Characterization of a putative sensory [FeFe]-hydrogenase provides new insight into the role of the active site architecture†

Henrik Land, a,b Alina Sekretareva, a,b Ping Huang, a,b Holly J. Redman, a,b Moritz Senger, a,b† Brigitta Németh, § Nakia Polidori, § Livia S. Mészáros, a,b Moritz Senger, a,b bc Sven T. Stripp a,c and Gustav Berggren a,b*

[FeFe]-hydrogenases are known for their high rates of hydrogen turnover, and are intensively studied in the context of biotechnological applications. Evolution has generated a plethora of different subclasses with widely different characteristics. The M2e subclass is phylogenetically distinct from previously characterized members of this enzyme family and its biological role is unknown. It features significant differences in domain- and active site architecture, and is most closely related to the putative sensory [FeFe]-hydrogenases. Here we report the first comprehensive biochemical and spectroscopical characterization of an M2e enzyme, derived from Thermoanaerobacter marrani. As compared to other [FeFe]-hydrogenases characterized to-date, this enzyme displays an increased H2 affinity, higher activation enthalpies for H+/H2 interconversion, and unusual reactivity towards known hydrogenase inhibitors. These properties are related to differences in active site architecture between the M2e [FeFe]-hydrogenase and “prototypical” [FeFe]-hydrogenases. Thus, this study provides new insight into the role of this subclass in hydrogen metabolism and the influence of the active site pocket on the chemistry of the H-cluster.

Introduction

Hydrogenase enzymes play a central role in hydrogen metabolism, where they catalyze the interconversion between protons and molecular hydrogen (H2). The [FeFe]-hydrogenases are generally considered the most active, operating close to the thermodynamic limit with reported H2 production rates exceeding 9000 s−1.1,2 Consequently, they have been intensively studied, both for their biotechnological potential and as a model system for the design of synthetic catalysts.3,4 Phylogenetically, [FeFe]-hydrogenases can be broadly divided into four main groups, denoted group A, B, C, and D, which in turn contain numerous subclasses.3,5 Considering the well-conserved nature of the auxiliary proteins involved in cofactor assembly (HydEFG),6 they all arguably share a dependence on the same hexanuclear iron cofactor, the “H-cluster”. This biologically unique cofactor consists of a canonical [4Fe–4S] cluster ([4Fe–4S]4) connected to a low valent dinuclear iron complex ([2Fe]3).10–11 The [2Fe]3 subsite is coordinated by CO and CN− ligands, and bridged by an azadithiolate ligand (adt = “SCH2NHCH2S−”). The overwhelming majority of biochemically characterized [FeFe]-hydrogenases belong to group A, with a primary focus on the “prototypical” [FeFe]-hydrogenases, e.g., Cr HydA1 from Chlamydomonas reinhardtii,14,15 Dd HydAB from Desulfovibrio desulfuricans,12,13,16,17 Cpl from Clostridium pasteurianum,18 as well as the multimeric electron bifurcating [FeFe]-hydrogenase from Thermotoga maritima.19–20 Studies of these enzymes form the foundation for our understanding of [FeFe]-hydrogenase biochemistry. Spectroscopy has identified numerous redox and protonation states of the H-cluster, around which various mechanistic proposals have been put forth.21–24 In short, the active-ready resting state (Hun) features a mixed valence Fe(u)Fe(ν) form of the [2Fe]3 subsite and an oxidized [4Fe–4S]4 cluster (2+). One-electron reduction results in either the Hred or Hred state, where Hred features a reduced [4Fe–4S]4 cluster while Hred features a reduced and protonated [2Fe]3 subsite.25 Further reduction results in the formation of the so-called Hhyd state featuring a terminal hydride on the [2Fe]3 subsite.26–28 Protonation of Hhyd results in H2 release, potentially proceeding via a discrete
intermediate \((\text{H}_{\text{hyd}}\text{H}^+)\),\textsuperscript{27-29} and returns the H-cluster to the \(\text{H}_{\text{ox}}\) state. Additionally, CO can reversibly bind to the H-cluster, giving rise to the inhibited \(\text{H}_{\text{ox}}\text{-CO}\) and \(\text{H}_{\text{red}}\text{-CO}\) states.\textsuperscript{30}

Considering the diverse nature of [FeFe]-hydrogenase, both with regards to structure as well as function, it is clear that characterization of representative examples from other subclasses is necessary to complete our understanding of this enzyme family and H-cluster chemistry. It has repeatedly been shown that [FeFe]-hydrogenases can operate at minimal over-potentials, albeit specific enzymes generally display a bias for either H\(^+\) reduction or H\(_2\) oxidation.\textsuperscript{31-34} Indeed, even in the relatively narrow selection of enzymes studied to-date significant differences in catalytic rates, stability of different H-cluster states and reactivity towards inhibitors \(\text{e.g., CO and O}_2\) have been observed.\textsuperscript{22,31,35-39} On a fundamental level, further insight into subclass-specific reactivities is critical for our understanding of hydrogen metabolism, and elucidating the interplay between the H-cluster and the protein. It will also serve to strengthen efforts related to biotechnological energy applications and potentially facilitate the development of selective antibiotics.\textsuperscript{8,9}

We recently reported the whole-cell characterization of an [FeFe]-hydrogenase from the thermophilic firmicute \textit{Thermoaerobacter mathranii} in \textit{E. coli}.\textsuperscript{40} The enzyme belongs to the hitherto uncharacterised M2e subclass, which displays a number of well-conserved differences in amino acid sequence as compared to the prototypical group A hydrogenases; namely in the active site cavity and the proton transfer pathway (Fig. 1).\textsuperscript{41} The M2e subclass has been proposed to form a distinct group of [FeFe]-hydrogenases, group D, and their physiological function is

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\caption{(A) Structural view of the active site and proton transfer pathway of a prototypical [FeFe]-hydrogenase. Structure and numbering based on \textit{Cpl} (PDB ID: 4XDC). Shown amino acid residues are either involved in interactions with the H-cluster or in the proton transfer pathway and show large variations between groups A, C and D. Potential interactions are shown with dashed lines. (B) Amino acid sequence comparison of \textit{Cpl}, \textit{Tm HydS} and \textit{Tam HydS} (\textit{Cpl} numbering) based on a Clustal\textsuperscript{\(\Omega\)} sequence alignment\textsuperscript{80} of sequences retrieved from Greening et al. 2016 (refer. 7) and homology modeling. H-cluster interacting cysteine residues are highlighted in bold. (C) Normalized consensus logos of [FeFe]-hydrogenase groups A, C and D generated in Jalview using the sequence alignment in (B). Coloring is based on the Clustal X color scheme. Amino acid residues involved in H-cluster interaction and proton transfer that show variation between the groups are highlighted in blue and red, respectively.}
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unknown. It is phylogenetically most closely related to the M2f subclass of group C, which has been identified as “putative sensory” and includes the recently characterized Tam HydS enzyme from Thermotoga maritima. In addition to the H-domain, which harbors the H-cluster, both subclasses feature an N-terminal domain with two [4Fe-4S] cluster-binding motifs as well as a C-terminal domain with a four-cysteine motif (C4x2-Cx4-Cx4-C) indicative of iron-sulfur (Fes) cluster binding. Additionally, the highly conserved cysteine residue initiating the proton transfer pathway from the adt amine in group A [FeFe]-hydrogenase is not conserved in the M2e and M2f subclasses (C308, Cpi number is used throughout the text unless otherwise noted). Indeed, all residues that have previously been shown to be crucial for proton transfer in group A are missing in these subclasses (Fig. 1). Moreover, the genes encoding M2e and M2f enzymes are often located on the same operon as group A hydrogenases. In light of these similarities, we have now denoted the enzyme as Tam HydS (previously Tam HydA). However, there are key differences between the two subclasses. In contrast to the M2f enzymes, the M2e subclass does not feature a Per-Arnt-Sim (PAS) domain, which was used to propose the sensing function of group C, as it is usually involved in the regulation of histidine kinases associated with signal transduction. In addition, the active site architecture displays distinct differences between the two subclasses (Fig. 1). The two methionine residues framing the diiron site, M497 and M533, are exchanged against serine and glycine in Tam HydS; and leucine and serine, respectively, in Tam HydS. These methionine residues are considered critical for modulating the reactivity of prototypical [FeFe]-hydrogenases, and it is noteworthy that the exchange of M533 to a hydroxyl donor (serine or threonine) appears to be a well-conserved property of M2e enzymes. On the other hand, M497 shows a low level of conservation, both for group C and D enzymes.

Herein, we report the first detailed characterization of an M2e enzyme, and by extension a group D [FeFe]-hydrogenase. The aforementioned Tam HydS enzyme has been isolated following heterologous expression, and characterized by electron paramagnetic resonance (EPR) spectroscopy, attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy, and protein film electrochemistry (PFE). Despite lacking several amino acid residues considered critical for the activity of prototypical [FeFe]-hydrogenases, Tam HydS shows reversible H+/H2 interconversion close to the thermodynamic potential, with a slight bias for H+ reduction. These findings show that the active site pocket can be significantly altered while still retaining the catalytic function of the H-cluster. However, in contrast to previously characterized [FeFe]-hydrogenases, an over-potential is observed at low temperatures; and the overall catalytic rates are low. Moreover, the M2e enzyme displays significant differences in its reactivity towards CO and O2, as compared to previously characterized [FeFe]-hydrogenases. We propose that the catalytic rates are influenced by intramolecular proton transfer. The study, furthermore, highlights the importance of the active site pocket in modulating the reactivity towards known inhibitors, and the stability of specific H-cluster states. Based on the aforementioned properties of Tam HydS which resemble those of known regulatory [NiFe]-hydrogenases, in combination with analysis on a genome level, we hypothesize that group D [FeFe]-hydrogenases serve a sensory rather than catalytic function.

Results and discussion

Isolation and characterization of apo-Tam HydS

Aerobic heterologous expression of Tam HydS in E. coli, in the absence of the HydEFG maturation enzymes, has been shown to result in soluble apo-protein that could be activated in vivo using [2Fe2S] subsite mimics. In this study, anaerobic isolation of apo-Tam HydS resulted in a purified protein (Fig. S1†) containing 13.9 ± 0.85 Fe/protein. Additional reconstitution of the FeS clusters resulted in a final Fe content of 16.1 ± 0.29 Fe/protein, as expected from the incorporation of four [4Fe-4S] clusters. Isolation of apo-Tam HydS also under aerobic conditions provided a protein with an Fe content of 10.6 ± 0.06 Fe/protein.

All three different purified forms of apo-Tam HydS displayed hydrogenase activity in vitro with sodium dithionite/methyl viologen (NaDT/MV+) enzymatic assays, following anaerobic incubation with the synthetic [2Fe2S]-analogue [Fe2(adt)(CO)4(CN)2]2−. Activation of aerobically and anaerobically purified apo-Tam HydS samples resulted in H2 production activities of 0.068 ± 0.001 and 0.077 ± 0.009 μmol H2 per min per mg, respectively. A further increase in the H2 production activity, to 0.090 ± 0.005 μmol H2 per min per mg was observed after activation of the reconstituted samples (Fig. 2).

X-band EPR spectra recorded on samples of as-isolated and reconstituted apo-Tam HydS are essentially silent, indicating that all FeS-clusters are present at a [4Fe-4S]2− oxidation state with spin s = 0, thus diamagnetic. Reduction of the reconstituted enzyme by NaDT at pH 8 brought the sample to an EPR active state, displaying a broad rhombic EPR spectrum near the g ∼ 2 region, typical of s = 1/2 [4Fe-4S]2− species (Fig. 3). Spin
HydS, known prototypical [FeFe]-hydrogenases, it is similar to Tm HydS with [2Fe]adt resulted in spontaneous formation of in vitro enzyme. Results were recently reported for the putative sensory M2f cluster coordinated by the C-terminal Cx2Cx4Cx16C motif is in contrast, the biochemical and physiological role of the [4Fe-4S] clusters in the reconstituted protein, including the active-site [4Fe-4S]H cluster. This assignment is further supported by EPR spectroscopy. Albeit only two of the clusters were clearly discernable, no signal attributable to [2Fe-2S] clusters was observed upon reduction, and only traces of a [3Fe-4S] cluster signal were present in the non-reduced samples. Similar results were recently reported for the putative sensory M2f enzyme Tm HydS, and it appears to be a shared trait between the two subclasses. This FeS cluster composition is also supported by the linear increase in activity as iron content is increased up to 16 Fe/protein. Although the H2 production activity of Megasphaera elsdenii HydS, observed in in vitro assays, is low compared to most known prototypical [FeFe]-hydrogenases, it is similar to Tm HydS. As the two N-terminal [4Fe-4S] clusters are well conserved across numerous [FeFe]-hydrogenases, including the aforementioned Dd HydAB as well as the M2 enzyme from Megasphaera elsdenii this arguably represents the catalytic electron transfer pathway also in the M2e enzymes. In contrast, the biochemical and physiological role of the [4Fe-4S] cluster coordinated by the C-terminal Cx4Cx4C motif is unknown. As it is located between the H-domain and the PAS domain in M2f [FeFe]-hydrogenases, it is likely to be involved in a signaling process.

EPR characterization of holo-Tam HydS

The in vitro enzymatic assays revealed that treating apo-Tam HydS with [2Fe]adt resulted in spontaneous formation of holo-Tam HydS on a minute time-scale, similar to what was previously observed under whole-cell conditions. In the absence of reductant (NaDT), this treatment is expected to yield an oxidized form of the H-cluster, either the Hox state or the Hox-CO state. Both states are paramagnetic and best described as [4Fe-4S]H2+[2Fe(μ-I)] (s = 1/2) species, thus EPR spectroscopy can be employed to monitor H-cluster assembly. It is well established that the EPR spectra of Hox and Hox-CO generally display rhombic and axial signals, respectively, with small anisotropy and spin transitions at g ≈ 2.22

Expecting a mixture of Hox and Hox-CO, the X-band EPR spectra collected on solution samples of as-prepared holo-Tam HydS, generated under an inert argon atmosphere, displayed a surprisingly complex pattern. As seen in Fig. 4A (spectrum a), quantification resulted in two spin centers per protein, suggesting that two of the [4Fe-4S] clusters are susceptible to NaDT reduction.

The iron quantification supports the presence of four [4Fe-4S] clusters in the reconstituted protein, including the active-site [4Fe-4S]H cluster. This assignment is further supported by EPR spectroscopy. Albeit only two of the clusters were clearly discernable, no signal attributable to [2Fe-2S] clusters was observed upon reduction, and only traces of a [3Fe-4S] cluster signal were present in the non-reduced samples. Similar results were recently reported for the putative sensory M2f enzyme Tm HydS, and it appears to be a shared trait between the two subclasses. This FeS cluster composition is also supported by the linear increase in activity as iron content is increased up to 16 Fe/protein. Although the H2 production activity of Megasphaera elsdenii HydS, observed in in vitro assays, is low compared to most known prototypical [FeFe]-hydrogenases, it is similar to Tm HydS. As the two N-terminal [4Fe-4S] clusters are well conserved across numerous [FeFe]-hydrogenases, including the aforementioned Dd HydAB as well as the M2 enzyme from Megasphaera elsdenii this arguably represents the catalytic electron transfer pathway also in the M2e enzymes. In contrast, the biochemical and physiological role of the [4Fe-4S] cluster coordinated by the C-terminal Cx4Cx4C motifs is unknown. As it is located between the H-domain and the PAS domain in M2f [FeFe]-hydrogenases, it is likely to be involved in a signaling process.

EPR characterization of holo-Tam HydS

The in vitro enzymatic assays revealed that treating apo-Tam HydS with [2Fe]dt resulted in spontaneous formation of holo-
at least seven features were resolved. The spectrum displayed characteristics suggesting the presence of $H_{\text{ox}}$ and $H_{\text{ox}}$-CO but the unusual complexity of the spectrum shows that more than two species contribute to the overall spectrum. Spin quantification of a representative spectrum resulted in 0.64 spin per protein, indicating that a fraction of the enzyme also resided in an EPR silent state, assigned by FTIR spectroscopy to the $H_{\text{red}}$ state (see below). The relaxation behavior of the signal(s) for holo-Tam HydS was estimated by monitoring the dominant $g \approx 2.04$–2.02 feature (Fig. S4†). All components indicated in the spectrum followed similar saturation trends, and displayed low $P_{1/2}$ values (73 $\mu$W and 1.15 mW at 15 and 21 K, respectively). This suggests that spin relaxation is a slow process, most likely due to isolation of the H-cluster from the lattice.

Flushing of holo-Tam HydS solutions with N$_2$ prior to freezing resulted in approx. 50% decrease of total spin density, reflected in a minor decrease in amplitude of the low-field features ($g \approx 2.1$) while a pseudo-axial component represented by a feature at $g = 2.022$ was almost completely lost (Fig. 4A, spectrum b). Subtraction of the spectrum obtained following N$_2$ flushing (spectrum b) from the spectrum of the as-prepared sample (spectrum a) provided a “pure” pseudo-axial spectrum (Fig. 4A, spectrum c) with $g_{||} = 2.041$ and $g_{\perp} = 2.022$. This signal is assigned to the $H_{\text{ox}}$-CO state (Fig. 4A, component “A2, $H_{\text{ox}}$-CO”). Similarly, the $H_{\text{ox}}$-CO component was diminished in samples of holo-Tam HydS isolated following H-cluster assembly in the presence of NaDT (Fig. S5†). The residual spectrum (spectrum b) appears to feature two sets of rhombic signals (components R1 and R2), in combination with an additional narrow axial signal (component A1). The $g_{||} = 2.034$ tensor of the latter signal is readily apparent while the $g_{\perp}$ tensor is tentatively assigned to 2.023 from simulations (indicated in dark brown in Fig. 4A, see Fig. S5† for simulation details). With regards to the rhombic signals, the $g$-values are 2.109 and 2.099 for the two features at the lower field wing, 2.053 and 2.044 in the centre region and 2.010 in the high field wing. The overall spectral shape was highly similar between biological repeats prepared at pH 8. The relative signal amplitudes observed in spectrum (b) were also retained at pH 5 (Fig. S5†) but this acidification resulted in a 2–3 gauss downshift of one set of the rhombic EPR signals, which facilitated separation into two sets of separate $g$-tensors ($g_{\text{Fxx}} = 2.109, 2.053, 2.010, \text{“component R1”}$; and $g_{\text{Fxx}} = 2.099, 2.044, 2.010, \text{“component R2”}$; Fig. 4A). It should be noted that the assignment of $g_{\perp} = 2.010$ is speculative due to its overlap with the adjacent axial signal.

To further clarify the EPR spectrum observed for Tam HydS, the holo-enzyme was generated using the modified cofactor $[2\text{Fe}][\text{pdt}](\text{CO})_4(\text{CN})_2$ – *([2Fe]pdt)*. pdt = “$\text{SC}2\text{H}_2\text{CH}_2\text{CH}_2\text{S}$”; pdt-Tam HydS). It has been shown for both group A and C [FeFe]-hydrogenases that replacing the amine-bridgehead of the adt ligand with a methylene group destabilizes the $H_{\text{ox}}$-CO state. Thus, analogous samples where [2Fe]$^{\text{pdt}}$ replaced [2Fe]$^{\text{adt}}$ were examined by EPR under the same recording conditions. The obtained spectrum showed a rhombic anisotropy with $g$-values of 2.106, 2.051 and 2.010 (Fig. 4A, spectrum d). This signal is attributed to the formation of a single pure $H_{\text{ox}}$ state, in good agreement with FTIR spectroscopy (Fig. S6†), as well as earlier studies of Tam HydS under whole-cell conditions. A comparison between holo-Tam HydS generated with [2Fe]$^{\text{adt}}$ (Fig. 4A, spectra a and b) and [2Fe]$^{\text{pdt}}$ (Fig. 4A, spectrum d) reveals that the rhombic features observed in spectra (a) and (b) display a significant overlap with the signal observed for pdt-Tam HydS (indicated with dashed lines in Fig. 4A). Consequently, the rhombic components R1 and R2 are assigned to two distinct $H_{\text{ox}}$-like species. The non-overlapping features of the spectrum correspond to the pseudo-axial components (A1 and A2), in agreement with the assignment of A2 to the $H_{\text{ox}}$-CO state.

Reduction of holo-Tam HydS with NaDT resulted in disappearance of the aforementioned H-cluster signals with concomitant appearance of a broader rhombic EPR spectrum (Fig. 4B, spectrum NaDT). The loss of the $H_{\text{ox}}$-CO and $H_{\text{ox}}$-like signals is attributed to a one-electron reduction of the H-cluster to the diamagnetic $H_{\text{red}}$ state (see below). The new signal partially resembles that observed for reduced apo-Tam HydS (Fig. 3), and spin quantification showed one spin per protein. Thus, one [4Fe–4S] cluster, with an EPR signature of $g_{\text{Fxx}} = 2.06, 1.94$ and 1.89, is susceptible to NaDT reduction in holo-Tam HydS. Subtraction of the holo-Tam HydS signal from that of apo-Tam HydS revealed another broad rhombic EPR signal ($g_{\text{Fxx}} = 2.08, 1.94$ and 1.85, see Fig. S3† green spectrum). As this signal was present in the apo-protein but lost upon H-cluster formation, it is tentatively attributed to the [4Fe–4S]$_n$ cluster of apo-Tam HydS. Reduction of holo-Tam HydS with H$_2$ provided a similar result compared to reduction with NaDT, although a larger fraction of the $H_{\text{ox}}$-CO state remained (Fig. 4B, spectrum H$_2$).

In summary, the combined EPR data from as-prepared and gas-flushed solution samples reveal an unusually complex mixture of oxidized states. Still, three of the contributing species can be assigned with relatively high certainty. Comparison of as-prepared and N$_2$ flushed samples show that a standard $H_{\text{ox}}$-CO species can form also in holo-Tam HydS. Conversely, the “split” $H_{\text{ox}}$-like signal observed in holo-Tam HydS is suggestive of the formation of two distinguishable $H_{\text{ox}}$-like states. Based on FTIR spectroscopy (see below), R2 is attributed to a state highly similar to the well-known $H_{\text{ox}}$ state of prototypical [FeFe]-hydrogenases ($g_{\text{Fxx}} = 2.099, 2.044, 2.010$), and the second rhombic EPR signal, R1, to a state similar to $H_{\text{red}}$ ($g_{\text{Fxx}} = 2.109, 2.053, 2.010$). A similar downshift of the $H_{\text{ox}}$ signal upon formation of $H_{\text{red}}$ has recently been reported for C$_r$ HydA1. As comparing spectra of samples prepared at mildly basic and acidic conditions did not reveal significant changes in the relative amplitudes of the rhombic signals their interconversion appears to be more complicated than an acid–base equilibrium. The structural details of this $H_{\text{ox}}$-H-like state in Tam HydS remains to be fully elucidated. Still, both appear catalytically competent, as they were both lost upon exposure to H$_2$.

**FTIR characterization of holo-Tam HydS**

The H-cluster of holo-Tam HydS was further investigated using ATR FTIR spectroscopy at different pH values and in the presence of H$_2$, N$_2$, CO, or O$_2$. The enzyme adopted $H_{\text{red}}$ as a semi-
The absorbance spectra of the H-cluster states identified in Fig. 5 were fitted and used to describe the interconversion reactions as a function of gas composition and time. For comparative purposes, analogous experiments were performed with the prototypical [FeFe]-hydrogenases Dd HydAB or Cr HydA1. Fig. 6A depicts the rapid conversion of $\text{H}_{\text{oxy}}$ into $\text{H}_{\text{red}}$ for Tam HydSs and Dd HydAB at 1%, 10%, and 100% $\text{H}_2$ over $\text{N}_2$. Dd HydAB was chosen for comparison because it shows a similar, albeit not identical, composition of reduced H-cluster states under $\text{H}_2$ (Fig. S10†). The identity of $\text{H}_{\text{red}}$ as resting state in Tam HydSs is illustrated by the pronounced persistence of $\text{H}_{\text{red}}$ when $\text{H}_2$ was removed from the gas phase ($t > 16.5$ min) whereas $\text{Dd}$ HydAB immediately converted into $\text{H}_{\text{oxy}}$. This accumulation of $\text{H}_{\text{oxy}}$ is a consequence of auto-oxidation, i.e. due to $\text{H}_2$ release. In

Table 1 Vibrational frequencies observed for the CN− and CO ligands

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the next step, the influence of temperature on the $H_{\text{red}}$ to $H_{\text{ox}}$ transition of Tam HydS and $Dd$ HydAB was investigated. We addressed the kinetics of auto-oxidation for five temperature points in the range between 20–40 °C. The enzymes were reduced in the presence of 1% $H_2$ and subjected to pure $N_2$ for 10 min, before they were re-reduced with 1% $H_2$. Fig. 6B depicts the changing population of $H_{\text{ox}}$ in Tam HydS as a function of gas, time, and temperature. Higher temperature increased the rate of $H_{\text{ox}}$ formation, upon removal of $H_2$ from the atmosphere, and induced a higher percentage of $H_{\text{ox}}$ accumulation (i.e., after 10 min). The same set of experiments was performed for $Dd$ HydAB. Albeit apparent instability of the H-cluster in $Dd$ HydAB. Fig. 6B depicts the HCluster transition of Tam HydS (Fig. S11†). Moreover, it should be noted that an increase in temperature also resulted in an increase in steady-state concentration of $H_{\text{ox}}$ already when Tam HydS equilibrated under 1% $H_2$, highlighting a positive entropy contribution for the $H_{\text{red}}$ to $H_{\text{ox}}$ transition (Fig. 6B).

The reactivity towards known [FeFe]-hydrogenase inhibitors was probed by exposing protein films to CO or $O_2$. Fig. 7A depicts the conversion of $H_{\text{ox}}$ into $H_{\text{ox}}$-CO for Tam HydS and $Dd$ HydAB at 1%, 10%, and 100% CO over $N_2$. Here, Tam HydS displays a notable lack of CO inhibition. Even under 100% CO, only 60% of the H-cluster population converted into $H_{\text{ox}}$-CO. Similar trends were observed with 10% $H_2$ in the $N_2$ carrier gas (Fig. S10†). In the absence of CO gas, $H_{\text{red}}$ recovered quickly. Adjusted for the CO-insensitive contamination of ~30% $H_{\text{inact}}$ (Fig. S10†), $Dd$ HydAB showed immediate, complete, and enduring CO inhibition. Fig. 7B depicts the reaction of oxidized Tam HydS and Cr HydA1 with 1 atm air. We chose Cr HydA1 for comparison because $Dd$ HydAB partly converted into unready states like $H_{\text{inact}}$ in the presence of $O_2$ whereas $O_2$ exposure rapidly destroyed the H-cluster in Cr HydA1 (Fig. S12†). In contrast, Tam HydS converted into an unprecedented species, denoted $H_{\text{air}}$, and the formation of this state was observed regardless of whether the H-cluster resided in the $H_{\text{red}}$ or $H_{\text{ox}}$ state upon $O_2$ exposure (Fig. S9†). As seen in Fig. 5A (blue

Fig. 6 The reactivity of Tam HydS and prototypical [FeFe]-hydrogenase ($Dd$ HydAB) towards $H_2$ (A) and temperature dependence of the auto-oxidation activity (B) monitored by time-resolved ATR FTIR spectroscopy. (A) Kinetic traces of $H_{\text{ox}}$ for Tam HydS (blue) and $Dd$ HydAB (red) that show the reaction with different concentrations of $H_2$ (RT). Note the persistence of $H_{\text{inact}}$ in the absence of $H_2$ for Tam HydS. (B) Change of $[H_{\text{ox}}]$ as a function of gas, time, and temperature. Representative data set for Tam HydS, recorded at pH 7. The equilibrium under 1% $H_2$ is slightly shifted in favour of $H_{\text{ox}}$ over $H_{\text{red}}$ as temperature is increased, and so does rate of $[H_{\text{ox}}]$ formation as the atmosphere is changed to pure $N_2$.

Fig. 7 The reactivity of Tam HydS and prototypical [FeFe]-hydrogenase ($Dd$ HydAB or Cr HydA1) towards CO (A) and $O_2$ (B) monitored by time-resolved ATR FTIR spectroscopy. (A) Kinetic traces of $H_{\text{ox}}$-CO for Tam HydS (blue) and $Dd$ HydAB (red) that show the reaction with different concentrations of CO. About 30% of the $Dd$ HydAB sample were arrested in the CO-insensitive $H_{\text{inact}}$ state. Thus, the observed accumulation of ~70% $H_{\text{ox}}$-CO can be considered complete. (B) Kinetic traces for Tam HydS (blue) and Cr HydA1 (red) that show the reaction with ~21% $O_2$ (air). Solid traces depict $H_{\text{ox}}$. While nearly 100% of the H-cluster is lost in Cr HydA1, a stable Fe(CO)$_2$CN species prevails in Tam HydS (dashed traces). The proposed reaction between the oxidized H-cluster ($H_{\text{ox}}$) and $O_2$ is depicted. FeS = [4Fe–4$S$]$_4$.
The catalytic properties of Tam HydS

The catalytic properties of holo-Tam HydS were investigated using protein film electrochemistry (PFE), revealing pH and temperature dependent catalytic currents for both H+ reduction and H2 oxidation (Fig. 8A). Four different procedures were tested for electrode immobilization of the enzyme with the best method being absorption on a pyrolytic graphite electrode in the presence of the polycationic polymyxin B sulfate (Fig. S14†). Spectro-electrochemistry also suggests that this mononuclear complex is bound to the [4Fe–4S]₄ cluster and that the modified H-cluster displays at least one redox transition, enabling accumulation of “H₄-reduced” and “H₄-oxidized” (Fig. 5B, S14 and S15†).

It is important to note that in all experiments even at large over-potentials for both H+ reduction and H2 oxidation the catalytic current does not reach a steady-state value, but increases almost linearly with over-potential. Such behavior has been rationalized by disorder among the adsorbed enzyme molecules, resulting in a dispersion of interfacial electron transfer rate constants.58-60 In this case, the steady-state limiting current (iₜₘₜ) can be estimated from a linear fit of the high driving force part of the cyclic voltammograms (CVs), where the slope (di/dE) is:

\[
\frac{di}{dE} = \frac{i_{\text{lim}}}{\beta d_0} \frac{F}{2RT}
\]  

Eqn (1) predicts that the product of the slope and temperature is proportional to the limiting current and therefore to the activity.

We first evaluated the enzyme affinity towards H2. It has been noted earlier that \( K_{\text{M,H,}} \) values determined from PFE experiments can be potential-dependent.52-63 Therefore, we recorded CVs at various concentrations of H2 in a broad potential window (Fig. S17†). For \( K_{\text{M,H}} \) estimation it is important to measure the current response under conditions where it is limited by the catalytic rate of the enzyme, i.e. proportional to the catalytic rate rather than mass transport or interfacial electron transfer.64 Thus, \( K_{\text{M,H}} \) values at various over-potentials were calculated, and the measurements were performed at 30 and 60 °C. Moreover, to ensure that the catalytic rate is not limited by mass transport, CVs were recorded at rotation rates of 2000 and 3000 rpm at each concentration of H2. At over-potentials starting from 200 mV (30 °C) and 100 mV (60 °C), calculated \( K_{\text{M,H}} \) values were identical within error (Table 2), indicating that the observed current is dominated by the catalytic reaction. Similar \( K_{\text{M,H}} \) values were obtained from linear fits of the high driving force part of the cyclic voltammograms at various H2 concentrations, further confirming prevalence of the catalytic reaction over interfacial electron transfer at high driving forces (Table S3†).

We further scrutinized the effect of temperature and pH on the catalytic activity of holo-Tam HydSD under conditions where the catalytic current for H2 oxidation is not limited by mass transport (3000 rpm rotation speed and 1 atm H2). Fig. 8A and C displays CVs recorded at various temperatures (10–70 °C) at pH 7, and different pH values at 30 °C, respectively. Fig. 8B shows the temperature dependence of the CVs at high driving force (eqn (1)) for H+ reduction and H2 oxidation. Fig. 8D shows the

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**Fig. 8** CVs obtained at a rotating disc PGE modified with Tam HydS under 1 atm H2 at (A) various temperatures in the 10–70 °C range at pH 7 and (C) various pH values from 5 to 9 at 30 °C. The scan rate is 2 mV s⁻¹, the rotation rate is 3000 rpm. The data shown in (A) and (C) are obtained from single films cycled up and down in temperature and pH, respectively. Film stability was verified at the end of each experiment by returning the solution to its starting state (pH 7 solution at 30 °C). Dependence of the high driving force slopes of the voltammograms (eqn (1)) times the temperature for H2 oxidation and H+ reduction on (B) temperature and (D) pH. Error bars show standard deviation between three films.
corresponding data as a function of pH. When pH is decreased, the catalytic activity towards H\textsuperscript{+} reduction increases and the catalytic wave is shifted to more anodic potentials, consistent with higher H\textsuperscript{+} concentration (Fig. 8C). Conversely, the magnitude of the catalytic current for H\textsubscript{2} oxidation does not vary smoothly with pH. The oxidation process is pH independent between pHs 8–9, increases at pH 6–7 and remains stable at this higher current down to pH 5. Moreover, over-potential for both H\textsubscript{2} oxidation and H\textsuperscript{+} reduction is lowest at pH 5 (Fig. S18\textsuperscript{†}). The origin of this pH switching behavior for H\textsubscript{2} oxidation is currently not identified.

Temperature was found to have a strong influence on H\textsubscript{2} oxidation and H\textsuperscript{+} reduction between 10–60 °C at pH 7 (Fig. 8A). Increasing the temperature not only resulted in higher overall currents, but also a significant decrease in over-potential. At room temperature, an over-potential of 50–60 mV was observed in both catalytic directions, in contrast to previously characterized group A [FeFe]-hydrogenases. With the exception of specific mutants the latter enzymes generally display rapid increase in currents around the thermodynamic midpoint potential.\textsuperscript{45,65,66} The over-potential requirement of Tam HydS is in line with the quasi-reversible nature of the H\textsubscript{ox}/H\textsubscript{red} transition observed by FTIR spectro-electrochemistry (Fig. S8\textsuperscript{†}). At temperatures of 50–60 °C the over-potential decreased to approx. 10 mV. The decrease of catalytic currents at 70 °C is attributed to the deactivation of the protein, since we did not observe any significant protein loss during the experiment at temperatures up to 60 °C (Fig. S19\textsuperscript{†}). It should also be noted that no oxidative inactivation\textsuperscript{67,68} was observed when cycling up to ±0 mV vs. SHE. Stability and increased catalytic activity of the protein at elevated temperatures is not surprising considering the thermophilic nature of \textit{T. mathranii}.\textsuperscript{69} The activation enthalpies (ΔH\textsuperscript{‡}) of the H\textsubscript{2} oxidation and H\textsuperscript{+} reduction reactions were estimated through Eyring plots based on the change of the high potential slope as a function of temperature (Fig. 9), and found to be similar in both catalytic directions (Table 3). In the case of the prototypical [FeFe]-hydrogenases \textit{Cr} HydA1 and \textit{Cal} (Table 3),\textsuperscript{70} distinctly lower activation enthalpies (ΔH\textsuperscript{‡}) for either H\textsuperscript{+} reduction or H\textsubscript{2} oxidation, respectively, have been reported, suggesting that \textit{Tam} HydS is exceptionally well balanced for bidirectional catalysis. Moreover, albeit the activation enthalpies are higher for \textit{Tam} HydS than \textit{Cr} HydA1 and \textit{Cal} (Table 3),\textsuperscript{70} the low specific activity observed for \textit{Tam} HydS (Fig. 2) cannot be explained by differences in activation enthalpies alone. Rather, the low catalytic rate of \textit{Tam} HydS is governed by mass transfer, e.g. proton- or H\textsubscript{2} transfer within the protein. Impaired proton transfer could also, at least partially, explain the over-potential observed at low temperature.\textsuperscript{44} Finally, it is noteworthy that films prepared of the enzyme exposed to air, to induce the formation of the H\textsubscript{air} state, displayed limited capacity for H\textsuperscript{+} reduction but a complete loss of H\textsubscript{2} oxidation function (Fig. S20\textsuperscript{†}). The catalytic properties of H\textsubscript{air} was further supported by \textit{in vitro} assays, showing a H\textsuperscript{+} reduction activity approximately hundred-fold lower than the native enzyme.

### Conclusions

This report represents the first biochemical and biophysical characterization of a group D [FeFe]-hydrogenase. As described herein, \textit{Tam} HydS features a number of properties similar to regulatory [NiFe]-hydrogenases,\textsuperscript{71} including a relatively low K\textsubscript{M}\textsuperscript{H\textsubscript{2}} and an increased tolerance against CO inhibition. This is in line with a potential sensory function, as expected from its close...
relationship to the putatively sensory group C [FeFe]-hydrogenases. Still, a $K_{M}^{H_{2}}$ of around 0.1 mM is 5–10 times higher than what has been reported for regulatory [NiFe]-hydrogenases,71,72 but is arguably in agreement with the overall higher $K_{M}^{H_{2}}$ and $K_{M}^{H_{2}}$ reported for prototypical [FeFe]-hydrogenase.73 Moreover, analysis on a genomic level shows that Tam HydS is encoded upstream of a heterotrimeric bifurcating group A [FeFe]-hydrogenase. Thus, we hypothesize that M2e represents an alternative type of sensory [FeFe]-hydrogenase. This raises the question of how signal transduction is achieved. In the case of group C putative sensory [FeFe]-hydrogenase, a PAS-domain is fused to the H-domain. Similarly, to the best of our knowledge, all regulatory [NiFe]-hydrogenases identified to-date feature the so-called HoxJ subunit, encoding a PAS domain-like sequence. A PAS-domain cannot be identified in the operon encoding for Tam HydS but the gene is flanked by a histidine kinase-like ATPase and an AraC family transcriptional regulator. These genes are commonly involved in signal transduction and regulation of transcription, which further suggests a regulatory role for the M2e hydrogenases.74,75

In the context of the H-cluster function, Tam HydS displays a number of diverging properties. As reflected in the tolerance towards CO, the unusual mixture of $H_{ox}$-like states, reaction enthalpies ($\Delta H^\ddagger$) that were practically identical for both $H^+$ reduction and $H_{2}$ oxidation, and a stabilization of the $H_{red}$ state, Moreover, it is noteworthy that its high tolerance towards CO Tam HydS reacted rapidly with $O_{2}$ to form a stable mononuclear version of the [2Fe]$_{3}$ subsite (Hred). This argues against gas diffusion (mass transfer) to the active site as the main defense mechanism towards CO inhibition, which we instead attribute to the unusual hydrogen-bonding network around the H-cluster afforded by variations of e.g. Cys299, Met353 and Met497 (Fig. 1). Indeed, an increased resistance towards CO has also been reported for the putative-sensory group C [FeFe]-hydrogenase Tm HydS, featuring different alterations of the same residues.38 The influence of the hydrogen-bonding network on CO affinity has also been suggested by studies of cofactor variants and mutants of Cr HydA1, in which the nearby cysteine was replaced by an alanine residue.39

The effect of $O_{2}$ on prototypical group A [FeFe]-hydrogenases has been intensively studied, nevertheless formation of Hair has not been reported for any other [FeFe]-hydrogenase. Instead, the reaction generally causes complete H-cluster degradation or formation of reversibly inhibited states like H inactive by coordination of thiol ligands to the [2Fe] subsite.37,76 Whether Hair has a physiological role remains uncertain. However, as this state appears unreactive towards $H_{2}$ while retaining a limited $H_{2}$ production capacity, it is unlikely to be relevant in $H_{2}$ sensing. Still, its formation provides a striking example of how the active site environment modulates the reactivity of the H-cluster. It should be noted that formation of Hair is dependent on $O_{2}$ as the equivalent state does not seem to be formed under oxidizing electrochemical conditions. As $O_{2}$ acts as a chemical oxidant it can lead to an alternative pathway that remains to be fully elucidated. Arguably, access to electrons and protons has a large influence on the reaction between the H-cluster and $O_{2}$ and several factors need to be considered to explain the formation of Hair in Tam HydS.77,78 It has been shown that in the case of [NiFe]-hydrogenase, $O_{2}$ can be efficiently reduced to $H_{2}O$ through rapid electron injection to the [NiFe] cofactor involving an unusual [4Fe–4S] cluster.79 The presence of a C-terminal [4Fe–4S] cluster and a disrupted proton transfer pathway differentiates M2e enzymes from prototypical [FeFe]-hydrogenases. Still, formation of Hair was not reported in the case of Tm HydS which features the same differences.80 This suggests that the reaction between the H-cluster and $O_{2}$ is significantly influenced by other variations in the active site architecture.

We presume that the lack of a cysteine in position 299 also influences the catalytic activity of Tam HydS, as hindered proton release could stabilize Hred kinetically, following reduction of the H-cluster with $H_{2}$. A similar stabilization of Hred has been noted for Tm HydS.80 However, while the latter enzyme is suggested to form an unusual, unprotonated, CO-bridged form of Hred (Hred ($H_{\text{red}}^\ddagger$)), Tam HydS appears to form an Hred state highly similar to that of prototypical [FeFe]-hydrogenase. Also, the complete lack of the well-conserved proton transfer pathway found in group A [FeFe]-hydrogenases raises the question of where an alternative pathway could reside. In the homology model of Tam HydS, a conserved glutamate residue close to the H-cluster (Glu252) is located in a preferable position for initiating proton transfer (Fig. S21f). However, there is no clear continuation of this potential pathway. In addition to changes in the proton transfer pathway, variation of Met353 and Met497 certainly also modulates the electronic properties of the H-cluster. It has been previously proposed that Met353 is important for tuning $K_{M}^{H_{2}}$ as well as the catalytic bias of prototypical [FeFe]-hydrogenase, in favor of either $H^+$ reduction or $H_{2}$ oxidation.80,81 Still, the exact contributions of the Met353Ser variation on the activity of Tam HydS remain to be fully elucidated.

In closing, this study shows that the Tam HydS M2e enzyme displays significant differences in reactivity as compared to previously studied group A as well as group C [FeFe]-hydrogenases. Thus, in addition to proposing a biological function for the group D [FeFe]-hydrogenases, it underscores how mutations of known [FeFe]-hydrogenases need to be complemented with further studies of the biodiversity to fully realize the chemical space of this fascinating family of enzymes. Mapping out the reactivity of these diverse enzymes is certainly critical for our understanding of hydrogen metabolism and envisioned biotechnological applications. It is also of high relevance in the context of bioinspired catalyst design, as it will provide new model systems for elucidating the influence of the protein environment and the outer coordination sphere on the reactivity of the H-cluster.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

