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# Indications for a universal hydrogen catalysis mechanism in [FeFe]-hydrogenases of different phylogenetic groups†

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[FeFe]-hydrogenases are metalloenzymes catalysing bidirectional hydrogen (H<sub>2</sub>) turnover. These enzymes are generally considered to be extremely efficient and fast catalysts. However, [FeFe]-hydrogenases constitute a very diverse enzyme family that can be divided into several distinct phylogenetic groups, denoted as groups A–G. Very little is known about the properties of [FeFe]-hydrogenases outside of the intensively studied group A, but recent studies on putatively sensory group C and D enzymes have revealed distinct differences in reactivity. The variation in structure, reactivity and physiological function observed between phylogenetic groups raises the question if all [FeFe]-hydrogenases follow the same mechanism for H<sub>2</sub> turnover. Here, we provide the first detailed spectroscopic investigation of a slow-acting putatively sensory group D [FeFe]-hydrogenase from *Thermoanaerobacter mathranii* (TamHydS). Photo-reduction enabled us to characterize redox states in group D [FeFe]-hydrogenase via infrared spectroscopy under catalytic conditions. The sequential population of redox states similar to group A [FeFe]-hydrogenases supports the notion that group A and D [FeFe]-hydrogenases follow a universal catalytic mechanism. However, clear differences between enzymes from different phylogenetic groups become evident when comparing the relative stability and protonation state of suggested key catalytic intermediates. Moreover, the spectroscopic data collected on TamHydS provides new insight into the structure of the reduced active site, lending further support for the notion of a retained bridging CO ligand throughout the entire catalytic cycle.

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## Introduction

[FeFe]-hydrogenases are metalloenzymes involved in hydrogen metabolism. They are a structurally and functionally highly diverse enzyme family and can be classified into phylogenetic groups (denoted group A–G).<sup>1</sup> Outside of group A, there is limited data available on [FeFe]-hydrogenases. Still, group C and D [FeFe]-hydrogenases stand out as they are proposed to serve a H<sub>2</sub> sensory function rather than a catalytic one.<sup>2,3</sup> The different phylogenetic groups likely share the same organometallic catalytic cofactor but differ in the active-site pocket and substrate transport pathways.<sup>2,4</sup> The active site consists of a

unique diiron site with a terminal CO and CN<sup>−</sup> ligand at each iron ion. The iron ions are further coordinated by two bridging ligands, a bidentate azadithiolate (−SCH<sub>2</sub>NCH<sub>2</sub>S−, ADT) and an additional CO molecule (μCO). The diiron site is linked *via* a cysteine residue to a [4Fe–4S] cluster ([4Fe–4S]<sub>H</sub>) to form the so-called H-cluster (Fig. 1A). Many [FeFe]-hydrogenases harbour additional [4Fe–4S] clusters in the N terminal domain involved in electron transfer, commonly denoted as F-clusters. In addition, group C and D enzymes often harbour an additional FeS cluster in the C-terminal domain called C-clusters. Due to their outstanding catalytic properties, [FeFe]-hydrogenases and the H-cluster have received significant attention from the bioinorganic chemistry and solar fuel research communities.<sup>5–8</sup>

To-date, mechanistic investigations have focused almost exclusively on group A [FeFe]-hydrogenases, for which several protonation and oxidation states of the H-cluster have been characterized. Albeit the mechanism remains debated, all currently proposed catalytic cycles include oxidized, single and double reduced H-cluster states.<sup>15–17</sup> Multiple spectroscopic signals associated with such states have been identified by Fourier-transform infrared (FTIR) and electron paramagnetic

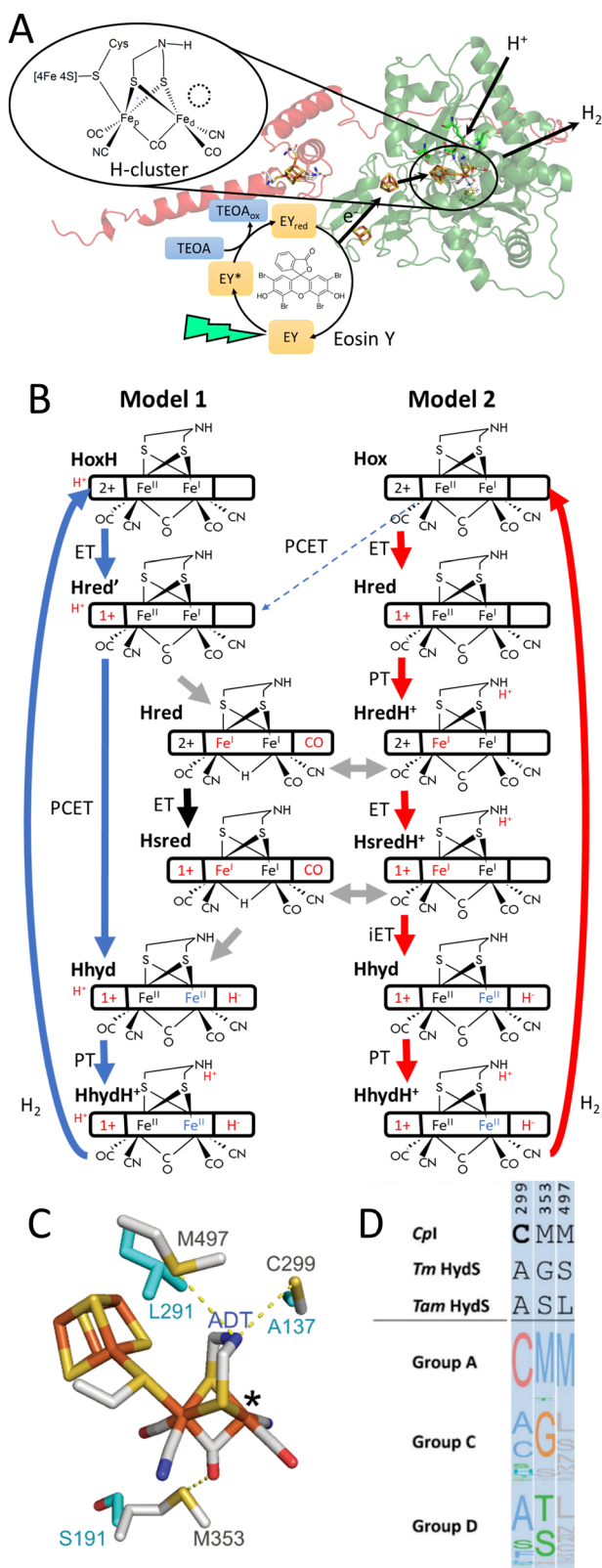
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**Fig. 1** (A) *TamHydS* protein structure, H-cluster and photo-reduction scheme. YASARA-generated homology model (green cartoon) of *TamHydS*<sup>9,10</sup> based on the crystal structure of Cpl (PDB ID 4XDC<sup>11</sup>). The [4Fe-4S] clusters and the H-cluster are shown as sticks (C: gray, Fe: orange, S: yellow, N: blue, O: red). The additional C-terminal domain was predicted with AlphaFold (red cartoon) and the associated [4Fe-4S] cluster inserted

manually. Details are described in the *TamHydS* structure section in the ESI.† Eosin Y (EY) is excited by visible light (EY\*) and reductively quenched by triethanolamine (TEOA). The reduced Eosin Y (EY<sub>red</sub>) can donate electrons to *TamHydS* resulting in population of reduced states and eventually H<sub>2</sub> production. (B) Two main catalytic cycle models. Model 1 blue arrows, model 2 red arrows, grey arrows indicate ligand rotation. The CO ligand occupying the apical vacancy and bound protons are indicated in red. Electron transfer (ET), internal electron transfer (iET), proton transfer (PT) and proton coupled electron transfer (PCET) steps are indicated at the arrows. Red letters indicate the site of reduction, blue letters the site of oxidation relative to the oxidized state (H<sub>ox</sub>). Note that for model 2 the nomenclature introduced by Sommer *et al.*<sup>12</sup> is used which leads to different redox states having the same abbreviation. (C) Group A and D cofactor second coordination sphere differences. Amino acids of Cpl interacting with the H-cluster (C299, M353, and M497) are shown in sticks (grey: C, yellow: S). The azadithiolate (ADT) bridgehead is labelled as Fe<sub>d</sub> (\*). The corresponding residues from the homology model of *TamHydS* are overlaid (cyan: C, red: O). (D) Normalized consensus logos of groups A, C, and D [FeFe]-hydrogenase generated in Jalview using ClustalΩ<sup>13</sup> sequence alignment of sequences retrieved from Greening *et al.*<sup>14</sup> The numbering is based on the sequence of Cpl. The conservation of the amino acid in a position is proportional to its font size.

resonance (EPR) spectroscopy. The oxidized state (H<sub>ox</sub>) features a [4Fe-4S]<sub>H</sub><sup>2+</sup> cluster and a Fe(II)Fe(I) diiron site. The H<sub>ox</sub> state and its potentially protonated equivalent H<sub>ox</sub>H, feature the aforementioned bridging CO ligand.<sup>18</sup> This ligation results in an open coordination site in an apical position at the distal iron atom (Fe<sub>d</sub>), a structure defining the “rotated” diiron site geometry.<sup>19–22</sup> The first reduction occurs at the [4Fe-4S]<sub>H</sub> cluster forming the H<sub>red</sub> state.<sup>12,18,23</sup> Protonation coupled to intramolecular electron transfer from the [4Fe-4S]<sub>H</sub> cluster to the diiron site has been proposed to give rise to a diiron site reduced state denoted H<sub>red</sub> (sometimes H<sub>red</sub>H<sup>+</sup>). Addition of a second electron to the H-cluster gives rise to the “super-reduced” state H<sub>sred</sub> (or H<sub>sred</sub>H<sup>+</sup>), with a reduced [4Fe-4S]<sub>H</sub><sup>+</sup> cluster. The hydride state, H<sub>hyd</sub>, is a tautomer of H<sub>sred</sub> featuring a terminal hydride at the former apical vacancy giving a formally di-ferrous diiron site.<sup>24–26</sup> Protonation of H<sub>hyd</sub> at the amine of the ADT ligand is proposed to yield the still only partially characterized state, H<sub>hyd</sub>H<sup>+</sup>.<sup>27,28</sup> The hydride and the proton may form H<sub>2</sub>, returning the H-cluster to the oxidized state, H<sub>ox</sub>. The exact structure and catalytic relevance of these states are subject to ongoing discussion, which has resulted in competing mechanistic models, at least two of which are extensively reviewed in recent literature.<sup>2,5,16,29–31</sup>

In short, the two mechanistic models diverge in the interpretation of the spectroscopic data, both with regards to ligand geometry of the diiron subsite as well as protonation status of the H-cluster (Fig. 1B). In model 1, two of the aforementioned reduced states, H<sub>red</sub> and H<sub>sred</sub>, are interpreted as inhibited states adopting an “inverted” diiron site geometry. Critically, here protonation of the diiron subsite is proposed to occur at the Fe ions yielding a bridging hydride (μH), while the former μCO ligand shifts to a terminal position that otherwise provides the apical vacancy. The expected thermodynamic stability of the μH geometry,<sup>32,33</sup> and the additional ligand rotation necessary to form a terminal hydride disfavour their involvement in fast H<sub>2</sub> catalysis.<sup>30,34–36</sup> Conversely, in model 2, the same

spectroscopic signals are attributed to species protonated at the ADT-amine instead of the iron ions, and commonly denoted  $H_{red}H^+$  and  $H_{sred}H^+$ . As a consequence,  $H_{red}H^+$  and  $H_{sred}H^+$  are proposed to retain the rotated geometry of the  $H_{ox}$  state and to be catalytically relevant.<sup>12,16</sup> In favour of model 1, the absence of a  $\mu CO$  band for  $H_{red}$  has been noted in numerous independent studies. An observation which, coupled with comprehensive DFT calculations and isotope editing, supports the notion of an inverted geometry in  $H_{red}$ .<sup>34,35</sup> However, low temperature experiments provide support for model 2. Albeit the  $\mu CO$  ligand band expected for the  $H_{red}H^+$  state has proven challenging to observe at room temperature it has been detected *via* FTIR spectroscopy at cryogenic temperatures.<sup>37–39</sup>

In the following, we will use the  $H_{red}/H_{red}/H_{sred}$  nomenclature to define specific spectroscopic fingerprint signals, without implying a specific ligand geometry and/or protonation status.

It is well-established that the protein environment has a large effect on the H-cluster.<sup>17,40–42</sup> In group A [FeFe]-hydrogenases both the active-site pocket and a proton transfer pathway (PTP), composed of conserved amino acids and water molecules, are supposed to play a key role in fast catalysis.<sup>43–45</sup> The H-bonding partners of the  $CN^-$  ligands of the H-cluster are conserved in the vast majority of groups of [FeFe]-hydrogenases.<sup>1,46</sup> Beyond these immediate interactions, several outer-coordination sphere amino acid residues are strictly conserved in the active-site pocket of group A [FeFe]-hydrogenases. Variation of these conserved residues through site directed mutagenesis has been shown to significantly impair turnover rates and in some cases even prevent H-cluster assembly.<sup>40</sup> However, the putatively sensory enzymes in groups C and D display variations of several conserved amino acids constituting the outer-coordination sphere in group A [FeFe]-hydrogenases (Fig. 1C and D).<sup>3,9</sup> Moreover, the PTP of group A is not conserved in either group C or group D [FeFe]-hydrogenases, and a distinct alternative PTP has been proposed for group D.<sup>47</sup>

As expected from these structural differences, the reactivity of group C and D enzymes is clearly different from the well-known group A [FeFe]-hydrogenases. Activity assays have shown that group C and group D enzymes are significantly slower than any reported group A enzyme.<sup>3,9</sup> Introducing the PTP and active-site of group A [FeFe]-hydrogenase into a group D enzyme has been shown to increase the catalytic rate more than 100-fold, underscoring the impact of the protein scaffold on H-cluster reactivity.<sup>42</sup> Moreover, an alternative diiron site reduced state  $Fe(I)Fe(I)$ , denoted  $H_{red}^*$ , has so far been detected exclusively in spectro-electrochemistry studies of the group C [FeFe]-hydrogenase from *Thermotoga maritima* (*TmHydS*).<sup>3,48</sup> Compared to  $H_{red}H^+$ ,  $H_{red}^*$  is proposed to lack the additional protonation at the ADT while featuring the rotated,  $\mu CO$ , geometry (compare Fig. S1, ESI†). In the only characterized group D [FeFe]-hydrogenase, from *Thermoanaerobacter mathranii* (*TamHydS*), catalysis is bidirectional but thermodynamically irreversible.<sup>9</sup> Kinetic modelling of cyclic voltammetry traces recorded under varied pH and  $H_2$  pressure supports the notion that *TamHydS* nevertheless follows the same mechanism as group A hydrogenases.<sup>49,50</sup> However, there is

currently very limited spectroscopic data available for *TamHydS* under turnover conditions to support this hypothesis. With regards to possibly catalytically active states, previously observed in group A enzymes, only oxidized ( $H_{ox}/H_{ox}H$ ) and a one-electron reduced redox state ( $H_{red}$ ) of the diiron site have been reported to-date.<sup>9,10</sup>

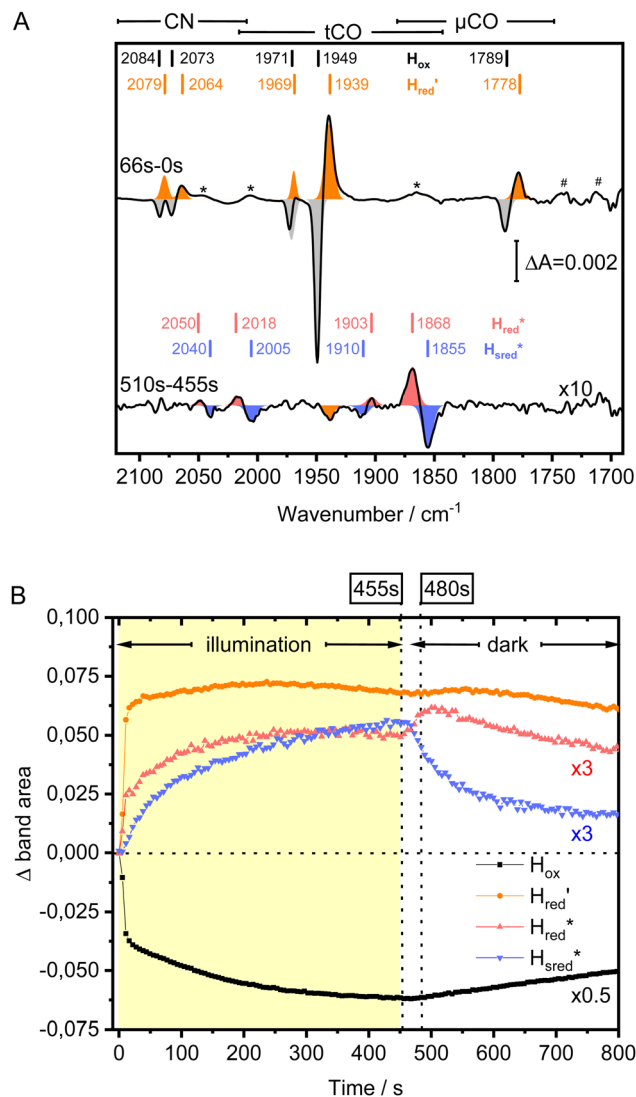
[FeFe]-hydrogenases serve as model systems for probing general concepts such as thermodynamically reversible *vs.* irreversible catalysis.<sup>51</sup> Thus, spectroscopic insight into the mechanism of these “non-prototypical” [FeFe]-hydrogenases is required to support or refute the suggestion arising from kinetic modelling work that the irreversible group D enzyme *TamHydS* still follows the same mechanism as the reversible group A enzymes.<sup>50</sup> Furthermore, considering how the active site of [FeFe]-hydrogenases inspire the design of synthetic catalysts for sustainable  $H_2$  catalysis,<sup>5,6</sup> a more detailed characterization of the group C and D enzymes is arguably critical to improve our understanding of how the outer-coordination sphere modulates the reactivity of the H-cluster.

Our initial efforts using spectro-electrochemistry and chemical reductants to probe the H-cluster of *TamHydS* have only revealed interconversion between the  $H_{red}$  and  $H_{ox}$  states.<sup>9</sup> To gain further insight into the H-cluster chemistry under turnover conditions we instead applied photo-reduction. [FeFe]-hydrogenases are not intrinsically photoactive, but catalysis can be photo-triggered *via* a suitable photosensitizer (Fig. 1A). Such photo-reduction has been shown to induce  $H_2$  catalysis *in vitro*<sup>52–55</sup> and in living cells.<sup>56,57</sup> Moreover, this technique has facilitated investigations into the population of the reduced state ( $H_{red}$ ) of the H-cluster as well as associated changes in the proton transfer pathway (PTP) of group A [FeFe]-hydrogenases under both steady-state and time-resolved conditions.<sup>45,57,58</sup>

Here, we investigate the group D [FeFe]-hydrogenase *TamHydS* *via* attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy, complemented by electron paramagnetic resonance (EPR) spectroscopy. Through photo-reduction, we now have detected a variety of additional states that feature similar redox levels as in group A [FeFe]-hydrogenases. We observe the accumulation of four distinct diiron site reduced states that most likely differ in protonation chemistry, of which two were exclusively observed in the group C [FeFe]-hydrogenase *TmHydS* before.<sup>3</sup> In turnover experiments, we can confirm a sequential population of redox states, namely from the oxidized state to one-electron reduced to two-electron reduced and putatively terminal hydride bound intermediates. Despite notable differences in active site cofactor environment, PTP, as well as catalytic activity rates, our findings provide support for a conserved catalytic mechanism in group A and D [FeFe]-hydrogenases. Additionally, the study sheds new light on the contested  $H_{red}$  intermediate.<sup>16,29</sup> The detection of a potential bridging CO ligand in the spectroscopic signature of the  $H_{red}$  state indicates that the catalytically important rotated cofactor geometry can be conserved for the reduced diiron site intermediate at ambient conditions.







**Fig. 2** (A) ATR-FTIR difference spectra of the *TamHydS*<sup>PDT</sup>/Eosin Y/TEOA mixture. Reduction upon illumination (66 s of illumination minus the initial pre-illumination “0 s” spectrum, top spectrum) and auto-oxidation after illumination was stopped (480 seconds minus the last illuminated spectrum at 455 seconds, bottom spectrum). The peaks of terminal cyanide (CN<sup>-</sup>), terminal (tCO) and bridging carbon monoxide (μCO) ligands for different redox states are colour coded and the band patterns indicated by coloured bars. (Top spectrum) Upon photo-reduction the oxidized state (H<sub>ox</sub>, 2084, 2073, 1971, 1949 and 1789 cm<sup>-1</sup>) depopulates, predominantly in favour of the one-electron reduced state (H<sub>red</sub>', 2079, 2064, 1969, 1939 and 1778 cm<sup>-1</sup>). Potential bands indicated by # do not correlate with any of the band patterns observed here. (Bottom spectrum) The 10-fold magnified difference spectrum displays the changes during re-oxidation after illumination. The minor redox species indicated by an \* in the top spectrum can be assigned to H<sub>red</sub>\* (2050, 2018, 1903 and 1868 cm<sup>-1</sup>) and H<sub>sred</sub>\* (2040, 2005, 1910 and 1855 cm<sup>-1</sup>). Compare with (B) for the kinetics of (de)population during and after photo-reduction. (B) Redox state populations over time during and after photo-reduction. The time traces represent the sum of the difference peak area associated with a given redox state. To facilitate comparison the population of H<sub>red</sub>' and H<sub>sred</sub>\* are multiplied by a factor of 3. The yellow box indicates the illumination period. Within 22 seconds most of the H<sub>ox</sub> population (black) is converted into H<sub>red</sub>' (orange). Half the H<sub>red</sub>' population (red) is formed on the same timescale. Subsequently, H<sub>sred</sub>\* (blue) starts to accumulate. Directly after illumination H<sub>ox</sub> and H<sub>red</sub>' appear to remain constant while H<sub>sred</sub>\* decays and H<sub>red</sub>' gets transiently populated. On a longer timescale > 150 seconds after illumination, all reduced species convert into H<sub>ox</sub>. Compare Fig. S4 (ESI<sup>†</sup>) for zoom-ins on the direct changes upon illumination and after illumination.

## Results and discussion

### Spectroscopic investigation of group D [FeFe]-hydrogenase *TamHydS*<sup>PDT</sup>

We initially studied an artificially matured variant of *TamHydS* where the secondary amine of the ADT bridgehead is replaced by a methylene group (PDT) denoted *TamHydS*<sup>PDT</sup> to avoid the complexity arising from protonation of the diiron subsite. For PDT cofactor variants of group A [FeFe]-hydrogenases, H<sub>ox</sub> and H<sub>red</sub>' are the main redox states reported.<sup>59,60</sup> A mixture of *TamHydS*<sup>PDT</sup> [FeFe]-hydrogenase, photosensitizer (Eosin Y), and sacrificial electron donor (triethanolamine (TEOA)) was applied to the surface of the ATR crystal, dried, and subsequently rehydrated with pH 8 Tris buffer. During continuous white light illumination, the photo-reduction of *TamHydS*<sup>PDT</sup> was followed in real time by ATR-FTIR difference spectroscopy. Fig. 2A (top spectrum) displays the “light minus dark” difference spectrum in the region of the CO and CN<sup>-</sup> ligands of the diiron site. Before illumination, the sample adopted the oxidized resting state, H<sub>ox</sub>, with its characteristic bands at 2084, 2073, 1971, 1949 and 1789 cm<sup>-1</sup> (negative grey bands Fig. 2A). Upon photo-reduction, H<sub>ox</sub> depopulates, converting into the commonly observed [4Fe-4S]<sub>H</sub> cluster reduced state, H<sub>red</sub>', with bands at 2079, 2064, 1969, 1939 and 1778 cm<sup>-1</sup> (positive orange bands Fig. 2A). The absolute spectra indicate that the transition is nearly quantitative (Fig. S2, ESI<sup>†</sup>). However, minor unassigned peaks in the difference spectrum indicate the formation of additional reduced species (marked by an \* in Fig. 2A, top).

The origin of these minor contributions becomes clearer when studying the auto-oxidation of the enzyme, spontaneously occurring once illumination was stopped. The difference spectrum computed from a dark spectrum collected 55 s after stopping illumination and the last illuminated spectrum (time-points indicated by dashed lines in Fig. 2B) clearly shows that the unassigned features can be attributed to two additional interconverting states (Fig. 2A bottom spectrum). We observe positive bands at 2050, 2018, 1903 and 1868 cm<sup>-1</sup> and negative bands at 2040, 2005, 1910 and 1855 cm<sup>-1</sup>. In both band patterns, the lowest wavenumber band is the most intense, which is a characteristic of diiron site reduced states.<sup>12,18,19</sup> Since the PDT ligand cannot be protonated and the cofactor cannot yield H<sub>red</sub> and H<sub>sred</sub>, we assign the minor species with a pronounced absorbance feature at 1868 cm<sup>-1</sup> to H<sub>red</sub>\* (Fig. 2A, bottom spectrum, positive bands), in agreement with data reported for the group C [FeFe] hydrogenase *TmHydS*<sup>PDT</sup>.<sup>3</sup> It follows that the second minor component, which is lost upon incubation in darkness, is attributable to H<sub>sred</sub>\* (compare Fig. S1, ESI<sup>†</sup>) additionally reduced at the [4Fe-4S]<sub>H</sub> cluster (Fig. 2A, bottom spectrum, negative bands). We do not observe the photolysis of CO ligands during photo-reduction.<sup>61,62</sup> Repeating the experiment with *TamHydS*<sup>PDT</sup> samples including sodium dithionite led to practically indistinguishable results (Fig. S3, ESI<sup>†</sup>). An overview of all *TamHydS* infrared signatures is given in Table 1 and Fig. S9 (ESI<sup>†</sup>). The detailed fit parameters for all infrared signatures can be found in the ESI<sup>†</sup>.



**Table 1** Infrared band patterns of different redox states in *TamHydS* (group D) and *CrHydA1* (group A) or *TmHydS* (group C) [FeFe]-hydrogenase

	<i>TamHydS</i> (group D) bands in $\text{cm}^{-1}$	<i>CrHydA1</i> (group A) bands in $\text{cm}^{-1}$
$\text{H}_{\text{ox}}$	2082, 2074, 1970, 1948, 1787 <sup>9</sup>	2088, 2072, 1964, 1940, 1800 <sup>25</sup>
$\text{H}_{\text{oxH}}$	n.d., n.d., 1978, 1954, 1795 <sup>9</sup>	2092, 2072, 1970, 1946, 1812 <sup>18</sup>
$\text{H}_{\text{red}}$	2062, 2030, 1961, 1922, 1895 <sup>9</sup>	2072, 2033, 1961, 1915, 1891 <sup>35</sup>
$\text{H}_{\text{red}}^*$	2062, 2032, 1922, 1896, 1803 (this study)	At 90 K, n.d., 1919, 1891, 1817 <sup>39</sup>
$\text{H}_{\text{red}}^{\text{PDT}}$	2052, 2012, 1903, 1875, n.d. (this study)	# <i>TmHydS</i> <sup>ADT</sup> (group C) 2055, 2022, 1894, 1871, 1763 <sup>3</sup>
$\text{H}_{\text{red}}^{\text{'}}$	2050, 2018, 1903, 1868, n.d. (this study)	# <i>TmHydS</i> <sup>PDT</sup> (group C) 2048, 2012, 1883, 1862, n.d. <sup>3</sup>
$\text{H}_{\text{red}}^{\text{'PDT}}$	n.d., n.d., 1960, 1939, 1782 (this study)	2084, 2066, 1962, 1933, 1792 <sup>15</sup>
$\text{H}_{\text{sred}}$	2079, 2064, 1969, 1939, 1778 (this study)	2090, 2072, 1966, 1941, 1810 <sup>59</sup>
$\text{H}_{\text{sred}}^*$	n.d., n.d., 1912, 1887, n.d. (this study)	2068, 2026, 1953, 1918, 1882 <sup>35</sup>
$\text{H}_{\text{sred}}^{\text{PDT}}$	n.d., n.d., n.d., 1865, n.d. (this study)	# <i>TmHydS</i> <sup>ADT</sup> (group C) 2047, 2013, 1900, 1861, 1751 <sup>3</sup>
$\text{H}_{\text{sred}}^{\text{'PDT}}$	2040, 2005, 1910, 1855, n.d. (this study)	# <i>TmHydS</i> <sup>PDT</sup> (group C) 2042, 2007, 1894, 1853, n.d. <sup>3</sup>
$\text{H}_{\text{hyd}}$	2082, 2075, 1981, 1970, 1848 (this study)	2082, 2068, 1978, 1960, 1860 <sup>24</sup>

Bands are indicated in the order CN, CN, tCO, tCO,  $\mu/\text{tCO}$ . #Data reported for the group C enzyme *TmHydS* is listed for  $\text{H}_{\text{red}}^*$  and  $\text{H}_{\text{sred}}^*$  to compare the observed band positions since this is the only [FeFe]-hydrogenases previously reported to adopt these states.

Using the aforementioned redox state assignment, we can monitor the redox state population over time during and after photo-reduction (Fig. 2B). A rapid conversion of  $\text{H}_{\text{ox}}$  primarily into  $\text{H}_{\text{red}}^*$  is evident during the first 22 seconds of illumination.  $\text{H}_{\text{red}}^*$  also starts to accumulate rapidly, reaching approximately half of the final  $\text{H}_{\text{red}}^*$  population on the same timescale (Fig. S4 for zoom in, ESI†). However, while the  $\text{H}_{\text{red}}^*$  population appears to plateau, we observe further population of  $\text{H}_{\text{red}}^*$  on a longer timescale. A lag-phase of about 20 seconds is observed for the population of  $\text{H}_{\text{sred}}^*$ . The fact that  $\text{H}_{\text{sred}}^*$  starts to accumulate when a large fraction of the enzyme has already converted to  $\text{H}_{\text{red}}^*$  and  $\text{H}_{\text{red}}^*$  is in line with the proposed two-electron reduced nature of this species. Note that the redox state populations plotted in Fig. 2 are normalized, and that the  $\text{H}_{\text{red}}^*$  and  $\text{H}_{\text{sred}}^*$  populations remain minor species when compared to the  $\text{H}_{\text{ox}}$  to  $\text{H}_{\text{red}}^*$  populations throughout the entire experiment (Fig. S5, ESI†). After photo-reduction, the  $\text{H}_{\text{red}}^*$  population appears to stay constant for *ca.* 150 seconds. The  $\text{H}_{\text{sred}}^*$  population decays fastest and most probably converts into  $\text{H}_{\text{red}}^*$  whose population is transiently increased before decaying as well (Fig. S4, ESI†). The  $\text{H}_{\text{ox}}$  population remains constant for *ca.* 30 seconds after illumination and recovers subsequently. Similar relatively sluggish conversion from one-electron reduced states to  $\text{H}_{\text{ox}}$  has been observed in group A [FeFe]-hydrogenases before.<sup>63</sup> Thus, the study of *TamHydS*<sup>PDT</sup> has allowed us to observe three reduced states ( $\text{H}_{\text{red}}^*$ ,  $\text{H}_{\text{red}}^*$ ,  $\text{H}_{\text{sred}}^*$ ), complementing the observation of the  $\text{H}_{\text{red}}$  state previously reported for *TamHydS*<sup>ADT</sup>.<sup>9,10</sup>

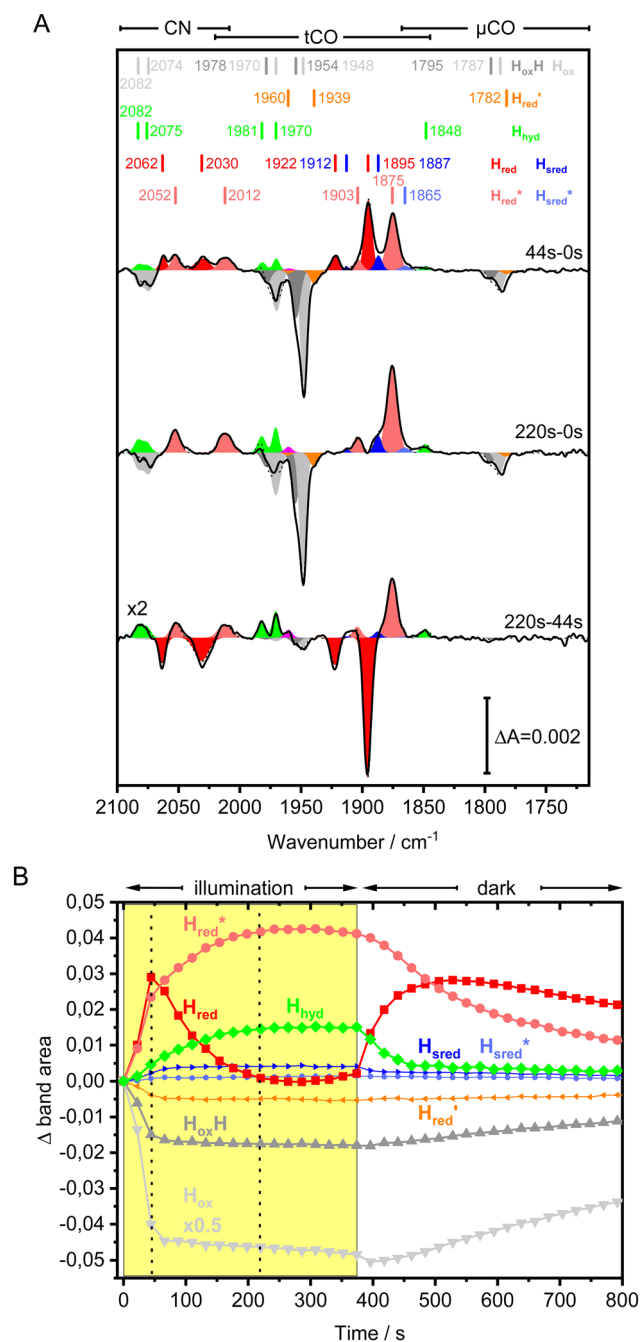
### New redox states for group D [FeFe]-Hydrogenase *TamHydS*<sup>ADT</sup>

Performing the analogous photo-reduction experiment on the catalytically active form *TamHydS*<sup>ADT</sup> (in the following *TamHydS*) resulted in more complex spectral changes. In Fig. 3A, difference spectra reflecting changes after 44 seconds (44s–0s, top spectrum) and 220 seconds (220s–0s, middle spectrum) of continuous illumination relative to the starting “pre-illumination” (“0”) spectrum are displayed (absolute and full series of difference spectra in Fig. S6, ESI†). As expected, we observe a decrease of bands associated with the oxidized states,  $\text{H}_{\text{ox}}$  and  $\text{H}_{\text{oxH}}$  (grey and dark grey bands). The difference

spectra also suggest the de-population of an additional minor species with small negative bands detected at 1960, 1939 and 1782  $\text{cm}^{-1}$  (orange bands). These bands are reminiscent of  $\text{H}_{\text{red}}^*$  when compared to *TamHydS*<sup>PDT</sup> (Fig. 2) and group A enzymes,<sup>12,15</sup> implying that this reduced species is present in low amounts at pH 8.

In contrast to the reduction of *TamHydS* by  $\text{H}_2$  gas and electrochemistry as reported earlier,<sup>9,10</sup> we detect two distinct terminal distal CO bands at 1895 and 1875  $\text{cm}^{-1}$ . To fit these bands, additional bands at 1887 and 1865  $\text{cm}^{-1}$  were necessary, overall indicative of four separate diiron site reduced states. The assignment to four distinct H-cluster states is further strengthened by their independent (de-)population kinetics during the photo-reduction experiment (Fig. 3B). The highest wavenumber band at 1895  $\text{cm}^{-1}$  is attributable to the previously observed protonated and reduced diiron site state,  $\text{H}_{\text{red}}$ , with an additional CO band detected at 1922  $\text{cm}^{-1}$  and CN<sup>−</sup> bands at 2030 and 2062  $\text{cm}^{-1}$ . The band at 1887  $\text{cm}^{-1}$ , which increases together with a band at 1912  $\text{cm}^{-1}$ , is attributed to the two electron reduced state  $\text{H}_{\text{sred}}$ , based on its position relative to that of  $\text{H}_{\text{red}}$  at 1895  $\text{cm}^{-1}$  and comparison to the infrared signatures of group A [FeFe]-hydrogenases.<sup>19,25</sup> The possibility that the signals at 1887 and 1895  $\text{cm}^{-1}$  instead reflect two  $\text{H}_{\text{red}}$  state populations with different redox states of neighbouring F-clusters is highly unlikely to rationalize the here observed shift of *ca.* 10  $\text{cm}^{-1}$ .<sup>64,65</sup> The band at 1875  $\text{cm}^{-1}$ , which follows the same kinetics as the bands at 2052, 2012 and 1903  $\text{cm}^{-1}$ , can be assigned to  $\text{H}_{\text{red}}^*$ , based on the  $\text{H}_{\text{red}}^*$  state signature observed in *TamHydS*<sup>PDT</sup>. Finally, based on the assignment of  $\text{H}_{\text{red}}^*$ , we can assign the small band detected at 1865  $\text{cm}^{-1}$  to  $\text{H}_{\text{sred}}^*$ . We note that this implies a blue-shift of the band by about 8  $\text{cm}^{-1}$ , when the dithiolate ligand featured an amine bridgehead group relative to the methylene analogue. This would be in good agreement with what has been reported for the group C [FeFe]-hydrogenase *TmHydS*, for which the band pattern of  $\text{H}_{\text{red}}^*$  in *TmHydS*<sup>ADT</sup> shifted to higher wavenumbers by *ca.* 10  $\text{cm}^{-1}$  when compared to *TmHydS*<sup>PDT</sup>.<sup>3</sup> Chongdar *et al.* attributed this shift to a change in electron density at the diiron core induced by the nature of the bridging dithiolate ligand.





**Fig. 3** (A) Light minus dark ATR-FTIR difference spectra of the *TamHydS*/Eosin Y/TEOA mixture after 44 seconds (top spectrum) and 220 seconds of illumination (middle spectrum). The bottom difference spectrum is calculated between 44 and 220 seconds of illumination (timepoints indicated by vertical dashed lines) where only small changes in oxidized redox states population occur (compare dashed lines in Fig. 3B) enabling the detection of the hydride state bands. The peaks of terminal cyanide (CN), terminal (tCO) and bridging carbon monoxide ( $\mu$ CO) ligands for each different redox state are colour coded, and the band patterns indicated by coloured bars. The difference spectrum is indicated in black and the fit by a dashed line. Upon photo-reduction the oxidized states ( $H_{ox}$ ,  $H_{oxH}$ ) and  $H_{red}$  depopulate in favour of the one-electron and two electron reduced states ( $H_{red}$ ,  $H_{sred}$ ,  $H_{red}^*$ ,  $H_{sred}^*$  and  $H_{hyd}$ ). Compare to Fig. 3B for the kinetics of (de)-population during and after photo-reduction. An unassigned band is indicated in magenta. (B) Redox state population kinetics during and after photo-reduction of *TamHydS*. The summed band area of each individual

redox state is plotted for visual comparison of the (de)-population kinetics. Within the first 40–60 seconds we observe a depopulation of the oxidized states ( $H_{ox}$ ,  $H_{oxH}$ ) as well as the one-electron reduced state  $H_{red}$ . The population of  $H_{red}$  reaches its maximum after 44 seconds and depopulates subsequently in the expense of  $H_{red}^*$  and the two electron reduced states ( $H_{sred}$ ,  $H_{sred}^*$ ,  $H_{hyd}$ ).  $H_{hyd}$  seems to accumulate the slowest reaching its maximum around 200 seconds. After photo-reduction, the two-electron reduced protonated redox states  $H_{hyd}$  and  $H_{sred}$  depopulate at the same time scale as we observe an increase of  $H_{red}$ ,  $H_{red}^*$  (and  $H_{sred}^*$ ) decay on a slower timescale. The population of  $H_{ox}$ ,  $H_{oxH}$  and  $H_{red}$  remains nearly constant for 80 seconds after photo-reduction. The yellow box indicates the illumination period. Each point is extracted from a difference spectrum ( $x=0$  seconds) (Fig. S6, ESI†) and difference spectra for 44–0, 220–0 and 220–44 seconds are shown in Fig. 3A.

To test this latter hypothesis, regarding the influence of the bridgehead group on the FTIR signatures of the H-cluster, we studied a series of diiron site mimics ( $Fe(i)Fe(i)$ ) featuring ADT and PDT ligands with different combinations of carbonyl, phosphine and  $CN^-$  ligands (Fig. S7, ESI†). The effect of the bridgehead group of the dithiolate ligand was found to vary depending on the rest of the ligand sphere of the model complexes. It was most distinct in the dicyanide complexes, where the mimic featuring the PDT ligand displayed a shift of certain CO bands by  $8\text{ cm}^{-1}$  relative to the ADT ligated analogue. When comparing the infrared signatures of *TamHydS*<sup>PDT</sup> and *TamHydS*<sup>ADT</sup>, a clear effect by H-cluster redox state was found (overview of infrared signatures in Table 1 and Fig. S9, ESI†). The two *TamHydS* versions seem to be nearly identical for  $H_{ox}$  with 0–2  $\text{cm}^{-1}$  shifts of the cofactor ligand bands. Conversely, for  $H_{red}$ , we observe a  $9\text{ cm}^{-1}$  shift on the proximal CO (pCO) band while the distal CO (dCO) band is found at an identical position. While for the  $H_{red}^*$  and  $H_{sred}^*$  states, the dCO band is shifted by 7–10  $\text{cm}^{-1}$  to higher energies in *TamHydS*<sup>ADT</sup> relative to *TamHydS*<sup>PDT</sup>. In combination, these observations support the idea that the nature of the bridgehead can influence the FTIR signature of H-cluster states. However, evidently, the effect is difficult to predict as the effect varies both with oxidation state and the remaining ligand sphere.

As the concentration of  $H_{ox}$  states remains practically constant after 44 seconds (Fig. 3B) the difference spectrum calculated between 220 and 44 seconds of illumination (220s–44s, Fig. 3A bottom) has no significant negative contributions from the oxidized state bands between 1980–1948  $\text{cm}^{-1}$ . This allows assigning the additional positive bands at 2082, 2075, 1981, 1970 and 1848  $\text{cm}^{-1}$  to the  $CN^-$  and CO ligands of a redox state with a super-oxidized diiron site when compared to  $H_{ox}$ . The inhibited states,  $H_{inact}$  and  $H_{trans}$ , feature a super-oxidized diiron site. However, reduction reactivates these inhibited states, thus they are highly unlikely to accumulate under photo-reduction.<sup>19,66</sup> Instead we attribute the band pattern to the hydride state,  $H_{hyd}$  (positive green bands, Fig. 3 bottom spectrum), which exhibits similar band patterns in group A [ $FeFe$ ]-hydrogenases.<sup>24,55</sup> However, due to the low population of the putative  $H_{hyd}$  species when performing the experiment in  $D_2O$  we refrain from a definite assignment.<sup>24,28</sup> Attempts at enriching this proposed essential catalytic intermediate in





*TamHydS* via our group A [FeFe]-hydrogenase specific protocols (low pH value, optional high reductant concentration, and exposure to  $H_2$  gas<sup>24,26</sup>) led to the population of  $H_{red}$  only, even though a significant fraction of  $H_{ox}H$  was present.<sup>9</sup>

To conclude, we observe similar redox states in group A and D [FeFe]-hydrogenases. The H-cluster in *TamHydS* can clearly adopt the oxidized state ( $H_{ox}$ ), the one-electron reduced states  $H_{red}'$  and  $H_{red}$  with the reducing equivalent on the  $[4Fe-4S]_H$  or the diiron site respectively, the super reduced state ( $H_{sred}$ ) with one reducing equivalent on the  $[4Fe-4S]_H$ , and the diiron site each, and appears to form also the di-ferrous terminal hydride state ( $H_{hyd}$ ).

To further investigate the interconversion of these redox states during and after photo-reduction, we again analysed the redox state population changes as a function of time (Fig. 3B). The quantification was done through summation of the areas of the bands associated with each state. The oxidized states  $H_{ox}$  and  $H_{ox}H$  as well as for the one-electron reduced state,  $H_{red}'$  depopulate mainly during the first 44 seconds, and reach a steady concentration after 66 seconds. The protonated one-electron reduced state  $H_{red}$  reaches its maximum population after 44 seconds, after which time the population of this species clearly decreases again. The alternative one-electron reduced state  $H_{red}^*$  instead increases on a slower timescale (ca. 50% populated at 44 s) and reaches a steady-state population after about 220 seconds. As can be seen in Fig. 3A,  $H_{red}$  and  $H_{red}^*$  are the main reduced states formed during photo-reduction. The two-electron reduced state  $H_{sred}$  rise to a plateau population within 120 seconds, while the  $H_{hyd}$  population takes approximately double the time (220 seconds, compare as well bottom spectrum Fig. 3A). Notably, the rise of  $H_{hyd}$  coincides with the decay of  $H_{red}$ . The time-dependency of the  $H_{sred}^*$  population is difficult to track with certainty due to the low signal intensity but seem to continuously increase slowly during illumination. Overall, these trends are in good agreement with an increasingly negative solution potential upon extended illumination. After illumination, we observe the converse transitions between H-cluster state populations. The protonated two-electron reduced states,  $H_{sred}$  and  $H_{hyd}$ , depopulate rapidly (negligible residual signal at  $t \approx 420$  s and 460 s, respectively; where illumination is stopped at  $t = 374$  s). On a much longer timescale compared to the decay of  $H_{sred}$  and  $H_{hyd}$  the populations of  $H_{red}^*$  (50% de-populated at 500 s) and  $H_{sred}^*$  decrease. The population of  $H_{red}$  varies in a more complex fashion. Again, coinciding with the opposite kinetics of  $H_{hyd}$ ,  $H_{red}$  increases shortly after illumination is arrested (maximum population at ca. 500 s) before it slowly starts to decrease. Similar to *TamHydS*<sup>PDT</sup> the population of the oxidized states,  $H_{ox}$  and  $H_{ox}H$ , remain nearly constant for ca. 80 seconds and repopulate slowly afterwards. The rapid appearance of  $H_{red}$  but more sluggish reformation of the  $H_{ox}/H_{ox}H$  after illumination agrees with  $H_{red}$  as a quasi-stable resting state as previously noted in  $H_2$  flushing experiments.<sup>9</sup>

Considering the more recently identified  $H_{red}^*$  and  $H_{sred}^*$  states, and assuming that the structural assignment of  $H_{red}^*$  proposed by Chongdar *et al.* is correct,<sup>3</sup> there are at least two

possible scenarios that can explain the kinetics observed in this study.  $H_{red}^*$ , and the associated  $H_{sred}^*$  state could reflect inhibited states, potentially related to the reductive inhibition phenomena previously noted by Léger and co-workers,<sup>67</sup> or represent catalytically relevant states closely related to the  $H_{red}'$  state.

If  $H_{red}^*$  would represent an inhibited, off-pathway, species,  $H_{red}$  and  $H_{hyd}$  would be the main reduced catalytic species interconverting during and after photo reduction. Indeed, the decay of  $H_{red}$  during prolonged illumination (photo-reduction) coincides with the population of  $H_{hyd}$ . Similar but opposite behaviour is observed directly after photo-reduction;  $H_{red}$  gets populated while  $H_{hyd}$  de-populates on the same timescale. The kinetics of  $H_{red}^*$  do not seem to be following the kinetics of either of these states besides getting populated alongside  $H_{red}$  at the beginning of the photo-reduction experiment. During the post-illumination phase,  $H_{red}^*$  decays slowly, potentially interconverting into  $H_{red}$  after the  $H_{hyd}$  state already decayed (around 500 seconds). The accumulation of a large population of an inhibited state under reducing conditions could also partially rationalize the low catalytic activities observed for group C and D [FeFe]-hydrogenases.<sup>3,9</sup> Still, we note that this model would require that the inhibition is reversible on the time-scale of the experiment, as otherwise the  $H_{red}^*$  state population would be expected to continuously increase during the photo-reduction. Arguing against this scenario is the fact that no reductive inhibition has been reported from earlier electrochemical studies of *TamHydS*.<sup>9,49,50</sup> The alternative rationale would be that  $H_{red}^*$  vs.  $H_{red}$  populations are governed by the relative rates of electron transfer and protonation. Here, pure electron transfer would yield  $H_{red}^*$ , while coupled (but not necessarily concerted) electron transfer and protonation yields  $H_{red}$ . During extended illumination we can expect electron transfer rates to increase as the population of photo-reduced EY increases, while proton transfer rates remain unchanged. This would potentially result in an increase in population of the reduced unprotonated state  $H_{red}^*$  relative to  $H_{red}$ . As illumination is arrested, the solution potential decreases and the protonation step can now compete, resulting in a decrease of  $H_{red}^*$  relative to  $H_{red}$ , as observed in our experiments on a longer timescale after illumination is stopped (starting around 500 seconds). This would instead make  $H_{red}^*$  a catalytically relevant intermediate analogous but not identical to  $H_{red}'$ , with still unidentified structural aspects of *TmHydS* and *TamHydS* favoring reduction of the di-iron site instead of the  $[4Fe4S]$  cluster during the initial electron injection into the H-cluster. The currently available data does not allow us to strictly distinguish these two hypotheses.

We note that when analysing the kinetics of the photo-reduction experiment in isolation, the population of the redox state assigned *via* spectroscopic analysis to  $H_{red}^*$  would be intuitively rather assigned to a two electron reduced species *e.g.*  $H_{sred}$ . However, such an assignment would imply a down shift of  $20\text{ cm}^{-1}$  of the main band of  $H_{red}$  ( $1895\text{ cm}^{-1}$ ) to the putative  $H_{sred}$  main band ( $1875\text{ cm}^{-1}$ ), contradicting all previous studies involving these two redox states reporting a shift of  $\approx 10\text{ cm}^{-1}$ .<sup>12,19,23,25,35,37</sup>



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temperatures for group A [FeFe]-hydrogenases,<sup>37–39</sup> and correlation of this experimental data with Density Functional Theory calculations favour its attribution to a Fe(I)Fe(I) species with strict rotated  $\mu\text{CO}$  geometry and a protonated ADT ligand.<sup>38,72</sup> Summing up, the fact that a band is now observed at this position provides compelling support for the notion that  $\text{H}_{\text{red}}$  can harbour a  $\mu\text{CO}$  ligand at room temperature. We further note that the absence of any  $\mu\text{CO}$  band in our spectra for  $\text{H}_{\text{red}}^*$  and  $\text{H}_{\text{sred}}^*$  in both the ADT and PDT version of *TamHydS* differs somewhat from what has been reported for the group C representative *TmHydS*.<sup>3</sup> In the latter case, the fully functional ADT version of the enzyme did show a distinct  $\mu\text{CO}$  band, while no corresponding band was observed for the PDT variant. Evidently, the relative amplitude of the  $\mu\text{CO}$  band is strongly dependent on a range of factors including temperature and nature of the bridgehead group.

## Conclusions

Besides  $\text{H}_{\text{ox}}/\text{H}_{\text{oxH}}$  and  $\text{H}_{\text{red}}$ , this study confirms that the H-cluster in *TamHydS* forms additional redox states analogous to the ones observed for group A [FeFe]-hydrogenases (Table 1 and Fig. S9, ESI†).<sup>5,72</sup> This lends spectroscopy support to the notion that group A and group D [FeFe]-hydrogenases follow the same catalytic cycle, as previously proposed from kinetic fitting of electrochemical data.<sup>49</sup> The idea of a shared catalytic mechanism is further supported by the observed sequential population of key redox states. In this scenario, the slow catalytic kinetics of *TamHydS* are attributable to an increased stability of  $\text{H}_{\text{red}}$  in the putatively sensory enzymes group C and D hydrogenases, relative to the group A. In the current study, this enhanced stability of  $\text{H}_{\text{red}}$  is reflected in its fast formation upon illumination and relatively slow conversion back to  $\text{H}_{\text{ox}}$  once illumination is stopped (Fig. 3). The stability of the one-electron reduced intermediate over a wide potential range is further in agreement with the spectro-electrochemistry data reported for the group C [FeFe]-hydrogenase *TmHydS*.<sup>3,48</sup>

However, different mechanisms operating in different groups of [FeFe]-hydrogenases cannot be fully excluded at this stage. The fact that  $\text{H}_{\text{red}}^*$  and  $\text{H}_{\text{sred}}^*$  are found to accumulate in both group C and D [FeFe]-hydrogenases, while no corresponding states have been observed for group A enzymes, underscores that the protein environment induces distinct differences in the properties of the H-cluster between groups. As noted above, one possible explanation for the accumulation of  $\text{H}_{\text{red}}^*$  and  $\text{H}_{\text{sred}}^*$  in these slow [FeFe]-hydrogenases could be that these are inhibited states, which would explain why mainly  $\text{H}_{\text{red}}/\text{H}_{\text{hyd}}$  populations interconvert during and after photo-reduction. On the other hand, the diiron site is clearly much more easily reduced in these two putatively sensory hydrogenases, as it accumulates without concomitant protonation and the slow interconversion of  $\text{H}_{\text{red}}/\text{H}_{\text{red}}^*$  after photo-reduction would favour  $\text{H}_{\text{red}}^*$  to be a potential catalytic relevant intermediate. When comparing group D and A enzymes the differences in H-cluster reactivity are further evidenced by the fact that still

incompletely characterized spectroscopic features have been observed in *TamHydS* under  $\text{H}_2$  pressure.<sup>9,47</sup> Beside reporting numerous H-cluster states previously unidentified in group D, our study sheds new light on the common  $\text{H}_{\text{red}}$  intermediate. Besides group C, this species appears to be shared across all [FeFe]-hydrogenases studied to date, but its structural details remain contested. The data reported herein does not allow for definite distinction between models 1 or 2, as direct experimental proof of either a  $\mu\text{H}$  ligand or a protonation of the ADT ligand (Fig. 1B) remains elusive. Still, when the diiron site reduced state,  $\text{H}_{\text{red}}$ , is induced *via* exposure to  $\text{H}_2$  gas in *TamHydS*, we now observe a band in the  $\mu\text{CO}$  band region that appears to be associated with  $\text{H}_{\text{red}}$ , indicating the existence of a bridging CO ligand in the  $\text{H}_{\text{red}}$  state at room temperature in line with the recent report of a  $\mu\text{CO}$  band for photo-reduced *TamHydS*.<sup>68</sup> We note that in earlier reports of group A [FeFe]-hydrogenases traces of a  $\mu\text{CO}$  bands potentially attributable to the  $\text{H}_{\text{red}}$  state could be visible at room temperatures as well, but to the best of our knowledge this is the first reported instance where the band is so clearly discernible. We show that  $\text{H}_{\text{red}}$  can adopt different F-cluster redox configurations, and the retained rotated diiron site geometry implied from the  $\mu\text{CO}$  band at ambient temperature would render  $\text{H}_{\text{red}}$  a potential catalytic intermediate.

In closing, we stress that many details surrounding the catalytic mechanism(s) of [FeFe]-hydrogenase remain to be resolved. However, the fact that  $\text{H}_{\text{ox}}$ ,  $\text{H}_{\text{red}}'$ ,  $\text{H}_{\text{red}}$ ,  $\text{H}_{\text{sred}}$  and  $\text{H}_{\text{hyd}}$ , have now all been observed in both group A and D [FeFe]-hydrogenase, arguably suggests a shared catalytic cycle. In addition, the here observed  $\mu\text{CO}$  ligand in  $\text{H}_{\text{red}}$ , favours a catalytic cycle involving diiron site reduced intermediates. Overall, these findings highlight how studies of [FeFe]-hydrogenases from alternative groups can provide new insight into the catalytic cycle, underscoring the need for continuous exploration of this diverse and fascinating enzyme family.

## Author contributions

M. S. designed experiments, performed the FTIR spectroscopy, analysed data and wrote the first draft of the manuscript. C. S. and P. H. performed EPR spectroscopy and analysed data. A. Z. performed the synthesis work. C. S., P. R. C. and H. L. isolated the proteins. G. B. contributed to the data analysis and manuscript preparation. All authors were involved in the analysis and revision of the manuscript and have given approval to the final version of the manuscript.

## Data availability

The methods and data supporting the findings of this study are available in the ESI.†

## Conflicts of interest

There are no conflicts to declare.



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