

**Title:** **Current State of [FeFe]-Hydrogenase Research: Biodiversity and Spectroscopic Investigations**



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1 **Current State of [FeFe]-hydrogenase Research - Biodiversity and Spectroscopic**  
2 **Investigations**

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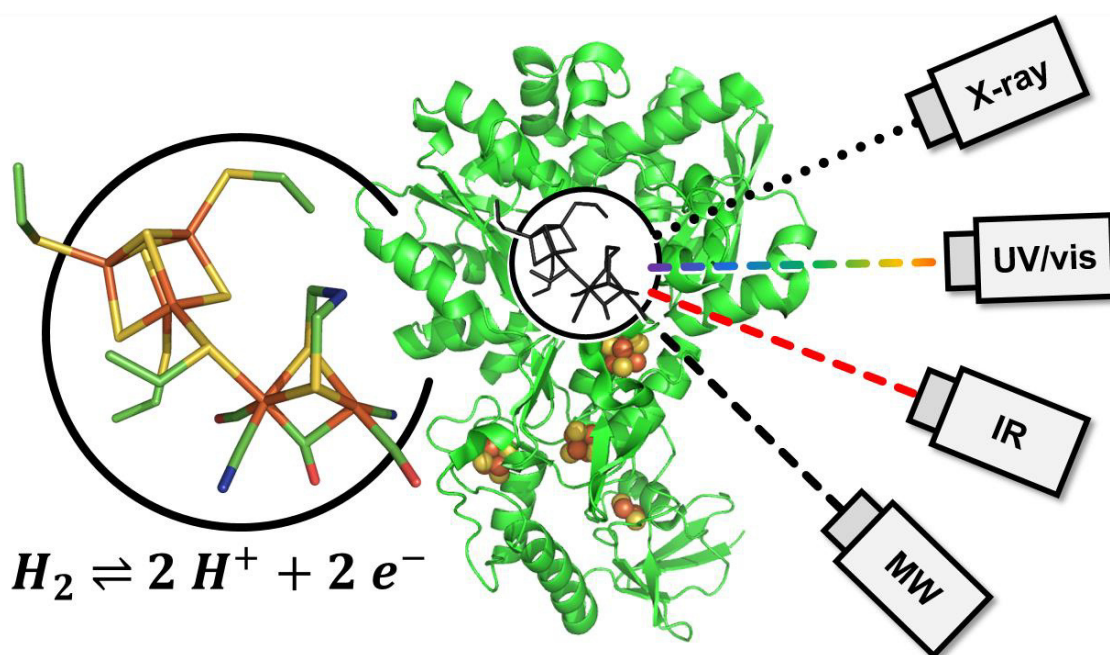
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14 Metalloenzymes, Iron-Sulfur Enzymes, Catalysis, Hydrogen Turnover, Proton-coupled  
15 Electron Transfer, Phylogenetics, Bioinorganic Chemistry, Biophysics

16

17 **Table of Contents**



18

19 **Abstract**

20 Hydrogenases are redox enzymes that catalyze the conversion of protons and molecular  
21 hydrogen (H<sub>2</sub>). Based on the composition of the active site cofactor, the monometallic [Fe]-  
22 hydrogenase is distinguished from the bimetallic [NiFe]- or [FeFe]-hydrogenase. The latter has  
23 been reported with particularly high turnover activities for both H<sub>2</sub> release and H<sub>2</sub> oxidation,  
24 notably at neutral pH, ambient temperatures, and negligible electric overpotential. Due to these  
25 properties, [FeFe]-hydrogenase represents the ‘gold standard’ in enzymatic hydrogen turnover.  
26 Understanding hydrogenase chemistry is crucial for the design of transition metal complexes  
27 that serve as potentially sustainable proton reduction or H<sub>2</sub> oxidation catalysts, *e.g.* in  
28 electrolytic devices or fuel cells.

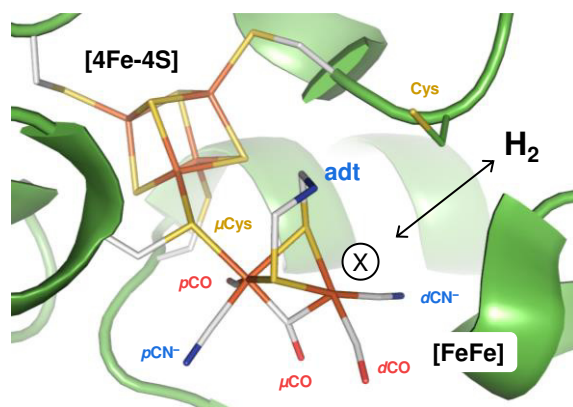
29 Even 20 years after the crystal structures of [FeFe]-hydrogenase have been published, several  
30 aspects of biological hydrogen turnover are heatedly discussed. In this perspective, we give an  
31 overview on how the diversity of naturally occurring and artificially prepared, semi-synthetic  
32 [FeFe]-hydrogenases deepens our understanding of hydrogenase chemistry. In parallel, we  
33 cover recent results from biophysical techniques that go beyond the scope of conventional X-  
34 ray diffraction, EPR, and FTIR spectroscopy. Taking into account both proton transfer and  
35 electron transfer as well as the notorious sensitivity of [FeFe]-hydrogenases towards carbon  
36 monoxide, the discussion further touches upon the molecular proceedings of biological  
37 hydrogen turnover.

38

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## 60 **1. Introduction**

61 Molecular hydrogen (H<sub>2</sub>) is widely recognized as a green energy carrier with the potential to  
62 replace fossil fuels.<sup>1-3</sup> Considering our interest in a ‘hydrogen society’, it is important to realize  
63 that evolution has already developed an efficient economy based on H<sub>2</sub>.<sup>4</sup> Iron-sulfur enzymes  
64 called hydrogenases are central to the hydrogen metabolism of numerous microorganisms as  
65 they catalyze the reversible conversion between protons and H<sub>2</sub>.<sup>5</sup> Depending on the nature of  
66 the metal cofactor, hydrogenases are divided into three main classes denoted as [Fe]-,  
67 [NiFe]-, or [FeFe]-hydrogenase. The latter are particularly efficient catalysts with H<sub>2</sub> evolution  
68 activities surpassing 10,000 H<sub>2</sub>/s.<sup>6</sup> Phylogenetically, there are indications that [FeFe]-  
69 hydrogenases could be the most recently evolved class of hydrogenase as archaea rely on [Fe]-  
70 and [NiFe]-hydrogenase whereas certain unicellular algae encode for [FeFe]-hydrogenase  
71 exclusively.<sup>7-11</sup> All [FeFe]-hydrogenases share a biologically unique cofactor, the hydrogen-  
72 activating ‘H-cluster’. This organometallic moiety consists of a [4Fe-4S] cluster connected to  
73 a diiron site *via* a bridging cysteine ([Figure 1](#)). The low valence iron ions of the diiron site are  
74 bridged by an azadithiolate ligand (adt) and coordinated by the  $\pi$ -accepting, strong-field ligands  
75 cyanide (CN<sup>-</sup>) and carbon monoxide (CO).<sup>12,13</sup> Details of the electronic structure and potential  
76 catalytic intermediates are discussed in **Chapter 3**. The biosynthesis of the H-cluster is a  
77 complex process that depends on a minimum of three [FeFe]-hydrogenase specific auxiliary  
78 enzymes.<sup>7</sup> Through the activity of two radical SAM enzymes (HydE and HydG), a pre-catalyst  
79 is assembled on a scaffold protein (HydF) and transferred to the hydrogenase apo-protein,  
80 generating the complete H-cluster.<sup>14-17</sup>



81

82 **Figure 1 - The active site cofactor of [FeFe]-hydrogenase.** The H-cluster comprises a [4Fe-  
 83 4S] cluster connected via a bridging cysteine ( $\mu\text{Cys}$ ) to a diiron site ([FeFe]) that is  
 84 coordinated by two  $\text{CN}^-$  ligands and three CO ligands. At the open coordination site of the  
 85 distal iron ion (X), gases like  $\text{H}_2$ , CO, and  $\text{O}_2$  react with the H-cluster. The azadithiolate (adt)  
 86 ligand mediates proton transfer between diiron site and protein fold, via a conserved cysteine  
 87 residue (Cys) in prototypical [FeFe]-hydrogenases. Drawn using pdb coordinates 4XDC (ref.  
 88 87). Legend: p – proximal; d – distal;  $\mu$  - bridging

89

90 Stephenson & Stickland were the first to report enzymatic hydrogen activation in 1931<sup>18</sup> and  
 91 the efforts aimed at unravelling Nature's principles for biological hydrogen conversion have  
 92 accelerated quite significantly over the past 20 years. In this perspective, we cover the state-of-  
 93 the-art with an emphasis on recent insights gained by studying 'new' [FeFe]-hydrogenases,  
 94 derived from both biodiversity and artificial enzymes featuring synthetically modified  
 95 cofactors (**Chapter 2**). Furthermore, we review trends in the spectroscopic analysis of [FeFe]-  
 96 hydrogenase including transient approaches and whole-cell spectroscopy (**Chapter 3**). Our  
 97 perspective will close on a brief note on the catalytic mechanism of [FeFe]-hydrogenase  
 98 (**Chapter 4**).

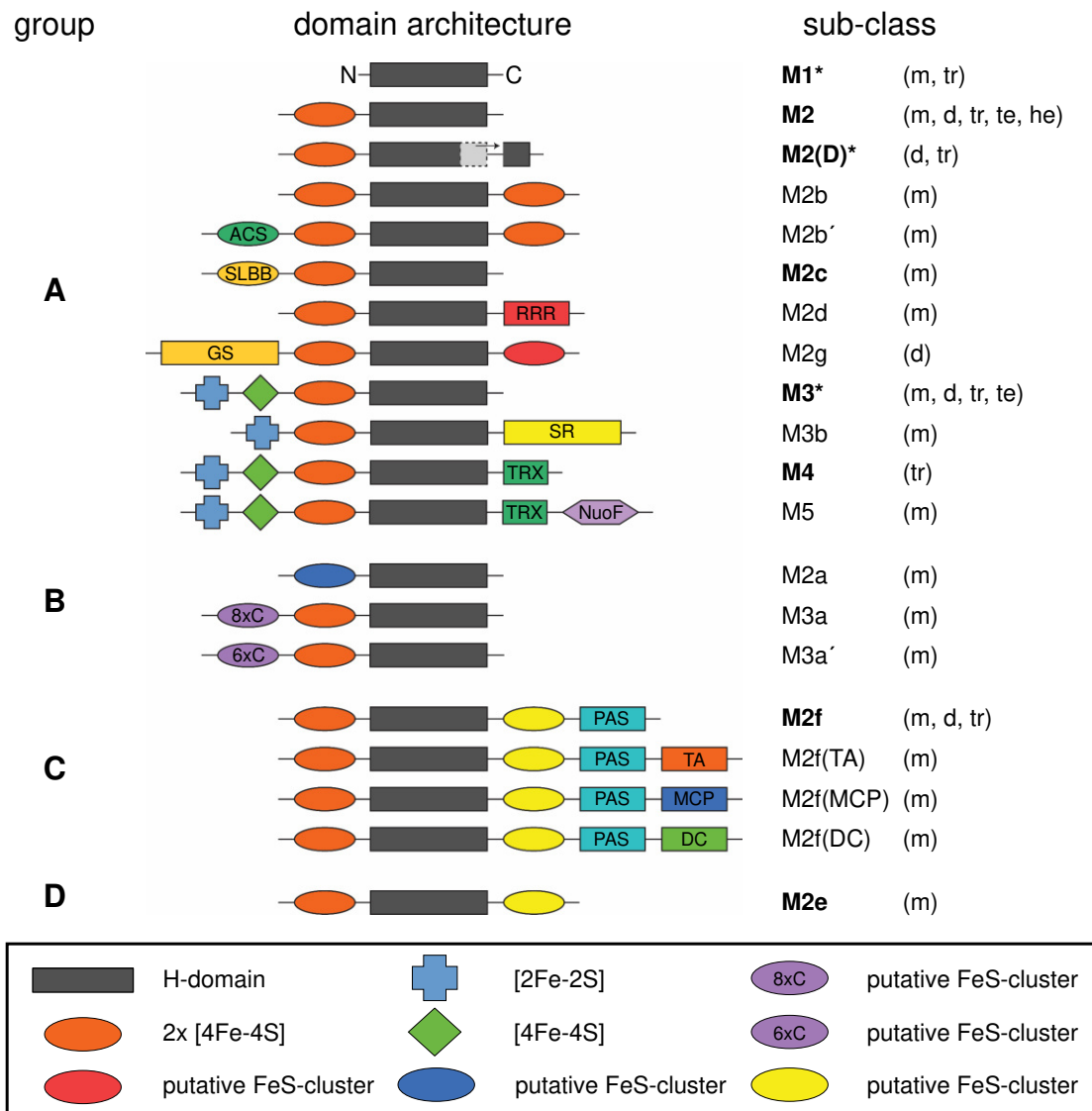
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## 100 **2. Novel Hydrogenases**

101 [FeFe]-hydrogenases are widely used as inspiration for the design of molecular catalysts and it  
102 is crucial to understand their catalytic design principles. However, only a fraction of the known  
103 [FeFe]-hydrogenases have been characterized and our knowledge on how the reactivity of the  
104 H-cluster is influenced by the protein fold in general and the active site pocket in particular is  
105 fragmentary at best. Truncation and site-directed mutation studies have been a powerful tool  
106 in this context but typically result in a loss of function.<sup>19</sup> In order to achieve a deeper  
107 understanding of biological hydrogen turnover, the biodiversity of [FeFe]-hydrogenase needs  
108 to be explored.

### 109 **2.1.1 On the Diversity of [FeFe]-hydrogenase**

110 Several studies of [FeFe]-hydrogenase biodiversity have been published, unfortunately with  
111 inconsistent naming conventions.<sup>7-9,20-23</sup> Herein, we provide a comprehensive summary of all  
112 [FeFe]-hydrogenase sub-classes identified to date, organized based on their domain structure  
113 and following the naming convention put forward by Jacques Meyer, Calusinska *et al.*, and  
114 Peters *et al.* (Figure 2).<sup>7-9</sup>



115

116 **Figure 2 - Biodiversity of [FeFe]-hydrogenase.** Domain architecture of the catalytic subunit  
 117 of all known [FeFe]-hydrogenase groups A – D. Sub-classes are named after their catalytic  
 118 subunit where ‘M’ stands for monomeric and the number is indicative of size. The lower-case  
 119 letter reflects that many sub-classes have been added over the years. Sub-classes in bold  
 120 indicate some degree of characterization whereas sub-classes with a crystal structure are  
 121 marked (\*). Abbreviations in parenthesis show in which quaternary structural variations the  
 122 sub-class occurs (i.e., monomeric (m), dimeric (d), trimeric (tr), tetrameric (te) and hexameric  
 123 (he)). Legend: acetyl-CoA synthase binding domain (ACS), soluble-ligand-binding  $\beta$ -grasp  
 124 binding domain (SLBB), rubredoxin-rubredoxin binding domain (RRR), glutamate synthase (GS),  
 125 sulfite reductase (SR), thioredoxin (TRX), NADH:ubiquinone  
 126 oxidoreductase chain F (NuoF), Per-Arnt-Sim domain (PAS), transcriptional activator (TA),  
 127 methyl-accepting chemotaxis protein (MCP), diguanylate cyclase (DC).



128 [FeFe]-hydrogenases can be separated into four distinct phylogenetic groups A – D (Figure 2).

129 Group A consists of prototypical and bifurcating [FeFe]-hydrogenases. In nature, prototypical

130 [FeFe]-hydrogenases perform hydrogen turnover using ferredoxin as redox partner while

131 bifurcating [FeFe]-hydrogenases perform the same reaction using both ferredoxin and NAD(H)

132 as electron donor/acceptor.<sup>24–26</sup> Electron bifurcation is a mechanism of biological energy

133 conservation in which exergonic and endergonic redox reactions are coupled to circumvent

134 thermodynamic barriers<sup>27–29</sup>, *e.g.* in the hydrogen turnover of the [FeFe]-hydrogenase from

135 *Thermotoga maritima* (*TmHydABC*)<sup>30</sup> or *Desulfovibrio fructosovorans* (*DfHnd*).<sup>31</sup> Group A

136 comprises the best characterized and catalytically most active enzymes such as the [FeFe]-

137 hydrogenase from *Chlamydomonas reinhardtii* (*CrHydA1*, sub-class M1),<sup>32</sup> *Desulfovibrio*

138 *desulfuricans* (*DdHydAB*, often referred to as *DdH*, sub-class M2(d)),<sup>33</sup> and *Clostridium*

139 *pasteurianum* and *acetobutylicum* (*CpHydA1* and *CaHydA1*, referred to as *CpI* and *CaI*, sub-

140 class M3).<sup>34</sup> Group B is phylogenetically distinct although its three sub-classes show similar

141 amino acid motifs around the H-cluster as Group A [FeFe]-hydrogenases. As no representative

142 example of Group B has been characterized so far, distinct differences between Group A and

143 Group B [FeFe]-hydrogenase remain to be identified. Group C has been classified as ‘sensory’

144 based on the presence of a PAS domain (Figure 2).<sup>8,22</sup> Moreover, genes encoding Group C

145 enzymes are commonly found upstream from known H<sub>2</sub>-producing [FeFe]-hydrogenases. The

146 biochemical characterization of the M2f-type [FeFe]-hydrogenase from *T. maritima* (*TmHydS*)

147 further supports the notion of a sensory function as it shows modest catalytic rates and an

148 apparent high sensitivity towards H<sub>2</sub>.<sup>35</sup> The closely related sub-class M2e (Group D) have a

149 similar gene localization and domain structure to M2f-type enzymes, suggestive of a similar

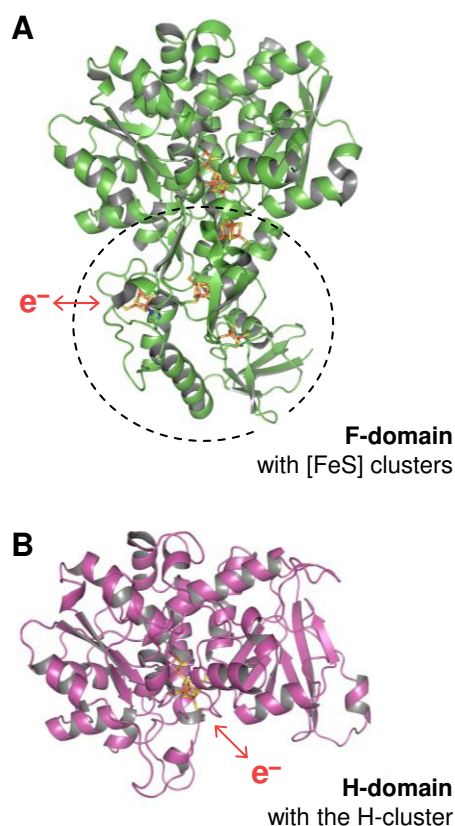
150 physiological function (Figure 2). However, the lack of a PAS domain in combination with

151 several differences in the active site amino acid composition makes their biological function

152 unclear at the moment.<sup>8</sup> One enzyme from this sub-class, derived from *Thermoanaerobacter*  
153 *mathranii* (*TamHydA*) has recently been characterized but further investigation is needed.<sup>36</sup>

### 154 **2.1.2 The Influence of F-clusters and Protein Environment**

155 The protein environment has a dramatic impact on the activity of the H-cluster, *e.g.* achieved  
156 through the influence of additional iron-sulfur clusters, well-controlled proton transfer, Lewis  
157 acid/base interactions in the active site, and through modulations of the electron density  
158 distribution across the active site. [FeFe]-hydrogenase carries up to five additional iron-sulfur  
159 clusters including the M4 sub-class that carries an additional C-terminal cluster in the TRX  
160 domain (Figure 2).<sup>22</sup> These ferredoxin-type ‘F-clusters’ act as a molecular wire through the  
161 protein and ensure efficient electron transfer between biological redox partners and the H-  
162 cluster (Figure 3). The part of the enzyme that binds the F-clusters is commonly referred to as  
163 the ‘F-domain’ in contrast to the ‘H-domain’ that exclusively comprises the H-cluster. The F-  
164 clusters have a significant influence on the overall reactivity of the hydrogenase. This has been  
165 elucidated in the case of the two closely related M3-type enzymes from *CpI* and *CaI* as well as  
166 the sub-class M2 enzymes from *Megasphaera elsdenii* (*MeHydA*) and *DdH*.<sup>37–40</sup> In the latter  
167 case, redox and pH titrations combined with FTIR and EPR spectroscopy revealed that the  
168 redox equilibrium of the diiron site is influenced by the oxidation state of the F-clusters.<sup>39</sup>  
169 Parallel studies of *CaI* and *MeHydA* have shown that the F-clusters affect the catalytic bias of  
170 the enzyme. In both cases, removal of the F-domain resulted in enzymes favoring H<sub>2</sub> release  
171 following a significant drop in H<sub>2</sub> oxidation rates.<sup>37,38</sup>



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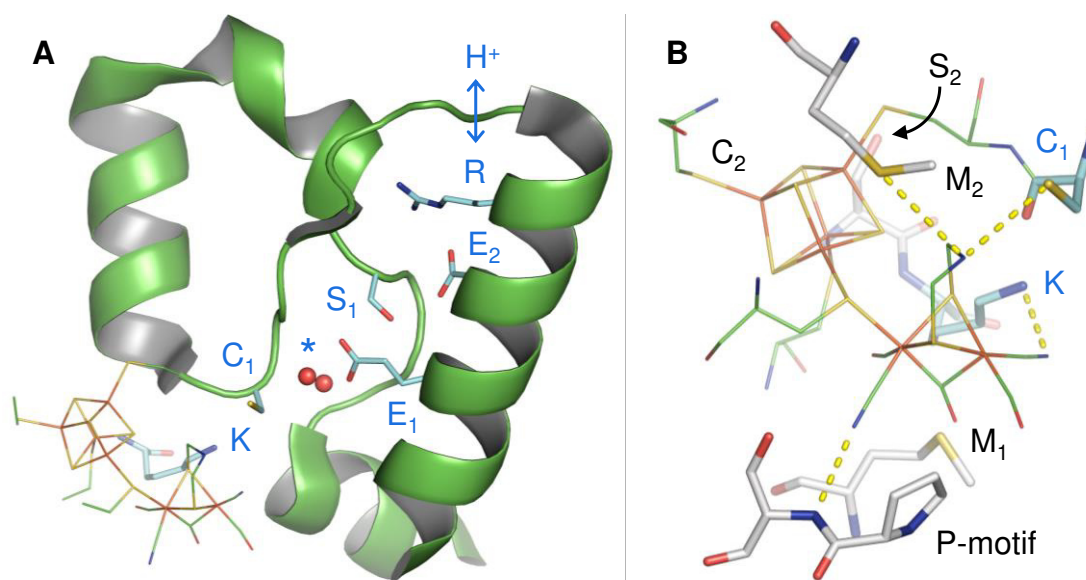
173 **Figure 3 - Electron transfer and domain architecture of [FeFe]-hydrogenase. (A)** Crystal  
 174 structure of CpI (M3) drawn using pdb coordinates 4XDC (ref. 87). The F-domain (dashed  
 175 circle) carries three [4Fe-4S] clusters and a single [2Fe-2S] clusters that facilitate electron  
 176 transfer between H-cluster and the protein surface. **(B)** Homology model of CrHydA1 (M1).  
 177 Here, the F-domain is missing and electrons are injected directly into the H-cluster.

178

179 The activity of [FeFe]-hydrogenase is additionally influenced by the mass transfer of gases  
 180 (e.g., H<sub>2</sub>, O<sub>2</sub>, or CO) and proton transfer. Molecular dynamics simulations proposed a number  
 181 of putative gas channels<sup>41</sup> and site-directed mutagenesis could slow down O<sub>2</sub> inactivation in  
 182 individual studies.<sup>42</sup> However, experimental proof verifying a main trajectory for gas transfer  
 183 has yet to be obtained.<sup>43</sup> There are indications that H<sub>2</sub> diffuses more freely through the protein  
 184 than bulky gases, which suggests that there might be no such thing as a specific H<sub>2</sub> channel.

185 The proton transfer pathway in Group A [FeFe]-hydrogenase comprise a series of well-  
 186 conserved amino acid and water residues that enable proton transfer between the H-cluster and

187 the enzyme surface (Figure 4).<sup>44-46</sup> It starts at the H-cluster with a cysteine residue (C<sub>1</sub>)  
 188 responsible for proton transfer to the H-cluster and continues with a serine (S<sub>1</sub>), glutamate (E<sub>1</sub>,  
 189 E<sub>2</sub>), and an arginine residue (R).<sup>47,48</sup> A methionine ‘above’ the H-cluster (M<sub>2</sub>) was discussed  
 190 as hydrogen-bonding partner to the adt ligand possibly providing an alternative proton transfer  
 191 trajectory.<sup>49</sup> However, in Group C [FeFe]-hydrogenases neither cysteine C<sub>1</sub> nor methionine M<sub>2</sub>  
 192 are conserved.<sup>35</sup> Another putative proton transfer pathway was suggested to involve a  
 193 conserved lysine residue (K) close to the distal iron ion.<sup>13</sup> Together, this implies that more  
 194 investigations on possible proton transfer pathways in [FeFe]-hydrogenase are needed, in  
 195 particular to understand the chemistry of the Group C and Group D enzymes.



196

197 **Figure 4 - Proton transfer pathway and active site niche of [FeFe]-hydrogenase.** Crystal  
 198 structure of *CpI* (M3), drawn using pdb coordinates 4XDC (ref. 87). (A) A conserved set of  
 199 amino acid residues (blue sticks and labels) and water molecules (\*) facilitates bidirectional  
 200 proton transfer. Alternatively, lysine residue K was discussed to be involved in proton transfer.  
 201 (B) At the diiron site, two methionine residues (M<sub>1</sub>, M<sub>2</sub>) and a backbone contact including a  
 202 proline residue (‘P-motif’) influence the H-cluster geometry and catalytic bias. The identity  
 203 and protonation state of amino acid residues at the [4Fe-4S] cluster (including cysteine C<sub>2</sub> and  
 204 serine S<sub>2</sub>) has been shown to affect the electrochemical properties of the H-cluster.

205

206 The amino acid environment of the H-cluster is suspected to play a role beyond modulating  
207 gas access and proton transfer. For example, the CN<sup>-</sup> ligands of the H-cluster were modelled  
208 according to potential hydrogen-bonding partners, *i.e.* lysine K and a backbone contact  
209 involving a proline residue ('P-motif', *e.g.* APA in *CrHydA1* and *DdH* or APS in *CpI* and *CaI*)  
210 close to the proximal iron ion, Fe<sub>p</sub> (Figure 4).<sup>19</sup> Hydrogen bonding between K and Fe<sub>d</sub>-CN<sup>-</sup> is  
211 a reasonable assumption and likely to be relevant in cofactor insertion but has not been verified  
212 experimentally. In contrast, site-directed mutagenesis of the 'P-motif' had significant impact  
213 on catalytic bias and spectroscopic properties.<sup>50</sup> This demonstrated how the protein modulates  
214 the electron density of the diiron site through Lewis acid/base interactions with the CN<sup>-</sup> ligands.  
215 At the [4Fe-4S] cluster, amino acid exchanges<sup>51</sup> and protonation differences (C<sub>2</sub> in Figure 4)<sup>52</sup>  
216 have been shown to affect the electrochemical properties of the H-cluster, and a similar effect  
217 was discussed for the orientation of a conserved serine (S<sub>2</sub> in Figure 4).<sup>53</sup> Moreover, a  
218 methionine residue below the H-cluster was suggested to promote the release of μCO into a  
219 semi-bridging or terminal position upon reduction of the diiron site (M<sub>1</sub> in Figure 4).<sup>35</sup> The  
220 conservation of this methionine is a key difference between groups A/B and C/D and has  
221 important implications for the catalytic mechanism (**Chapter 4**). Finally, it has also been  
222 proposed that differences in the 'polarity' of the active site, enables tuning of [FeFe]-  
223 hydrogenases towards either H<sub>2</sub> release or H<sub>2</sub> oxidation.<sup>53</sup>

### 224 **2.1.3 Isolation of Functional [FeFe]-hydrogenase**

225 Historically, the preparation of pure [FeFe]-hydrogenase samples has been limited by several  
226 factors. In the 1950s, enzyme isolation was limited to homologous expression, causing  
227 significant challenges depending on the source organism.<sup>54</sup> During the 1970s and 1980s,  
228 heterologous (over-) expression in easy-to-handle host organisms such as *Escherichia coli* or  
229 *Pichia pastoris* was developed and became the method of choice.<sup>55,56</sup> However, the successful  
230 expression of an active [FeFe]-hydrogenase in *E. coli* was not published until 2004, when

231 Posewitz *et al.* reported the co-expression of CrHydA1 with the auxiliary maturases HydEFG  
232 from *C. reinhardtii*.<sup>14</sup> The method was then refined by using the *E. coli* strain BL21(DE3) $\Delta$ IscR  
233 in which deletion of the gene encoding the transcriptional regulator IscR stimulates iron sulfur  
234 cluster biosynthesis. This resulted in a ten-fold increase in yield of active [FeFe]-hydrogenase  
235 compared to previous reports.<sup>57,58</sup> Expressing [FeFe]-hydrogenase in *C. acetobutylicum* or  
236 *Shewanella oneidensis* (*i.e.*, bacteria natively expressing HydEFG) suffered from  
237 complications in handling these organisms.<sup>59–61</sup> In 2013, it was shown that inactive [FeFe]-  
238 hydrogenase apo-protein can be heterologously overexpressed and artificially activated using  
239 a synthetic mimic of the cofactor (**Chapter 2.2**).<sup>62,63</sup> This breakthrough paved the way for a  
240 new era in [FeFe]-hydrogenase discovery. By taking advantage of the increasing amount of  
241 sequenced genomes<sup>64</sup>, bioinformatics can identify any [FeFe]-hydrogenase encoding gene of  
242 interest, regardless of the source organism. The genes can then be codon-optimized for  
243 expression in common hosts and synthesized in a matter of days. This approach was recently  
244 explored when eight putative [FeFe]-hydrogenase genes from a range of different sub-classes  
245 were synthesized and expressed in conventional *E. coli* expression strains, notably without co-  
246 expression of HydEFG.<sup>36</sup> Active hydrogenase was generated through addition of synthetic  
247 cofactor mimics to the cell suspension.

248 **Outlook.** Several aspects motivate the discovery of novel [FeFe]-hydrogenases. *Firstly*,  
249 mechanistic studies of [FeFe]-hydrogenase are necessary to understand their complex  
250 chemistry and to aid the development of efficient biomimetic catalysts. As previously  
251 mentioned, the vast majority of the characterized [FeFe]-hydrogenases originate from Group  
252 A. Although the active site architecture in this group is conserved, these enzymes still exhibit  
253 clear differences in catalytic behavior. Rates for H<sub>2</sub> release and H<sub>2</sub> oxidation differ by a factor  
254 of 500 and 15,000, respectively, underscoring the influence of the non-catalytic domains on  
255 the activity of the enzyme. But also between the closely related [FeFe]-hydrogenases within

256 the same sub-class of Group A H<sub>2</sub> release rates differ up to 40 times,<sup>53,65–67</sup> suggesting that even  
257 a detailed discrimination based on differences in domain structure and active site architecture  
258 does not allow for an impeccable prediction of catalytic activity. Clearly, aspects of the  
259 molecular determinants of [FeFe]-hydrogenase turnover kinetics are poorly understood.  
260 Moving forward, comparative studies of different [FeFe]-hydrogenases from the same  
261 organism, as exemplified by a recent report on *CpI – III* by Artz *et al.*, can be expected to shed  
262 light into factors fine-tuning the reactivity of [FeFe]-hydrogenase.<sup>53</sup> In the context of molecular  
263 design, studies of point mutants and truncated [FeFe]-hydrogenases has provided critical  
264 insight into protein/cofactor interplay primarily through loss-of-function observations.<sup>49,68</sup>  
265 Other variations critically affect the catalytic bias.<sup>50,69</sup> Complementing such work with gain-  
266 of-function studies is expected to provide a wealth of additional data, *e.g. via* domain-swapping  
267 and/or the transformation of low-activity Group C or Group D enzymes to an enzyme  
268 displaying activities *on par* with Group A [FeFe]-hydrogenase.

269 *Secondly*, the understanding of biological hydrogen metabolism is still rather limited. From an  
270 environmental and biotechnological perspective, the organisms capable of metabolizing H<sub>2</sub>  
271 needs to be better understood. This is also relevant from a medical perspective since many of  
272 these organisms are involved in human pathogenesis.<sup>11</sup> Beyond hydrogen turnover activity, the  
273 bifurcating, multimeric [FeFe]-hydrogenases of Group A are interesting for the study of  
274 metabolic networks, *e.g.* involving NAD(P)H and CO<sub>2</sub> conversion.<sup>26–29</sup> Moreover, *DfHnd* (sub-  
275 class M3) and the [FeFe]-hydrogenase from *Clostridium beijerinckii* (*CbA5H*, sub-class M2c)  
276 appears to display a unique tolerance towards O<sub>2</sub>.<sup>31,70</sup> These observations underscore the  
277 potential of exploring hitherto uncharacterized sub-classes in efforts to discover enzymes with  
278 unprecedented activities and properties.

279 *Thirdly*, there is a great need to increase the toolbox of available [FeFe]-hydrogenases so that  
280 the best possible candidates can be identified with regards to catalytic performance and O<sub>2</sub>

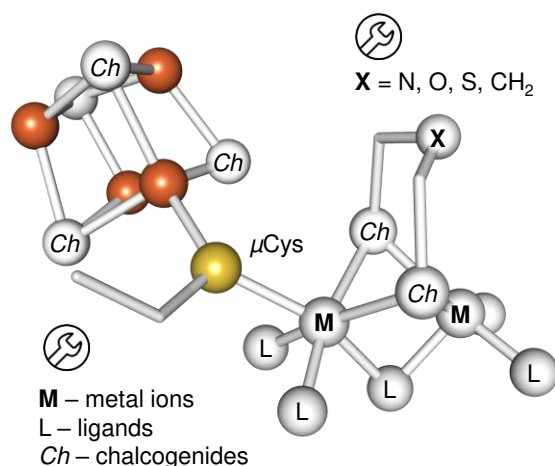
281 tolerance. These enzymes can then be optimized, *e.g.* through directed evolution and applied  
282 in industrial H<sub>2</sub> production or in biotechnological devices such as fuel cells. In the next chapter,  
283 we will discuss modification of the active site cofactor beyond the scope of nature.

## 284 **2.2 Artificial Maturation and Organometallic Variants**

285 The biosynthesis of the H-cluster proceeds *via* a discrete intermediate containing the [4Fe-4S]  
286 cluster but lacking the diiron site.<sup>71,72</sup> This intermediate is commonly referred to as ‘apo-  
287 protein’ despite carrying at least one metal center already. As hydrogenase apo-protein is  
288 readily isolated *via* heterologous expression in the absence of HydEFG, the assembly line could  
289 be hijacked through the introduction of synthetic analogues of the diiron site (‘artificial  
290 maturation’) enabling the preparation of semi-synthetic [FeFe]-hydrogenase (Figure 5).<sup>62,63</sup>  
291 More recently, this strategy has been utilized to identify possible intermediates in the assembly  
292 of the pre-catalyst.<sup>73–75</sup> Arguably, the main impact of artificial maturation to-date has been the  
293 simplified preparation of the active enzyme<sup>30,66</sup> including site-selective isotopologues,<sup>76–78</sup>  
294 which also facilitates rapid screening protocols.<sup>36</sup> Another important aspect is the preparation  
295 of [FeFe]-hydrogenases in which the diiron site is synthetically modified in order to generate  
296 non-natural H-clusters and enzymes with new properties.<sup>79–82</sup>

297





298

299 **Figure 5 - The H-cluster as a toolbox.** Several organometallic variants comprising non-  
 300 natural cofactors have been reported. Modifications include different metal ions ( $M$ ) and metal  
 301 ligands ( $L$ ), the replacement of sulfur against selenium (chalcogenides,  $Ch$ ), and different  
 302 variations of the dithiolate headgroup ( $X$ ).

303

304 In the case of Group A [FeFe]-hydrogenase, the active site cavity has been extensively explored  
 305 through site-directed mutagenesis (**Chapter 2.1.2**).<sup>49–52</sup> Of particular note in the context of  
 306 organometallic variants is the critical importance of hydrogen-bonding interactions between  $C_1$   
 307 (**Figure 4**) and the central nitrogen atom of the adt group.<sup>45</sup> Artificial maturation has enabled  
 308 detailed studies of this proton transfer pathway and facilitated the discovery of a number of  
 309 proton-coupled electron transfer events relevant to the catalytic cycle of the H-cluster (**Chapter**  
 310 **4**), *i.e.* through manipulations of the diiron site rather than the protein (**Figure 5**). For example,  
 311 diiron complexes that carry propanethiolate (pdt) or oxodithiolate (odt) ligands instead of the  
 312 natural adt ligand can be incorporated into the apo-protein with good yields. Such variants  
 313 showed very specific turnover characteristics and allowed locking the H-cluster in specific  
 314 oxidation states (**Chapter 3.1**).<sup>62,63</sup> Numerous other organometallic variants have been  
 315 reported, documenting modifications all across the H-cluster (**Figure 5**). The iron ions have  
 316 been replaced by ruthenium, resulting in the formation of a [RuRu] terminal hydride species  
 317 that appears highly stable.<sup>83</sup> The chalcogens have been changed from sulfur to selenium in both

318 the [4Fe-4S] cluster as well as the diiron site.<sup>84,85</sup> The latter variant showed a shift in catalytic  
319 bias towards H<sub>2</sub> release, but also suffered from a significant decrease in cofactor stability.  
320 Modifications of the diatomic ligands revealed that monocyanide variants of the H-cluster  
321 retain remarkable residual activity both *in vitro* and in whole cells.<sup>85-89</sup>

322 **Outlook:** The preparation of organometallic variants will undoubtedly continue contributing  
323 to our mechanistic understanding of [FeFe]-hydrogenase. The preparation of modified H-  
324 clusters outcompeting the native cofactor remains a significant challenge, although the  
325 selenium analogue appears to improve H<sub>2</sub> release activity.<sup>84,85</sup> It is noteworthy that synthetic  
326 modification of the diiron site have a dramatic effect on the reactivity towards known  
327 inhibitors.<sup>89</sup> Thus, it arguably provides a convenient route towards more stable catalysts. In  
328 parallel to the enzymology aspect, the generation of highly active, semi-synthetic [FeFe]-  
329 hydrogenases is of particular relevance in the context of designing small molecule systems.  
330 Since the structural elucidation of the H-cluster two decades ago,<sup>12,13</sup> the design and  
331 characterization of synthetic diiron complexes has been a highly active research field, and  
332 several comprehensive reviews have been published on the topic.<sup>90-92</sup> Historically, this work  
333 has been critical, *e.g.* in assigning the nature of the diatomic ligands, identifying the nature of  
334 the bridgehead atom, highlighting the importance of acid/base residues in the vicinity of the  
335 metal sites, and mapping out the reactivity of terminal *vs.* bridging hydrides. In solution, such  
336 small molecule mimics generally display limited catalytic efficiencies; however, upon  
337 incorporation into the enzyme unstable complexes are transformed into highly active catalysts.  
338 This immediately underscores the importance of the [4Fe-4S] cluster and the protein  
339 environment for activity (**Chapter 2.1.2**). Analogously, the catalytic properties of small  
340 molecule systems can be significantly improved through the addition of redox and acid/base  
341 functionalities<sup>91-94</sup>, or by incorporating them into polymers.<sup>95-97</sup> In order to disentangle the  
342 different effects of the protein fold on the reactivity of synthetic diiron complexes, it is highly

343 relevant to complement studies of different sub-classes of [FeFe]-hydrogenase with model  
344 systems featuring redox active ligands in combination with a more elaborate second  
345 coordination sphere.

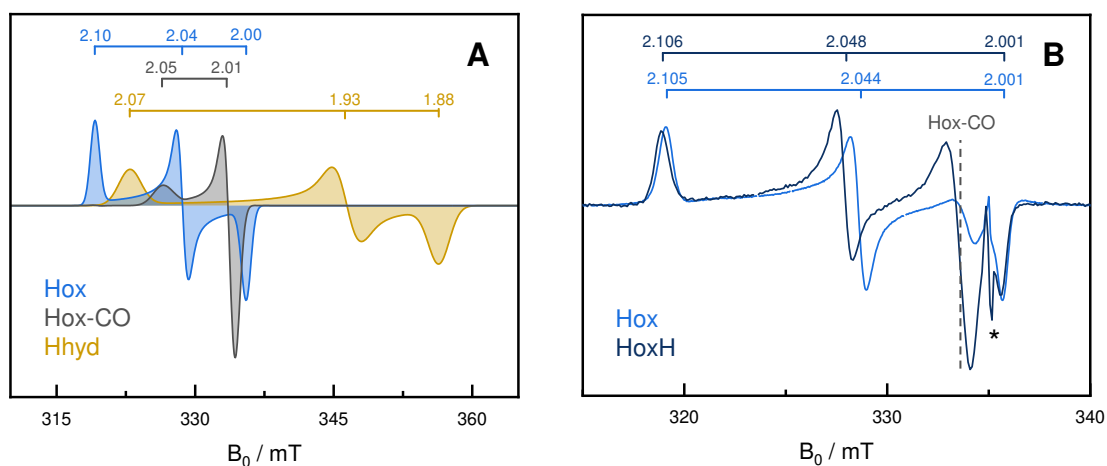
### 346 **3. Novel Methods**

347 The H-cluster has been investigated by continuous-wave and pulsed EPR spectroscopic  
348 techniques as well as Mössbauer spectroscopy, nuclear resonance vibrational spectroscopy  
349 (NRVS), and nuclear magnetic resonance spectroscopy (NMR). Similarly, the CO and CN<sup>-</sup>  
350 ligands of the H-cluster provide exquisite marker bands for FTIR spectroscopy. FTIR and EPR  
351 spectroscopy have arguably become the methods-of-choice in the field. Albeit not novel  
352 methods, these techniques are continuously employed in the identification of new states of  
353 potential catalytic relevance. In the following section, we will outline how spectroscopy has  
354 provided the foundation for our current mechanistic understanding, before describing how  
355 certain methods are used in new ways to provide even more detailed insight into H-cluster  
356 chemistry. In addition, recent data from synchrotron methods are discussed.

#### 357 **3.1.1 Electronic Properties of the H-cluster**

358 The paramagnetic resting state **Hox** gives rise to a rhombic signal ( $g=2.10, 2.04, 2.00$ ; see  
359 [Figure 6](#)) representing the oxidized [4Fe-4S] cluster (+2) and a mixed-valence diiron site  
360 (Fe<sub>p</sub>(II)-Fe<sub>d</sub>(I), +3).<sup>98-100</sup> The symmetry of the formally diamagnetic [4Fe-4S] cluster (*i.e.*, a  
361 pair of Fe(II)Fe(III) with  $S = 9/2$ ) is broken due to spin exchange coupling with the diiron site  
362 ( $S = 1/2$ ). Lubitz *et al.* noted that the strong electronic contact facilitates electron transport  
363 between [4Fe-4S] cluster and diiron site.<sup>101</sup> Accordingly, the putative protonation of the [4Fe-  
364 4S] cluster in **HoxH** (see below) is observable by EPR spectroscopy ([Figure 6](#)). Upon CO  
365 inhibition, spin exchange coupling is significantly enhanced in favor of the [4Fe-4S] cluster  
366 leading to an axial EPR spectrum with  $g = 2.05$  and  $2.01$  in **Hox-CO** ([Figure 6](#)).<sup>102,103</sup> The spin  
367 polarization was recently calculated to fit an apical CO model.<sup>104</sup> Studying the <sup>14</sup>N hyperfine

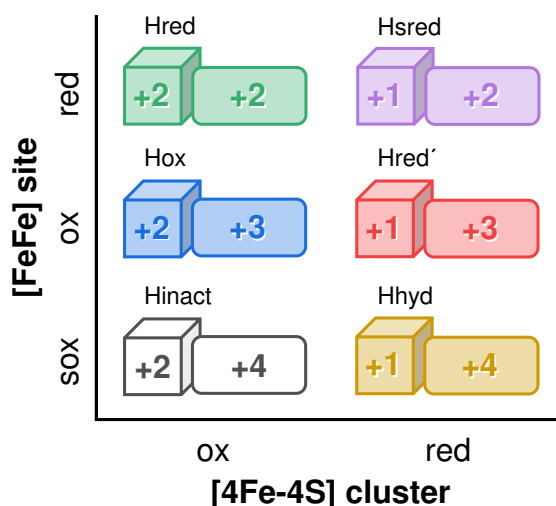
368 interactions, Silakov *et al.* revealed the chemical nature of the dithiolate group resolving a long-  
369 running controversy whether its central atom is an oxygen (odt), carbon (pdt) or nitrogen atom  
370 (adt) in favor of the latter.<sup>105</sup> Proton-coupled electron transfer involving the amine bridgehead  
371 of the adt ligand results in the formation of the diamagnetic state **Hred** (Fe<sub>p</sub>(I)-Fe<sub>d</sub>(I), +2).  
372 Here, the [4Fe-4S] cluster remains oxidized.<sup>98</sup> Further reduction of this species gives the  
373 rhombic EPR spectrum of a [4Fe-4S] cluster (+1) characteristic for the ‘super-reduced’ H-  
374 cluster, **Hsred**. Often, these states are referred to as HredH<sup>+</sup> and HsredH<sup>+</sup> in literature.  
375 Recently, the EPR signature of **Hsred** (g = 2.08, 1.94, 1.87)<sup>106,107</sup> was re-interpreted to stem  
376 from Hhyd:red, a hydride-like H-cluster intermediate. Lorent *et al.* suggest an alternative axial  
377 EPR signature (g = 2.15, 1.86) for **Hsred**.<sup>108</sup> Due to spin exchange coupling, the H-cluster is  
378 EPR-silent when *only* the [4Fe-4S] cluster is reduced (**Hred’** and **Hred’-CO**).<sup>109</sup> More  
379 recently, an H-cluster species with a terminal hydride was discovered, the so-called hydride  
380 state (**Hhyd**).<sup>110-112</sup> EPR, Mössbauer, and FTIR spectroscopy identified the latter as a ‘super-  
381 oxidized’ diiron site (Fe<sub>p</sub>(II)-Fe<sub>d</sub>(II), +4) coupled to a reduced [4Fe-4S] cluster (+1), giving rise  
382 to a rhombic EPR spectrum (g = 2.07, 1.93, 1.88, see [Figure 6](#)).<sup>110</sup> Further, the EPR spectrum  
383 of **Hhyd** bears similarities to the inactive **Htrans** state (g = 2.06, 1.96, 1.89 as observed in  
384 *DdH*).<sup>106</sup> Direct proof for the presence of a terminal hydride was presented by Reijerse *et al.*  
385 who applied NRVS on <sup>57</sup>Fe-enriched samples of *CrHydA1*-odt investigating the low-frequency  
386 Fe<sub>d</sub>-H<sup>-</sup> vibration at ~730 cm<sup>-1</sup> and ~670 cm<sup>-1</sup>.<sup>113</sup> The terminal hydride was additionally  
387 identified by <sup>1</sup>H NMR.<sup>114</sup> [Figure 7](#) depicts the electronic configuration of key H-cluster  
388 intermediates.



389

390 **Figure 6 - EPR signatures of the H-cluster in key redox states.** All data for CrHydA1. (A)  
 391 Simulations of EPR spectra for the oxidized states **Hox** and **Hox-CO** as well as the reduced  
 392 state **Hhyd**. The g-values are indicated. The EPR spectrum of **Hsred** is under debate. (B)  
 393 Experimental EPR spectra of the oxidized state **Hox** and the oxidized protonated state **HoxH**  
 394 displaying a subtle shift in g-tensors. Both samples show a contamination with **Hox-CO** and  
 395 artifacts of the quartz cuvette (\*). Experimental conditions:  $T = 15\text{ K}$ , frequency = 9.4 GHz,  
 396 microwave power = 4  $\mu\text{W}$ , modulation frequency/ amplitude = 100 kHz/ 0.5 mT, and  
 397 conversion time = 60 ms.

398



399

400 **Figure 7 - Electronic properties of key redox states.** The  $[4\text{Fe-4S}]$  cluster is drawn as a cube,  
 401 the diiron site is represented by a rectangular box. The abbreviations refer to either reduced  
 402 (red,  $\text{Fe}_p(\text{I})\text{-Fe}_d(\text{I})$ ), oxidized (ox,  $\text{Fe}_p(\text{I})\text{-Fe}_d(\text{II})$ ), or super-oxidized (sox,  $\text{Fe}_p(\text{II})\text{-Fe}_d(\text{II})$ ) diiron  
 403 site (y-axis) and either reduced (red, +1) or oxidized (ox, +2)  $[4\text{Fe-4S}]$  cluster (x-axis). The

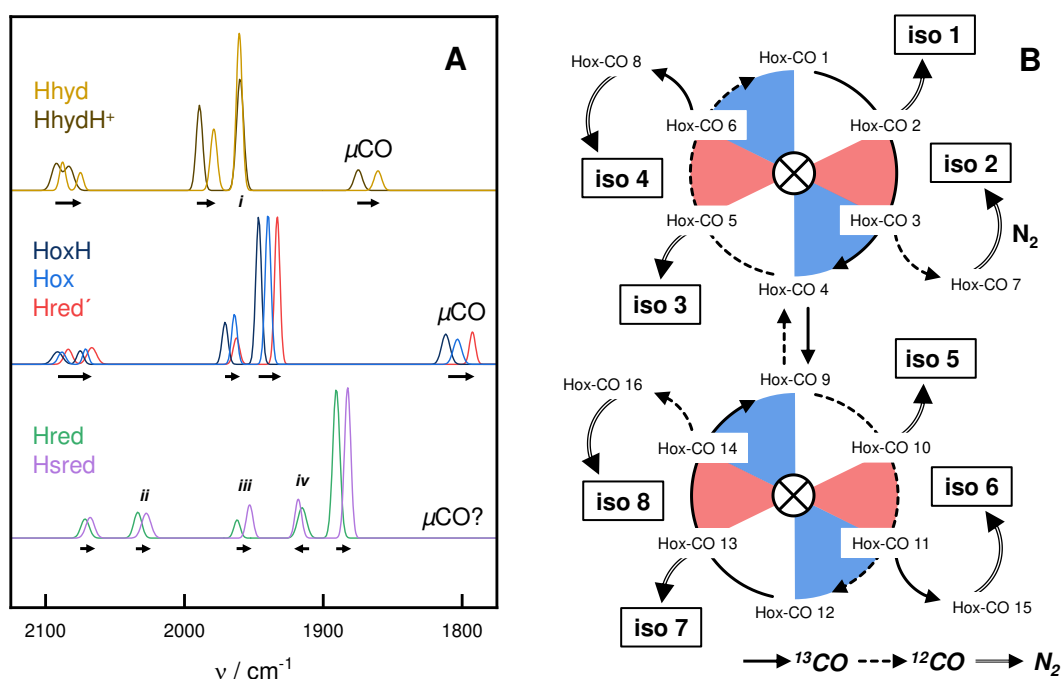
404 following details are not shown (see text): The electronic configuration of **Hox-CO** and **Hred'-**  
405 **CO** is the same as in **Hox** and **Hred'**, respectively. **Hhyd** binds a terminal hydride, which  
406 contributes two additional electrons. Similarly, **Hred** and **Hsred** can be described as with an  
407 Fe(II)-Fe(II) site and a bridging hydride. An isoelectronic transition from **Hsred** to **Hhyd** might  
408 be possible. **Htrans**, the one-electron reduced form of **Hinact**, would occupy the same position  
409 as **Hhyd** in this diagram.

410

### 411 3.1.2 Vibrational Properties of the H-cluster

412 The CO and CN<sup>-</sup> ligands preserve the low-spin character of the diiron site<sup>115,116</sup> and couple the  
413 H-cluster to the protein environment.<sup>50</sup> Making use of FTIR spectroscopy, the CO/CN<sup>-</sup>  
414 stretching frequencies can be addressed as intrinsic marker bands (Figure 8). They are  
415 characteristic for the geometry and the protonation state of all H-cluster intermediates  
416 independent of magnetic properties and <sup>57</sup>Fe labelling. For example, the **Hox** state shows a  
417 clear pattern of five bands that were assigned to uncoupled stretching vibrations of the CO and  
418 CN<sup>-</sup> ligands at each iron ion and a Fe-Fe bridging carbonyl ( $\mu$ CO) vibration at lower  
419 frequencies.<sup>117</sup> Under acidic conditions, the small linear *upshift* of vibrational frequencies in  
420 the protonated resting state **HoxH** (mean off-set ~6 cm<sup>-1</sup> relative to **Hox**) was assigned to a  
421 protonation event at the [4Fe-4S] cluster (as discussed above). Uniform shifts were observed  
422 between **Hred'** and **Hred'H**<sup>118</sup>, and a recent X-ray free electron laser (XFEL) structure  
423 suggests that changes in the orientation of a serine near the [4Fe-4S] cluster (S<sub>2</sub> in Figure 4)  
424 may have an effect similar to an adjacent protonation.<sup>53</sup> In contrast, the small linear *downshift*  
425 of frequencies in the transition from **Hox** to **Hred'** (mean off-set ~5 cm<sup>-1</sup>) suggested reduction  
426 to the [4Fe-4S] cluster most likely concerted with a protonation event at the same site.<sup>118,119</sup>  
427 The hydride state **Hhyd** represents an H-cluster species with most of the electron density  
428 located at the Fe<sub>a</sub>-H<sup>-</sup> bond and the [4Fe-4S] cluster formally rendering the diiron site a super-  
429 oxidized Fe(II)-Fe(II) species.<sup>110-113</sup> This results in a mean *upshift* of the CO/CN<sup>-</sup> signature by

430  $\sim 20\text{ cm}^{-1}$  relative to **Hox**, which is comparable to **Htrans**.<sup>120</sup> Apparently, the terminal hydride  
 431 species and the reduced [4Fe-4S] cluster damp the frequency increase expected from the  
 432 oxidation of the diiron site. In comparison to **Hox/HoxH** and **Hred'/Hred'H**, the irregular shift  
 433 of frequencies in **HhydH<sup>+</sup>** against **Hhyd** may stem from protonation of the amine headgroup  
 434 of the adt ligand.<sup>110,121</sup> Other hydride-like H-cluster intermediates (**Hhyd:ox** and **Hyd:red**) were  
 435 identified by laser irradiation of reduced [FeFe]-hydrogenase under cryogenic conditions.<sup>108</sup>



436

437 **Figure 8 - IR signature of the H-cluster in key redox states and isotope editing.** All data for  
 438 *CrHydA1*. (A) Gaussian fits of experimental IR spectra for Fe(II)-Fe(II), Fe(I)-Fe(II), and  
 439 Fe(I)-Fe(I) species of the H-cluster (top to bottom). Arrows highlight spectral shifts that were  
 440 assigned to protonation and/or redox differences at the [4Fe-4S] cluster. Note the following  
 441 irregularities: (i) Relative to **Hox/HoxH** and **Hred'/Hred'H** the shift between **Hhyd** and  
 442 **HhydH<sup>+</sup>** is less regular. (ii) One of the CN<sup>-</sup> ligands is strongly downshifted in **Hred** and **Hsred**.  
 443 (iii) The conversion of  $\mu\text{CO}$  into a terminal CO ligand upon reduction of the diiron site is under  
 444 discussion. (iv) Note the inversion of band frequencies. (B) Site-selective <sup>13</sup>CO isotope editing  
 445 of [FeFe]-hydrogenase under different illumination conditions (white area, dark; red area,  
 446 640 nm; blue area, 460 nm). The technique allows accumulation of all 16 CO isotopomers of  
 447 **Hox-CO** and all 8 non-inhibited H-cluster states (**Hox**). See ref. 123 for details.

448 The vibrations of the CO/CN<sup>-</sup> ligands are largely uncoupled in the ‘Hox-like’ states that are  
449 characterized by a  $\mu$ CO geometry and an open coordination site or terminal hydride ligand at  
450 the distal iron ion. Other H-cluster states exhibit pronounced vibrational coupling, *e.g.* upon  
451 reduction of the diiron site or CO inhibition.<sup>109,117,120</sup> Here, site-selective <sup>13</sup>CO isotope editing  
452 (Figure 8) in combination with quantum mechanical (QM) calculations allows unraveling the  
453 structure of H-cluster intermediates with great precision, *i.e.* simulating the coupling patterns  
454 for a given structure with respect to the change in mass.<sup>122</sup> The statistics obtained in such  
455 experiments suggested the presence of an apical CN<sup>-</sup> ligand in **Hox-CO** and **Hred'-CO**<sup>123,124</sup>  
456 hydrogen-bonded to adt-NH in a similar fashion as the apical hydride of **Hhyd**.<sup>89</sup> Moreover,  
457 the vibrational energy transfer in **Hred** and **Hsred** at room temperature hints at a cofactor  
458 geometry with an apical *d*CO ligand and a Fe-Fe bridging hydride ( $\mu$ H).<sup>125</sup> Cryogenic FTIR,  
459 on the other hand, suggested a diverging band pattern for these states (**Chapter 3.3**)  
460 incompatible with a  $\mu$ H ligand. Independent of temperature, **Hred** and **Hsred** show a number  
461 of further irregularities, *e.g.* the large frequency difference between *p*CN<sup>-</sup> and *d*CN<sup>-</sup> and the  
462 *upshift* of the *p*CO frequency upon reduction of the [4Fe-4S] cluster. The conflicting proposals  
463 for the geometry of **Hred** and **Hsred** are discussed in the next chapter.

### 464 **3.2 Biophysical Investigations *in cristallo***

465 The crystal structure of the [FeFe]-hydrogenase from *C. pasteurianum* and *D. desulfuricans* by  
466 X-ray diffractometry (XRD) laid the foundation for a molecular understanding of hydrogen  
467 catalysis.<sup>12,13</sup> Hot on the heels of the oxidized resting state **Hox**, *CpI* was crystallized in the  
468 presence of CO<sup>126-128</sup> and *DdH* was crystallized in the presence of H<sub>2</sub><sup>129</sup> resulting in H-cluster  
469 geometries that served as models for **Hox-CO** and **Hred**, respectively. Even though those  
470 crystal structures provide an exceptionally strong starting point for understanding of [FeFe]-  
471 hydrogenase chemistry, detailed insight into the dynamic geometry of the H-cluster is  
472 dependent on spectroscopy and QM calculations.<sup>130-134</sup> As discussed in the preceding chapter,



473 alternative ligand orientations in **Hox-CO**, **Hred**, and **Hsred** have been proposed. The limited  
474 spatial resolution of XRD on protein crystals impedes a distinction between the diatomic  
475 ligands at the H-cluster, which is why CO and CN<sup>-</sup> were assigned according to potential  
476 hydrogen-bonding contacts with the protein fold in the oxidized state.<sup>12,13</sup> At the proximal iron  
477 ion, the original CO/CN<sup>-</sup> assignment was found to be correct.<sup>50</sup> But while the crystal structure  
478 of CO-inhibited enzyme was modelled with an apical CO ligand (position 'X' in Figure 1),<sup>126-</sup>  
479 <sup>128</sup> vibrational coupling clearly suggested two equatorial carbonyls and an apical CN<sup>-</sup> ligand at  
480 the distal iron ion in **Hox-CO**.<sup>122-124</sup> This implies pronounced rotational freedom of ligands.  
481 Such dynamics are likely to play a role in the reaction with molecular oxygen<sup>135</sup> and the  
482 accumulation of the diiron-site reduced states **Hred** and **Hsred**.

483 The crystal structure of the reduced [FeFe]-hydrogenase *DdH* showed changes in the electron  
484 density of the H-cluster that were interpreted as a 'semi-bridging' CO ligand in a terminal,  
485 equatorial position at the distal iron ion.<sup>129</sup> At first glance, this interpretation is supported by  
486 room temperature IR spectroscopy as neither *DdH* nor *CrHydA1* show a low frequency peak  
487 in the IR signature of **Hred** (compare Figure 8).<sup>120,136</sup> But the vibrational coupling observed in  
488 **Hred** and **Hsred** is not compatible with a 'semi-bridging' CO geometry and rather suggests an  
489 apical CO ligand.<sup>125</sup> Whether the protection against CO inhibition in these states<sup>89</sup> is related to  
490 the reduction of the diiron site or the presence of an apical CO ligand remains to be clarified.  
491 Interestingly, cryogenic IR spectroscopy indicated a bridging ligand for both **Hred** and  
492 **Hsred**.<sup>108,137,138</sup> This is in agreement with a recent study by Artz *et al.* comparing cryogenic  
493 *CpI* crystal structures solved with both synchrotron radiation and the free electron laser (XFEL)  
494 light source at Stanford.<sup>53</sup> The authors were able to quantify the extent of photoreduction  
495 suggesting that in the presence of strong reductant up to 50% of *CpI* was found in a "more  
496 reduced conformation" featuring the H-cluster in a  $\mu$ CO geometry.

497 **Outlook.** Crystallized in the presence of H<sub>2</sub>, the structure of *DdH* provides insight into the  
498 reduced form(s) of the H-cluster.<sup>129</sup> However, a pure accumulation of **H<sub>red</sub>** has never been  
499 proven *in crystallo* and the coordinates as reported by Nicolet *et al.* in 2001 most likely  
500 represent an ensemble of different reduced H-cluster intermediates. To assign XRD structural  
501 data, it will be necessary to investigate protein crystals by spectroscopy. In this context, single-  
502 crystal EPR spectroscopy is an exceptionally powerful tool for probing paramagnetic  
503 intermediates. As nicely exemplified by work on [NiFe]-hydrogenase<sup>139</sup>, it provides a method  
504 not just for verification of XRD sample status but also generates additional highly detailed  
505 experimental insight on electronic and geometric structure of the cofactor. Recent  
506 improvements in resonator design enabled its application also on [FeFe]-hydrogenase by  
507 Sidabaras *et al.*<sup>140</sup> Unfortunately, EPR spectroscopy is not applicable for all H-cluster states  
508 (**Chapter 3.1.1**) creating a need for additional spectroscopic methods. To this end, Ash *et al.*  
509 reported a FTIR spectro-electrochemical setup that allows probing single protein crystals by *in*  
510 *situ* IR microscopy as a function of applied potential.<sup>141</sup> The authors could demonstrate how a  
511 crystalized [NiFe]-hydrogenase switches from a reduced state (Ni-R) to an oxidized state (Ni-  
512 B), and *vice versa*. In the future, similar experiments will facilitate a validation of [FeFe]-  
513 hydrogenase redox states formed *in crystallo*, *e.g.* in the process of crystal growth under H<sub>2</sub> or  
514 CO. More elegantly, the enzyme could be adjusted to the redox state of interest  
515 electrochemically *in situ*, *i.e.* directly ahead of the XRD experiment. Within a relatively narrow  
516 time-frame, combining IR absorbance and XRD in a single setup will be feasible.<sup>142</sup> The  
517 influence of temperature is discussed in the next chapter.

### 518 **3.3 The Influence of Temperature**

519 While many techniques demand refrigeration or cryogenic temperatures < 90 K (*e.g.*,  
520 synchrotron XRD and XAS/XES, NRVs, Raman, EPR, and Mössbauer spectroscopy) others  
521 are compatible with ambient conditions (*e.g.*, FTIR and UV/vis spectroscopy, protein film

522 electrochemistry, and biochemical analyses). With respect to [FeFe]-hydrogenase, recent results  
523 indicate that a direct comparison is not always possible, in particular regarding the geometry  
524 of the reduced H-cluster. Making use of FTIR spectro-electrochemistry under ambient  
525 conditions, several groups demonstrated the apparent disappearance of the  $\mu\text{CO}$  band together  
526 with the appearance of a new band in the region associated with terminal CO ligands. This was  
527 interpreted as a shift of the  $\mu\text{CO}$  ligand into a terminal position upon accumulation of **Hred**  
528 and **Hsred**.<sup>120,125,129,136</sup> These findings were supported by investigations on H<sub>2</sub>- and/or  
529 dithionite-reduced enzyme under ambient conditions, albeit such experiments generally result  
530 in a greater variety of redox states (*i.e.*, including **Hhyd**).<sup>89,143</sup> Co-accumulation with **Hred'**  
531 complicated the assignment of **Hred** and led to the incorrect conclusion that **Hred** featured a  
532  $\mu\text{CO}$  band in *CrHydA1*.<sup>107</sup> Once **Hred'** had been properly identified<sup>52,118</sup>, the absence of a  
533 classic  $\mu\text{CO}$  ligand in **Hred** and **Hsred** was largely agreed upon. The crystal structure of  
534 reduced *DdH* seemed to support this, as discussed above (**Chapter 3.2**).<sup>129</sup>

535 Making use of FTIR spectroscopy on H<sub>2</sub>- and dithionite-reduced hydrogenase under cryogenic  
536 conditions, three recent studies identified low-frequency bands that were not observed at  
537 ambient temperatures. For *DdH*, *CrHydA1*, and *CaI* these bands were assigned to a  $\mu\text{CO}$  ligand  
538 in **Hred** ( $\sim 1809 \pm 8 \text{ cm}^{-1}$ ) and **Hsred** ( $\sim 1800 \pm 3 \text{ cm}^{-1}$ ).<sup>108,137,138</sup> Surprisingly, these  $\mu\text{CO}$   
539 frequencies are in the same range as **Hox** and **Hred'** (**Figure 8**), which would hint at a mixed  
540 valence rather than a fully reduced configuration of the diiron site. Under ambient conditions,  
541 the  $\mu\text{CO}$  stretching frequency is well conserved (**Table 1**) and has proven to be an excellent  
542 reporter of the electron density distribution across the diiron site (**Chapter 3.1.2**), *i.e.* due to  
543 his symmetric position between the proximal and distal ion ion. Additionally, the  $\mu\text{CO}$   
544 vibration is uncoupled from the terminal CO ligands<sup>52</sup>, which allows interrogating the  $\mu\text{CO}$   
545 vibration for protonation and redox differences independent of H-cluster geometry. Between  
546 Fe(II)-Fe(II) in **Hhyd** and Fe(I)-Fe(II) in **Hox** the difference is  $\sim 60 \text{ cm}^{-1}$ , for example (**Figure**

547 8).<sup>112</sup> In the Group C [FeFe]-hydrogenase from *T. maritima* **Hred** and **Hsred** display a distinct  
 548  $\mu\text{CO}$  ligand at ambient conditions, which is the reason Chongdar *et al.* introduced the  
 549 nomenclature Hred\* and Hsred\*. Here, the  $\mu\text{CO}$  frequency difference between Fe(I)-Fe(II) in  
 550 **Hox** and Fe(I)-Fe(I) in **Hsred** is  $\sim 50\text{ cm}^{-1}$ .<sup>35</sup> It should be mentioned that a similar low frequency  
 551  $\mu\text{CO}$  band has been assigned to the mixed valence diiron site of **Hox** in *CpII*, a representative  
 552 of the Group A [FeFe]-hydrogenases (sub-class M2).<sup>53</sup>

553 **Table 1.** Infrared signature of **Hox** for prototypical Group A [FeFe]-hydrogenases at room  
 554 temperature. The CO/CN<sup>-</sup> frequencies are given in  $\text{cm}^{-1}$ .

enzyme	<i>p</i> CN	<i>d</i> CN	<i>p</i> CO	<i>d</i> CO	$\mu\text{CO}$	ref.
<i>DdH</i>	2093	2078	1965	1940	1802	120
<i>CrHydA</i>	2088	2070	1964	1940	1802	107
<i>CbA5H</i>	2091	2080	1964	1940	1800	70
<i>MeHydA</i>	2087	2079	1964	1937	1803	66
<i>CpI</i> *	2069	2081	1970	1947	1801	53
<i>CaI</i> *	2070	2082	1970	1947	1802	137

\* Due to difference in the active site niche (P-motif), the assignment of *p*CN<sup>-</sup> and *d*CN<sup>-</sup> has been found to be inverted in *CpI* and *CaI* (see ref. 50).

555

556 Based on the observation that the terminal CO ligands do not significantly change with  
 557 temperature, Birrell *et al.* speculate that the  $\mu\text{CO}$  ligand in **Hred** and **Hsred** is present under  
 558 ambient conditions as well, *i.e.* with a reduced absorption coefficient that impedes detection.<sup>138</sup>  
 559 Arguably, the small frequency differences for the terminal CO ligands upon cooling hint at  
 560 structural changes in the H-cluster environment, *i.e.* involving residues directly interacting with  
 561 the cofactor (**Chapter 2.1.2**), rather than independent H-cluster states with a very similar IR  
 562 signature. However, more detailed studies on the vibrational line broadening as a function of  
 563 temperature are required. Clarifying the influence of temperature, additional experiments will  
 564 be necessary, *e.g.* addressing the glass transition from ambient to refrigeration temperatures.

565 **Outlook.** Thermodynamic considerations exclude H-cluster intermediates with larger  
566 structural differences from the catalytic cycle<sup>130,134,144</sup>, which raised the question whether **Hred**  
567 and **Hsred** can play a role in the rapid hydrogen turnover of [FeFe]-hydrogenase (**Chapter**  
568 **4**).<sup>145</sup> Today, ambient and cryogenic measurements show both the presence of an additional  
569 terminal CO ligand at room temperature<sup>120,125,129,136</sup> and the presence of a  $\mu$ CO ligand at  
570 cryogenic temperature.<sup>108,137,138</sup> Moreover, the geometry of **Hred** and **Hsred** under ambient  
571 conditions is hotly debated, with regards to protonation sites and whether the diiron site adopts  
572 an H<sub>2</sub>-inhibited,  $\mu$ H geometry or an active-ready, ‘Hox-like’ geometry with a  $\mu$ CO ligand.<sup>125</sup>

573 In order to understand the interconversion of ambient and cryogenic species of the H-cluster,  
574 it will be important to consider the influence of rotational freedom and protein environment as  
575 well as proton transfer and proton-coupled electron transport in the accumulation and ‘shaping’  
576 of redox states as a function of temperature. The lack of compatibility between recent models  
577 underscores the inadequate comprehension of hydrogenase catalysis in general.<sup>145</sup> These  
578 considerations are of utmost importance when it comes to the interpretation of crystal structures  
579 derived from conventional, cryogenic XRD and spectroscopic investigation *in crystallo*  
580 (**Chapter 3.2**). Exploiting XFEL radiation to solve the crystal structure of oxidized and reduced  
581 [FeFe]-hydrogenase under ambient conditions is an exciting prospect towards a unification of  
582 models. Here, it will be important to investigate enzyme with clear preferences for specific  
583 redox states, either natural or semi-synthetic [FeFe]-hydrogenases (**Chapter 2**). Moreover,  
584 XFELs can be used for serial femtosecond crystallography (SFX) that allows investigating the  
585 structure of short-lived, catalytic intermediates.<sup>146–148</sup> As [FeFe]-hydrogenases are not easily  
586 activated, we discuss suitable trigger concepts in the next chapter.

587

### 588 **3.4 Beyond Steady-state Spectroscopy**

589 Hydrogenases have been characterized by steady-state spectroscopy (**Chapters 3.1**) and XRD  
590 (**Chapter 3.2**). In the case of [FeFe]-hydrogenase, various *operando* or *in situ* methods  
591 facilitated recording spectral data or activity profiles as a function of electrochemical  
592 potential<sup>149–151</sup>, gas composition<sup>89</sup> and reactant concentration<sup>52</sup>, visible light irradiation,<sup>100,152</sup>  
593 and temperature.<sup>137,138</sup> While *in situ* methods typically provide a time resolution between  
594 seconds and hours, transient spectroscopy allows following short-lived cofactor intermediates  
595 that are difficult to stabilize under steady-state conditions.

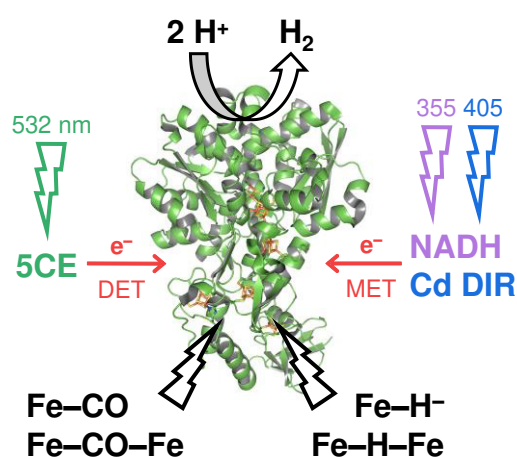
596 UV/vis and IR spectroscopy are well established for the analysis of transient processes in  
597 retinal-, porphyrin-, or flavin-binding proteins.<sup>153–155</sup> This is due to advantageous absorption  
598 properties (good signal-to-noise) and the ease of handling visible and infrared light. Here, laser  
599 sources are exploited to trigger and trace the natural reactivity of the chromoprotein, *i.e.* in a  
600 ‘flash photolysis’ setup comprising separate beams (as opposed to ultrafast ‘pump/probe’  
601 spectroscopy).<sup>156,157</sup> Hydrogenases lack a dedicated chromophore thus triggering activity is  
602 not trivial. To this end, absorption of laser light can be exploited to induce changes in  
603 temperature (‘T-jump’), pH, or redox potential, the latter in combination with suitable dyes.<sup>158–</sup>  
604 <sup>160</sup> With the development of tunable quantum cascade lasers (QCL), a powerful tabletop  
605 infrared light source became available that can be exploited as broadband probing light in a  
606 flash photolysis-type set-up.<sup>156</sup>

607 Transient infrared spectroscopy on hydrogenases was initially demonstrated on [NiFe]-  
608 hydrogenase. Greene *et al.* reported the first characterization of the soluble [NiFe]-hydrogenase  
609 from *Pyrococcus furiosus* by QCL flash photolysis in the mid-IR.<sup>161</sup> The authors used either  
610 NADH or Cd nanorods (DIR, dot-in-rod) as reductant, transferring an electron to the oxidized  
611 hydrogenase upon ionization at 355 nm or 405 nm, respectively (Ni-S → Ni-C).<sup>162,163</sup> In a  
612 complementary approach, Greene *et al.* addressed the photolability of a catalytic bridging

613 hydride intermediate (Ni-C  $\rightarrow$  Ni-S) at 530 nm, efficiently exploiting the bimetallic cofactor  
614 as ‘caged proton’.<sup>164</sup> These studies confirmed the kinetic competence of all observed  
615 intermediates. In a proof-of-concept study, the first QCL flash photolysis on [FeFe]-  
616 hydrogenase was reported by Mirmohades *et al.* in 2016.<sup>165</sup> Here, the authors followed the re-  
617 binding of an inhibiting CO molecule to the H-cluster of *CrHydA1* after photolysis at 355 nm  
618 (**Hox**  $\rightarrow$  **Hox-CO**) making use of the natural photosensitivity of iron-carbonyl complexes. The  
619 kinetics of CO re-binding were confirmed for several organometallic variants of *CrHydA1*.<sup>89</sup>  
620 A photo triggered transition from **Hox** to **Hred** has been reported for *CaI* adsorbed to Cd-Se  
621 nanocrystals,<sup>166</sup> revealing a distinct H/D isotope effect that is related to proton-coupled electron  
622 transfer.<sup>48</sup> Proceeding more deeply into the catalytic cycle, Greene *et al.* exploited NADH as  
623 reductant to the electron-bifurcating [FeFe]-hydrogenase *TmHydABC* when activated at 355  
624 nm.<sup>167</sup> The transient accumulation of **Hsred** and **Hred'** (incorrectly assigned to **Hred** in the  
625 publication) over **Hox** was found to be compatible with the relatively low turnover frequency  
626 of the enzyme ( $\sim 10$  H<sub>2</sub>/s).<sup>168</sup> However, whether these states are relevant to the fast hydrogen  
627 turnover of Group A [FeFe]-hydrogenases like *DdH* ( $>1,000$  H<sub>2</sub>/s) remained a matter of  
628 speculation. Eventually, Sanchez *et al.* demonstrated the use of Cd-doped nanorods<sup>162,163</sup> for  
629 the photoreduction of *CrHydA1* *via* mediated electron transfer (MET).<sup>169</sup> A great variety of  
630 redox states were observed, including **Hred** and **Hsred** as well as **Hred'** and **Hhyd**.

631 **Outlook.** As outlined above, numerous states have been identified under steady-state  
632 conditions, which inspired the formulation of different mechanistic proposals (**Chapter 4**).  
633 Undoubtedly the next step in mechanistic studies is to investigate the transient catalytic  
634 proceedings in [FeFe]-hydrogenase at different temperatures and pH conditions. This will  
635 facilitate a temporal separation of redox states. Based on the data reported by Sanchez *et al.*<sup>169</sup>,  
636 all key redox states (**Hred**, **Hred'**, **Hsred**, and **Hhyd**) may participate in catalytic turnover;  
637 however, pH titrations under both oxidizing and reducing conditions indicate significant

638 differences in the steady-state accumulation of redox states.<sup>170</sup> Such difference will have an  
 639 influence on kinetic parameters. Making use of eosin Y as a photosensitizer facilitates direct  
 640 electron transfer (DET) to the hydrogenase<sup>48</sup>, which may have an effect on the transient kinetics  
 641 as well. The photoexcitation at ~530 nm allows using light that does not affect the iron-carbonyl  
 642 bonds of the H-cluster on short time scales. Cross-coupling of hydrogenase molecules to  
 643 electron relay compounds or redox dyes represents another option to induce DET.<sup>171</sup> An  
 644 alternative approach would be addressing the ‘natural’ photosensitivity of certain redox states,  
 645 *i.e.* as shown by Greene *et al.* for the bridging hydride intermediate of [NiFe]-hydrogenase<sup>164</sup>  
 646 and Lorent *et al.* for **Hred** and **Hsred** of CrHydA1 at cryogenic temperatures.<sup>108</sup> In the future,  
 647 it will be exciting to see how these triggering concepts are utilized in transient UV/vis, FTIR,  
 648 or X-ray spectroscopy as well as SFX diffraction experiments. Figure 9 summarizes the  
 649 different trigger concepts discussed and suggested in this chapter.



650

651 **Figure 9 - Triggering hydrogen turnover or catalytic changes by visible light.** NADH and Cd-  
 652 doped nanorods (dot-in-rod, DIR) were used as redox dyes to follow the transient increase and  
 653 decrease of several [FeFe]-hydrogenase redox states. In comparison to mediated electron  
 654 transfer (MET, *e.g.* using methyl viologen or bipyridinium groups), direct electron transfer  
 655 (DET) may influence kinetic parameters, *e.g.* exploiting 5'-carboxy eosin Y (5CE). Addressing  
 656 the ‘natural’ photosensitivity of certain redox states represents an alternative approach. Here,  
 657 iron-carbonyl or iron-hydride bonds are interrogated by visible light of different color.

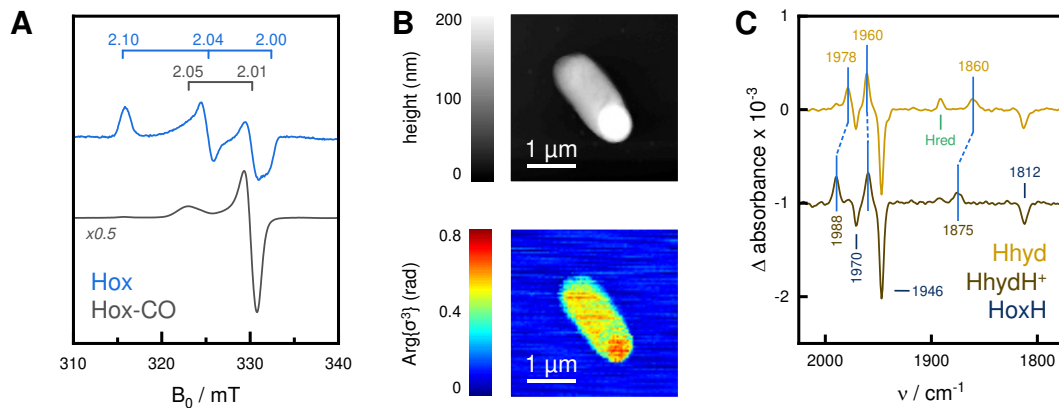


### 658 3.5 Biophysical Investigations *in vivo*

659 Mössbauer, EPR, and FTIR spectroscopic measurements with a good signal-to-noise ratio  
660 demand purified protein samples, often in high concentration and/or larger quantities.  
661 However, the properties of purified hydrogenase may deviate from the enzyme in its native  
662 environment, and several aspects of hydrogen turnover were first observed *in vivo*.<sup>23</sup> In 2010,  
663 Horch *et al.* reported whole-cell EPR and FTIR spectra of the soluble [NiFe]-hydrogenase from  
664 *Ralstonia eutropha* that helped elucidating the mechanism of O<sub>2</sub> tolerance of this particular  
665 enzyme.<sup>172</sup> Similar studies on [FeFe]-hydrogenase were not available until 2017 when  
666 Berggren *et al.* adopted the protocol of artificial maturation<sup>62,63</sup> to bacterial cells heterologously  
667 expressing [FeFe]-hydrogenase apo-protein.<sup>88</sup> This paved the way for the first whole-cell EPR  
668 study on CrHydA1 in recombinant *E. coli* cells.<sup>173</sup>

669 The paramagnetic states **Hox** and **Hox-CO** give relatively unperturbed signals in whole-cell  
670 EPR, comparable with purified enzyme (Figure 10).<sup>174</sup> These signatures have been exploited  
671 by Land *et al.* to screen uncharacterized [FeFe]-hydrogenases in *E. coli*, circumventing the  
672 means of protein purification for preliminary identification and characterization.<sup>36</sup> Moreover,  
673 this latter study comprises the first whole-cell FTIR evaluation of [FeFe]-hydrogenase, which  
674 proved to be challenging due to low signal intensity in the CO/CN<sup>-</sup> frequency regime of the H-  
675 cluster and significant overlap with water absorption. Investigating the Group D [FeFe]-  
676 hydrogenase TamHydA, for the first time an EPR-silent reduced H-cluster state (*i.e.*, **Hred**)  
677 could be identified in a living organism. Cell lysates containing catalytically active [FeFe]-  
678 hydrogenases like CrHydA1 and the M2-type [FeFe]-hydrogenase HYDA from *Solobacterium*  
679 *moorei* (M2) even showed a clear current response in chronoamperometric experiments  
680 depending on whether H<sub>2</sub> was available for oxidation or not.<sup>36</sup> Recently, Mészáros *et al.* used  
681 near-field optimal microscopy (sSNOM) to map the protein content of recombinant *E. coli* cells  
682 (Figure 10).<sup>121</sup> No protein was detected outside the cell bodies indicating that all spectroscopic

683 results stem from hydrogenase inside the bacteria. Varying the redox conditions, pH value, and  
 684 gas atmosphere, EPR and FTIR spectroscopy verified the formation of reactive hydride states  
 685 under cellular conditions, including **Hhyd** and **HhydH<sup>+</sup>** (Figure 10). Note that Mulder *et al.*  
 686 suggested the existence of **HhydH<sup>+</sup>** previously, albeit with an incomplete IR signature.<sup>110</sup>



687

688 **Figure 10 - Biophysical investigations in vivo.** (A) Whole-cell EPR spectra recorded of cells  
 689 incubated in the absence (upper spectrum) and presence of CO gas (lower spectrum). The  
 690 signatures of **Hox** and **Hox-CO** are clearly detectable. (B) Atomic force microscopy (upper  
 691 panel) and scattering-type scanning near-field optical microscopy (sSNOM) on recombinant  
 692 *E. coli* cells. The sSNOM map was recorded at  $1660 \text{ cm}^{-1}$ , indicating the lack of protein-specific  
 693 signal anywhere outside the cell. (C) Whole-cell ATR FTIR difference spectroscopy. At low pH  
 694 and in the presence of  $\text{H}_2$ , a pattern characteristic of **Hhyd** - **HoxH** (upper spectrum) was  
 695 observed. When the concentration of dithionite in the sample was increased, **HhydH<sup>+</sup>** was  
 696 found to be accumulated over **HoxH** (lower spectrum).

697

698 **Outlook.** The first generation of whole-cell biophysical investigations agrees with most  
 699 measurements performed on purified enzyme. Recombinant hydrogenase was found to react  
 700 surprisingly fast to changes in buffer, redox potential, pH, and gas composition. H-cluster states  
 701 **Hox**, **Hred**, and **Hhyd** could be detected in living cells, even under mildly alkaline  
 702 conditions.<sup>121</sup> However, elusive species like **Hred'** and **Hsred** were barely observed. This is  
 703 indicative of oxidation and docking to the energy metabolism of host cells, *i.e.* via the bacterial

704 ferredoxin as demonstrated by Barstow *et al.* before.<sup>175</sup> In the future, it will be exciting to study  
705 the physiological involvement of hydrogenase in recombinant bacteria and green algae, *e.g.* in  
706 the context of bifurcation or photosynthesis.<sup>176–178</sup> Using suitable dyes, fluorescence  
707 microscopy could give further insight in pH changes or H<sub>2</sub> release of single cells.<sup>179–181</sup>  
708 Methodically, EPR and FTIR spectroscopy proved to be powerful tools for the analysis of  
709 recombinant whole cells. It may be possible to optimize the chemical sensitivity and spatial  
710 resolution of near-field techniques to reveal the intracellular location of hydrogenase based on  
711 the CO/CN<sup>-</sup> signature of the H-cluster.<sup>182–185</sup> Film electrochemistry on intact *E. coli* cells  
712 suffered from low catalytic currents; however, introducing electron relays in the outer  
713 membrane may establish electrophysiological measurements of hydrogenase activity (for  
714 comprehensive reviews on bioelectrochemistry, see Armstrong *et al.* and Del Barrio *et*  
715 *al.*<sup>150,186</sup>).

#### 716 **4. The Catalytic Mechanism**

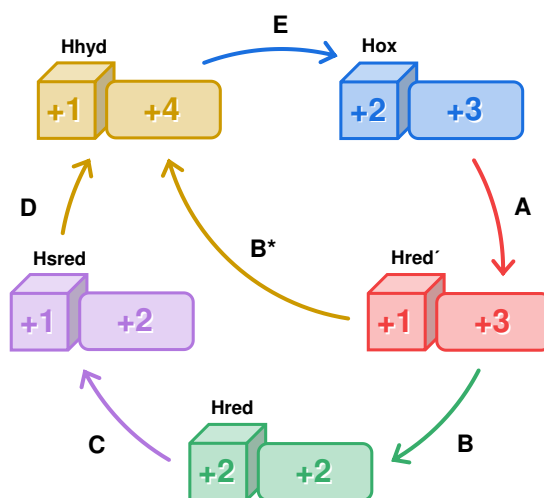
717 In **Chapter 2.1.1**, we suggested grouping the different [FeFe]-hydrogenases based on their  
718 phylogenetic relationship (Group A – D) and domain architecture (M1 – M5). Considering the  
719 well-conserved nature of the maturation enzymes (HydEFG), all hydrogenases arguably carry  
720 the same H-cluster. However, the presence of up to five F-clusters and deviations in the identity  
721 of amino acid residues in proton transfer pathway and active site niche brings forth significant  
722 differences in catalytic behavior. Additionally, the advances in artificial maturation allowed  
723 exploring variations in cofactor composition beyond the scope of nature (**Chapter 2.1.2**).  
724 These findings form the basis to which speculations on the catalytic proceedings of hydrogen  
725 turnover must obey.

726

727 In **Chapter 3.1** we introduced the huge variety of electronic intermediates adopted by the H-  
728 cluster. While some of these intermediates are rather well understood (**Hox**, **Hred'**, **Hhyd**)  
729 others show controversial characteristics (**Hred**, **Hsred**, **Hox-CO**). Understanding the latter  
730 states, we suggested combining X-ray diffraction with spectroscopic investigations of  
731 crystallized enzyme, *e.g.* as grown under H<sub>2</sub> or adjusted to a certain electrical potential  
732 (**Chapter 3.2**). Further complicating the situation, recent findings demonstrated the influence  
733 of temperature on the geometry of the H-cluster. This raised general concerns regarding the  
734 relevance of cryogenic methods in understanding [FeFe]-hydrogenase. In **Chapter 3.3**, we  
735 reviewed the key differences between ambient and cryogenic measurements, suggesting  
736 diffraction experiments with XFELs as a powerful technique harvesting structural information  
737 under ambient conditions. Besides investigating [FeFe]-hydrogenase with transient  
738 spectroscopy methods (**Chapter 3.4**), serial femtosecond crystallography at XFEL sources  
739 may also provide time-resolved structural information.

740 Based on the wealth of biochemical, structural, and spectroscopic data, different catalytic  
741 cycles have been suggested.<sup>145</sup> These are subject to constant evolution; therefore, we refrain  
742 from reviewing any details. Figure 11 depicts a simplified catalytic cycle that shows the two  
743 main models. Going from A – E in the direction of H<sub>2</sub> release, both Birrell *et al.* and Ratzloff  
744 *et al.* propose the involvement of **Hred** and **Hsred** (5-step model).<sup>137,138</sup> In the first step, **Hred'**  
745 is formed by one-electron reduction of **Hox** (A). The charge resides at the [4Fe-4S] cluster.  
746 Upon protonation of the adt ligand (<sup>+</sup>NH<sub>2</sub>), it is conceivable that the electron tunnels to the  
747 diiron site forming **Hred** (B). In a second step of one-electron reduction, **Hred** is converted  
748 into **Hsred** (C). Afterwards, the extra proton at the adt ligand migrates to the open binding site  
749 of the distal iron ion and forms **Hhyd**, formally oxidizing the diiron site by two electrons (D).  
750 Light-induced FTIR difference spectroscopy demonstrated the conversion of **Hred** into  
751 Hhyd:ox and **Hsred** into Hhyd:red, which indicates that the formation of **Hhyd** over **Hsred** is

752 formally allowed.<sup>108</sup> The reduced [4Fe-4S] cluster and hydrogen bonding between adt and Fe<sub>a</sub>-  
 753 H<sup>-</sup> stabilizes the super-oxidized diiron site.<sup>89</sup> Upon proton transfer *via* the adt ligand, the  
 754 terminal hydride combines to H<sub>2</sub>, presumably including transient H-cluster states like **HhydH**<sup>+</sup>  
 755 and **Hox-H<sub>2</sub>** (not shown).<sup>108</sup> Eventually, release of H<sub>2</sub> restores the oxidized resting state **Hox**  
 756 (E).



757  
 758 **Figure 11 – Possible succession of redox intermediation in biological hydrogen turnover.**  
 759 *The 5-step model (A – E) includes **Hred** and **Hsred**, presuming the H-cluster retains the  $\mu$ CO*  
 760 *ligand upon reduction of the diiron site. The 3-step model (A – B\* – E) suggests a ‘short-cut’*  
 761 *from **Hred'** to **Hhyd** as both species share the same geometry. The geometry of **Hred** and **Hsred***  
 762 *under ambient conditions is debated. See text for details.*

763  
 764 The 5-step model in Figure 11 is based on the interpretation of **Hred** and **Hsred** as H-cluster  
 765 intermediates with an open coordination site at the distal iron ion, not unlike **Hox** or **Hred'**. As  
 766 discussed in **Chapter 3.2**, this was concluded from the XRD structure of reduced enzyme,  
 767 showing a ‘semi-bridging’ CO ligand and no electron density in the apical position.<sup>129</sup>  
 768 However, recent FTIR investigation under *cryogenic* conditions by both Birrell *et al.* and  
 769 Ratzloff *et al.* instead suggest fully-bridged models for **Hred** and **Hsred** (**Chapter 3.3**).  
 770 Investigating H-cluster reduction under ambient conditions, Mebs *et al.* proposed an alternative

771 interpretation of the diiron site geometry in **Hred** and **Hsred**.<sup>125</sup> Here, the presence of a apical  
772 CO ligand at the distal iron ion alongside stabilization of charge *via* a bridging hydride species  
773 puts **Hred** and **Hsred** in the position of ‘H<sub>2</sub>-inhibited’ intermediates.<sup>89</sup> The 3-step model in  
774 [Figure 11](#) starts with the conversion of **Hox** into **Hred**’, including reduction of the [4Fe-4S]  
775 cluster and protonation of a coordinating cysteine residue (A). In a second proton-coupled  
776 electron transfer step, **Hhyd** is formed from **Hred**’ directly (B\*). Stabilizing the reduced [4Fe-  
777 4S] cluster *via* protonation in both species impedes electrons ‘leaking’ into the diiron  
778 site.<sup>52,112,118</sup> Dihydrogen release and restoration of **Hox** is believed to occur in a similar fashion  
779 as described above (E).

## 780 **5. Concluding Remarks**

781 We only just scratched the surface of hydrogenase biodiversity. Moving forward, investigating  
782 novel [FeFe]-hydrogenases will help understanding the molecular proceedings of hydrogen  
783 turnover. Even less comprehensive is our knowledge about how amino acid patterns shape  
784 proton transfer, gas channels, and the dynamic geometry of the H-cluster. While organo-  
785 metallic variants are not likely to enhance catalytic efficiency, interfering with nature will  
786 facilitate our comprehension of the inner workings of [FeFe]-hydrogenase. Future experiments  
787 must facilitate connections between data collected under ambient or cryogenic conditions, on  
788 isolated enzyme or whole cells, and in-between species. Critically, steady-state measurements  
789 must also be complemented with transient spectroscopy studies and high-level QM calculations  
790 to verify the catalytic relevance of identified states. Fortunately, the community is not shy  
791 tackling such problems with novel techniques, *e.g.* two-dimensional infrared spectroscopy  
792 (2DIR),<sup>187</sup> Fourier-transformed alternating current voltammetry (FTacV)<sup>188</sup>, or scattering-type  
793 scanning near-field optimal microscopy (sSNOM).<sup>121</sup> More will follow, from the biological,  
794 chemical, and physical corners of the community, and it will be exciting to participate in the  
795 progress.

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806

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