{H}_2 \) binding more weakly to a more reduced state.

**Reactions with CO.** Cyclic voltammograms of the electrocatalytic activities of \( \text{DdHydAB} \), \( \text{CrHydA1} \), and \( \text{CaHydA} \) at pH 6.0 under a flow of 100% \( \text{H}_2 \) are shown in Figure 3 (A, C, E—left-hand column). These voltammograms show that all the enzymes are bidirectional, with \( \text{CaHydA} \) being particularly biased in the direction of \( \text{H}_2 \) production. The traces in either potential direction cut through the zero current axis at the cell potential (see vertical line) for the \( 2\text{H}^+/2\text{H}_2 \) couple. At high potentials, all three enzymes undergo inactivation to give a species that is most likely \( \text{H}_4 \) \text{inact} although this process is very slow for \( \text{CaHydA} \). The voltammograms in the right-hand column (B, D, F) were recorded during the efflux of CO that had been introduced by injecting a saturated solution (giving a concentration of 100 \( \mu \)M CO in the cell for the experiment on \( \text{CrHydA1} \), 30 \( \mu \)M for the experiment on \( \text{DdHydAB} \)) or flowing 100% CO briefly through the cell (for \( \text{CaHydA} \)) as the potential was cycled between \(-0.55 \) and 0 V prior to the scans shown in panels B, D, and F.

Informative changes in the voltammograms of \( \text{DdHydAB} \), \( \text{CrHydA1} \) and \( \text{CaHydA} \) occur upon removal of CO during continuous cycling (B, D, F). In all the voltammograms show that once the potential is made sufficiently positive to start \( \text{H}_2 \) oxidation, there is an initial increase in current which is followed by a decrease (see the dashed arrow indicating the oxidative sweep). The magnitude of this effect is most apparent after a certain time has elapsed, dependent upon the hydrogenase, and finally the voltammograms resume the expected shapes for \( \text{H}_2 \) oxidation and production at pH 6 under 1 bar \( \text{H}_2 \) analogous to those shown in the left-hand column. This cyclic ‘inhibitor-on/inhibitor-off’ behavior shows that CO binding to the active site is favorable and fast during \( \text{H}_2 \) oxidation but relatively weak during \( \text{H}_2 \) production. Thus CO reinitiates strongly as the potential is raised to oxidize \( \text{H}_2 \) which addresses a more oxidized form of the enzyme (above \(-0.3 \) V). The

![Figure 4. Inhibition of DdHydAB (red lines), CrHydA1 (blue lines) and CaHydA (black lines) by injection of 4 \( \mu \)M CO at (A) \(-0.05 \) V (\( \text{H}_2 \) oxidation) and (B) \(-0.4 \) V (\( \text{H}_2 \) production). Experimental conditions: pH 6.0, 10 °C, 1 bar \( \text{H}_2 \), electrode rotation 3000 rpm. The final level of the current reached upon recovery of \( \text{H}_2 \) oxidation activity of CaHydA is marked by the dashed line in A. The exponential decrease in concentration of dissolved CO is shown by the gray trace (details given in Supporting Information, Figure SI.2).](image-url)

The voltammogram for \( \text{CrHydA1} \) also shows an oxidation peak soon after commencing the scan in the negative direction. A similar observation was reported in previous experiments on \( \text{DdHydAB} \) where it arises from the reactivation of some \( \text{H}_4 \) \text{inact} and its rapid inactivation by CO.\(^{47} \)

Further insight into CO binding and release under different conditions is provided by Figure 4. The CO inhibition profiles for \( \text{DdHydAB} \), \( \text{CrHydA1} \) and \( \text{CaHydA} \) were obtained for \( \text{H}_2 \) oxidation (Panel A) or \( \text{H}_2 \) production (Panel B) by injecting an aliquot of CO-saturated solution and then recording the catalytic current as the CO is removed by flushing. The same experimental conditions (pH 6.0, 10 °C, 1 bar \( \text{H}_2 \), electrode rotation 3000 rpm) were used for all experiments. In each case, injection of CO-saturated buffer gives an immediate initial CO concentration of 4 \( \mu \)M which decreases exponentially to zero,\(^{48} \) as depicted by the gray trace (right y-axis) which represents the dependence of [CO] on time throughout the experiment; < 0.2 \( \mu \)M CO remains in solution after 500 s. (See Supporting Information (Figure SI.2) for how this dependence is determined.) In the case of \( \text{CaHydA} \), inhibition continues to increase even though most of the CO has been flushed out of the cell, indicating that the rate of reaction with CO is slow. For \( \text{H}_2 \) oxidation, the rates and extent of inhibition reached after CO injection decrease in the order \( \text{DdHydAB} > \text{CrHydA1} > \text{CaHydA} \). The reactivation rates are slow and strikingly similar for all enzymes. For \( \text{H}_2 \) production (panel B) the rates and extent of inhibition by CO again decrease in the order \( \text{DdHydAB} > \text{CrHydA1} > \text{CaHydA} \).


The term $K_{IC}^{CO}$ (equil) depends on $H_2$ concentration and potential, but we kept these variables constant (80% $H_2$, −0.05 V). These titrations were similar in design to those shown in Figure 2C and are described in more detail in Supporting Information (Figure S1.3). Values of $K_{IC}^{CO}$ (equil) are shown in Table 2. Comparable data for $Dd$HydAB could not be obtained because the enzyme was fully inhibited at the lowest practical $CO$ concentrations.

Panel B of Figure 5 shows that CO inhibition of $H_2$ production of these enzymes is only partially reversible. This observation was consistently made with $Ca$HydA and $Cr$HydA but the instability of $Dd$HydAB films prevented us from making similar measurements with this enzyme. By comparison, CO inhibition is fully reversible when measuring $H_2$ oxidation at −0.05 V. In all cases, the background decrease in current that we refer to as film loss (traced by the dashed line) was checked through control experiments carried out without introducing $CO$. The rates determined for the inactivation of $H_2$ production by CO are therefore approximate. The potential dependence of the rate of inactivation of $H_2$ production was not investigated further, although we noted that the inactivation process became noticeably biphasic as the potential was lowered below −0.4 V. We raise this issue later in the Discussion.

Panels C and D of Figure 5 show that: (i) $Dd$HydAB is always the fastest to react with CO and $Ca$HydA the slowest, and (ii) for all three enzymes, inhibition of $H_2$ oxidation measured at −0.05 V is substantially faster than inhibition of $H_2$ production at −0.4 V, but the same order $Dd$HydAB > $Cr$HydA > $Ca$HydA is observed in both catalytic directions. The second-order rate constants ($k_{react}$) are provided in Table 2 (see later).

In all cases, the reactivation rates ($k_{react}$) are the same, within reasonable error, for $H_2$ oxidation and $H_2$ production. Experiments carried out with $Cr$HydA1 and $Ca$HydA showed that $k_{react}$ is strongly light sensitive (see Supporting Information, Figure S1.1) in agreement with earlier electrochemical observations made with $Dd$HydAB.\textsuperscript{47} In addition, $k_{react}$ increased significantly when the temperature was raised to 25 °C. From the ratio of rate constants for the reactivation and inactivation reactions at 10 °C, we derived the kinetic inhibition constants $K_{IC}^{CO}$ (kin) = $k_{react}$/[$H_2$]. In Table 2, $K_{IC}^{CO}$ (kin) values are compared with the equilibrium values, $K_{IC}^{CO}$ (equil), obtained by titration for $Cr$HydA1 and $Ca$HydA. The two values for $Cr$HydA1 are in good agreement, although for $Ca$HydA, $K_{IC}^{CO}$ (equil) is rather higher than $K_{IC}^{CO}$ (kin). The ratio of the $k_{react}$ values for CO inhibition of $H_2$ oxidation and $H_2$ production—the "catalytic direction discrimination"—is about 49 for $Dd$HydAB, 22 for $Cr$HydA1 and 3 for $Ca$HydA: these variations clearly arise from differences in the rate that CO binds because $k_{react}$ is similar for all three enzymes.

Reactions with $O_2$: Figure 6 shows experiments in which each enzyme is subjected to 5% $O_2$ during $H_2$ oxidation at −0.05 V vs SHE. As before, the $H_2$ concentration was kept constant throughout the entire time-course. Each experiment began with 80% $H_2$, 20% $N_2$ flushing through the cell headspace. The gas mixture was then switched to 80% $H_2$, 15% $N_2$, 5% $O_2$ for the duration of the reaction (this involved simultaneous injection of an aliquot of gas-equilibrated buffer for $Dd$HydAB and $Cr$HydA1; the kinetics of $O_2$ inactivation of $Ca$HydA were so slow that the injection was unnecessary). The gas was finally changed back to 80% $H_2$, 20% $N_2$ once most of the activity (>90%) had been eliminated. We noted that the rate of inactivation of $O_2$ showed a dependence on $H_2$ concentration, with inactivation occurring more rapidly at lower levels, thus
Table 2. Compilation of $k_{\text{kin}}$ and $k_{\text{equil}}$ Values for CO Inhibition of H$_2$ Production and H$_2$ Oxidation for DdHydAB, CrHydA1 and CaHydA, with the Corresponding Catalytic Direction Discrimination Factors and Values of $K^{(\text{CO})}$ Determined by Kinetic and Equilibrium Methods$^*$

<table>
<thead>
<tr>
<th></th>
<th>DdHydAB</th>
<th>CrHydA1</th>
<th>CaHydA</th>
</tr>
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<tbody>
<tr>
<td>H$<em>2$ oxidation $k</em>{\text{meas}}$/$s^{-1}$$\mu$M$^{-1}$</td>
<td>$3.9 \times 10^{-1}$</td>
<td>$1.9 \times 10^{-2}$</td>
<td>$1.1 \times 10^{-3}$</td>
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<tr>
<td>H$<em>2$ production $k</em>{\text{meas}}$/$s^{-1}$$\mu$M$^{-1}$</td>
<td>$8.0 \times 10^{-3}$</td>
<td>$4.8 \times 10^{-4}$</td>
<td>$3.6 \times 10^{-4}$</td>
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<tr>
<td>Catalytic direction discrimination H$<em>2$ oxidation $k</em>{\text{meas}}$/$s^{-1}$</td>
<td>49</td>
<td>22</td>
<td>3</td>
</tr>
<tr>
<td>H$<em>2$ production $k</em>{\text{meas}}$/$s^{-1}$</td>
<td>$1.9 \times 10^{-3}$ $\pm 2 \times 10^{-4}$</td>
<td>$2.3 \times 10^{-3}$ $\pm 1 \times 10^{-3}$</td>
<td>$1.8 \times 10^{-3}$ $\pm 1 \times 10^{-3}$</td>
</tr>
<tr>
<td>$K^{(\text{CO})}$ (equil)/$\mu$M for H$_2$ oxidation (at $-0.05$ V)</td>
<td>-</td>
<td>1.0 $\times 10^{-1}$</td>
<td>$2.2 \times 10^{-1}$</td>
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<tr>
<td>$K^{(\text{CO})}$ (kin)/$\mu$M for H$_2$ production (at $-1.0$ V)</td>
<td>$4.8 \times 10^{-3}$</td>
<td>$1.2 \times 10^{-1}$</td>
<td>1.6</td>
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<tr>
<td>$K^{(\text{CO})}$ (kin)/$\mu$M for H$_2$ production (at $-0.40$ V)</td>
<td>0.34</td>
<td>1.4</td>
<td>5.6</td>
</tr>
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</table>

$^*$ All values pertaining to H$_2$ production were measured at $-0.40$ V.

Figure 6. Inactivation of H$_2$ oxidation by DdHydAB, CrHydA1 and CaHydA by 5% O$_2$. The headspace mixture is composed of 80% H$_2$ and the remaining 20% is as indicated. For DdHydAB and CrHydA1 a 0.67 mL aliquot of 20% O$_2$/80% H$_2$-saturated buffer was injected into the cell which initially contained 2 mL of buffer, to give an instant concentration of 5% O$_2$ at the points marked by the dashed arrows. For CaHydA, the reaction was sufficiently slow that it could be initiated simply by changing the headgas mixture. Other conditions: pH 6.0, 10 °C, electrode rotation 3000 rpm, −0.05 V vs SHE, headspace gas mixture composed of 80% H$_2$, 20% mixture of N$_2$ and O$_2$.

indicating that O$_2$ and H$_2$ are competitive.$^{34}$ We also noted that after removing O$_2$ from the cell, a very small amount of activity was consistently recovered for all three enzymes. Oxygen undergoes very slow reduction at graphite at $-0.05$ V, therefore control experiments (Figure S1, Supporting Information) were performed to assess the contribution to the current from O$_2$ reduction. This contribution to the current was then subtracted from the experiments to verify the end point and the activity remaining. In separate anaerobic experiments we determined that small quantities of hydrogen peroxide that would be formed during electrolytic O$_2$ reduction did not cause inactivation, by injecting aliquots of H$_2$O$_2$ solution and monitoring the effect on the H$_2$ oxidation current at $-0.05$ V (10 °C). This decrease in current was observed to be much slower than that due to equivalent concentrations of O$_2$ for all three enzymes.

Figure 7. Dependence of rate constants ($k_{\text{meas}'}$) for inactivation of enzymatic H$_2$ oxidation by O$_2$, on O$_2$ concentration, for DdHydAB, CrHydA1 and CaHydA (shown as an expanded scale). Experimental conditions: pH 6.0, 10 °C, electrode rotation 3000 rpm, $-0.05$ V vs SHE, headspace gas mixture composed of 80% H$_2$, 20% mixture of N$_2$ and O$_2$,and this...

The headspace mixture is composed of 80% H$_2$ and the remaining 20% is as indicated. For DdHydAB and CrHydA1 a 0.67 mL aliquot of 20% O$_2$/80% H$_2$-saturated buffer was injected into the cell which initially contained 2 mL of buffer, to give an instant concentration of 5% O$_2$ at the points marked by the dashed arrows. For CaHydA, the reaction was sufficiently slow that it could be initiated simply by changing the headgas mixture. Other conditions: pH 6.0, 10 °C, electrode rotation 3000 rpm, −0.05 V vs SHE, headspace gas mixture composed of 80% H$_2$, 20% mixture of N$_2$ and O$_2$. We also noted that after removing O$_2$ from the cell, a very small amount of activity was consistently recovered for all three enzymes. Oxygen undergoes very slow reduction at graphite at −0.05 V, therefore control experiments (Figure S1, Supporting Information) were performed to assess the contribution to the current from O$_2$ reduction. This contribution to the current was then subtracted from the experiments to verify the end point and the activity remaining. In separate anaerobic experiments we determined that small quantities of hydrogen peroxide that would be formed during electrolytic O$_2$ reduction did not cause inactivation, by injecting aliquots of H$_2$O$_2$ solution and monitoring the effect on the H$_2$ oxidation current at −0.05 V (10 °C). This decrease in current was observed to be much slower than that due to equivalent concentrations of O$_2$ for all three enzymes.

Figure 7 shows how rate constants for O$_2$-inactivation of H$_2$ oxidation activity vary with O$_2$ concentration for all three [FeFe]-hydrogenases. In each case the rate of inactivation is first-order in O$_2$ concentration. Table 3 shows the rate constants for inactivation by O$_2$ alongside the rate constants for inhibition of H$_2$ oxidation by CO. For each enzyme, the rate of reaction with CO is much faster (80–200-fold) than with O$_2$ and this ratio, which we refer to as the “gas identity discrimination”, is also included in Table 3.
Table 3. Comparison of the Second-Order Rate Constants for Inhibition by CO ($k_{\text{inact}}(\text{CO})$) and Inactivation by O$_2$ ($k_{\text{inact}}(\text{O}_2)$) of DdHydAB, CrHydA1 and CaHydA and Evaluation of the Gas Identity Discrimination—the Ratio $k_{\text{inact}}(\text{CO})/k_{\text{inact}}(\text{O}_2)$

<table>
<thead>
<tr>
<th>enzyme</th>
<th>$k_{\text{inact}}(\text{CO})$/(s)$^{-1}$</th>
<th>$k_{\text{inact}}(\text{O}_2)/(s)^{-1}$</th>
<th>$k_{\text{inact}}(\text{CO})/k_{\text{inact}}(\text{O}_2)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DdHydAB</td>
<td>$3.9 \times 10^{-1}$</td>
<td>$1.8 \times 10^{-1} \pm 3 \times 10^{-4}$</td>
<td>217</td>
</tr>
<tr>
<td>CrHydA1</td>
<td>$1.9 \times 10^{-2}$</td>
<td>$2.2 \times 10^{-2} \pm 1 \times 10^{-4}$</td>
<td>86</td>
</tr>
<tr>
<td>CaHydA</td>
<td>$1.1 \times 10^{-3}$</td>
<td>$5.1 \times 10^{-4} \pm 1 \times 10^{-4}$</td>
<td>216</td>
</tr>
</tbody>
</table>

H$_2$ Production in the Presence of O$_2$. Finally, we carried out experiments to estimate the extent to which the rate of inactivation by O$_2$ depends on whether the hydrogenase is operating in the direction of H$_2$ production or H$_2$ oxidation. We used a procedure described recently, in which the problem of distinguishing the current due to enzymatic H$_2$ reduction from that due to electrochemical O$_2$ reduction is resolved by adding an inhibitor. The decrease in current observed when the inhibitor is added provides a direct measure of the component of the current due to enzyme-catalyzed H$_2$ reduction. The experiments conducted for DdHydAB, CrHydA1 and CaHydA are shown in Figure 8.

The experiment performed on DdHydAB started under an atmosphere of N$_2$, and a current corresponding to enzymecatalyzed H$_2$ production was recorded (stage 1). The headgas was then switched to 1% CO in N$_2$, and a rapid and almost complete loss of current was observed. When the CO was flushed from the cell, the current increased and reached a steady level (albeit not the same level as prior to CO introduction, due to the partial irreversibility of this reaction, see Figure 4). The current that was recovered during CO efflux was adopted as the normalization unit for the next stage. In stage 2, O$_2$ was introduced: the current initially increased due to direct reduction of O$_2$ at the graphite electrode but then began to decrease as the enzyme became inactivated. Introduction of 1% CO after 2000 s under O$_2$ resulted in a rapid loss of current, the magnitude of which reports on the enzyme-catalyzed current component prior to CO inhibition. Removal of CO caused the current to increase again, but a further decrease in current was observed, as expected, when O$_2$ was removed from the cell after $t = 8500$ s. Finally in stage 3 the inhibition step with CO was repeated to establish the extent of survival of the hydrogenase. Similar sequences of steps were used in the experiments with CrHydA1 and CaHydA, except that 99% and 95% CO, respectively, were used instead of 1% CO in order to compensate for the much slower kinetics and lower CO affinity of these hydrogenases compared to DdHydAB; in addition, 5% O$_2$ was used to obtain a higher rate of inactivation for CaHydA. To estimate the half-life for inactivation in each case, the decrease in current upon addition of CO after reaction with O$_2$ for $t$ seconds (during stage 2) was divided by the original increase in current observed when CO was removed during stage 1. The half-life was calculated using $t_{1/2} = -t \ln(2)/\ln(x)$ where $x$ is the fraction of H$_2$ production current surviving after $t$ seconds. From control experiments such as those previously described, it was estimated that the concentrations of O$_2$ experienced by the enzyme at $-0.4$ V under headgas conditions of 1% and 5% were 0.7% and 4.5%, respectively. The corresponding half-lives for H$_2$ oxidation activity under similar O$_2$ concentrations were calculated from the rate constants in Table 3. The results and comparisons are shown in Table 4.

All enzymes remained at least 30% active after 2000 s under 1% bulk O$_2$ (i.e., at least 0.7% O$_2$ at the electrode) for DdHydAB and CrHydA1 and even under 5% bulk O$_2$ (around 4.5% O$_2$ at the electrode) for CaHydA. More significantly, there were important differences among the three enzymes when comparing their survival to O$_2$ exposure.
During \( H_2 \) production with the data obtained for \( H_2 \) oxidation. In the case of \( DdHydAB \), the \( H_2 \)-production activity remaining is about sixty-fold greater than expected on the basis of the results described above for \( O_2 \) inactivation of \( H_2 \) oxidation. The enhancement is also observed for \( CrHydA1 \), but to a lesser extent than \( DdHydAB \). On the other hand, no clear difference was observed for \( CaHydA \) between the rates of inactivation observed when monitoring \( H_2 \) production at \(-0.4 \) V or \( H_2 \) oxidation at \(-0.05 \) V.

**Discussion**

The three hydrogenases we have investigated include two with potential applications in large-scale \( H_2 \) production (\( CrHydA1 \) for photosynthesis and \( CaHydA \) for fermentation) and one of known crystal structure (\( DdHydAB \)). In addition, \( CaHydA \) is closely related to the Fe protein for which the structure is known. Some important comparisons have been made, exploiting the unique ability of protein film electrochemistry to measure, simultaneously, the rates and extent of changes in catalytic activities under well-defined potentials (driving force). A summary of the quantitative observations and interrelationships is provided in Figure 9.

The reactions with CO are highly informative, and we and others have noted that CO protects \([FeFe]-hydrogenases\) from \( O_2 \) degradation, suggesting both inhibitors target the same site. In all cases we could use CO as a strong inhibitor of both \( H_2 \) oxidation and \( H_2 \) production, helped by the fact that binding of \( H_2 \) under both conditions is much weaker than CO binding (see Table 1).

Light sensitivity of CO inhibition is a well-established property of \([FeFe]-hydrogenases\), and originates from the photolability of the Fe-CO bond. A particularly useful result is the similarity in the rates of dark reactivation of the CO-inhibited hydrogenases. In all cases, the rate is accelerated by illumination (as reported in an earlier study for \( DdHydAB \)), and this suggests strongly that the reaction being observed in all cases is an elementary dissociation of the Fe-CO bond. This result demonstrates an intrinsic property of the H-cluster, maintained regardless of the slightly differing protein environments among the \([FeFe]-hydrogenases\). Evidently, all that is required for reactivation is to liberate the coordination site and ensure that CO escapes from the pocket before it can recombine.

Our \( K^{CO} \) data for \( CaHydA \) determined during \( H_2 \) oxidation lie broadly in the range of values (around 1 \( \mu \)M) obtained by Thauer and co-workers for CO binding to the related enzyme from \( C. pasteurianum \), although those experiments also used a higher temperature and we found a consistently higher value during \( H_2 \) production with the data obtained for \( H_2 \) oxidation. In the case of \( DdHydAB \), the \( H_2 \)-production activity remaining is about sixty-fold greater than expected on the basis of the results described above for \( O_2 \) inactivation of \( H_2 \) oxidation. The enhancement is also observed for \( CrHydA1 \), but to a lesser extent than \( DdHydAB \). On the other hand, no clear difference was observed for \( CaHydA \) between the rates of inactivation observed when monitoring \( H_2 \) production at \(-0.4 \) V or \( H_2 \) oxidation at \(-0.05 \) V.

**Figure 9.** Bar charts representing various comparisons between \( DdHydAB \), \( CrHydA1 \), and \( CaHydA \). (A) Comparative rates of CO-inhibition of \( H_2 \) oxidation \((k_{\text{inact}}(CO/H_2), H^+ \) reduction \((k_{\text{inact}}(CO/H^+) \) and rates of recovery from CO-inhibition \((k_{\text{re-act}}(CO)) \). (B) Discrimination factors characterizing the favorability of binding CO over \( O_2 \) \((k_{\text{inact}}(CO/O_2)/k_{\text{inact}}(O_2/H^+) \) and binding CO when the enzyme is catalyzing \( H_2 \) oxidation compared to \( H_2 \) production \((k_{\text{inact}}(O_2/H^+)/k_{\text{inact}}(O_2/H^+) \) for \( DdHydAB \), \( CrHydA1 \) and \( CaHydA \). As the rate of reactivation from CO inhibition is essentially independent of the process being catalyzed, it is simply represented by the term \( k_{\text{inact}}(CO) \). The \( k_{\text{inact}}(O_2/H^+)/k_{\text{inact}}(O_2/H^+) \) ratios are approximate because the values of \( k_{\text{inact}}(O_2/H^+) \) are estimates. This ratio is approximated to 1 for \( CaHydA \).


(49) This allowed for the same concentration of \( H_2 \) to be employed in the experiments at \(-0.4 \) and \(-0.05 \) V, thus ensuring that the experiments were comparable. For other hydrogenases, strong \( H_2 \) inhibition of \( H_2 \) production at \(-0.4 \) V would have prevented experiments performed at this potential from being performed under \( 80\% \) \( H_2 \) as they were at \(-0.05 \) V.


Recognizing this is a simplistic model, we now consider the following limiting scenarios: (i) if \( k_{out} \leq k_2 \), \( k'_{inact} \sim k_{out}[\text{X}] \) so the rate of inhibitor binding depends only on the external concentration and rate of internal transport of \( \text{X} \); in this case little discrimination is expected based on the redox state of the H-cluster. Alternatively, (ii), if \( k_{out} \gg k_2 \), i.e. if the protein’s internal structure does not provide an effective barrier to transport of \( \text{X} \), it follows that \( k_{inact} = k_{out}[\text{X}][k_{out}] \). In this scenario, the rate of inhibition depends not only on the nature of \( \text{X} \) but also upon \( k_2 \) and therefore should also be faster for conditions favoring \( H_{\text{ox}} \) (\( H_2 \) oxidation) compared to \( H_{\text{red}} \) (\( H_2 \) production).

Reactivation follows the reverse sequence, and for \( k_{out}[\text{X}] = 0 \) (because CO is removed from the solution) we obtain

\[
\text{rate of re-activation} = \frac{k_{out} k_{-2} [\text{X}-\text{Fe}]}{k_{out} + k_2} \tag{4a}
\]

and the first-order rate constant (as measured experimentally) is given by

\[
k_{\text{re-act}} = \frac{k_{out} k_{-2}}{k_{out} + k_2} \tag{4b}
\]

Under the limiting condition \( k_{out} \ll k_2 \), \( k_{\text{re-act}} = k_{out} k_{-2}/k_2 \), whereas if \( k_{out} \gg k_2 \), the rate of reactivation reduces to \( k_{-2} \), reflecting the likelihood that CO escapes from the enzyme (\( k_{out} \) before it can recoordinate \( k_2 \)). Our data suggest that the latter situation must generally be the case, with a more intermediate situation (a smaller \( k_{out} \) relative to \( k_2 \)) applying for \( Ca\text{HydA} \) (see below). Overall, the dissociation constant is given by \( K_{2}^{\text{O2}}(\text{kin}) = k_{\text{re-act}}/k_{\text{inact}} = k_{out} k_{-2}/k_{out} k_2 \), which always depends on the kinetics of making and breaking the Fe-CO bond.

This analysis can be extended to the reaction of \( [\text{FeFe}] \) hydrogenases with \( O_2 \), although this reaction is essentially irreversible. Table 3 and Figure 9 show that trends among the hydrogenases as observed for their reactions with \( O_2 \) are mirrored in their reactions with \( O_2 \); for example, \( D/H\text{HydAB} \) shows the highest rates of inhibition in both cases and the greatest discrimination based on catalytic direction.

In mechanistic terms, the minuscule protection that \( D/H\text{HydAB} \) possesses against attack by \( O_2 \) is provided only within the active-site pocket in which \( O_2 \) is able to discriminate between different catalytic states of the enzyme (a strong \( k_2 \) dependence, according to the model). In contrast, the small catalytic direction discrimination observed for \( Ca\text{HydA} \) can be interpreted in terms of it showing a less excessive value of \( k_{out} \) (a more restrictive tunnel or filter) in relation to \( k_2 \). Values for \( k_{out} \) should correlate closely with those for \( k_2 \); thus, it is significant that \( Ca\text{HydA} \) also shows the slowest rates of reaction with \( O_2 \) and, with a half-life of several minutes under atmospheric \( O_2 \) levels at 10 °C, looks to be a promising model for aerobic biohydrogen production even though it stems from a strict anaerobe.

The evidence (strong light enhancement) that the rate-determining step in reactivation is the elementary scission of the Fe-CO bond in the \( H_{\text{ox}}-\text{CO} \) state, and the observation that the rate measured in the dark is quite similar for all enzymes (which share only 40% sequence similarity) shows that the kinetics of reactivation are governed more by the intrinsic properties of the H-cluster than by the nature of the surrounding enzyme. Lubitz and colleagues have proposed that the H-clusters in \( Cr\text{HydA1} \) and \( D/H\text{HydAB} \) are similar, although they differ slightly in electronic detail.\(^{28}\) Note that were CO to coordinate...
to different states of the H-cluster at $-0.4$ and $-0.05$ V, we would expect $k_{\text{on,act}}$ to depend significantly on potential, but it does not. This supports the view that CO (and by extension, O$_2$) binds to Hox but not Hred. The other comparison in Figure 9 which is reasonably constant among all three enzymes is the gas identity discrimination (CO vs O$_2$). This again may reflect intrinsic behavior of the H-cluster because CO is a superior ligand to O$_2$ in terms of its $\pi$-acceptor capability. Dominant intrinsic effects are not unexpected, given the unusually independent status of the 2Fe subcluster, which was described in the Introduction as an enzyme cofactor resembling an organometallic compound buried in a protein. Clearly our model is an oversimplification, albeit necessary at this stage, and to understand this observation more fully we are undertaking theoretical calculations, including predictions of relative transport rates through the enzyme.

The notion of a filter or a tunnel connecting the H-cluster to the molecular surface is supported by the two available [FeFe]-hydrogenase structures. In DdHydAB and Cpf there is a ‘static’ tunnel that can be revealed using a cavity-searching program. The tunnel in each enzyme has a central cavity that can bind a Xe atom and a narrower path leading to Fe$_2$. Figure 10B depicts the experimentally observed tunnel and Xe site in DdHydAB. The tunnel connects the molecular surface to the active site, and one possibility is that the Xe atom occupies a cavity in which dissociated CO could reside before rebinding to Fe$_2$ or escaping to the medium. Molecular dynamics simulations based upon the Cpf structure revealed a second tunnel that also connects to the central cavity. What can be concluded from both crystallographic and theoretical studies is that dynamic fluctuations are important for intramolecular gas diffusion in [FeFe]-hydrogenases.

Two further mechanistic points emerge from this study. First, we always observed that CO inhibition of H$_2$ production is only partially reversible. At present we have no explanation for this, although Adams reported in 1987 that CO binds irreversibly to a catalytic intermediate of Cpf. Further investigations including a full study of the potential dependence of CO binding during H$_2$ production are clearly required to resolve this issue, which may have mechanistic relevance. Second, we always recorded a small proportion of activity returning after O$_2$ inactivation, an observation in line with those reported by Baffert et al.

Also in studies on CaHydA. Such a part-reversal is consistent with the mechanism proposed by Stripp et al. in which O$_2$ must first bind in a reversible manner at the distal Fe of the 2Fe subcluster before causing irreversible damage to the [4Fe-4S]$_{10}$ subcluster.

From a biological perspective, the precise, quantitative data that we have been able to extract and compare for the three different hydrogenases should be understandable in terms of the lifestyles of the organisms that express them. This is true, in part. Recent studies on the O$_2$ detoxification mechanism in C. acetobutylicum have shown that this fermentative bacterium can survive limited exposure to air and can even undergo cell division at surprisingly high concentrations of O$_2$. The relative O$_2$ stability of CaHydA may therefore be a consequence of concerted evolutionary adaptation to an O$_2$-rich atmosphere. However, certain species of the Desulfovibrio genus have also been reported to exhibit short-term survival when exposed to O$_2$, thus, from a microbiological viewpoint the large disparity in O$_2$ sensitivity between CaHydA and DdHydAB is puzzling. The ‘intermediate’ degree of O$_2$ sensitivity displayed by CrHydA1 is consistent with the observation that although it is only expressed in C. reinhardtii under anaerobiosis, it is likely to be in contact with at least trace amounts of O$_2$ that are produced by photosystem II.

Acknowledgment. Research in the group of FAA was supported by the UK BBSRC (Grant BB/D52222X) and EPSRC. C.C. and J.C.F.C. thank the CEa and the CNRS (France) for institutional funding. S.S. and T.H. were supported by the Deutsche Forschungsgemeinschaft (SFB 480) and EU/energy network SolarH2 (FP7 418 contract 212508). We thank Dr. Alison Parkin for providing part of Figure 3 and for helpful advice, and Dr. A. Volbeda for preparing Figure 10B.

Supporting Information Available: Photolability of the CO-bound state: determination of the variation of the concentration of CO with time in the experiments shown in Figure 4; determination of $K^\text{Th}$(equil); control experiments for experiments investigating O$_2$ inactivation of H$_2$ oxidation. This material is available free of charge via the Internet at http://pubs.acs.org.
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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.² Years later, Hans Gaffron observed that green algae can either oxidize hydrogen in concert with CO₂ fixation in the “dark reaction”¹,² or evolve hydrogen gas upon illumination.³ 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 CO₂ 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 CO₂ 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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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.\textsuperscript{12-14} Unlike PSII, the PSI complex is essential to hydrogen evolution.\textsuperscript{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.\textsuperscript{20,21} This PSII-independent hydrogen evolution, which utilizes fermentative oxidation of organic substrates, is referred to as “photofermentation”.\textsuperscript{25,26}

Hydrogenases are ubiquitous in strict and facultative anaerobes, and the vast majority is found in prokaryotes.\textsuperscript{25,26} Hydrogenases are transition metal enzymes, likely to be developed in a pre-photosynthetic, reducing atmosphere.\textsuperscript{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.\textsuperscript{24} According to the composition of the bimetallic active site cofactor, [NiFe], [FeFe] and hydrogen-forming methylenetetrahydromethanopterin (Hmd) [Fe] hydrogenases are distinguished.\textsuperscript{25,29} Under physiological conditions, [NiFe] hydrogenases generally act as uptake hydrogenases while [FeFe] hydrogenases often catalyze hydrogen evolution.\textsuperscript{30,31} Hydrogen release with [FeFe] hydrogenases is fast and in most cases controlled by diffusion of substrates and products.\textsuperscript{25,29} [NiFe] hydrogenases were shown to exhibit much higher affinities for hydrogen (as a substrate in uptake) than [FeFe] hydrogenases.\textsuperscript{24} [NiFe], [FeFe] and [Fe] hydrogenases (Hmd) are not homologs and give a model for convergent mechanistic evolution.\textsuperscript{32}

[FeFe] hydrogenases have been described for pro- and eu- karyotes, [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.\textsuperscript{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.\textsuperscript{34} 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.\textsuperscript{35} 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.\textsuperscript{36,38} Moreover, hydrogen catalysts can also be coupled to photosensitizers and give a model for convergent mechanistic evolution.\textsuperscript{32}

During the past fifteen years, traditional physiological and biochemical studies have yielded information on photobiological hydrogen evolution in green algae.\textsuperscript{6,8} Several review articles summarize the discovery of hydrogen turnover under sulfur deprivation and the isolation of genes encoding for different algal hydrogenases.\textsuperscript{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 \textit{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.\textsuperscript{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.\textsuperscript{42} However, thirty years elapsed between the first observation of a “Cell-free Hydrogenase from \textit{Chlamydomonas}” by Frederick B. Abeles\textsuperscript{43} and the purification of the \textit{C. reinhardtii} hydrogenase by Happe and Naber in 1993.\textsuperscript{44}

Abeles could show in his pioneering experiments that the cell-free preparations of \textit{Chlamydomonas} \textit{euganetos} evolve hydrogen when the hydrogenase fraction was incubated with reduced methyl viologen as electron mediator.\textsuperscript{45} 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 \textit{C. reinhardtii}.\textsuperscript{46} The trick was to use an affinity chromatography with immobilised ferredoxin which is the electron donor to the hydrogenase \textit{in vivo}. The preparation was 40% pure and the specific hydrogen evolution capacity of the enzyme was calculated to be 1800 \(\mu\)mol H\(_2\) min\(^{-1}\) mg\(^{-1}\).\textsuperscript{47} Additional experiments on HydA1 showed that “activation and de novo synthesis” of the protein was inhibited by cycloheximide but not chloramphenicol.\textsuperscript{48} 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 \textit{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 hydrogenase 6100-fold and determined the specific activity for hydrogen evolution as 935 \(\mu\)mol H\(_2\) min\(^{-1}\) mg\(^{-1}\).\textsuperscript{47} 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 methyl viologen reducing activity. Another study showed that the protein contains iron but no nickel.\textsuperscript{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 cyanobacteria, the free-living precursors of plastids, encode exclusively for [NiFe] hydrogenases. Schnackenberg \textit{et al.} published the isolation of an ostensible [NiFe] hydrogenase of the green alga \textit{Scenedesmus obliquus}.\textsuperscript{48} While it was not yet established that the \textit{C. reinhardtii}
Hydrogenase is encoded in the nucleus,66 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.69 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.61,68 Based on these elementary results, the hydA genes of further algal species were isolated in the following years.32,33 It turned out that the hydrogenase proteins of algae represent a novel class of [FeFe] hydrogenases.57 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).33 Moreover, the reported occurrence of [NiFe] hydrogenases in green algae68 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 Roesler and Lien as follows: “More detailed analysis of the active site of C. reinhardtii hydrogenase by the use of electron spin resonance (ESR) spectroscopy would be highly desirable for comparative purposes, but the low quantity of hydrogenase present in this organism makes this a difficult task.”48 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.44 Second, a heterologous expression system for [FeFe] hydrogenases in the fermentative bacterium Clostridium acetobutylicum was established.59 Using Escherichia coli or Shewanella oneidensis as hosts, synthesis led to only low amounts of recombinant [FeFe] hydrogenases80 or high amounts of protein but comparably low specific activities.97 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.84 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”.97 In vivo, [FeFe] hydrogenases are usually found in hydrogen evolution.58 Essayed in situ, catalysis is mostly bidirectional. Enzyme activity is easily inactivated by oxygen and CO59,60 although the characteristics of inactivation differ in the reduction (evolution) and oxidation (uptake) directions.44 [FeFe] hydrogenases from organisms like Clostridium pasteurianum and acetobutylicum, Desulfovibrio desulfuricans and Megasphaera elsdenii have been described in detail.55,59 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 CpI, DdH and HydA of M. elsdenii release hydrogen at exceptionally high rates (5000–8000 µmol H2 min⁻¹ mg⁻¹).

Most [FeFe] hydrogenases consist of a single peptide chain. The structures of [FeFe] hydrogenases of D. desulfuricans (DdH) and C. pasteurianum (CpI) have been resolved by X-ray crystallography.50,59 CpI represents the typical bacterial-type [FeFe] hydrogenase. Two domains can be distinguished. The C-terminal “H-domain” carries the H-cluster, an electronically “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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Name</th>
<th>M.²</th>
<th>Vmax ³</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium pasteurianum</td>
<td>CpI</td>
<td>63.8</td>
<td>5500</td>
<td>Adams 1990 (25)</td>
</tr>
<tr>
<td>Clostridium acetobutylicum</td>
<td>HydA</td>
<td>64.3</td>
<td>1750</td>
<td>von Abendroth et al. 2008 (58)</td>
</tr>
<tr>
<td>Desulfovibrio desulfuricans</td>
<td>DdH</td>
<td>46.1+14.0</td>
<td>8820</td>
<td>Hatchikian et al. 1992 (62)</td>
</tr>
<tr>
<td>Megasphaera elsdenii</td>
<td>HydA</td>
<td>53.6</td>
<td>7000</td>
<td>Adams 1990 (25)</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>HydA1</td>
<td>47.5</td>
<td>935</td>
<td>Happe and Naber 1993 (44)</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>HydA2</td>
<td>47.3</td>
<td>n.d.</td>
<td>Forester et al. 2003 (66)</td>
</tr>
<tr>
<td>Scenedesmus obliquus</td>
<td>HydA</td>
<td>44.6</td>
<td>630</td>
<td>Girbal et al. 2005 (55)</td>
</tr>
<tr>
<td>Chlamydomonas moewusii</td>
<td>HydA1</td>
<td>45.4</td>
<td>1600</td>
<td>Kamp et al. 2008 (54)</td>
</tr>
<tr>
<td>Chlorococcum submarinum</td>
<td>HydA</td>
<td>45.3</td>
<td>640</td>
<td>Kamp et al. 2008 (54)</td>
</tr>
<tr>
<td>Chlorella fusca</td>
<td>HydA</td>
<td>45.1</td>
<td>1000</td>
<td>Winkler et al. 2002a (17)</td>
</tr>
</tbody>
</table>

² M, Molecular weight in kDa as derived from protein primary structure. ³ Vmax, Specific hydrogen evolution activity expressed as µmol H2 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. 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. 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. Therefore, chlorophyll-type [FeFe] hydrogenases have been termed “photosynthetic hydrogenases”. 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. 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 *CpI* and *DdH*. 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 chlorophyll-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. The binding niche for ferredoxin is formed by bulky, basic amino acid residues (K and R in Fig. 1) which are conserved in chlorophyll-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 chlorophyll-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 (*CpI*). Fig. 2 compares the cartoon model crystal structures of *CpI* (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 *CpI* 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. 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. 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. 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 chlorophyll-type hydrogenases, lacks the F-domain. The putative binding niche of ferredoxin is marked in Fig. 2, as well as the insertion region discussed by Winkler and co-workers.

**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]”. Each iron atom of the [2Fe] cluster is coordinated with one cyanide group (CN⁻) and one or two carbon monoxide groups (CO). In respect to the position of the [4Fe-4S] subcluster, the [2Fe] iron...
atoms are labelled ‘proximal’ and ‘distal’. Catalysis is thought to take place at a free binding site of the distal iron atom.60,69,70

Different redox states have been described for the H-cluster. The oxidized, catalytically active ‘H\textsubscript{ox}’ state is paramagnetic and EPR-active. The distal iron atom of the [2Fe]\textsubscript{0} moiety Fe\textsubscript{2} is less reduced than the proximal iron atom Fe\textsubscript{1}, giving the characteristic [4Fe-4S]\textsuperscript{2+}–Fe\textsubscript{1}(I)Fe\textsubscript{2}(II) assignment.71 One CO is found in a bridging position as identified by its typical vibrational absorption around 1800 cm\textsuperscript{-1} (see below). H\textsubscript{ox} can bind a molecule CO at the Fe\textsubscript{2} binding site. The paramagnetic state ‘H\textsubscript{ox}–CO’ is annotated as [4Fe-4S]\textsuperscript{2+}–Fe\textsubscript{1}(I)Fe\textsubscript{2}(II)–CO.70,72,73 Carbon monoxide is a potent inhibitor of [FeFe] hydrogenase activity.74,75 Furthermore, all [FeFe] hydrogenases are sensitive to oxygen inactivation, and oxygen competes with CO for the same binding site.25,76,77 In contrast to oxygen inactivation, inhibition by CO is largely, but not entirely, reversible.71,76,77,79 Reduction of the distal iron atom gives ‘H\textsubscript{red}’. This diamagnetic state is assigned as [4Fe-4S]\textsuperscript{2+}–Fe\textsubscript{1}(I)Fe\textsubscript{2}(I) or hybrid species [4Fe-4S]\textsuperscript{2+}–Fe\textsubscript{1}(I)Fe\textsubscript{2}(II)–H\textsubscript{2}, alternatively, and not detectable by EPR spectroscopy.72,78,79

The [FeFe] hydrogenases of the Desulvovibrio genus Dd/H and DdH 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\textsubscript{inact}’ has been characterized for aerobically isolated Dd/H and DdH. 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\textsubscript{2}-binding site is thought to be occupied by either OH\textsuperscript{−} or H\textsubscript{2}O.70,71 By means of a reductive treatment, H\textsubscript{inact} is converted to the active form H\textsubscript{ox} via a state ‘H\textsubscript{inact}–CO’. 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\textsubscript{inact} and H\textsubscript{inact}–CO are not defined for hydrogenases like HydA1 of C. reinhardtii or Cpl 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\textsubscript{air}’ to avoid confusion with H\textsubscript{inact}. The active site composition and precise redox state of H\textsubscript{air} remains a matter of speculation.79

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\textsubscript{ox} and H\textsubscript{ox}–CO.84 The CO-inhibited form of HydA1 of C. reinhardtii, 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

<table>
<thead>
<tr>
<th>Organism</th>
<th>H\textsubscript{ox}</th>
<th>H\textsubscript{ox}–CO</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium acetobutylicum</td>
<td>n.d.</td>
<td>2.075, 2.009, 2.009</td>
<td>Von Abendroth 2008 (58)</td>
</tr>
<tr>
<td>Clostridium pasteurianum</td>
<td>n.d.</td>
<td>2.072, 2.006, 2.006</td>
<td>Bennet 2000 (117)</td>
</tr>
<tr>
<td>Desulfovibrio desulfuricans</td>
<td>2.100, 2.040, 1.999</td>
<td>2.065, 2.007, 2.001</td>
<td>Silakov 2007 (71)</td>
</tr>
<tr>
<td>Chlamydomonas reinhardtii</td>
<td>2.102, 2.040, 1.998</td>
<td>2.052, 2.007, 2.007</td>
<td>Kamp 2008 (54)</td>
</tr>
<tr>
<td>Chlamydomonas moewusii</td>
<td>2.103, 2.038, 1.998</td>
<td>2.052, 2.008, 2.008</td>
<td>Kamp 2008 (54)</td>
</tr>
<tr>
<td>Chlorococcum submarinum</td>
<td>2.100, 2.040, 1.999</td>
<td>2.056, 2.008, 2.008</td>
<td>Kamp 2008 (54)</td>
</tr>
</tbody>
</table>
[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.24,75,77 Table 2 summarizes the EPR characteristics for some relevant [FeFe] hydrogenases.

Configuration of the H-cluster

The diamagnetic Hox 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 easily attributable to a Fe CO H-cluster.

EXAFS can not directly detect electronic states, this elongation an increased number of CO ligands at the [2Fe] cluster could have been distinguished for [Fe-4S] cluster could have been distinguished.

An overall geometry similar to that of bacterial hydrogenases. The H-cluster remains essentially unperturbed upon hydrogen gas treatment, but oxidation with CO (giving Hox-CO) revealed an increased number of CO ligands at the [2Fe] moiety and a —0.1 Å elongation of the Fe(I)—Fe(II) distance. Although EXAFS can not directly detect electronic states, this elongation is essentially attributable to a Fe(I)—Fe(I) attraction in Hox lifted upon oxidation.70 In bacterial [FeFe] hydrogenases, formation of a bridging CO between the [2Fe] iron atoms was observed as a consequence of oxidative treatment.25,70,71,81 The intensified attribution of CO ligands in Hox-CO can be explained alike. Fig. 3 shows the EXAFS analysis in a structural model for the oxidized Hox-CO H-cluster.

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 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.77,78 Modified noble metal electrodes present tailor-made binding surfaces and, in case of gold, provide
the possibility for concerted spectro-electrochemical analyses. 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. 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. 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. 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. However, immobilization of a chlorophyll-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. This is not trivial as electrons need to tunnel directly into the active site due to the lack of accessory clusters in HydA1. From a recent EXAFS analysis, it was observed that the [4Fe-4S] cluster and subsequent loss of iron. The Fe K-edge of oxygen inactivation. The catalytically active [2Fe] unit is initially left intact. As CO is not known to bind to cubane clusters, it must bind to the [2Fe] moiety. Thus, the electrochemical demonstration that CO protects the active site from oxygen indicates that oxygen does not directly attack the cubane cluster. 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. 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. 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*. PetF is discussed to be the central branching point of reducing power in sulfur-deprived algae.\textsuperscript{55,108,109} 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.\textsuperscript{54,108}

A recent study examines the interaction of *C. reinhardtii* proteins HydA1 and PetF with the help of site directed mutagenesis.\textsuperscript{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.\textsuperscript{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, \( V_{\text{m}} \) 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.\textsuperscript{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.”\textsuperscript{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]\(_{\text{II}}\) moiety and translocation of the prosthetic group onto the hydrogenase apo-protein.

In *C. reinhardtii*, HydE and HydF form a single-chain protein complex.\textsuperscript{87,82} 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.\textsuperscript{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,\textsuperscript{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.\textsuperscript{51,39} One approach is to immobilize both PSI and PetF on electrically linked gold electrodes.\textsuperscript{114} On the anodic site, PSI is bound to a special carbohydrate polymer which has been shown to work best for large protein complexes.\textsuperscript{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 PSI and PetF 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 PSI, a stable D1 variant is of interest, *e.g.* from a thermophilic organisms.\textsuperscript{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.

**Acknowledgements**

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**References**

DISCUSSION

3.1 Heterologous expression and synthesis of [FeFe] hydrogenases

Section (2.1) reports on recent improvements in heterologous synthesis and purification of CrHydA1, the [FeFe] hydrogenase of *C. reinhardtii*. Isolation yield was increased ten-fold (up to 1.000 µg L\(^{-1}\)) by adapting the eukaryotic hydA1\(_{Cr}\) gene to the *C. acetobutylicum* codon usage. Furthermore, elongation of the strep-tag was found to optimize binding to the StrepTactin column. EPR confirmed the integrity of the active site H-cluster. Furthermore, expression and protein synthesis of streptagged CaHydA in its native host was established by homologous recombination. Protein yield was doubled to 800 µg L\(^{-1}\).

The \(V_{\text{max}}\) for CaHydA was found to be 1.750 µmol H\(_2\) min\(^{-1}\) mg\(^{-1}\) which is 175-fold higher than published by Girbal et al. in 2005 [35] and in agreement with recent publications by other authors [74, 133]. The sound conditions of the H-cluster in CaHydA were proven by EPR spectroscopy.

In recent years, three different systems emerged that allow for heterologous synthesis and genetic engineering of [FeFe] hydrogenases. These can be categorized according to the host organism: *Clostridium acetobutylicum* [34, 35], *E. coli* [134, 135], and *S. oneidensis* [70]. *Clostridium acetobutylicum* is a strict anaerobe that exhibits a fermentation metabolism. *Escherichia coli* and *S. oneidensis* are facultative anaerobes that run on a “surviving” metabolism if deprived from O\(_2\) [136, 137]. In Table 1 a comparison of the most important parameters can be found.

<table>
<thead>
<tr>
<th>host organism</th>
<th>genetic handling</th>
<th>promoter</th>
<th>abs. yield [µg L(^{-1})]</th>
<th>rel. yield (oD = 1)</th>
<th>max. oD</th>
<th>(V_{\text{max}}^\ast)</th>
<th>% (V_{\text{max}}^\ast)</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. acetobutylicum</em></td>
<td>–</td>
<td>thl</td>
<td>1.000</td>
<td>250</td>
<td>4</td>
<td>625</td>
<td>67</td>
<td>[34, 35]</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>+</td>
<td>T7</td>
<td>1.000</td>
<td>2.000</td>
<td>0.5</td>
<td>150</td>
<td>16</td>
<td>[134, 135]</td>
</tr>
<tr>
<td><em>S. oneidensis</em></td>
<td>++</td>
<td>lac</td>
<td>500</td>
<td>500</td>
<td>1</td>
<td>700</td>
<td>75</td>
<td>[70]</td>
</tr>
</tbody>
</table>

All three systems display specific advantages and drawbacks. The closely related proteobacteria *E. coli* and *S. oneidensis* are genetically well-characterised. A wide variety of protocols for transformation and synthesis of recombinant proteins can be found. Moreover, both microorganisms display great flexibility when it comes to codon usage. *Shewanella oneidensis* is suitable for the translation of eukaryotic genes in particular [70]. Electro-transformation and establishment of recombinant genetic material in *C. acetobutylicum* [138] is far less efficient. Codon usage adaptation was proven necessary for the successive optimisation of protein output. Thus, it is illustrated how a pronounced downside is overcome rather easily.
The absolute yield of protein is comparatively low in *C. acetobutylicum*, *E. coli* and *S. oneidensis*. This reflects the physiologic effort, biosynthesis of [FeFe] hydrogenases means to the host cell. Due to the most recent improvements for the isolation protocol of hydrogenase from *C. acetobutylicum* host cell culture, maximum yield is increased to 2.000 µg L⁻¹ [139, 140]. However, normalised to oD₆₀₀ₙₘₖₜ = 1 the protein yield of *C. acetobutylicum* is still lower than with *E. coli* and *S. oneidensis*. Expression is under control of strong promoters and the recombinant hydrogenase is isolated via strep-tag affinity chromatography in each case. What makes a difference is that the clostridial host organism co-synthesises an intrinsic [FeFe] hydrogenase (CaHydA) in addition to the recombinant one [35]. It is reasonable to assume that this dual burden restricts the overall yield of CrHydA1 in *C. acetobutylicum*.

The *S. oneidensis* and *C. acetobutylicum* systems produce [FeFe] hydrogenases with high specific H₂ evolution activity. Both bacteria encode for intrinsic [FeFe] hydrogenases and have been shown to posses the specific maturation apparatus HydE/ F/ G. While *C. acetobutylicum* synthesises both CaHydA and CrHydA1, in case of *S. oneidensis*, a hydrogenase-deficient mutant is exploited. The double mutant *S. oneidensis* AS52 [137] is devoid of the [FeFe] and [NiFe] hydrogenase genes and completely lacks hydrogen activity. The transcription and protein level of HydE/ F/ G is not affected. The capability to produce [FeFe] hydrogenase with high specific activity is the most important argument in favour of the *S. oneidensis* and *C. acetobutylicum* systems.

*Escherichia coli* uses [NiFe] hydrogenases in mixed acid fermentation. No [FeFe] hydrogenase is found with *E. coli* [136]. To qualify the organism for maturation of [FeFe] hydrogenases, co-expression of HydE/F/G had to be achieved. At first, cloning the genes of *C. reinhardtii* HydEF, HydG, and CrHydA1 resulted in negligible yield of hydrogenase protein (the maturases HydE and F are coupled to one protein HydEF in *C. reinhardtii*) [69]. King et al. eventually established the stable co-expression of *C. acetobutylicum* HydE/ F/ G and CrHydA1 in *E. coli* [134]. The absolute yield was found twice that of *S. oneidensis* but only minor values for Vₘₐₓ have been reported. Apparently, the cellular interplay of CrHydA1 apoprotein and HydE/ F/ G gives active enzyme in only 16% of all cases. Although *E. coli* can not compete with *S. oneidensis* and *C. acetobutylicum* in terms of efficiency, the possibility to probe maturation with different combinations of HydE/ F/ G is an important and unique feature. The individual maturation enzymes and in vivo biogenesis of the H-cluster is best studied with *E. coli* [71, 72].

In summary, *S. oneidensis* AS52 hold the biggest potential in [FeFe] hydrogenase biosynthesis. The organism is genetically versatile and easily modified. Cell growth is performed in sealed vessels with no elaborate fermentation reactor necessary. *Shewanella oneidensis* AS52 transformants can be screened in a cell-based in vitro essay – the gram-negative organism readily disrupts upon osmotic shock. Mutants with maximal H₂ evolving activity can be chose for the production of recombinant
DISCUSSION

[FeFe] hydrogenases [139]. However, the successful isolation of a recombinant [FeFe] hydrogenase in *Shewanella* has been shown only once and the system needs essential optimisation before it can compete with the established *C. acetocutyllicum* system. By now, *C. acetobutylicum* is the host cell system with the biggest outcome. Thanks to the improvements conducted in the last three years, an in-depth characterisation of *CaHydA* and, above all, *CrHydA1* was made possible.

3.2 On the electronic structure of the H-cluster

Section (2.3) presents a closer look at the electronic structure of the H-cluster by X-ray absorption spectroscopy (XAS) at the k-edge of iron, more specifically extended X-ray absorption fine structure (EXAFS). The [FeFe] hydrogenase of *C. reinhardtii* facilitates an XAS analysis of the active site prosthetic group due to the absence of any ferredoxin-type [FeS] clusters. The overall structure of the *CrHydA1* H-cluster resembles the cofactor modelled into the crystal structures of *CpI* and *DdH* [32, 33, 55]. Sub-angstrom changes upon oxidative inhibition (CO) and reduction (*H*$_2$) were probed. Furthermore, we followed the effects of O$_2$ inactivation. The EXAFS characterisation of *CrHydA1* produced intricate structural data of high interest for synthetic chemistry and DFT modelling of the H-cluster [141-144].

It has been suggested that one of the terminal CO ligands at the distal iron atom of [2Fe]$_{H}$ shifts in a bridging position Fe$_p$–Fe$_d$ upon oxidation of *H*$_{red}$ to *H*$_{ox}$ [32, 38]. After adding external CO to the reduced sample, the Fe$_p$–Fe$_d$ distance increased about 0.1 Å supporting the formation of a “stiff” CO bridge in *H*$_{ox}$-CO (see Figure 10 for illustration). External CO is supposed to block the Fe$_d$ binding site as discussed by various authors [31, 32, 38, 145] but not binding the Fe$_p$–Fe$_d$ bridging position directly. The Fe–Fe distances in the [4Fe-4S] cluster are not affected by CO inhibition at the di-iron site.

Oxidation of the H-cluster with O$_2$ resulted in a quite surprising finding. Apparently, the contribution of the cubane cluster (Fe–Fe $\approx$ 2.7 Å) decreases faster upon inactivation than the absorption recorded for [2Fe]$_{H}$ (Fe–Fe $\approx$ 2.5/ 2.6 Å). The discrimination of this 0.1 Å difference was achieved by XAS examination for the first time and allowed following O$_2$ oxidation of both parts of the H-cluster separately. A discussion of this behaviour can be found in chapter (3.4).

![Figure 10](image-url) – Changes upon CO oxidation of the H-cluster as monitored by Fe–Fe distances (dashed lines). (A) *H*$_{red}$ in the Fe$_d$(I)-Fe$_d$(I) notation and (B) *H*$_{ox}$-CO with external CO bound to the vacant Fe$_d$ site (X). While the distances of the iron atoms in the [4Fe]$_{H}$ cluster remain constant throughout the samples, CO binding causes a $\sim$0.1 Å elongation of Fe–Fe in [2Fe]$_{H}$. 
DISCUSSION

There is some uncertainty regarding the precise conformation of $H_{\text{red}}$. Successive gassing of dithionite-reduced $CrHydA1$ with $H_2$ did not result in distinct changes of the Fe–Fe distances. Binding of substrate $H_2$ in a Fe$_p$–Fe$_d$ bridging position has been proposed [48, 146]. Two observations would have been indicative for this: (a) elongation of the Fe$_p$–Fe$_d$ distance as in $H_{\text{ox}}$–CO and (b) no difference between $H_{\text{red}}$ and $H_{\text{ox}}$–CO regarding the Fe$_p$–Fe$_d$ [49, 146] distance. The former was not observed experimentally. On the contrary, the difference between $H_{\text{red}}$ and $H_{\text{ox}}$–CO was remarkable (~ 0.1 Å). Thus, cooperative $H_2$ bonding to Fe$_p$–Fe$_d$ bridging position can be ruled out.

Silakov et al. published a FTIR-voltammetric study of $CrHydA$ using a “Moss” cell [147]. Upon reduction, the authors surprisingly found the bridging CO in $H_{\text{ox}}$ still present in $H_{\text{red}}$. This notion has important impact for the discussion of the reaction mechanism as both oxidised and reduced states are catalytically active [29]. An intact CO bridge in $H_{\text{red}}$ argues against the bridging position as hotspot of catalysis. This is in line with the observation of external CO being competitive with $H_2$ and $O_2$ [42] but contrasts with the structure of $H_{\text{red}}$ as deducted from XAS. However, in vitro state-pure $H_{\text{red}}$ is difficult to achieve. It is likely to assume that protein samples as derived from chemical and electro-chemical reduction are not the exactly same state. Note that the “super-reduced” state [45, 147, 148] has not been observed by protein film electrochemistry with $CrHydA1$.

### 3.3 Immobilisation of [FeFe] hydrogenases on conductive surfaces

**Protein film electrochemistry** (PFE) is used to study the activity of redox proteins in contact with a conductive surface. The [FeFe] hydrogenases $DdH$, $CrHydA1$, and $CaHydA$ have been subject to elaborate protein film electrochemistry analyses. This is a representative examination of all variants of [FeFe] hydrogenases: *Desulfovibrio*, *Chlorophyta* and *Clostridia*-type enzymes are probed [31, 32, 60, 149]. Section (2.2) reports on immobilisation of $CrHydA1$ on a modified gold electrode and initial spectro-electrochemical investigations. Production of $H_2$ was probed by gas chromatography. Exploiting another system, protein film electrochemistry on **pyrolytic graphite edge** (PGE) is presented in (2.4) and (2.5). Figure 11 shows a comparison of the different approaches.

![Figure 11](image-url) — Schematic comparison of the set-up in SEIRAS (left) and graphite-based electrochemistry (right). (A) Working buffer, (B) protein film, (C) SAM, and (D) electrode. Infrared beam and plasmon enhancement is shown in red. The shuttling methylviologen (MV) is drawn reduced (blue) and oxidised (orange). On graphite, direct electron transfer takes place. Readings on graphite are performed with a RDE: While products (e.g., $H_2$) accumulate on stationary electrodes, rotation guarantees for diffusion-adjusted kinetics. Note the orientation in the protein film in the SEIRAS system.
Spectro-electrochemical analysis of hydrogenase films

Immobilisation on a gold surface brings forth the chance to survey a protein film both by electrochemistry and Fourier-transform infrared spectroscopy (FTIR). The method applied is referred to as surface-enhanced IR absorption spectroscopy (SEIRAS) [150]. It makes use of plasmon enhancement of the IR beam at rough gold surfaces and the specific change of the absorption patterns if protein is bound to the surface [151]. Thus, electrochemical activity can directly be linked to structural / molecular changes of the protein. Infrared spectroscopy is a powerful technique in hydrogenase research in particular. The CN\(^{-}\) and CO ligands of the cofactors in [NiFe] and [FeFe] hydrogenases show specific absorbance from 2100 to 1800 cm\(^{-1}\). The [FeFe] hydrogenases \textit{DdH} and \textit{Cpl} have been characterised by FTIR [43, 45, 152], as well as \textit{DvH} and the enzyme of \textit{M. elsdenii} [153, 154]. Overall, bacterial [FeFe] hydrogenases share similar IR characteristics. Applying an in-solution spectro-electrochemical setup, \textit{CrHydA1} has been shown to behave distinctively different in FTIR [147].

\textit{CrHydA1} was probed on a self-assembled monolayer (SAM) of mercaptopropionic and mercaptoundecanoic acid. No direct electron transfer was observed – the activity of \textit{CrHydA1} relies on the presence of methylviologen as electron shuttle. Modification of gold surfaces is sufficient to avoid protein degradation. Making good benefit of this need, gold surfaces can be designed at will by using a wide range of linkers [150, 155], e.g. to help binding in a certain orientation. Surface plasmon resonance (SPR) was used to determine the concentration of bound protein. With mercaptopropionic acid as bio-compatible modification, H\(_2\) evolution efficiency corresponds to about 15% of supplied electrons. Formation of the protein layer was followed by SEIRAS. However, the specific infrared signals of the H-cluster CN\(^{-}\) and CO ligands [43, 152] were not resolved.

The SEIRAS approach holds important advantages for the electrochemical-controlled infrared analysis of hydrogenases. Just recently, we were able to bind \textit{CrHydA1} to a short-length mercapto linker with viologen redox activity (unpublished experiments). Although this is still different from direct electron transfer, the potential control of bound protein is decoupled from methylviologen diffusion and allows for kinetic experiments with an improved temporal resolution. As most hydrogenases are able to accept electrons from methylviologen [16] we expect the presented surface-modification to work with hydrogenases from many species.

Direct electrochemistry of hydrogenase films on graphite

Other than with noble metal surfaces, proteins immediately bind to bio-compatible graphite [156]. Direct electron transfer occurs if the redox-active centre of the protein is in tunnelling distance to the conductive surface. Given this, current recorded as function of applied potential is a direct measure of
DISCUSSION

protein (redox) activity [157]. Armstrong et al. established protein film electrochemistry for many [NiFe] and [FeFe] hydrogenases using pyrolytic graphite edge rotating disc electrodes (see [24, 25] for review). In this set-up, hydrogenases bind the rough pyrolytic graphite surface without preset orientation. Direct electron transfer has been observed in most cases and the exposition of the protein film on the electrode surface allows for manifold chemical treatments.

Alternative systems have been introduced. Hambourger et al. used pyrolytic graphite and carbon felt electrodes [158] to construct a simple device for the photobiological production of H₂ [159]. Their approach incorporates a porphyrine-sensitised TiO₂ anode in electric contact with a cathode immobilised with CaHydA. In 2005, Rüdiger et al. came up with an approach that allows directed protein bonding to pyrolytic graphite surfaces. They modified an electrode with aminophenyl groups that can bind the negatively charged part of D. gigas [NiFe] hydrogenase. This domain is responsible for electron transfer [160]. More stable films and enhanced redox activity were achieved according to this modification.

The immobilisation of DdH, CrHydA1, and CaHydA on blank pyrolytic graphite rotating disc electrodes is reported in sections (2.4) and, more detailed, (2.5). The bacterial DdH and CaHydA enzymes readily respond to the applied potential with sharp proton reducing activity. CaHydA shows only minor leaning towards H₂ uptake. DdH and CrHydA1 display both reduction and oxidation activity. Down to -600 mV vs. NHE, indications for a second electron transfer step (H₇red → H₇red as in [45, 147]) were absent. Kinetic parameters support the notion that [FeFe] hydrogenases are typically not inhibited by H₂ as a product [13]. Interestingly, an area of sigmoid-shaped infliction was found around the midpoint potential of CrHydA1. This reflects the special character of CrHydA1 – due to the absence of the accessory [FeS] clusters, a little over-potential is necessary to drive electron transfer around -340 mV vs. NHE. Furthermore, CrHydA1 and DdH inactivate at potentials more positive than 0 V vs. NHE. “Anaerobic inactivation” at positive potentials is far less pronounced for CaHydA. This might indicate a correlation from catalytic activity and the extent of anaerobic inactivation. The process is reversible to different extent and typical for the hydrogenase probed [13]. A state “H₇oxinact” is defined [161, 162] to distinguish this poorly understood state from H₇inact and H₇oxair [29].

3.4 Mechanisms of O₂ inactivation in [FeFe] hydrogenases

Oxygen sensitivity of hydrogenases is not a surprising thing to find if the general liability of [FeS] proteins to oxidative damage is taken into account (1.2). Any low-spin iron is oxidised rapidly upon contact with strong ligands like O₂ or CO. In parts this is because why ferrous iron has become a limiting nutrient in Plant and bacterial growth – most Fe(II) has been oxidised to Fe(III) under the influence of constantly increasing O₂ levels [163]. Iron-sulphur proteins have to deal with the same problem, so do hydrogenases.
DISCUSSION

“The appearance of oxygen on earth led to two major problems: the production of potentially deleterious reactive oxygen species and a drastic decrease in iron availability. In addition, iron, in its reduced form, potentates \( \text{O}_2 \) toxicity by converting (…) the less reactive \( \text{H}_2\text{O}_2 \) to more reactive oxygen species.” Touati, 2000 [164]

The question is raised how hydrogenases modulate \( \text{O}_2 \) sensitivity. **First**, a structural comparison between [NiFe] and [FeFe] hydrogenases stresses the existence of gas channel filters in hydrogenases [76]. Carbon monoxide and \( \text{O}_2 \) are used to distinguish diffusion and reaction kinetics. **Second**, the molecular mechanisms of \( \text{O}_2 \) inactivation in [FeFe] hydrogenases are discussed. Inactivation has never been assayed intricately due to the small rate constant of inactivation \( k_{\text{inact}} \) – in our hands, as fast as \( 1.8 \times 10^3 \) s\(^{-1}\) mM\(^{-1}\) for \( \text{DdH} \). On the other hand, oxidation of [FeS] clusters is an effect that not only affects the H-cluster but all iron in the protein.

**[NiFe] and [FeFe] hydrogenases display different levels of \( \text{O}_2 \) sensitivity**

For the design of protein variants less sensitive to \( \text{O}_2 \) it is crucial to learn about the structural and molecular mechanisms of \( \text{O}_2 \) inactivation. Some strategies to circumvent \( \text{O}_2 \) damage in [FeS] proteins were presented in the introduction. Regarding their \( \text{O}_2 \) sensitivity, [NiFe] hydrogenases display the greatest versatility among all hydrogenases. Isolated under aerobiosis, standard-type, *Desulfovibrio* [NiFe] hydrogenases are insensitive to \( \text{O}_2 \) damage. The states “Ni-A” and “Ni-B” have been defined by EPR [29]. Due to an oxygen species blocking the Ni-Fe binding site the enzyme is inactive in this state [165]. Subsequent reduction gives the active “Ni-C” state that binds a hydride ion – and is prone to irreversible oxidative inhibition [166]. An important difference between [NiFe] and [FeFe] hydrogenases is that \( \text{O}_2 \) inhibits the enzyme. [FeFe] hydrogenases are inactivated and irreversibly unfolded, as suggested by circular dichroism spectroscopy (unpublished experiments). Carbonmonoxid blocks [NiFe] hydrogenases by attacking the terminal nickel site. “Ni-CO” refers to Ni-C as inhibited by CO [167]. In [FeFe] hydrogenases, \( \text{O}_2 \) and CO bind the same site (see below).

Remarkably, a certain subclass of [NiFe] hydrogenases is capable of hydrogen turnover in the presence of \( \text{O}_2 \) [168]. The membrane bound and soluble [NiFe] hydrogenases of *Knallgas* bacteria *Ralstonia eutropha* H16 and *R. metallidurans* CD34 were proven to function in \( \text{H}_2 \) cycling at ambient \( \text{O}_2 \) levels [13, 169]. The term “\( \text{O}_2 \) tolerance” was introduced; however it is not clear how these enzymes achieve to cope with \( \text{O}_2 \). An “oxidase activity” of the Ni-Fe cofactor has been discussed to be crucial in \( \text{O}_2 \) tolerance [25, 75].

The [NiFe] hydrogenases of *Ralstonia* species have been subject to intensive engineering. No crystal structure could have been obtained but sequence alignments suggest that *Knallgas* hydrogenases differ from “standard” [NiFe] hydrogenases in the position of two bulky residues
narrowing a gas channel that points to the catalytic nickel atom of the cofactor [168, 170, 171]. Replacing corresponding residues in the standard [NiFe] hydrogenases did result in a delayed inactivation but not O₂ tolerance [74, 172, 173]. Ludwig and co-workers tried to re-sensitise the O₂-tolerant [NiFe] hydrogenases of *R. eutropha* and *metallidurans* only to find these enzymes two orders more sensitive against O₂ than the wild type enzyme [174]. The authors conclude,

“Tolerance to O₂ is clearly a complex factor and is determined by a well adapted spatial and electronic structure of the active site rather than a simple restriction of diffusion of inhibitory gases such as O₂.” Ludwig et al., 2009 [174]

While connecting cavities are necessary to supply hydrogenases with substrate, a direct relation “tunnel width / O₂ sensitivity” is obviously not feasible [76]. In [FeFe] hydrogenases, only one gas channel (“A”) has been found [31, 32]. Another is supported by DFT simulations (“B”) but was not observed in crystal structures available [175]. In *Clostridia, Desulfovibrio*, and *Chlorophyta*-type hydrogenases, gas channel A is a conserved structural element [15]. This channel points to the distal iron atom in *DdH* and *CaHydA*, allowing for selective entrance only. Figure 12 shows the vicinity of the H-cluster in *CpI*; note the enclosed surroundings of the [4Fe]₄ moiety. The flexibility of protein channels is subject to quite some discussion in gas-processing proteins [176, 177]. It might be likely that in hydrogenases, cavities form and deflate as a function of provided substrate – whether this is H₂, O₂ or CO [26, 76]. These considerations are important for a discussion of the pronounced variability in O₂ sensitivity we found with [FeFe] hydrogenases *DdH*, *CrHydA1*, and *CaHydA*. The functional differences are not immediately evident from the (crystal) structures.

![Figure 12](https://example.com/figure12.png)

*Figure 12 – Selective access to the active site in [FeFe] hydrogenase CpI. (A) [4Fe]₄ subcluster with its coordinating cysteine residues (white). (B) [2Fe]₂ site facing the end of the gas channel “A” as found in the crystal structure. The azadithiolate ligand and the catalytic part of [2Fe]₂ (Fe₆h here blocked by CO) is oriented to the point where the cavity connects to the channel. (C) A look into the cavity from outside the immediate peptide environment of the H-cluster. Fe₆-CO and the azadithiolate nitrogen atom (purple) are visible.*
Oxygen sensitivity of three [FeFe] hydrogenases is probed by CO inhibition \((2.5)\). Certain trends have been observed. Throughout all experiments, the notoriety for gas induced oxidation (expressed as \(k_{\text{inact}}\)) follows \(\text{DdH} > \text{CrHydA1} \gg \text{CaHydA}\). The statement of Baffert et al. that “activity does not correlate with \(O_2\) sensitivity” \([133]\) was found being correct. The clostridial [FeFe] hydrogenase is less sensitive against oxidation by three orders of magnitude compared to \(\text{DdH}\). Only minor discrimination between \(O_2\) and CO was found. Note that CO inhibition is about 200-fold faster than \(O_2\) inactivation in all three enzymes. The trend outlined above is mirrored in the differences between \(O_2\) and CO when the catalytic direction is assayed. Oxygen inactivation is the same for \(\text{CaHydA in \(H_2\) oxidation and \(H^+\) reduction direction (}\(k_{\text{inact}} (O_2/H_2) / k_{\text{inact}} (O_2/H^+) \approx 1)\) but \(\text{DdH is 100 times more rapidly inactivated in \(H_2\) oxidation. The same correlation was observed in CO inhibition. However, CO binds the H-cluster with greater affinity in the oxidised state. A preference of \(H_{\text{in}} (\text{Fe}_p (I) – \text{Fe}_d (II))\) in CO bonding was argued before \([25, 145]\). The minimum ratio was \(k_{\text{inact}} (\text{CO}/H_2) / k_{\text{inact}} (\text{CO}/H^+) \approx 5\) for \(\text{CaHydA}\).

It is likely to assume that the rate of catalysis and degree of susceptibility is not governed by gas channels. Despite the significant differences in inactivation and inhibition of the [FeFe] hydrogenases, \(O_2 / CO\) gas discrimination ratio is the same. Thus, not diffusion through the protein (\(k_{\text{in}} / k_{\text{out}}\) according to the kinetic model in \((2.5)\)) but the actual binding process (\(k_z / k_z\)) determines the reaction rate. Similar, the rate of re-activation \(k_{\text{re-act}}\) from CO inhibition upon illumination \([38, 148, 162]\) does not differ in \(\text{DdH, CrHydA1, and CaHydA. Light scission of Fe–CO is not influenced by the active site environment \([178]\) and \(k_{\text{re-act}}\) exclusively represents the rate of CO diffusing from the vicinity of the active site, and out of the protein. In Figure 13, \(k_{\text{inact}}\) for CO and \(O_2\) are compared to \(k_{\text{re-act}}\) after illumination of the CO-inhibited electrode (adapted from Figure 9 in section \((2.5)\)). Summed up, the different rates of inactivation in \(\text{DdH, CrHydA1, and CaHydA do not stem from variably sized gas channels but reflect intrinsic parameters of the active site environment and the H-cluster.}

![Figure 13](image-url) - Comparison of \(k_{\text{inact}}\) for CO and \(O_2\) and light-induced rate of reactivation after CO inhibition (\(k_{\text{re-act}}\)). Inactivation and inhibition was measured at \(H_2\) uptake potential to avoid oxidation of \(O_2\) on the graphite surface. While \(\text{DdH, CrHydA1, and CaHyd differ by up to \(x1000\) in \(k_{\text{inact}}, k_{\text{re-act}}\) is roughly the same for all hydrogenases. Note that inhibition (CO) is approximately 200-fold faster than inactivation (\(O_2\)).
DISCUSSION

A model for the molecular mechanism of O$_2$ inactivation

Not much is known about the exact mechanisms of O$_2$ inactivation. Given that the extended protein scaffold of the H-cluster is not determining for the rate of inactivation, residues in the close vicinity of the cofactor are likely to determine inactivation, inhibition, and the rate of catalysis as well [85, 179, 180]. To elucidate the part of certain amino acids in O$_2$ inactivation, it is important to get an idea what exactly happens to O$_2$ when it approaches the H-cluster. We used a concerted XAS / electrochemistry analysis to reveal the mechanisms.

The electronic structure of the H-cluster in CrHydA1 was probed by XAS (2.4). The average distance of the iron atoms in [4Fe]$_{II}$ is 2.7 Å but slightly shorter in [2Fe]$_{II}$ (2.5 Å). The Fourier-transform EXAFS spectrum now reveals the selectivity of O$_2$ damage. Upon O$_2$ inactivation, the contribution of the Fe–Fe distance in [4Fe]$_{II}$ is reduced. The peak that represents Fe–Fe in [2Fe]$_{II}$ is remarkably stable. Inhibition with CO did not result in a decrease of these bands; same is true for H$_2$ reduction.

The different effects of O$_2$, CO and H$_2$ were assayed by protein film electrochemistry on pyrolytic graphite electrodes (2.5). Key to our observations was that (a) O$_2$ inactivation is prevented by CO inhibition and (b) the rate of inactivation is decreased in the presence of H$_2$. Thus, O$_2$, CO and H$_2$ competitively bind the same site [26, 28]. It is well established that external CO attacks the distal iron atom of the [2Fe]$_{II}$ site [38, 43, 145]. Accordingly, the vacant coordination site of this atom is where O$_2$ and H$_2$ do bind as well. Carbonmonoxide exclusively binds the [2Fe]$_{II}$ moiety. Oxidation of [4Fe4S] cluster by CO is chemically unlikely and has never been reported under the “mild” potentials applied here [181]. Given a near 100% protection of CO against O$_2$, we assume that O$_2$ selectively attacks the [2Fe]$_{II}$ site and not the cubane cluster. This is not trivial as [4Fe-4S] clusters are easily disrupted upon O$_2$ oxidation (1.2). Strong support for the impassivity of the [4Fe]$_{II}$ cluster comes from experiments on [FeFe] maturation.

The assembly of the H-cluster relies on a specific protein apparatus. Three enzymes have been identified that catalyze synthesis and translocation of the H-cluster: HydE, F, and G (3.1). According to an accepted proposal [182], HydF acts as a scaffold protein that holds a premature H-cluster formed by the help of radical-SAM proteins HydE and HydG. The latter is discussed to catalyse the synthesis of the azadithiolate ligand [183].

Just recently, HydF of C. acetobutylicum has been shown to contain a [FeS] cluster that displays characteristic CN$^-$ and CO bands in the IR spectrum. This supports the theory of a premature H-cluster in HydF and the enzyme as a “construction site” of cofactor synthesis [73]. Mulder et al. could demonstrate that [FeFe] hydrogenase synthesised in E. coli (which naturally lacks the H-cluster maturation apparatus) contains not the H-cluster but a catalytically inactive [4Fe-4S] cluster [71]. This
DISCUSSION

apoprotein was shown to give nearly full hydrogen cycling activity after in vitro maturation with CaHydF [73]. Mulder et al. reported the maturation of CrHydA1 apoprotein as isolated under aerobiosis [71]. Obviously, the [4Fe-4S] cluster in the apoprotein is not perturbed upon O$_2$ contact. Maturation under anaerobiosis recovers not only activity but O$_2$ sensitivity as well. Active CrHydA1 enzyme as inactivated by O$_2$ is impossible to re-activate by HydF or iron-sulphur cluster reconstitution [184] (unpublished experiments). This indicates damage of the [2Fe]$_{III}$ site and the cubane cluster.

Oxygen inactivation in [FeFe] hydrogenases can be explained with a three-step process. First, O$_2$ attacks the distal iron atom of the catalytic part of the H-cluster. Second is reduction of O$_2$. Fraser and co-workers discussed the “oxidase activity” of Ralstonia-type [NiFe] hydrogenases under O$_2$-rich conditions, pointing out that a similar process might be at hand in [FeFe] hydrogenases [25]. The difference, however, would be how the proteins deal with the product of O$_2$ reduction – which is, reactive oxygen species like peroxide or superoxide. Production of oxygen species by [FeS] clusters is a frequently observed phenomenon [83, 185]. Third could be (a) migration of reactive oxygen species to the nearby [4Fe]$_{III}$ cluster and subsequent degradation [114] or (b) “through-bond” oxidation [4Fe]$_{III}^{2+} \rightarrow$ [4Fe]$_{III}^{3+}$ via the bridging cysteine. The [4Fe]$_{III}^{3+}$ state is typically not stable [186, 187] and loses an iron atom to form a transient [3Fe-4S]$^{1+}$ cluster. Both mechanisms initiate a stepwise degradation of [4Fe]$_{III}$ and, subsequently, the [2Fe]$_{III}$ cluster. A release of ferric iron (Fe(III)) to the medium is evident from the absorption edge in XAS for samples intensively treated with O$_2$. An example of controlled [4Fe-4S] cluster degradation via [3Fe-4S]$^{1+}$ can be found with FNR (1.2). Figure 14 illustrates the course of events in superoxide-mediated inactivation of [FeFe] hydrogenases.

If reactive oxygen species are discussed to be liable in O$_2$ inactivation, a comparison of [NiFe] and [FeFe] hydrogenases turns out to be helpful. Release of superoxide from the soluble [NiFe] hydrogenase of *R. eutropha* (“SH”) upon aerobisation has been reported by Schneider and Schlegel 30 years ago [188]. Superoxide dismutase stabilises SH, catalase does not.

![Figure 14](image_url) – Stepwise degradation of the H-cluster by superoxide. (A) Reduction of O$_2$ at the [2Fe]$_{III}$ site generates superoxide. (B) Superoxide (red) migrates and (C) oxidises the [4Fe]$_{III}$ cluster to an unstable +3 form. One iron atom (Fe(III), brown) is lost, and the [3Fe-4S]$^{1+}$ cluster dissolves spontaneously (not shown). Attack of the cubane subcluster is the first step in complete loss of the H-cluster. O$_2$ does not directly attack the [4Fe]$_{III}$ site.
DISCUSSION

“The correlation between O₂ concentration (…) and inactivation rates and the stabilization of hydrogenase by addition of superoxide dismutase indicated that superoxide radicals are responsible for enzyme inactivation.” Schneider and Schlegel, 1981 [188]

In contrast, the [FeFe] hydrogenase of *D. desulfuricans* is not protected against O₂ inactivation by superoxide dismutase or catalase [189]. Van der Westen et al. conclude that [FeFe] hydrogenases do not produce reactive oxygen species when gassed with O₂. We probed CrHydA1 and SH for superoxide production and found the same trend verified (unpublished experiments). However, taking in account the molecular mechanism sketched above, superoxide “release” is not likely in [FeFe] hydrogenases. Due to the close proximity of [2Fe]₉ and [4Fe]₉ disruption of clusters should be rapid and inevitable [110].

We assume that [FeFe] hydrogenase can not deal with reactive oxygen species formed at the active site. No mechanism for reactive oxygen emission could evolve. From an evolutionary viewpoint, this was not necessary at all as [FeFe] hydrogenases replace O₂ as terminal electron acceptor in anaerobic respiration [190, 191]. The leaning towards H₂ evolution (“electron valve”) reflects this function. [FeFe] hydrogenases have been found with strict anaerobes nearly exclusively [15, 16]. In green algae, they are synthesised only when O₂ is absent [59]. [FeFe] hydrogenases do not have to deal with O₂ – which is different in *Ralstonia*-type [NiFe] hydrogenases. These enzymes do not serve as “electron valves” but catalyse H₂ uptake under conditions of atmospheric O₂ [168]. Just recently, Saggu et al. published a spectroscopic study on the membrane bound [NiFe] hydrogenase of *R. eutropha* [192]. The authors found that,

> “The proposed high potential species is therefore consistent with the presence of an additional iron close to the position of the proximal [4Fe4S] cluster or, alternatively, with the presence of an oxidized high potential [4Fe4S]⁺³ at the proximal position.” Saggu et al., 2009 [192]

A stable, high potential [4Fe4S] cluster would provide an environment insensitive against reactive oxygen damage [97]. This has prominently been shown for HiPIP ferredoxins (1.2). Thus, *Ralstonia*-type [NiFe] might have found a way to make use of a one-electron transition metal cofactor in the presence of O₂. Albeit the enzyme displays oxidase activity, reactive oxygen is guided “safely” out of the protein. The gas channel differences in *Ralstonia* and standard-type [NiFe] hydrogenase [75] are likely to modulate O₂ tolerance. However, key to catalysis under O₂ should be the cofactor environment rather than gas accessibility.
Towards the O$_2$-tolerant [FeFe] hydrogenase

The biotechnological generation of an O$_2$-tolerant [FeFe] hydrogenase is of greatest interest in basic and applied research. Two major findings should facilitate the design of novel hydrogenase variants: (a) O$_2$ sensitivity is not governed by gas channels and (b) the capability of the hydrogenases to deal with reactive oxygen species as product of O$_2$ reduction at the active site determines the level of O$_2$ tolerance.

To understand the molecular background of O$_2$ sensitivity in [FeFe] hydrogenases, a comparison of the active site environment in CaHydA and DdH will be helpful. The difference in O$_2$ sensitivity is $10^3$ (2.5). Given that O$_2$ and CO reach the H-cluster in both enzymes at comparable rate, the underlying mechanism is not likely that of a gas filter. Three protein systems are considered to learn about O$_2$ sensitivity: High-potential ferredoxins (HiPIP), the soluble [NiFe] hydrogenase of *R. eutropha* (SH), and fumarate nitrate reduction (FNR). Scaffold-mediated stabilisation of the [4Fe-4S]$^{2+}$/$g198$[4Fe-4S]$^{3+}$ transition in HiPIPs [97] could serve as an ideal for the residue composition around the [4Fe]$_{\mu\mu}$ in [FeFe] hydrogenases. A high-potential subcluster would provide a tough environment for the release of reactive oxygen at the di-iron site – this is what has been discussed for the “Knallgas” hydrogenase SH [192]. Eventually, the course of event in cluster dismissal has to be taken in account again. The model depicted in (2.4) is quite similar to what has been reported for oxidative cluster degradation in FNR [132]. On the structural level, it is worthwhile to investigate how FNR is attacked by O$_2$ and subsequently deals with reactive oxygen species.

Due to the minimal [FeS] cluster composition, the [FeFe] hydrogenase of *C. reinhardtii* is the perfect subject for modifications towards an O$_2$ tolerant [FeFe] hydrogenase. Here, the heterologous synthesis in *S. oneidensis* AS52 holds potential for an efficient screening system. Other than with *C. acetobutylicum*, AS52 lacks any hydrogenase background so that a simple whole-cell *in vitro* test will yield immediate information on the catalytic profile of the recombinant hydrogenase.
SUMMARY

This work elaborates on the molecular background of oxygen sensitivity in [FeFe] hydrogenases. Hydrogenases are iron-sulphur proteins that catalyse hydrogen turnover in microorganisms. [FeFe] hydrogenases in particular are high-efficiency catalysts for hydrogen evolution but irreversibly inactivated by O\textsubscript{2}. Their unique [4Fe-4S]–[2Fe-2S] cofactor is referred to as “H-cluster”.

Three different [FeFe] hydrogenases were analysed by electrochemistry. These were the bacterial hydrogenases \textit{CaHydA} and \textit{DdH} as well as \textit{CrHydA1} of \textit{Chlamydomonas reinhardtii}, a unicellular green alga. The effects of \textit{O}_2 and CO were probed, the latter of which reversibly inhibits hydrogenases. \textit{DdH} is inactivated by \textit{O}_2 ten times more rapid than \textit{CrHydA1} (2.2 × 10\textsuperscript{-4} s\textsuperscript{-1} µM\textsuperscript{-1}) and three orders of magnitude faster than \textit{CaHydA} (5.5 × 10\textsuperscript{-6} s\textsuperscript{-1} µM\textsuperscript{-1}). The same correlation was found for CO inhibition, although reactivation is in the same range for all three enzymes. Moreover, CO was shown to prevent the H-cluster from \textit{O}_2 damage. Given that CO can not bind the [4Fe-4S] part of the cofactor, the capability of CO to protect the entire H-cluster proves that \textit{O}_2 attacks the [2Fe-2S] site exclusively, too.

The main focus of this study was the structural analysis of the H-cluster of \textit{CrHydA1}. For the first time, the H-cluster was analysed by X-ray absorption spectroscopy (XAS) at the K-edge of iron. Due to the accuracy of XAS, resolution even of closely spaced interatomic distances was achieved. The \textit{CrHydA1} H-cluster consists of a [4Fe4S] cluster (Fe–Fe ≈ 2.53 Å) and a di-iron site (Fe–Fe ≈ 2.73 Å) which both are similar to their crystallographically characterized counterparts. Treatment of \textit{CrHydA1} with CO resulted in a 0.1 Å increase of the Fe–Fe distance of the [2Fe-2S] site. This agrees well with formation of a bridging CO in H\textsubscript{ox}-CO. Surprisingly, XAS shows that reaction with \textit{O}_2 results in destruction of the [4Fe-4S] domain of the active site H-cluster while leaving the di-iron moiety essentially intact.

The XAS analysis of \textit{CrHydA1} represents the first experimental approach to the structural implications of \textit{O}_2 inactivation. Although electrochemistry has shown that \textit{O}_2 does not attack the [4Fe-4S] part of the active site cofactor, XAS data suggests specific degradation of the cubane cluster. Illustrating the course of events, a novel thesis on \textit{O}_2 inactivation of [FeFe] hydrogenases is devised.
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2008 Vortrag „Two distinct [FeFe] Hydrogenases in the Spotlight of biophysical & electrochemical Characterisation“ Sven T. Stripp, Martin Winkler, Ilka Husemann, and Thomas Happe. BMBF Tagung, Marburg


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