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

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[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 *Thermoaerobacter mathranii*. 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.⁵–⁹ 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]₄).¹⁰–¹¹ The [2Fe]₄ 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*,¹¹,¹⁶,¹⁷ 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 (H₉₀) features a mixed valence Fe(II)/Fe(III) form of the [2Fe]₄ subsite and an oxidized [4Fe–4S]₄ cluster (2+). One-electron reduction results in either the H₉₀ or H₉₋ state, where H₉₋ features a reduced [4Fe–4S]₄ cluster while H₉₋ features a reduced and protonated [2Fe]₄ subsite.²⁵ Further reduction results in the formation of the so-called H₉₋ state featuring a terminal hydride on the [2Fe]₄ subsite.²⁶–²⁸ Protonation of H₉₋ results in H₂ release, potentially proceeding via a discrete

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intermediate $\text{H}_{\text{ox}}\text{H}^{+}$, 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. 

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 $\text{H}^{+}$ reduction or $\text{H}_{2}$ oxidation. 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 (e.g., CO and O$_2$) have been observed. 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.

We recently reported the whole-cell characterization of an [FeFe]-hydrogenase from the thermophilic firmicute *Thermoaerobacter mathranii* in *E. coli*. 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). The M2e subclass has been proposed to form a distinct group of [FeFe]-hydrogenases, group D, and their physiological function is

**Fig. 1** (A) Structural view of the active site and proton transfer pathway of a prototypical [FeFe]-hydrogenase. Structure and numbering based on 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 Cpl, *Tm* HydS and *Tam* HydS (Cpl numbering) based on a ClustalO sequence alignment of sequences retrieved from Greening et al. 2016 (ref. 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. Still, 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 (Cx2–C4x3x16C) 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 (C299, Cpi numbering 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-Anti-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 M353, are exchanged against serine and glycine in *Tam HydS* and leucine and serine, respectively, in *Tam HydS*. These methionines are considered critical for modulating the reactivity of prototypical [FeFe]-hydrogenases, and it is noteworthy that the exchange of M353 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 [2Fe]4 m site 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 *in vitro* sodium dithionite/methyl viologen (NaDT/MV2+) enzymatic assays, following anaerobic incubation with the synthetic [2Fe]4 adt analogue [Fe2t(adt)(CO)4(CN)2]2– ([2Fe]4adt). 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
known prototypical [FeFe]-hydrogenases, it is similar to Tm
HydS with [2Fe]adt resulted in spontaneous formation of
in vitro The enzyme results were recently reported for the putative sensory M2f
cluster coordinated by the C-terminal Cx2Cx4Cx16C motif is
contrast, the biochemical and physiological role of the [4Fe
HydS, observed in in vitro EPR characterization of
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, sug-
gesting 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.45,46 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.46 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 Tm
HydS, observed in in vitro assays, is low compared to most
known prototypical [FeFe]-hydrogenases, it is similar to Tm
HydS.18 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 elsdonii this arguably represents the catalytic
electron transfer pathway also in the M2e enzymes.31,32,38 In
contrast, the biochemical and physiological role of the [4Fe–4S]
cluster coordinated by the C-terminal Cx3CxCxCx4C 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 previ-
ously observed under whole-cell conditions.46 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.13,47–49 Both states are paramagnetic and best described as
[4Fe–4S]H2+[2Fe{η1}] (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, observed under an inert argon atmosphere, displayed

Fig. 3 X-band EPR spectra of reconstituted apo-Tam HydS (50 μM).
Gray spectrum: as prepared; black spectrum: NaDT reduced. The
observed rhombic signal in the reduced sample is typical for [4Fe–4S]+
clusters. The g-tensors are indicated for the two contributing
components, identified through comparison to NaDT reduced holo-
Tam HydS (see Fig. S3f). The weak signal marked with * appearing at g
= 2.02 is attributed to a trace amount of [3Fe–4S]H+. EPR settings: T =
17 K; modulation frequency 100 kHz, amplitude 15 G; microwave
frequency 9.4 GHz, power 8 mW.

Fig. 4 (A) Signals observed for holo-Tam HydS and attributed to the
H-cluster, (a) holo-Tam HydS activated with [2Fe]adt as isolated; (b)
holo-Tam HydS flushed by N2 to remove Hox-CO contributions
(contains components R1, R2, A1 and A2); (c) difference spectra ob-
tained from subtraction of spectrum (b) from (a) revealing an axial
signal attributed to Hox-CO (`component A2, Hox-CO’); (d) Tam
HydS activated with [2Fe]adt (pdt-Tam HydS, as isolated). EPR settings:
T 21 K; modulation frequency 100 kHz, amplitude 10 G; microwave
frequency 9.4 GHz, power 16 μW. (B) Reduction of holo-Tam HydS
activated with [2Fe]adt by H2 and NaDT resulting in disappearance of
the H-cluster signals and appearance of a rhombic signal attributed to
a [4Fe–4S]+ cluster. EPR settings: T 10 K; modulation frequency 100
kHz, amplitude 15 G; microwave frequency 9.4 GHz, power 80 μW. g-
values indicated in the figure on horizontal bars, for details see main
text.
at least seven features were resolved. The spectrum displayed characteristics suggesting the presence of Hox and Hox-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 Hred state (see below). The relaxation behavior of the signal(s) for holo-Tam HydS was estimated by monitoring the dominant \( g = 2.04-2.02 \) feature (Fig. 4f). All components indicated in the spectrum followed similar saturation trends, and displayed low \( P_{1/2} \) values (73 \( \mu \)T and 1.15 mT 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 \( \text{N}_2 \) prior to freezing resulted in approx. 50% decrease of total spin density, reflected in a minor decrease in amplitude of the low-field features (\( g = 2.1 \)) 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 \( \text{N}_2 \) 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 Hox-CO state (Fig. 4a, component “A2, Hox-CO”). Similarly, the Hox-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 (\( g_{\text{R1}} = 2.109, 2.053, 2.010 \), “component R1”; and \( g_{\text{R2}} = 2.099, 2.044, 2.010 \), “component R2”; Fig. 4a). It should be noted that the assignment of \( g_{\parallel} = 2.010 \) 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 \([\text{Fe}_{2}\{\text{pdt}\}{\text{(CO)}}_{4}{\text{(CN)}}]^{2+}\) ([2Fe]pdt, pdt = “\text{SCH}_{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 Hox-CO state.\(^{11,16,18,51,52}\) Thus, analogous samples where \([\text{Fe}]^\text{pdt}\) replaced \([\text{Fe}]^\text{pdt}\) 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 pure Hox state, in good agreement with FTIR spectroscopy (Fig. S6†), as well as earlier studies of Tam HydS under whole-cell conditions.\(^{34,35}\) A comparison between holo-Tam HydS generated with [2Fe]pdt (Fig. 4a, spectra a and b) and [2Fe]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 Hox-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 Hox-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 Hox-CO and Hox-like signals is attributed to a one-electron reduction of the H-cluster to the diamagnetic Hred 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 \( g = 2.06, 1.94 \) 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 (\( g_{\text{R1}} = 2.08, 1.94 \) 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] cluster of apo-Tam HydS. Reduction of holo-Tam HydS with \( \text{H}_2 \) provided a similar result compared to reduction with NaDT, although a larger fraction of the Hox-CO state remained (Fig. 4b, spectrum H). 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 \( \text{N}_2 \) flushed samples show that a standard Hox-CO species can form also in holo-Tam HydS. Conversely, the “split” Hox-like signal observed in holo-Tam HydS is suggestive of the formation of two distinguishable Hox-like states. Based on FTIR spectroscopy (see below), R2 is attributed to a state highly similar to the well-known Hox state of prototypical [FeFe]-hydrogenases (\( g_{\text{R2}} = 2.099, 2.044, 2.010 \)), and the second rhombic EPR signal, R1, to a state similar to HoxH (\( g_{\text{R1}} = 2.109, 2.053, 2.010 \)). A similar downshift of the Hox-like signal upon formation of HoxH has recently been reported for Cr 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 Hox-like state in Tam HydS remains to be fully elucidated. Still, both appear catalytically competent, as they were both lost upon exposure to \( \text{H}_2 \).

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 \( \text{H}_2, \text{N}_2, \text{CO}, \text{or O}_2 \). The enzyme adopted Hred as a semi-
likely attributable to the M393S variation in signature is attributed to an HoxH-like state, albeit this state is generally not observed at neutral pH in prototypical [FeFe]-hydrogenases. This mixture of Hox-like states and their relative ratio is in agreement with the observation of two rhombic EPR signals under similar conditions (Fig. 4). The Hred to Hox transition was further analyzed by ATR FTIR spectro-electrochemistry, revealing two unusual properties of the Tam HydS enzyme (Fig. S8†). The reaction displayed a significant over-potential requirement, and while the quasi-reversible nature of the process prevented an exact assignment of the Hox/Hred reduction potential it was clearly shifted in an anodic direction as compared to previously studied prototypical [FeFe]-hydrogenase, with Em,s of ≈ −350 to −450 mVs reported for Cr HydA1 and Dd HydAB. During the reductive scan, Hox/Hred interconversion in Tam HydS was observed at Em ≈ −300 mV vs. SHE, while the re-oxidation did not occur until a potential of approx. +200 mV was applied (at pH 8). No intermediates were observed in the process. Thus, Hred appears to be both kinetically and thermodynamically stabilized in Tam HydS. A relatively anodic Hox/Hred midpoint potential has been reported also for the putative sensory Group C hydrogenase Tam HydS.8

Fig. 5A shows the IR signatures of Hox, Hox-CO, and Hred observed for Tam HydS. The assignment of the spectra to specific H-cluster states was facilitated by their similar similarities to spectra previously reported for prototypical [FeFe]-hydrogenases. Still, the frequencies of the terminal CO/CN–ligands are upshifted in comparison to Cr HydA1 and Dd HydAB and closer to Cpi and Cai from C. acetobutylicum.31,32,52,55 The high-frequency CO band of Hox-CO (2026 cm⁻¹) indicates a constrained geometry.31 In contrast to these upshifts, the μCO band of Hox (1788 cm⁻¹) and Hox-CO (1786 cm⁻¹) was found at lower frequencies than typically observed. These latter differences, as compared to group A and C [FeFe]-hydrogenases, are likely attributable to the M393S variation in Tam HydS as this residue is in close contact with the bridging μCO ligand. A distinct feature in the μCO region for the Hred state could not be discerned. At low pH and high concentrations of NaDT, accumulation of Hox-H over Hox was achieved (Fig. 5B), although the protein film never fully converted into Hox-H (Fig. S7†). Note that the signature of Hox-H at low pH is in excellent agreement with the hypothetically shifted Hox-like spectrum observed at pH 8. In contrast to what has been reported for prototypical [FeFe]-hydrogenases, no accumulation of Hox-H was observed, e.g., when low pH samples were exposed to H2.56 Moreover, Hoxred and Hox-air were never detected. Table 1 summarizes the IR signature of all observed H-cluster states.

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 Hox into Hred for Tam HydS and Dd HydAB at 1%, 10%, and 100% H2 over N2. Dd HydAB was chosen for comparison because it shows a similar, albeit not identical, composition of reduced H-cluster states under H2 (Fig. S10†). The identity of Hred as resting state in Tam HydS is illustrated by the pronounced persistence of Hred when H2 was removed from the gas phase (t > 16.5 min) whereas Dd HydAB immediately converted into Hox. This accumulation of Hox is a consequence of auto-oxidation, i.e. due to H2 release. In

| Table 1 | Vibrational frequencies observed for the CN– and CO ligands |
|---------|----------------------------------------------------------|
| CN– (cm⁻¹) | CO (cm⁻¹) |
| Hred | 2064 | 1972 | 1922 | 1896 |
| Hox | 2083 | 2073 | 1971 | 1947 | 1788 |
| Hox-H | 2088 | 2078 | 1980 | 1955 | 1800 |
| Hox-CO | 2088 | 2082 | 2026 | 1978 | 1966 | 1786 |
| Hox-air | 2095 | 2021 | 1972 |
| Hox-air-red | 2088 | 2013 | 1960 |
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, $Tam$ HydS equilibrated under 1% $H_2$ already when $Tam$ HydS was arrested in the CO-insensitive $H_{\text{inact}}$ state. Thus, the observed accumulation of $H_{\text{inact}}$ 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 $HydS_{\text{u}}$, 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

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†).
temperature dependent catalytic currents for both H+ reduction and H2 oxidation the over-potentials 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. S16†). The overall FTIR spectral features in combination with 13CO isotope editing clearly supports the assignment of a mononuclear Fe(CO)2CN species (Fig. S14†). Spectro-electrochemistry also suggests that this mononuclear complex is bound to the [4Fe–4S]ii cluster and that the modified H-cluster displays at least one redox transition, enabling accumulation of “Hair-red” and “Hair-ox” (Fig. 5B, S14 and S15†).

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 and EPR samples collected of Tam HydS exposed to air did not reveal any discernable EPR signal, apart from minor features at g = 4.3 and 2.02, attributable to small amounts of Fe^3+ ions (“junk iron”) and [3Fe–4S] cluster species, respectively. Similarly, a NaDT reduced anaerobic sample of Tam was also essentially EPR silent, albeit trace amounts of a [4Fe–4S]i species became discernable (Fig. S13†). A mononuclear version of the [2Fe]i subsite has previously been observed by X-ray crystallography in the prototypical CpI [FeFe]-hydrogenase, following extended O2 exposure of the enzyme in crystallo.57 The overall FTIR spectral features in combination with 13CO isotope editing clearly supports the assignment of a mononuclear Fe(CO)2CN species (Fig. S14†). Spectro-electrochemistry also suggests that this mononuclear complex is bound to the [4Fe–4S]ii cluster and that the modified H-cluster displays at least one redox transition, enabling accumulation of “Hair-red” and “Hair-ox” (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 (i_lim) can be estimated from a linear fit of the high driving force part of the cyclic voltammograms (CVs), where the slope (dE/di) is:

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

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_M'H2 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_M'H2 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_M'H2 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_M'H2 values were identical within error (Table 2), indicating that the observed current is dominated by the catalytic reaction. Similar K_M'H2 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 HydS 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

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.

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corresponding data as a function of pH. When pH is decreased, the catalytic activity towards H\(^+\) reduction increases and the catalytic wave is shifted to more anodic potentials, consistent with higher H\(^+\) concentration (Fig. 8C). Conversely, the magnitude of the catalytic current for H\(_2\) 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\(_2\) oxidation and H\(^+\) reduction is lowest at pH 5 (Fig. S18). The origin of this pH switching behavior for H\(_2\) oxidation is currently not identified.

Temperature was found to have a strong influence on H\(_2\) oxidation and H\(^+\) 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.\(^{44,45,46}\) The over-potential requirement of Tam HydS is in line with the quasi-reversible nature of the H\(_{\text{ox}}$/H\(_{\text{red}}\) transition observed by FTIR spectro-electrochemistry (Fig. 8B). 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†). It should also be noted that no oxidative inactivation\(^{67,68}\) 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 \(T.\) \(m\)athranii.\(^{69}\) The activation enthalpies (\(\Delta H^\theta\)) of the H\(_2\) oxidation and H\(^+\) 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 \(C\). \(r\)HydA\(_1\) and \(C\). \(a\)I (Table 3),\(^{70}\) distinctly lower activation enthalpies (\(\Delta H^\theta\)) for either H\(^+\) reduction or H\(_2\) oxidation, respectively, have been reported, suggesting that Tam HydS is exceptionally well balanced for bidirectional catalysis. Moreover, albeit the activation enthalpies are higher for Tam HydS than \(C\). \(r\)HydA\(_1\) and \(C\). \(a\)I, they are still significantly lower than what has been reported from the \(E.\) \(c\)ol [NiFe]-hydrogenases \(E\). \(c\)ol Hyd1 and \(E\). \(c\)ol Hyd2 (Table 3). Thus, the low specific activity observed for Tam HydS (Fig. 2) cannot be explained by differences in activation enthalpies alone. Rather, the low catalytic rate of Tam HydS is governed by mass transfer, e.g. proton- or H\(_2\) transfer within the protein. Impaired proton transfer could also, at least partially, explain the over-potential observed at low temperature.\(^{44}\) Finally, it is noteworthy that films prepared of the enzyme exposed to air, to induce the formation of the H\(_{\text{air}}\) state, displayed limited capacity for H\(^+\) reduction but a complete loss of H\(_2\) oxidation function (Fig. S20†).

### Conclusions

This report represents the first biochemical and biophysical characterization of a group D [FeFe]-hydrogenase. As described herein, Tam HydS features a number of properties similar to regulatory [NiFe]-hydrogenases,\(^{71}\) including a relatively low \(K_M^{H_2}\) and an increased tolerance against CO inhibition. This is in line with a potential sensory function, as expected from its close
A group C \([\text{FeFe}]\)-hydrogenase

Tm

electrochemical conditions. As O\(_2\) acts as a chemical oxidant it

Moreover, it is noteworthy that despite its high tolerance

higher than what has been reported for regulatory \([\text{NiFe}]\)-

hydrogenases, even though it displays a catalytic bias of prototypical \([\text{FeFe}]\)-hydrogenase.

Thus, we hypothesize that M2e represents an alternative type of sensory \([\text{FeFe}]\)-hydrogenase. This raises the question of how signal transduction is achieved. In the case of group C putative sensory \([\text{FeFe}]\)-hydrogenase, a PAS-domain is fused to the H-domain. Similarly, to the best of our knowledge, all regulatory \([\text{NiFe}]\)-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 \(\text{Tam HydS}\) 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 H-cluster function, \(\text{Tam HydS}\) displays a number of diverging properties. As reflected in the tolerance towards CO, the unusual mixture of H\(_{\text{ox}}\)-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\(_{\text{red}}\)-state. Moreover, it is noteworthy that despite its high tolerance towards CO \(\text{Tam HydS}\) reacted rapidly with O\(_2\) to form a stable mononuclear version of the \([2\text{Fe}]_\text{aq}\) subsite (H\(_{\text{red}}\)). 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 \([\text{FeFe}]\)-hydrogenase \(Tm\) HydS, featuring different alterations of the same residues.\(^{31,41}\) The influence of the hydrogen-bonding network on CO affinity has also been suggested by studies of cofactor variants and mutants of \(\text{Ct HydA1}\), in which the nearby cysteine was replaced by an alanine residue.\(^{32}\)

The effect of O\(_2\) on prototypical group A \([\text{FeFe}]\)-hydrogenases has been intensively studied, nevertheless formation of H\(_{\text{air}}\) has not been reported for any other \([\text{FeFe}]\)-hydrogenase. Instead, the reaction generally causes complete H-cluster degradation or formation of reversibly inhibited states like H\(_{\text{inact}}\) by coordination of thiol ligands to the \([2\text{Fe}]\) subsite.\(^{25,26}\) Whether H\(_{\text{air}}\) 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 H\(_{\text{air}}\) 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 H\(_{\text{air}}\) in \(\text{Tam HydS}\). It has been shown that in the case of \([\text{NiFe}]\)-hydrogenase, O\(_2\) can be efficiently reduced to H\(_2\)O through rapid electron injection to the \([\text{NiFe}]\) cofactor involving an unusual \([4\text{Fe}-4\text{S}]\) cluster.\(^{79}\) The presence of a C-terminal \([4\text{Fe}-4\text{S}]\) cluster and a disrupted proton transfer pathway differentiates M2e enzymes from prototypical \([\text{FeFe}]\)-hydrogenases. Still, formation of H\(_{\text{air}}\) was not reported in the case of \(Tm\) HydS which features the same differences.\(^{28}\) 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 \(\text{Tam HydS}\), as hindered proton release could stabilize H\(_{\text{red}}\) kinetically, following reduction of the H-cluster with H\(_2\). A similar stabilization of H\(_{\text{red}}\) has been noted for \(\text{Tm HydS}\).\(^{38}\) However, while the latter enzyme is suggested to form an unusual, unprotonated, CO-bridged form of H\(_{\text{red}}\) (H\(_{\text{red}}^\ddagger\)), \(\text{Tam HydS}\) appears to form an H\(_{\text{red}}\)-state highly similar to that of prototypical \([\text{FeFe}]\)-hydrogenase. Also, the complete lack of the well-conserved proton transfer pathway found in group A \([\text{FeFe}]\)-hydrogenases raises the question of where an alternative pathway could reside. In the homology model of \(\text{Tam HydS}\), 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 \(K^\ddagger_{\text{M}}\) as well as the catalytic bias of prototypical \([\text{FeFe}]\)-hydrogenase, in favor of either H\(^+\) reduction or H\(_2\) oxidation.\(^{30,44}\) Still, the exact contributions of the Met353Ser variation on the activity of \(\text{Tam HydS}\) remain to be fully elucidated.

In closing, this study shows that the \(\text{Tam HydS}\) M2e enzyme displays significant differences in reactivity as compared to previously studied group A as well as group C \([\text{FeFe}]\)-hydrogenases. Thus, in addition to proposing a biological function for the group D \([\text{FeFe}]\)-hydrogenases, it underscores how mutations of known \([\text{FeFe}]\)-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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