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## The missing pieces in the catalytic cycle of [FeFe] hydrogenases

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Hydrogen could provide a suitable means for storing energy from intermittent renewable sources for later use on demand. However, many challenges remain regarding the activity, specificity, stability and sustainability of current hydrogen production and consumption methods. The lack of efficient catalysts based on abundant and sustainable elements lies at the heart of this problem. Nature's solution led to the evolution of hydrogenase enzymes capable of reversible hydrogen conversion at high rates using iron- and nickel-based active sites. Through a detailed understanding of these enzymes, we can learn how to mimic them to engineer a new generation of highly active synthetic catalysts. Incredible progress has been made in our understanding of biological hydrogen activation over the last few years. In particular, detailed studies of the [FeFe] hydrogenase class have provided substantial insight into a sophisticated, optimised, molecular catalyst, the active site H-cluster. In this short perspective, we will summarise recent findings and highlight the missing pieces needed to complete the puzzle.

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## The big picture: renewable energy and the H<sub>2</sub> economy

The Industrial Revolution marked an incredible time of human ingenuity and progress. Yet, it has also caused huge amounts of carbon dioxide, methane, and other potent greenhouse gases to accumulate in the atmosphere, leading to global warming and

climate change. Now, efforts to curb greenhouse gas emissions require us to abandon fossil fuels and move to a completely circular energy economy. While electricity produced by renewable energy sources such as wind and solar power has the potential to supply all our current energy demands, the issue of energy storage remains a difficult problem to solve. Hydrogen could act as a suitable energy vector as it can be produced by water electrolysis and its combustion produces water as the sole byproduct (Fig. 1).<sup>1</sup> Advantageously, hydrogen is incredibly energy-dense (120 MJ kg<sup>-1</sup>),<sup>2</sup> however, its storage and transport prove problematic. Regardless, hydrogen is still a crucial reactant for several industrial processes, including oil refining and the Haber Bosch process for the reduction of nitrogen to ammonia, which each use around 30% of annual global

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Fig. 1 Schematic representation of a hydrogen economy.

hydrogen production.<sup>3</sup> Presently, 98% of hydrogen is produced from fossil fuels, and of the remaining 2% produced by electrolysis, only a fraction is produced using renewable electricity.<sup>3</sup>

There are currently three major technologies for producing hydrogen *via* electrolysis: alkaline electrolyzers, which use nickel-based electrodes and work at very large overpotentials, proton exchange membrane electrolyzers, which use platinum, and solid oxide electrolyzers, which use nickel-yttria-stabilised zirconia.<sup>4</sup> So, while water electrolysis is an established hydrogen production technology, there is still significant interest in developing more efficient, stable, and cheaper relevant materials. Since hydrogen has a relatively large bond dissociation energy ( $436 \text{ kJ mol}^{-1}$ ),<sup>2</sup> it is fairly inert and must be activated by a catalyst. As such, there has been incredible investment in understanding how nature uses hydrogen and catalyses this difficult reaction with high efficiency.

In nature, hydrogen is predominantly produced and consumed by hydrogenases, of which there are three phylogenetically distinct classes: the [NiFe] hydrogenases, the [FeFe] hydrogenases and the [Fe] hydrogenases. All three groups heterolytically split hydrogen, with [Fe] hydrogenases requiring a cosubstrate, methenyl-tetrahydromethanopterin, which they

hydrogenate by direct hydride transfer. Meanwhile, [NiFe] and [FeFe] hydrogenases reversibly oxidise hydrogen to protons and electrons and transfer these electrons to electron carriers like ferredoxin. Nitrogenases and a plethora of other enzymes have been demonstrated to oxidise or produce hydrogen, albeit at substantially lower rates than the true hydrogenases.<sup>5–11</sup>

Studying how hydrogenases work to understand the crucial mechanistic principles of efficient hydrogen activation has been an active area of research for well over 50 years. But how far have we come and what is left to understand? In this perspective article, we will address these questions, with a particular focus on [FeFe] hydrogenases. We will also discuss where we see the field going over the next years and what the main challenges are for uncovering the ‘missing pieces’ in the catalytic cycle.

## The unique structure of the [FeFe] hydrogenase active site

Since 1996 it has been known that [FeFe] hydrogenases contain an active site, referred to as the H-cluster, which is a unique type of iron-sulfur cluster coordinated by cyanide and carbon monoxide ligands.<sup>12</sup> However, its intricacies were unknown<sup>13</sup> until Peters and coworkers published the X-ray crystal structure of the [FeFe] hydrogenase from *Clostridium pasteurianum* (CpI) in 1998.<sup>14</sup> For the first time, a unique  $[2\text{Fe}]_{\text{H}}$  subcluster, coordinated by diatomic ligands and an additional unprecedented bridging ligand was observed covalently attached to a  $[4\text{Fe}-4\text{S}]_{\text{H}}$  cubane cluster. This was immediately suggested as the site of hydrogen activation. Initially, the unknown bridging ligand was identified to contain two sulfurs, which directly coordinate each Fe. The electron density between them was modelled as water. A later structure from Nicolet and coworkers of the [FeFe] hydrogenase from *Desulfovibrio desulfuricans* (DdHydAB) modelled the bridging ligand as a propane-1,3-dithiolate ligand (PDT).<sup>15</sup> A subsequent publication from the same group revised



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Fig. 2 Structure of the active site H-cluster of [FeFe] hydrogenase in (A) a ChemDraw style representation and (B) a Pymol style representation. Residue numbering is from the *C. pasteurianum* enzyme. The image was prepared using PDB ID 4XDC.<sup>24</sup>



Fig. 3 Proposed catalytic cycle of [FeFe] hydrogenase. The blue pathway indicates the active cycle, while the pink pathway indicates inactivation by binding to inhibitors like  $\text{H}_2\text{S}$  and  $\text{HCN}$ , the purple pathway indicates reversible inhibition by  $\text{CO}$ , and the gray pathway indicates an alternative route from the  $\text{H}_{\text{red}}\text{H}^+$  state to the  $\text{H}_{\text{hyd:red}}$  state under less reducing conditions. The yellow, orange and red rectangles represent the  $[\text{2Fe}]_{\text{H}}$  cluster in the  $\text{Fe}(\text{II})\text{Fe}(\text{II})$ ,  $\text{Fe}(\text{II})\text{Fe}(\text{I})$  and  $\text{Fe}(\text{I})\text{Fe}(\text{I})$  oxidation states, respectively, with the terminal  $\text{CO}$  and  $\text{CN}^-$  ligands omitted for clarity. The yellow and orange diamonds represent the  $[\text{4Fe-4S}]_{\text{H}}$  cluster in the +2 and +1 oxidation states, respectively.

this to a 2-azapropane-1,3-dithiolate ligand (ADT, also known as di(thiomethyl)amine or DTMA).<sup>16</sup> This latter idea was supported by some groups through density functional calculations<sup>17</sup> but others proposed a more likely bridging ligand to be 2-oxapropane-1,3-dithiolate (ODT, also known as di(thiomethyl) ether or DTME).<sup>18</sup> After years of intense debate, the composition of the dithiolate as ADT is now well established, both through spectroscopic studies<sup>19–22</sup> and reconstitution studies.<sup>23</sup> Thus, today we have a fairly clear picture of the active site structure of [FeFe] hydrogenases (Fig. 2).

The [FeFe] hydrogenase H-cluster provides a rare and interesting example of two metal clusters electronically coupled by a simple covalent linkage (Fig. 3). The  $[\text{4Fe-4S}]_{\text{H}}$  cluster in the

absence of the  $[\text{2Fe}]_{\text{H}}$  subcluster is stable and functions as a simple redox cofactor.<sup>23</sup> As is the  $[\text{2Fe}]_{\text{H}}$  subcluster which can also undergo protonation and deprotonation. However, the  $[\text{2Fe}]_{\text{H}}$  subcluster alone is an extremely poor hydrogen conversion catalyst; it is only by inserting it into the [FeFe] hydrogenase protein scaffold that high activity is achieved. Substitution of the sulfur in the  $[\text{4Fe-4S}]_{\text{H}}$  cluster with selenium alters the cluster redox potential but has very little effect on activity.<sup>25</sup> Meanwhile, mutation of various amino acids surrounding the  $[\text{2Fe}]_{\text{H}}$  subcluster produces highly inactive enzymes,<sup>26</sup> suggesting this factor is most crucial for enzyme activity. Interestingly though, mutation of the ligands coordinated to  $[\text{4Fe-4S}]_{\text{H}}$  (e.g., from cysteine to histidine) affects activity, shifting the catalytic bias and increasing the overpotential.<sup>27,28</sup>

## General mechanistic principles: proton-coupled electron transfer and hydride formation

In 2006, Roseboom and coworkers investigated the [FeFe] hydrogenase from *Desulfovibrio desulfuricans* using infrared (IR) spectroscopy coupled with electrochemistry.<sup>29</sup> This enzyme is unusual because it can be purified under air in an inactive over-oxidised state known as  $\text{H}_{\text{inact}}$ . In their titrations from high potential to low potential they observed conversion of  $\text{H}_{\text{inact}}$  to an intermediate state  $\text{H}_{\text{trans}}$  followed by formation of the active oxidised state,  $\text{H}_{\text{ox}}$ . Further reduction formed the one-electron-reduced  $\text{H}_{\text{red}}$  state, followed by the formation of a two-electron-reduced, or super-reduced,  $\text{H}_{\text{sred}}$  state. Based on the positions of the IR bands as well as the known electron paramagnetic resonance (EPR) properties of each state, the  $\text{H}_{\text{inact}}$  state was described as a  $[\text{4Fe-4S}]^{2+}\text{-}[\text{Fe}(\text{II})\text{Fe}(\text{II})]$  state. Reduction to  $\text{H}_{\text{trans}}$  involved reduction of the  $[\text{4Fe-4S}]_{\text{H}}$  cluster and yielded a  $[\text{4Fe-4S}]^+\text{-}[\text{Fe}(\text{II})\text{Fe}(\text{II})]$  state.  $\text{H}_{\text{ox}}$  was thought to be isoelectronic with  $\text{H}_{\text{trans}}$  with a  $[\text{4Fe-4S}]^{2+}\text{-}[\text{Fe}(\text{II})\text{Fe}(\text{I})]$  state, where the electron moved from the  $[\text{4Fe-4S}]_{\text{H}}$  cluster to the  $[\text{2Fe}]_{\text{H}}$  cluster. Reduction of  $\text{H}_{\text{ox}}$  to  $\text{H}_{\text{red}}$  was thought to occur at the  $[\text{2Fe}]_{\text{H}}$  cluster yielding a  $[\text{4Fe-4S}]^{2+}\text{-}[\text{Fe}(\text{I})\text{Fe}(\text{I})]$  state, and formation of  $\text{H}_{\text{sred}}$  was thought to involve reduction of the  $[\text{4Fe-4S}]_{\text{H}}$  cluster giving a  $[\text{4Fe-4S}]^+\text{-}[\text{Fe}(\text{I})\text{Fe}(\text{I})]$  state. The last transition was observed to be not completely reversible. These transitions were all pH-dependent, indicating the important role of proton-coupled electron transfer for the enzymes' high catalytic activities.

In 2009, Silakov and coworkers performed similar experiments with the smaller, simpler [FeFe] hydrogenase from *Chlamydomonas reinhardtii* (*CrHydA1*), made similar observations and came to similar conclusions.<sup>30</sup> However, one interesting finding was a large difference in the IR bands of the one-electron-reduced  $\text{H}_{\text{red}}$  state between the two enzymes. This issue was later resolved when it was discovered that the one-electron reduced state in *CrHydA1* consisted of two different states, whose population depended on the pH, having roughly equal populations of both at neutral pH.<sup>31</sup> Thus, these two states were dubbed the  $\text{H}_{\text{red}}$  and  $\text{H}_{\text{red}}\text{H}^+$  states, where the former was observed predominantly at high pH and the latter at low pH. The  $\text{H}_{\text{red}}$  state had small red-shifts in the IR bands relative to





the  $H_{ox}$  state whereas the  $H_{red}H^+$  state had large red-shifts, indicating that the  $H_{red}$  state had a  $[4Fe-4S]^+-[Fe(II)Fe(I)]$  electronic structure while the  $H_{red}H^+$  state had a  $[4Fe-4S]^{2+}-[Fe(I)Fe(I)]$  electronic structure. It was assumed that the ADT ligand bridging the two Fe atoms was protonated in the  $H_{red}H^+$  state facilitating electron transfer by charge neutralisation effects. Using a non-protonatable propane-1,3-dithiolate (PDT) ligand prevented the formation of  $H_{red}H^+$ , supporting the assignment.<sup>32</sup> Furthermore, photolysis experiments were able to enrich the  $H_{red}$  state.<sup>33</sup> Resonance Raman (RR) spectroscopy of *CrHydA1* revealed a species containing a reduced  $[4Fe-4S]^+$  cluster and a  $H_{ox}$ -like  $[2Fe]_H$  site, likely derived from  $H_{red}H^+$ . The RR experimental conditions, laser illumination at low temperature, could facilitate electron transfer from the  $[2Fe]_H$  subcluster to  $[4Fe-4S]_H$ , generating a  $[4Fe-4S]^+-[Fe(II)Fe(I)]$  state, which was named  $H_{red}'$  at the time. Katz *et al.* proposed a proton transfer between the H-cluster and a near base (Cys169)<sup>33</sup> as the driving force for electron transfer between the  $[4Fe-4S]_H$  and  $[2Fe]_H$  sites. Therefore, the populations of the isoelectronic states  $H_{red}$  and  $H_{red}H^+$  were controlled by the protonation states of the ADT ligand through charge compensation of the  $[FeFe]$  subcluster. This was later supported by IR spectroelectrochemical titrations.<sup>31</sup>

Interestingly, the  $[FeFe]$  hydrogenase from *D. desulfuricans* only shows the  $H_{red}H^+$  state at neutral pH.<sup>29</sup> More carefully analysed redox titrations revealed that initial reduction of the  $H_{ox}$  state generates a mixture of both  $H_{red}$  states but that further reduction converts the  $H_{red}$  state into the  $H_{red}H^+$  state. In this case, what appears to happen is the reduction of the accessory iron-sulfur clusters triggers the electron to move from the  $[4Fe-4S]_H$  subcluster to the  $[2Fe]_H$  subcluster by charge repulsion. This then increases the  $pK_a$  on the H-cluster forcing it to be completely protonated at neutral pH.<sup>34</sup>

Despite having very little catalytic activity the  $[FeFe]$  hydrogenases in which the ADT cofactor is substituted with PDT or oxapropane-1,3-dithiolate (ODT) have proved incredibly useful for understanding the catalytic mechanism. The PDT variant can only exist in two oxidation states: an  $H_{ox}$ -like state with  $[4Fe-4S]^{2+}-[Fe(II)Fe(I)]$  and an  $H_{red}$ -like state with  $[4Fe-4S]^+-[Fe(II)Fe(I)]$ . The missing nitrogen bridgehead seems to prevent the formation of states in which  $[2Fe]_H$  is reduced to  $Fe(I)Fe(I)$  as its reduction potential is too negative without coupled protonation due to charge imbalance. If, as has been suggested by others,<sup>35</sup> the  $H_{red}H^+$  and  $H_{sred}H^+$  states contain bridging hydrides rather than protonated amines, then it is not clear why these states would not form in the PDT variant; there may be slower kinetics involved but eventually, these states should form. It is also interesting to note that terminal hydrides have yet to be detected in the PDT variant, which hints that the differences between the ADT and PDT variants are more complicated than simply the loss of the bridging amine. It should also be noted that the redox potential for the  $H_{ox}/H_{red}$  transition has been reported to be pH-dependent, indicating that reduction of  $[4Fe-4S]_H$  involves PCET.<sup>36</sup> However, this result has been disputed<sup>37</sup> and argued to be an artefact due to the presence of sodium dithionite in the preparations.<sup>38</sup>

While the PDT variant was useful for understanding PCET at the H-cluster, the ODT variant has been useful for understanding the formation of terminal Fe-hydrides. The ODT variant forms a fairly stable state with the lowest CO-band at  $1867\text{ cm}^{-1}$ ,<sup>23</sup> which was thought to have a  $[4Fe-4S]^+-[Fe(II)Fe(II)]H^-$  structure. The reason for this is still not entirely clear. It has been argued that the ether group in ODT, being much less basic than the amine group of ADT, is a poor proton relay so allows kinetic trapping of the Fe-hydride intermediate.<sup>39</sup> Another possibility is that, like the PDT variant, the ODT variant does not form the  $H_{red}H^+$  state, but, unlike the PDT variant, the ODT variant undergoes PCET with protonation of  $Fe_d$ . Regardless, Reijerse *et al.* directly observed the terminal hydride on  $Fe_d$  by studying the  $^{57}Fe$  labeled *CrHydA1* ODT variant with nuclear resonance vibrational spectroscopy (NRVS).<sup>40</sup>

A final point that required investigation was the observation that both the  $H_{red}H^+$  and  $H_{sred}H^+$  states appeared to be lacking a bridging CO band in the IR spectra, and instead appeared to have an additional terminal CO at approximately  $1960\text{ cm}^{-1}$  or  $1950\text{ cm}^{-1}$ . This finding has been interpreted to mean that the bridging CO becomes terminal leading to the formation of bridging hydride states. Under some circumstances, however, the bridging CO appeared to be retained *e.g.* at low temperature<sup>41–43</sup> and in sensory enzymes.<sup>44</sup> Furthermore, the  $1960\text{ cm}^{-1}$  and  $1950\text{ cm}^{-1}$  bands have been suggested to be due to small amounts of terminal hydride-containing states, which can be populated at low temperatures by photoexcitation. Therefore, the structural assignments of the  $H_{red}H^+$  and  $H_{sred}H^+$  states remain contentious.

Overall, catalysis at the H-cluster appears to proceed as follows: in the proton reduction direction, the active oxidised  $H_{ox}$  state can be reduced by one electron. This gives an H-cluster with a  $pK_a$  of approximately 7.2, so, around pH 7, a protonated ( $H_{red}H^+$ ) and deprotonated ( $H_{red}$ ) form are observed. The  $H_{red}H^+$  state has an oxidised  $[4Fe-4S]_H$  cluster and accepts an electron to form the  $H_{sred}H^+$  state. Meanwhile, in the reverse direction, the  $H_{red}$  state can be oxidised to the  $H_{ox}$  state by electron transfer from the  $[4Fe-4S]_H$  cluster. This ensures a high level of catalytic reversibility as both redox events happen at very similar potentials, which are pH-dependent and close to the  $2H^+/H_2$  couple. In the  $H_{sred}H^+$  state, both electrons needed for hydrogen production are loaded at the H-cluster and a proton is bound to the  $[2Fe]_H$  subcluster. Electron and proton rearrangement occurs and the  $[2Fe]_H$  subcluster is transformed to an isomeric state containing a hydride – the  $H_{hyd}$  state. The intermediates of this process were studied by light-induced spectroscopy under cryogenic temperature<sup>43</sup> (detailed discussion in Section 7). The  $H_{hyd}$  state then accepts a second proton forming the transient  $H_{hyd}H^+$  state before hydrogen is formed and released from the active site. After  $H_2$  is released from  $H_{ox}H_2$ , the enzyme returns to  $H_{ox}$  and the next turnover begins. This cycle operates in the  $H_2$  oxidation direction by an exact reversal of each of these steps. In our interpretation of the data, proton-couple electron transfer (PCET) is essential for the catalytic cycle. The  $[4Fe-4S]_H$  cluster serves as an electron input module and protonation of the amine in the  $[2Fe]_H$  cluster triggers electron transfer from  $[4Fe-4S]_H$  to  $[2Fe]_H$ .



# Thermodynamic and kinetic considerations of the catalytic mechanism: pre-steady state and steady-state conditions

So far, most data used to understand the mechanism of [FeFe] hydrogenases have been collected under equilibrium (often near-equilibrium) or steady-state turnover conditions. More *operando* experiments are needed to confirm that the catalytic states identified are indeed catalytic. It has been argued that all states observed under equilibrium/steady-state conditions are thermodynamic sinks and, therefore, off-pathway non-catalytic intermediates.<sup>35,45,46</sup> This logic would seem to contend that an enzyme with only catalytic states and no off-pathways would be spectroscopically silent, which is extremely unlikely. The energy landscape during catalysis is unlikely to be entirely flat and homogenous, thus, the most abundantly observed intermediates will be those that fall into energy troughs. It should be noted that under equilibrium conditions, a relatively small difference in Gibbs free energy ( $\approx 2.7$  kcal mol<sup>-1</sup> – on the order of a hydrogen bond) is required for a state to be 100-fold more abundant than another (eqn (1)).

$$\Delta G = -RT \ln \left( \frac{\text{products}}{\text{reactants}} \right) = -8.314 \times 298 \times \ln(100) \\ = 11.4 \text{ kJ mol}^{-1} = 2.7 \text{ kcal mol}^{-1} \quad (1)$$

Small energy differences may be responsible for some catalytic intermediates remaining unobserved. However, that does not mean that every observed state is non-catalytic *a priori*. Steady-state turnover may reveal further catalytic states as rate-limiting steps start to populate higher energy intermediates, which accumulate faster than they decay. However, the ultimate proof of a state being catalytic will be its observation during pre-steady state kinetic analysis; the states that appear and disappear within a single turnover must, by definition, be involved in the catalytic cycle.

Despite the importance of such studies, relatively few publications have attempted to measure pre-steady state kinetics due to their experimental challenges. The [FeFe] hydrogenase from *D. desulfuricans* is suggested to turnover at least 10 000 times per second.<sup>47</sup> This indicates a catalytic cycle spanning 100  $\mu$ s and requires a time resolution of at least 1  $\mu$ s. This can be achieved by some spectroscopic techniques, including pump-probe IR spectroscopy to study the CO and CN band vibrations.<sup>48</sup>

On top of this, experiments must be performed under conditions that allow turnover. This precludes (to a certain extent) the use of many spectroscopies (e.g. EPR spectroscopy) that require frozen samples. Turnover cannot be limited by exogenous factors such as diffusion of substrates to and from the active site. This adds complication as, during proton reduction, both protons and electrons are required by the hydrogenase. The latter requires an electron mediator, while the former can be mediated by water and buffer salts. Typical

diffusion coefficients of small molecules in aqueous solution are on the order of  $10^{-10}$  to  $10^{-9}$  m<sup>2</sup> s<sup>-1</sup>, while for protons they are estimated to be 10<sup>5</sup>-fold higher based on the Grotthuss proton-hopping mechanism.<sup>49</sup> Therefore, for efficient (sub- $\mu$ s) electron transfer *via* an electron mediator, the average distance between the reduced mediator and the enzyme needs to be extremely short and the concentration of both the enzyme and electron mediator must be very high.

Several studies have attempted to measure pre-steady state kinetics in [FeFe] hydrogenases using photosensitisers and diffusible electron mediators for intermolecular electron transfer.<sup>48,50–55</sup> However, diffusion limitations may still be an issue. To overcome these limitations, the hydrogenase needs to be covalently attached to the photosensitiser so that rapid intramolecular electron transfer occurs. Several early studies indicated that this would be possible using thiol linkers<sup>56,57</sup> but so far time-resolved studies using this approach have not been published. An alternative approach is to use the photosensitivity of the H<sub>ox</sub>-CO state. [FeFe] hydrogenases are inhibited by CO, forming the H<sub>ox</sub>-CO state, which is also reducible to the H<sub>red</sub>-CO state. The H<sub>ox</sub>-CO state is known to be photosensitive (at least in the frozen state). An [FeFe] hydrogenase sample prepared under CO in the presence of H<sub>2</sub> (to initiate H<sub>2</sub> oxidation) or another reductant (to initiate H<sub>2</sub> production) might be expected to become catalytically active upon photolysis of the Fe-CO bond. An open question here is whether CO rebinding is more rapid than catalysis.

## Primary coordination sphere and contributions from the protein framework: from secondary coordination sphere to long-range interactions, proton transfer pathway and electron relay

Metallic Fe as well as various synthetic Fe-based materials do not come close to the efficiency or reversibility that is achieved by [FeFe] hydrogenases for hydrogen interconversion. The primary coordination sphere of the H-cluster finely tunes the electronic structure of the Fe ions in such a way that low oxidation states and spin states are stabilised in the enzyme. This is achieved through the combination of strong  $\sigma$ -donating (CN<sup>-</sup>) and  $\pi$ -accepting (CO) ligands, along with the covalent “soft” thiolate ligands. The  $\pi$  back-bonding interactions favor the lower valence Fe(I) and Fe(II) formal oxidation states as electron density is accepted from the metal 3d<sub>xy</sub>, yz, xz orbitals. Meanwhile the CN<sup>-</sup> ligands, which only weakly engage in  $\pi$  backbonding, but are much stronger  $\sigma$  donors, donate electron density into the metal 3d<sub>x<sup>2</sup>-y<sup>2</sup></sub> and 3d<sub>z<sup>2</sup></sub> orbitals, favoring higher valence formal oxidation states. It was recently observed that the binding of CN<sup>-</sup> to the H-cluster stabilised its overoxidised Fe(II)Fe(II) oxidation state,<sup>58,59</sup> whereas CO binding maintains the Fe(II)Fe(I) oxidation state observed for H<sub>ox</sub>. It has not yet been possible to synthesise a [2Fe] analogue with extra CN<sup>-</sup> ligands, however, it has been possible to integrate a [2Fe]



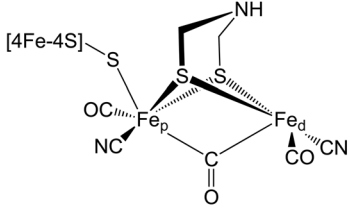
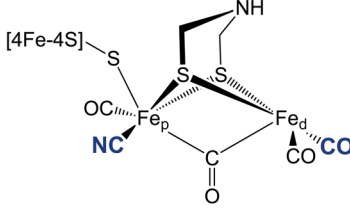
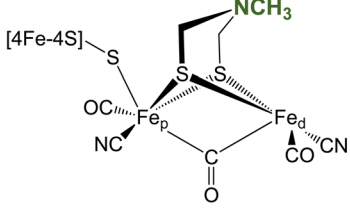
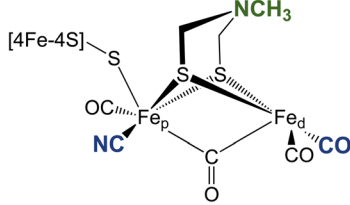
analogue where a  $\text{CN}^-$  ligand was replaced by a CO ligand (*i.e.* MonoCN).<sup>60,61</sup> In this case, spectroscopic data indicated stabilisation of the  $\text{Fe(I)Fe(I)}$  oxidation state. These results support the postulation that the balance of  $\text{CN}^-$  and CO ligands in the H-cluster dramatically affects its electronic structure. There is a balance to be struck between the  $\sigma$ -donating and  $\pi$ -accepting character of the ligands to tune the electron density on the metals such that the redox transitions (II/I) fall into a potential range suited for hydrogen production and oxidation.

Additional insight into this can be garnered from studies of synthetic complexes integrated into  $[\text{FeFe}]$  hydrogenases. In 2015, Siebel *et al.* attempted to reconstitute the H-cluster using 15 synthetic  $[\text{2Fe}]$  analogues, including the native precursor complex, as well as variations on the dithiolate bridging ligand and the number of CO/ $\text{CN}^-$  ligands.<sup>61</sup> Ten of these complexes were successfully inserted. Only two of the ten reconstituted enzymes showed significant catalytic activity, namely, the native ADT cofactor and a monocyanide (pentacarbonyl) form of the ADT complex, which presented with 47%  $\text{H}_2$  production activity and 41%  $\text{H}_2$  oxidation activity compared to the native enzyme

(Table 1). Evidence provided by Lorenzi *et al.* suggests that the  $\text{CN}^-$  ligand coordinates to  $\text{Fe}_p$  in the monocyanide variant of *CrHyaA1*.<sup>60</sup> So, although changing the relative  $\sigma$ -donating/ $\pi$ -accepting abilities of the ligands has a large effect on the electronic structure of the H-cluster, it seems to have no significant effect on the activity.<sup>60</sup>

The enzyme containing an *N*-methylazadithiolate (*N*-methyl-ADT) bridge possessed 1% activity for  $\text{H}_2$  production and oxidation. This may indicate that the ability to form an ammonium ion, with a doubly protonated nitrogen ( $\text{R-NH}_2^+$ ), could be crucial for activity. Interestingly, like the PDT variant, the as-isolated state of this  $[\text{FeFe}]$  hydrogenase appears to be the  $\text{H}_{\text{red}}$  state, whereas, under the same conditions, the native enzyme was in a mixture of  $\text{H}_{\text{ox}}$ ,  $\text{H}_{\text{red}}$ ,  $\text{H}_{\text{red}}\text{H}^+$  and  $\text{H}_{\text{sred}}\text{H}^+$  states. This indicates that the *N*-methylamine group has a lower  $\text{pK}_a$  and is unprotonated under these conditions (pH 8). This is counterintuitive as protonated tertiary amines are expected to be stabilised relative to protonated primary amines due to the inductive effect of the additional methyl carbon. However, the interaction with the protein matrix may play an additional role

Table 1 Structure and relative activities of different cofactors integrated into an  $[\text{FeFe}]$  scaffold

Cofactor	Structure	Relative activity (%)	
		$\text{H}_2$ oxidation	$\text{H}_2$ production
ADT		100	100
ADT MonoCN		41	47
<i>N</i> -Methyl-ADT		1	1
<i>N</i> -Methyl-ADT MonoCN		0.001	0.05



(discussed below). Another question regarding this variant is whether the methyl group points away from the open coordination site or toward it. The free complex is more stable with the methyl group pointing down toward the metals due to the anomeric effect and there will be reduced steric repulsion if the methyl group occupies space in the open coordination site. However, this would block the binding of  $H_2$ , possibly providing another explanation for the lack of activity. Ultimately, crystal structures could reveal why this variant is so inactive.

The monocyanoide form of the *N*-methyl-ADT variant was also tested and found to have 0.05% of the  $H_2$  production activity and 0.001% of the  $H_2$  oxidation activity of the native-like enzyme. This was surprising for two reasons. Firstly, *N*-methyl-ADT and monocyanoide substitutions independently have little effect on the catalytic bias, but together shift the bias much more in favor of  $H_2$  production. Secondly, the monocyanoide substitution of the ADT variant only halves the activity whereas this substitution on the *N*-methyl-ADT variant decreases the activity over 20-fold. Clearly, the factors affecting the enzyme activity are not well understood with multiple factors at play, so further investigation of cofactor variants using spectroscopic and electrochemical approaches could provide crucial insight.

The active site of an [FeFe] hydrogenase is intimately influenced by its interactions with the protein matrix. These interactions include the covalent attachment of  $[4Fe-4S]_H$  and  $[2Fe]_H$  via a cysteine residue, hydrogen-bonding interactions to the bridging thiolate, CO, and  $CN^-$  ligands, and longer-range electrostatic interactions. The study by Knözer and coworkers was the first to try and understand these effects by mutating the protein matrix surrounding the H-cluster in *CrHydA1* and *CpI*.<sup>26</sup> They found that several surrounding amino acids play important roles in catalysis. A cysteine residue (Cys299) located within hydrogen bonding distance of the ADT ligand (Fig. 4) and thought to make up part of the proton transfer pathway was mutated to serine giving a completely inactive enzyme with unusual spectroscopic properties. A methionine (Met497),

whose thioether group is positioned close to the ADT bridging ligand was mutated to leucine and gave a slightly less active enzyme. Spectroscopic analysis attributed this to a loss of the  $[2Fe]_H$  subcluster from the active site. Another methionine (Met353) whose thioether group is positioned close to the bridging CO ligand was mutated to leucine giving a less active enzyme. Spectroscopic analysis was consistent with a high level of intact H-cluster, indicating that the lowered activity was related to the loss of an interaction between the thioether of the methionine and the bridging CO ligand. Lastly, mutation of an arginine residue (whose guanidinium group is positioned very close to the distal  $CN^-$  ligand), when mutated to asparagine, gave an inactive enzyme which lacked both IR and EPR signals from the  $[2Fe]_H$  site. From this, it was established that some amino acids surrounding the H-cluster are catalytically relevant while others play a role in stabilising the  $[2Fe]_H$  subcluster.

Subsequent studies have further investigated the role of the cysteine in the proton transfer pathway, mutating it to serine,<sup>62–65</sup> alanine<sup>59,63,66</sup> and aspartic acid.<sup>62,63,66,67</sup> The cysteine likely forms a hydrogen bond with the ADT ligand. It is generally accepted that this amino acid is responsible for directly exchanging protons between the H-cluster and the proton transfer pathway. Recent studies have also suggested that it directly influences the H-cluster's electronic structure.<sup>59,62</sup> The IR spectrum of the as-isolated C169A mutant of *CrHydA1* is essentially identical to that of the  $H_{ox}$  state of wild-type *CrHydA1*,<sup>59</sup> but EPR spectroscopy shows that the state has one more electron than  $H_{ox}$ , and is  $H_{red}$ -like with IR bands shifted to higher energy. Oxidation of the C169A mutant gives an IR spectrum with higher energy IR bands and an EPR spectrum typical of  $H_{ox}$ . This suggests that the C169A mutant has a more electron-deficient  $[2Fe]_H$  core. Similar results have been reported for the C169S mutant<sup>65</sup> but interpreted differently.

A further interesting feature of the C169A and C169S mutants is that they both stabilise a terminal hydride intermediate ( $H_{hyd}$ ).<sup>39,64,65</sup> This state is extremely persistent in these mutants and is observed during spectroelectrochemical titrations,<sup>64</sup> confounding the initial kinetic proposal that a deficient proton transfer pathway slows the rate of transfer and traps the  $H_{hyd}$  state. By acting as a hydrogen bond acceptor C169 of the wild-type enzyme may increase the negative charge on the nitrogen of the ADT ligand, increasing its pKa and strengthening its basic properties. This would affect the thermodynamic hydricity at the H-cluster<sup>68,69</sup> stabilising the protonated amine ( $H_{red}H^+$  and  $H_{sred}H^+$  states) over the protonated Fe ( $H_{hyd}$ ).

A third argument in support of this idea is that the C169A mutants of *CrHydA1*, and the equivalent C178A mutant of *DdHydAB*, can tolerate the binding of  $CN^-$  to the apical coordination site of the H-cluster much more readily than wild-type enzymes, whose H-clusters cannot tolerate the additional electron density and partially decompose on addition of  $CN^-$ .<sup>59</sup> The C169A and C178A mutants have no hydrogen bonding interaction with ADT, removing additional negative charge on the nitrogen. As the ADT ligand is now more electron-withdrawing the H-cluster can accept the extra electron density introduced by the additional  $CN^-$  ligand; the free NH groups of their ADT



Fig. 4 Structure of the active site H-cluster of [FeFe] hydrogenase showing the interacting amino acid residues and proton transfer pathway. Distances shown are in Å. Residue numbering is from the *C. pasteurianum* enzyme. The image was prepared using PDB ID 4XDC.<sup>24</sup>





ligands may hydrogen bond to the exogenous  $\text{CN}^-$  ligand, further increasing the stability of the H-cluster.

Overall, the hypothesised hydrogen bond from the ADT ligand to the cysteine thiol helps explain several observations but is far from being conclusively demonstrated. Further experiments such as investigations of the C169A mutant containing the PDT or ODT cofactor are needed to provide additional insight.

The electron relay serves as a pathway for electrons to reach the active site but the properties of these clusters are thought to also influence catalytic activity, catalytic bias, and oxygen sensitivity. Removing the F-domain was achieved for the *Clostridium acetobutylicum*<sup>70</sup> and *Megasphaera elsdenii*<sup>71</sup> [FeFe] hydrogenases, leading to loss of activity. For the *M. elsdenii* enzyme, the catalytic bias was also affected. In the *D. desulfuricans* [FeFe] hydrogenase, it was observed that reduction of the F-clusters influenced the redox state of the H-cluster through electrostatic repulsion.<sup>34</sup> This effect destabilised states in which the  $[\text{4Fe-4S}]_{\text{H}}$  subcluster was reduced such as  $\text{H}_{\text{red}}$  and  $\text{H}_{\text{sred}}\text{H}^+$ . These states are still likely to form transiently during catalysis even if they are not observed during thermodynamic titrations.

Thus, it has been made clear that the H-cluster cannot be considered an isolated entity as it is intimately connected to the protein scaffold. This has an enormous influence on the electronic and geometric structure through various types of interactions. These interactions are only now beginning to be understood for a select group of model enzymes. It is very likely that studying the diversity of [FeFe] hydrogenases in nature will reveal additional insight and possibly specific evolutionary adaptations that modify the H-cluster for specific functions.

## The diversity of [FeFe] hydrogenases: prototypical, ancestral and sensory type

In recent years it has become clear that there is huge diversity of [FeFe] hydrogenases in nature and so far, only a tiny fraction of this diversity has been studied. In 2001, Vignais, Billoud and Jacques reviewed the classification and phylogeny of hydrogenases based on the available data.<sup>72</sup> They presented examples of [FeFe] hydrogenases containing only the catalytic H-domain (e.g. from the green algae *C. reinhardtii* and *Chlorella fusca*), containing accessory iron-sulfur cluster domains (e.g. from *M. elsdenii* and *C. pasteurianum*), possessing additional subunits (e.g. from *Desulfovibrio fructosovorans* and *Thermotoga maritima*) and even an enzyme with a similar composition to the *D. fructosovorans* and *T. maritima* enzymes but where the enzyme's subunits are fused into one polypeptide. Then, in 2007, Meyer classified [FeFe] hydrogenases into the more familiar M1–M5 subgroups, where M stands for monomeric.<sup>73</sup> M1 indicates the presence of the H-domain only and M2–M5 indicate the presence of additional iron-sulfur cluster domains (Fig. 5). In this article, Meyer performed extensive phylogenetic analysis with a subset of the [FeFe] hydrogenase sequences available and found that the majority of sequences cluster together and include sequences from the M1–M5 groups.

Later, Calusinska *et al.* performed a phylogenetic analysis of hydrogenases with a focus on *clostridia* and found that [FeFe] hydrogenases could be divided into several groups (A, B, C and D), of which the first two groups were further subdivided (A1–8 and B1–3).<sup>75</sup> Each of these groups corresponded well to phylogenetic groups identified previously by Meyer. Group C was remarked as consisting of dimeric PAS/PAC sensory domain containing hydrogenases and group D as consisting of putative hydrogenases. In 2016, Greening *et al.* carried out an even more extensive phylogenetic analysis on 1223 [FeFe] hydrogenase sequences and classified them similarly to Calusinska where group A was subdivided into four groups and group D was integrated into group C (Fig. 5).<sup>74</sup> Overall, these studies show an enormous diversity of [FeFe] hydrogenase sequences of which very few have been investigated.

Group A, also known as the prototypical group, includes the vast majority of [FeFe] hydrogenases studied to date, and the only group for which structures are available. It includes *CpI*<sup>14,76</sup> from *C. pasteurianum*, *DdHydAB*<sup>15,47</sup> from *D. desulfuricans* and *CrHydA1*<sup>77</sup> from *C. reinhardtii*. Also included is the class of electron-bifurcating [FeFe] hydrogenases<sup>78,79</sup> that reversibly reduce protons using NADH and ferredoxin synergistically, as well as a class of hydrogen uptake [FeFe] hydrogenases, A3, which includes *CpII*<sup>80,81</sup> from *C. pasteurianum*, and the uniquely oxygen-stable A5 [FeFe] hydrogenase, *CbA5H*, from *Clostridium beijerinckii*.<sup>82</sup>

Group B, also known as the ancestral group, is much more poorly studied, with only a few characterised examples including the functionally and spectroscopically characterised *CpIII* from *C. pasteurianum* and the less well-characterised *HydA2* from *Clostridium acetobutylicum*.

Group C, also known as the sensory group, is also poorly studied but two examples, *HydS* from *Thermotoga maritima* (*TmHydS*)<sup>44,83</sup> and *HydS* from *Thermoanaerobacter mathranii* (*TamHydS*)<sup>84–86</sup> have recently been produced and characterised both functionally and spectroscopically. These enzymes have very low activity and, at least in the case of *TamHydS*, show extremely irreversible electrochemical behavior.<sup>84</sup> They are theorised to have a sensing function, presumably hydrogen, but, as yet, the mechanism of signal transduction is unknown.

To provide one example of how different [FeFe] hydrogenases from different groups can be, *TmHydS* and *TamHydS* both lack the crucial amino acids of the proton-transfer pathway identified in group A [FeFe] hydrogenases including a cysteine that directly interacts with the ADT ligand and is thought to be the direct proton donor/acceptor of the H-cluster. In the case of *TamHydS* an alternative proton transfer pathway has been identified.<sup>87</sup> A proton transfer pathway is yet to be identified for *TmHydS* despite intense investigation.<sup>83</sup>

There is still much to learn about the wider diversity of [FeFe] hydrogenases. A crucial part of future research will be to characterise enzymes from groups B, C and the more diverse branches of group A. Understanding their unique physiological functions and how these influence their protein sequences and thus the structure and properties of the active site and accessory clusters is crucial to understanding how enzyme efficiency is maximised. By understanding these structure-function







Fig. 5 (A) Simplified view of the [FeFe] hydrogenases phylogeny diagram proposed by Greening *et al.* (2016).<sup>74</sup> (B) Biodiversity of [FeFe] hydrogenases represented by five main subclasses named as M1–5. M stands for monomeric and number indicates the size. Legend: TRX – thioredoxin, Nuof – NADH: ubiquinone oxidoreductase chain F.

relationships we will learn more about designing optimal  $H_2$  oxidation and  $H_2$  production catalysts based on iron.

## Future outlook: the missing pieces of the catalytic cycle

### The hydride state, $H_{hyd}$

As discussed above, the first steps of the catalytic cycle in the  $H_2$  production direction, namely reduction, protonation and further reduction have been comprehensively studied, and we are reaching a point where these stages of the catalytic cycle are understood. It has been proposed that another form of the two-electron reduced, protonated state exists, called  $H_{hyd}$ , in which there is a terminal hydride bound to the apical position on  $Fe_d$ . How exactly this state forms is not clear but the following observations have been made:

(1)  $H_{hyd}$  can be formed at low pH in the presence of high concentrations of sodium dithionite ( $Na_2S_2O_4$ ).<sup>39</sup> This was taken as evidence that this state is transiently formed under conditions of high turnover.

(2)  $H_{hyd}$  can be formed under equilibrium conditions in enzymes where there have been mutations made to the terminal cysteine in the proton-transfer pathway<sup>65</sup> or where the ADT bridging ligand is substituted with oxadithiolate (ODT).<sup>39</sup> It has been assumed that these modifications restrict proton transfer and so the terminal hydride that is formed as an intermediate cannot be protonated to make  $H_2$ .

(3) Alternative forms of  $H_{hyd}$  named  $H_{hyd:ox}$  and  $H_{hyd:red}$  can be formed upon illumination of  $H_{red}H^+$  and  $H_{sred}H^+$ , respectively, under cryogenic conditions.<sup>43</sup> These results may indicate that the  $H_{hyd}$  states are less stable, tautomeric forms of  $H_{red}H^+$  and  $H_{sred}H^+$ , and so energy must be supplied to enable proton transfer from the nitrogen base to  $Fe_d$ .

(4) Electrochemical titrations of *CpI* crystals have shown to populate hydride species.<sup>88</sup>

Another explanation for the formation of  $H_{hyd}$  in the disrupted proton-transfer mutants and ODT enzyme stands. Both have less basic bridgeheads; mutation of the cysteine may influence the basicity of the ADT amine through disruption of hydrogen bonding and the ether bridgehead in ODT is clearly less basic than the native amine. A follow-up question then is why the PDT enzyme, in which the amine is substituted with methylene, does not behave in the same way as the ODT enzyme. This may be explained by differences in the redox potential of the diiron clusters between PDT and ODT, where ODT ought to be more positive than PDT. One might then expect that PDT can indeed form  $H_{hyd}$  but at more negative potentials than ODT, so far not accessed experimentally.

But what is the difference between  $H_{hyd:ox}$  and  $H_{hyd:red}$  and the  $H_{hyd}$  states observed at low pH and in mutants? Table 2 compares the IR bands and EPR *g*-values of the  $H_{hyd}$  states so far identified in *CrHydA1*.

The first thing to note is some discrepancies between the IR band frequencies measured for what should be identical  $H_{hyd}$  states in different studies. In most cases these discrepancies are small ( $>3\text{ cm}^{-1}$ ) but for the  $CN^-$  bands they appear to be larger. In general  $H_{hyd:red}$  appears to have the most red-shifted IR bands indicating the most electron-rich  $[2Fe]_H$  site. Meanwhile,  $H_{hyd:ox}$  has more blue-shifted IR bands. The differences between these two states are thought to be due to the difference in the redox state of  $[4Fe-4S]_H$ , which is oxidised in  $H_{hyd:ox}$  and reduced in  $H_{hyd:red}$ . The  $H_{hyd}$  state formed at low pH, on the other hand, possesses IR bands that are blue-shifted relative to  $H_{hyd:red}$  but red-shifted relative to  $H_{hyd:ox}$ , yet is also proposed to have a reduced  $[4Fe-4S]_H$  like  $H_{hyd:red}$ . Clearly, there is something about the structure around the H-cluster in  $H_{hyd}$  that is different. Stripp and coworkers proposed that  $H_{hyd}$  has a protonated  $[4Fe-4S]_H$ ,<sup>36,66</sup> which would shift some of the electron density from  $[2Fe]_H$  back onto  $[4Fe-4S]_H$ . This idea agrees with the observation of this state at low pH,<sup>39</sup> although the protonation of the  $[4Fe-4S]_H$  cluster in other catalytic states



**Table 2** Spectroscopic signatures of  $H_{\text{hyd}}$  states reported in the literature for the HydA1 [FeFe] hydrogenase from *Chlamydomonas reinhardtii*

Name	IR						EPR				Ref.
	CN <sub>p</sub>	CN <sub>d</sub>	CO <sub>p</sub>	CO <sub>d</sub>	CO <sub>b</sub>	Average	Difference to $H_{\text{hyd}}$	$g_1$	$g_2$	$g_3$	
$H_{\text{hyd}}$	2082 <sup>a</sup> (2088)	2068 <sup>a</sup> (2075)	1978 (1979)	1960 (1961)	1860 (1861)	1990 (1993)	— +3	2.077 (2.080)	1.935 (1.941)	1.880 (1.884)	39 (89)
$H_{\text{hyd:ox}}$	2092	2086	1983	1954	1865	1996	+6 (+3)	n.a.	n.a.	n.a.	43
$H_{\text{hyd:red}}$	2087	2078	1972	1954	1851	1988	−2 (−5)	2.069	1.938	1.880	43
$H_{\text{hyd}}(\text{ODT})$	2081 <sup>a</sup> (2090)	2076 (2075)	1980 (1980)	1962 (1963)	1868 (1869)	1993 (1995)	+3 (0) +5 (+2)	(2.069)	(1.941)	(1.879)	39 (40)
$H_{\text{hyd}}(\text{C169A})$	2082 <sup>a</sup> (2089)	2068 <sup>a</sup> (2076)	1978 (1980)	1962 (1962)	1862 (1864)	1990 (1994)	0 (−3) +4 (+1)	(2.075)	(1.942)	(1.884)	39 (59)
$H_{\text{hyd}}(\text{C169S})$	n.r.	n.r.	1977	1960	1860			2.068	1.943	1.881	65
$H_{\text{hyd}}(\text{E279A})$	2082	2068	1984	1970	1858	1992	+2 (−1)	n.r.	n.r.	n.r.	39
$H_{\text{hydH}}(\text{C169S})$	n.r.	n.r.	1987	1967	1874			2.065 <sup>b</sup>	1.969 <sup>b</sup>	1.906 <sup>b</sup>	64

<sup>a</sup> Values reported in ref. 39 deviate substantially ( $>6\text{ cm}^{-1}$ ) from other ref. 40, 59, 66 and 89. <sup>b</sup> The same EPR spectrum has been assigned to a “ $H_{\text{trans}}$ -like” state in ref. 90 and a similar EPR spectrum was reported for the CN<sup>−</sup>-bound “ $H_{\text{trans}}$ -like” state in the C169A mutant of *CrHydA1* ( $g = 2.068$ , 1.977, 1.916) in ref. 59.

is still disputed.<sup>37,38</sup> The  $H_{\text{hyd}}$  states formed in the ODT enzyme and a C169A mutant have similar IR bands to the  $H_{\text{hyd}}$  state formed at low pH.<sup>39</sup> Again, these could represent states with protonated  $[4\text{Fe-4S}]_{\text{H}}$  clusters, however, these  $H_{\text{hyd}}$  states are stable at neutral pH. Since neither of these changes (substitution of ADT with ODT or mutation of Cys169 to Ala) should influence the  $\text{pK}_{\text{a}}$  of  $[4\text{Fe-4S}]_{\text{H}}$  it seems unlikely that these states would represent protonated  $[4\text{Fe-4S}]_{\text{H}}$  states. Instead, an alternative explanation could be that the removal of the hydrogen-bonding interaction between the ADT amine and the cysteine thiol group influences the electronic structure of the H-cluster in such a way that disfavors protonation of the bridging ADT and favours terminal hydride formation on Fe<sub>d</sub>. If this is the case, then could this also explain the stabilisation of  $H_{\text{hyd}}$  at low pH and the differences in the IR signatures of  $H_{\text{hyd}}$  vs.  $H_{\text{hyd:red}}$ ? Evidence in this direction comes from comparison of the  $H_{\text{ox}}$  and  $H_{\text{red}}$  states in the ODT enzyme and the C169A mutant (Table 3).

From Table 3, the  $H_{\text{ox}}$  spectra for ODT and C169A are blue-shifted compared to  $H_{\text{ox}}$  in WT ADT, while the  $H_{\text{red}}$  spectra for ODT and C169A are like  $H_{\text{ox}}$  in WT ADT but blue-shifted

compared to  $H_{\text{red}}$  in WT ADT. This indicates that IR spectra for the ODT and C169A enzymes are blue-shifted compared to their WT counterparts.

### Have we observed $H_{\text{hyd}}H^+$ and/or $H_{\text{ox}}(H_2)$ ?

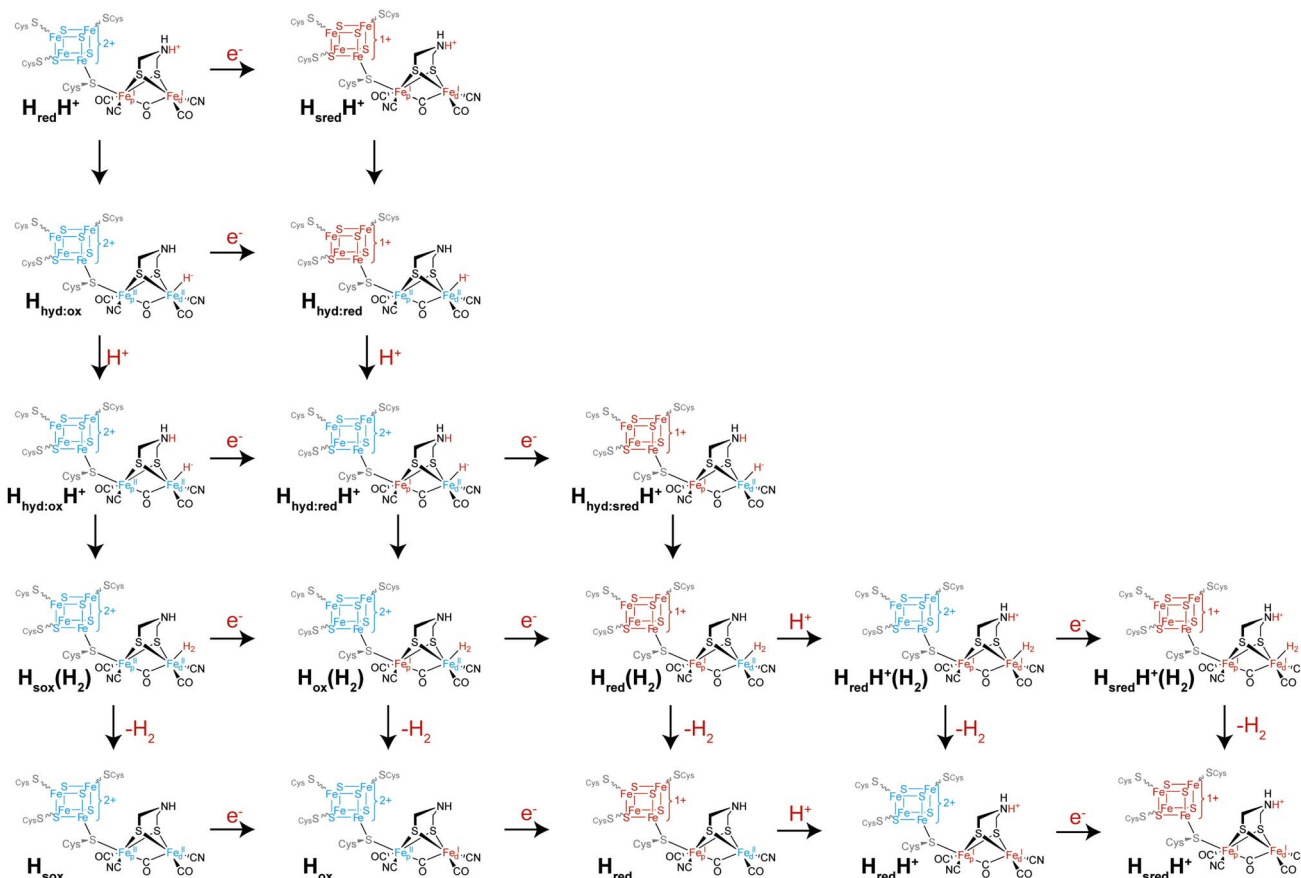
While there have been some reports of  $H_{\text{hyd}}H^+$  in the literature, these are yet to be substantiated with direct evidence and mostly rely on observed IR and EPR spectra that are somewhat similar to  $H_{\text{hyd}}$  but blue-shifted (in the case of the IR spectra). In 2014, Mulder observed IR bands at 1987, 1967 and  $1874\text{ cm}^{-1}$  in the C169S mutant of *CrHydA1*, where the bridging CO shifted to  $1964\text{ cm}^{-1}$  in  $D_2O$  and was associated with an EPR spectrum with  $g$ -values of 2.065, 1.969 and 1.906.<sup>65</sup> DFT calculations supported an assignment to  $H_{\text{hyd}}H^+$ , with a protonated ADT and a reduced  $[4\text{Fe-4S}]_{\text{H}}$  cluster. In 2021, Mészáros observed a similar IR spectrum with bands at 1988, 1959 and  $1975\text{ cm}^{-1}$  for WT *CrHydA1*, associated with an EPR spectrum with  $g$ -values of 2.073, 1.935 and 1.881 and assigned this to the same  $H_{\text{hyd}}H^+$  state.<sup>92</sup> While there are some similarities between the spectroscopic properties of these two putative  $H_{\text{hyd}}H^+$  states, they are clearly very different to the  $H_{\text{hyd}}$  states of WT and C169S

**Table 3** IR spectroscopic signatures of  $H_{\text{ox}}$  and  $H_{\text{red}}$  in *CrHydA1* WT ADT, ODT and C169A ADT

Name	IR						Difference to $H_{\text{ox}}$	Ref.
	CN <sub>p</sub>	CN <sub>d</sub>	CO <sub>p</sub>	CO <sub>d</sub>	CO <sub>b</sub>	Average		
$H_{\text{ox}}$ (WT/ADT)	2088	2072	1964	1940	1804	1974	—	91
$H_{\text{ox}}$ (WT/ODT)	2092	2076	1970	1947	1811	1979	+5	91
$H_{\text{ox}}$ (C169A/ADT)	2092 <sup>a</sup> (n.r.)	2075 <sup>a</sup> (n.r.)	1972 <sup>a</sup> (1972)	1946 <sup>a</sup> (1948)	1813 <sup>a</sup> (1815)	1980	+6	66 (59)
$H_{\text{red}}$ (WT/ADT)	2084	2066	1962	1933	1792	1967	−7	91
$H_{\text{red}}$ (WT/ODT)	2083	2070	1964	1943	1804	1973	−1	91
$H_{\text{red}}$ (C169A/ADT)	2089 <sup>b</sup>	2068 <sup>b</sup>	1971 <sup>b</sup>	1939 <sup>b</sup>	1804 <sup>b</sup>	1974	0	59

<sup>a</sup> This spectrum was actually assigned to  $H_{\text{ox}}H$  in ref. 66. However, in ref. 59 it was observed that this state formed under conditions typical for forming  $H_{\text{ox}}$  and was associated with an  $H_{\text{ox}}$ -like EPR spectrum. <sup>b</sup> This state was assigned to  $H_{\text{red}}$  in ref. 66. However, in ref. 59 it was observed that this state formed under conditions typical for forming  $H_{\text{red}}$  and was found to be an EPR-silent state, untypical for  $H_{\text{ox}}$ .





**Fig. 6** Possible alternative pathways taken by the H-cluster from the  $H_{red}H^+$  state (top left), which can proceed by reduction to the  $H_{sred}H^+$  state (horizontal arrow) or tautomerism to the  $H_{hyd:ox}$  state (vertical arrow). The  $H_{sred}H^+$  state can also tautomerise (vertical arrow), yielding the  $H_{hyd:red}$  state. Both forms of the  $H_{hyd}$  state can then be protonated (vertical arrows) and the two possible forms of  $H_{hyd}H^+$  can then undergo reduction (horizontal arrows) or tautomerism (vertical arrows), and so on.

While  $\text{H}_{\text{hyd}}\text{H}^+$  stabilisation in mutants may be achievable, it seems unlikely that it can be stabilised in WT enzymes due to their exceptionally fast turnover rates. The same is probable for any potential  $\text{H}_{\text{ox}}(\text{H}_2)$  intermediate. A possible way of capturing these states would be to use ultra-fast spectroscopic techniques such as pump-probe IR, which has been coupled to some kind of method for rapidly triggering catalysis. The Dyer group have pioneered methods for using CdS/CdSe dot-in-rod photosensitiser systems coupled to redox mediators for light-triggered reduction of [FeFe] hydrogenases among other enzymes.<sup>48,52</sup> Work with *CrHydA1* showed that the enzyme converted from  $\text{H}_{\text{ox}}$  to  $\text{H}_{\text{red}}$  on a timescale of 10 to 100  $\mu\text{s}$ ; over the same time-period,  $\text{H}_{\text{red}}\text{H}^+$  and  $\text{H}_{\text{sred}}\text{H}^+$  were also formed.  $\text{H}_{\text{hyd}}$  was formed between 50 and 500  $\mu\text{s}$ . All states eventually decayed reforming  $\text{H}_{\text{ox}}$  on timescales similar to those expected

based on the known catalytic rates for  $\text{H}_2$  production by *CrHydA1* ( $\approx 1000 \text{ s}^{-1}$  or  $1 \mu\text{s}^{-1}$ ). A caveat of this study was that the starting state of the enzyme was a mixture of  $\text{H}_{\text{ox}}$ ,  $\text{H}_{\text{red}}$  and  $\text{H}_{\text{red}}^+$ , which complicated the kinetic analysis as single electron reduction will convert  $\text{H}_{\text{ox}}$  to  $\text{H}_{\text{red}}/\text{H}_{\text{red}}^+$  and reduce  $\text{H}_{\text{red}}/\text{H}_{\text{red}}^+$  to  $\text{H}_{\text{sred}}^+/\text{H}_{\text{hyd}}$ , and so the kinetics of  $\text{H}_{\text{red}}/\text{H}_{\text{red}}^+$  formation are a convolution of both a formation and decay rate. Furthermore, in a subsequent study it was found that increasing the protein concentration led to faster kinetics indicating a diffusion limitation.<sup>51</sup> Accordingly, this method could be enhanced by engineering photosensitisers directly onto [FeFe] hydrogenases, as has been previously shown.<sup>55–57,93,94</sup>

As we are unsure of what we are looking for, that is entirely possible. What do we expect it to look like? Here, some clues can be acquired from investigation of synthetic  $\eta^2\text{-H}_2$  metal complexes.<sup>95</sup> Many  $\eta^2\text{-H}_2$  mono-metallic complexes have been studied over the years but examples of bimetallic complexes like  $[\text{2Fe}]_{\text{H}}$  that stably bind  $\eta^2\text{-H}_2$  are rarer.<sup>96–101</sup> Generally, dihydrogen complexes are identified through structural characterization (X-ray or neutron crystallography), vibrational



spectroscopy (IR, Raman, inelastic neutron scattering) and NMR spectroscopy. Free  $\text{H}_2$  in the gas phase has a vibrational frequency of around  $4100\text{ cm}^{-1}$  (ref. 102) and the transition is IR invisible due to the absence of a change in dipole moment. Binding of  $\text{H}_2$  to metals gives IR active modes due to the dipolar character of the metal-hydrogen bonds and the coupling of the H-H stretch to other vibrations. A range of complexes show vibrational modes in the  $2200\text{--}3200$ ,  $1100\text{--}1700$  and  $400\text{--}1000\text{ cm}^{-1}$  regions.<sup>95</sup> Due to the low intensity of these peaks and their potential overlap with other bands from the protein matrix, the possibility of observing a dihydrogen bound species by IR is low. In NMR, metal-bound dihydrogen presents with chemical shifts in the  $2.5$  to  $-31$  ppm region, which overlaps with the chemical shifts of metal-hydrides.<sup>95</sup> For reference, the [FeFe] hydrogenase  $\text{H}_{\text{hyd}}$  state gives a peak at  $-9.6$  ppm.<sup>103</sup> However, observation of  $J_{\text{HD}}$  coupling or short  $T_1$  relaxation times can provide support for a dihydrogen species. However, in reality, this can only be achieved with  $S = 0$  systems as unpaired electrons tend to produce NMR line-broadening, as would be expected for a putative  $\text{H}_{\text{ox}}(\text{H}_2)$  intermediate.

Specifically, for [FeFe] hydrogenases, EPR spectroscopy may provide a valuable tool for identifying and characterizing  $\text{H}_2$ -adducts. The Peters and Hoffmann groups measured EPR and ENDOR on an  $\text{H}_2$ -bound Fe(I) complex, which revealed a single electron-nuclear hyperfine interaction with a coupling tensor of  $[2.3, -40.6, -37.8]$  with both isotropic and anisotropic components.<sup>104</sup>

Returning to IR, it might be possible to observe the  $\text{H}_2$ -bound intermediate indirectly through changes to the CO and  $\text{CN}^-$  ligand vibrations upon  $\text{H}_2$  binding. Side-on binding of  $\text{H}_2$  has both a  $\sigma$ -donation and  $\pi$ -backdonation contribution. Binding of hydrogen to monometallic carbonyl complexes causes a small blue shift in the vibrational frequency of the carbonyl IR bands, likely reflecting the decreased backdonation due to some of this electron density being donated into the  $\text{H}_2$   $\sigma^*$ .<sup>95</sup> A similar effect is seen in CO binding to the [FeFe] hydrogenase H-cluster forming the  $\text{H}_{\text{ox}}\text{-CO}$  state; the IR band of the bridging CO is blue-shifted by around  $10\text{ cm}^{-1}$ .<sup>13</sup> So, it can be postulated that an  $\text{H}_{\text{ox}}(\text{H}_2)$  state may show similar blue shifted IR bands.

NRVS has been used extensively to probe the Fe-H vibrations of the  $\text{H}_{\text{hyd}}$  states but could also be a tool to explore potential  $\text{H}_{\text{ox}}(\text{H}_2)$  states. For an Fe- $\eta^2\text{-H}_2$ -hydride complex an intense NRVS symmetric Fe- $\text{H}_2$  stretching mode was observed at  $1052\text{ cm}^{-1}$  that shifts to  $781\text{ cm}^{-1}$  upon  $\text{D}_2$  substitution.<sup>105</sup> In addition, the weaker antisymmetric stretch mode was observed at  $1774\text{ cm}^{-1}$  and bending modes were observed at  $558$ ,  $584$ ,  $733$  and  $823\text{ cm}^{-1}$ . These results suggest that in principle, an  $\text{H}_2$  bound intermediate of an [FeFe] hydrogenase could be probed with NRVS provided it is sufficiently enriched.

Lastly, X-ray crystallography is generally not considered as an effective method for observing protons due to their low electron density and bond libration effects that give anomalous bond lengths. Despite this, it has been used to provide evidence for the location of hydrides and protons in [NiFe] hydrogenase enzymes due to sub-angstrom resolution quality of the data.<sup>106</sup> Alternatively, neutron diffraction could be employed, as

neutrons are scattered predominantly by the nucleus and the coherent neutron scattering length of hydrogen is similar to heavy atoms. However, due to the negative sign of the scattering length of  $^1\text{H}$  and the large amount of incoherent scattering it is much easier to obtain information on the location of deuterium. Regardless, due to the weak intensity of neutron sources very large crystals are needed and data collection times are extremely long. Recently, a neutron structure was published for an oxidised form of [NiFe] hydrogenase,<sup>107</sup> however, this technique is yet to reveal the locations of hydrides and protons from hydrogen splitting. The size of the crystals needed (at least  $1\text{ mm}^3$ ) on top of the requirement for deuteration continues to cause problems for neutron diffraction experiments. Nevertheless, it is likely that the next few years will see the publication of neutron and subangstrom resolution X-ray structures of [FeFe] hydrogenases, revealing the locations of protons. If these can be carried out on the  $\text{H}_{\text{hyd}}$  states or potential  $\text{H}_{\text{hyd}}\text{H}^+$  and  $\text{H}_{\text{ox}}(\text{H}_2)$  intermediates, these structures will provide unprecedented insight into our understanding of biological  $\text{H}_2$  activation.

### New tools: CryoEM and its potential in hydrogenase research

Electron imaging visualises electrostatic potential, which depends on the positions of both nuclei and electrons.<sup>108</sup> Thus, hydrogens can be observed more clearly in cryoEM than in X-ray crystallography. Another advantage of cryoEM is that samples do not need to be crystallised. However, there is currently a size limit of around  $50\text{ kDa}$ , below which obtaining cryoEM structures is exceptionally difficult, but still possible. In recent years, several cryoEM structures of hydrogenases have been published including various electron-bifurcating [FeFe] hydrogenases.<sup>109–113</sup> One structure of a [NiFe] hydrogenase reached  $1.52\text{ \AA}$  resolution, potentially paving the way toward resolving hydrogen atoms.<sup>113</sup>

### A multiple catalytic pathways scenario?

Another intriguing idea is that of multiple catalytic pathways. Earlier in this article, two hypotheses for the catalytic cycle of [FeFe] hydrogenase were presented, both with some evidence in support of them. However, it is possible that, to a certain extent, elements of both cycles are true, and that alternative pathways are taken by [FeFe] hydrogenases depending on (i) the conditions and (ii) which enzyme is being studied. It has already been demonstrated for *CrHydA1* that at least three different forms of the  $\text{H}_{\text{hyd}}$  state exist including the 'classical'  $\text{H}_{\text{hyd}}$  observed at low pH and  $\text{H}_{\text{hyd:ox}}$  and  $\text{H}_{\text{hyd:red}}$  observed at high pH, mainly under cryogenic conditions and illumination but also thought to contribute at room temperature. During proton reduction, from the  $\text{H}_{\text{red}}\text{H}^+$  state there are multiple potential pathways that can be taken (Fig. 6): if electron transfer is slow then tautomerization to  $\text{H}_{\text{hyd:ox}}$  may be faster than reduction to  $\text{H}_{\text{sred}}\text{H}^+$ .  $\text{H}_{\text{hyd:ox}}$  could then be reduced to  $\text{H}_{\text{hyd:red}}$  or at low pH become protonated to a putative  $\text{H}_{\text{hyd:ox}}\text{H}^+$  state, which could either become further reduced to a putative  $\text{H}_{\text{hyd:red}}\text{H}^+$  state or, again if electron transfer is slow, directly form  $\text{H}_2$ . Formation of  $\text{H}_2$  from a putative  $\text{H}_{\text{hyd:ox}}\text{H}^+$  state would leave the  $[\text{4Fe-4S}]_{\text{H}}$  cluster oxidised and the  $[\text{2Fe}]_{\text{H}}$  cluster in an overoxidised  $\text{Fe(II)Fe(II)}$



oxidation state, a putative “H<sub>sox</sub>” state, which may not be stable. Thus, it can already be seen that depending on the electron supply, pH, and enzyme properties such as redox potentials and pK<sub>a</sub> values, several pathways from H<sub>red</sub>H<sup>+</sup> may exist. Since H<sub>sred</sub>H<sup>+</sup> cannot be further reduced or protonated, it can only tautomerise to H<sub>hyd:red</sub>. H<sub>hyd:red</sub> is unlikely to be reducible but will become protonated to give H<sub>hyd:red</sub>H<sup>+</sup>. H<sub>hyd:red</sub>H<sup>+</sup> formation is likely to involve PCET where [4Fe–4S]<sub>H</sub> transfers an electron to [2Fe]<sub>H</sub>. In principle, H<sub>hyd:red</sub>H<sup>+</sup> could be further reduced to give H<sub>hyd:sred</sub>H<sup>+</sup> where both the [4Fe–4S]<sub>H</sub> and [2Fe]<sub>H</sub> clusters are reduced, which would in turn form H<sub>2</sub> bound to the H<sub>red</sub> state in a H<sub>red</sub>(H<sub>2</sub>) state. Likewise, it would also be possible to reduce the H<sub>ox</sub>(H<sub>2</sub>) state before releasing H<sub>2</sub> giving H<sub>red</sub>(H<sub>2</sub>). Such states could potentially be observed under conditions where there is a very fast electron supply. One could also envisage additional states such as the protonated H<sub>ox</sub> state (H<sub>ox</sub>H<sup>+</sup>) or the deprotonated H<sub>sred</sub>H<sup>+</sup> (H<sub>sred</sub>) state that could follow additional pathways. Whether any of these states are populated during turnover, even under extreme conditions, is not known but it is speculated to be the case based on electrochemical studies of amino acid variants.<sup>46</sup> It may also be possible that accessing some of these alternative pathways is responsible for phenomena observed under extreme conditions during electrochemistry such as high potential inactivation<sup>114</sup> and low potential inactivation.<sup>45</sup>

It could also be possible that different pathways are followed depending on the direction of catalysis. For example, if inter-conversion of the H<sub>red</sub>H<sup>+</sup> and H<sub>sred</sub>H<sup>+</sup> states with their H<sub>hyd</sub> tautomers is slow compared with electron transfer, then during proton reduction the pathway H<sub>red</sub>H<sup>+</sup> → H<sub>sred</sub>H<sup>+</sup> → H<sub>hyd:red</sub> would be followed while the H<sub>hyd:red</sub> → H<sub>hyd:ox</sub> → H<sub>red</sub>H<sup>+</sup> pathway would be followed during H<sub>2</sub> oxidation. Determining whether [FeFe] hydrogenases do indeed follow multiple pathways depending on conditions requires the ability to carefully control the rate of electron supply while monitoring the composition of states using spectroscopy. This is very difficult using standard spectroscopic approaches where electrons are exchanged with the enzyme using mediators. Instead, an approach is needed where the enzyme is directly attached to an electrode surface. The techniques of surface enhanced IR spectroscopy (SEIRAS) and protein film infrared electrochemistry (PFIRE) are designed to do this.<sup>115</sup> While [NiFe] hydrogenases have been quite extensively studied with SEIRAS<sup>116–119</sup> and also by PFIRE,<sup>120,121</sup> there are only two examples of SEIRAS being employed with an [FeFe] hydrogenase, specifically CrHydA1,<sup>122,123</sup> and no examples of PFIRE applied to [FeFe] hydrogenases. In both SEIRAS studies only the spectra of the protein backbone amides could be observed and there was no evidence of the active site CO and CN<sup>−</sup> ligands, even though catalytic activity was observed. Thus, there is potential for this technique and for PFIRE to be incredibly useful in future studies, to particularly allow *operando* conditions.

## Conclusion

[FeFe] hydrogenases are incredibly fascinating yet complex enzymes capable of extremely high turnover frequencies with

exceptional efficiency and reversibility. How this is achieved is still not completely understood despite decades of research. In this perspective we have provided a summary of where we are now in terms of our understanding, how we have gotten here and where we think the field is moving and how it is going to get there, particularly highlighting the main challenges. The catalytic cycle of [FeFe] hydrogenases has been a difficult puzzle to build, but we believe that enough pieces are in place to provide a good overview of the picture we are putting together. However, the next pieces of the puzzle will be the most challenging to assemble, requiring new techniques and approaches or combinations of existing techniques. The promise of being able to design new catalytic materials motivates scientists to keep working on this challenging puzzle and we expect the next few years will see substantial progress toward this goal.

## Author contributions

All authors contributed to the conceptualisation, literature search, writing and figure making of the perspective and were involved in revising, editing, and proofreading the initial and revised versions.

## Conflicts of interest

There are no conflicts to declare.

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